The role of p19 C-H-Ras protein in metastasis and proliferative pathways

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"THE ROLE OF P19 C-H-RAS PROTEIN IN METASTASIS AND PROLIFERATIVE PATHWAYS"

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AAMP	Angio associated migratory cell protein
ACA's	Adrenocortical adenomas
ACCS's	Adrenocortical carcinomas
ACR-ABL	A translocation oncogenic fusion protein generates by in lymphocytic leukemia
ADAM23	ADAM metalloproteinase 23
ADAM9	ADAM metallopeptidase domain 9, member membrane-anchored proteins and have been implicated in a variety of processes involving cell-cell and cell-matrix interactions, including fertilization, muscle development genesis.
Ago2	Argonaute protein
AKT	Also known as protein kinase B (PKB) is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration.
AKT (Thr 308)	Protein kinase phosphorylated in threonine 308
AKT (Ser 473)	Protein kinase phosphorylated in serine 473
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP-1	Activating protein 1, is a collective term reffering to dimeric transcription factors componed of Jun, Fos o r ATF (activating transcription factor) subunits that bind to a common DNA site
APC	Tumour suppressor gene which codifies for cell adhesión proteins
APL	Acute promyelocityc leukaemia
ApoE	Apolipoprotein E is a class o apolipoprotein found in the chylomicron and intermediate-densitiy lipoprotein (IDLs) that is essential for the normal catabolism of triglyceride rich lipoprotein constituents.
ARPCL5	Actin related protein 2/3 complex, subunit 5-like
BC	Breast cáncer
BCL-2	B-cell lymphoma 2 is the founding member of the Bcl-2 family of apoptosis regulador proteins encoged by the BCL2 gene
BCL-w	A novel member of the bcl-2 family, promotes cell survival.
BCRA1	A tumour suppressor gene that produces a protein called breast cancer type 1 susceptibility protein, responsable for repairing DNA.
BCRA2	A tumor supresor gene that produces a protein called breast cancer type 2 susceptibility protein, responsable for repairing DNA.
BCR-ABL	Philadelphia chromosome is a translocation which results of the oncogenic BCR-ABL gene fusion, located on the shorter derivate 22 chromosome. ABL gene located in chromosome 9 joined to BCR gene located in chromosome 22.
Birc5	Also known as a surviving is one of the genes located on chromosome arm 17 q in the region that is often gained in neuroblastoma. BIRC5 is a protein in the intrinsic apoptotic pathway that interacts with XIAP and DIABLO leading to caspase-3 and caspase-9 inactivation.
Blimp-1	Also known as PR domain zinc finger protein 1 is a protein that in humans is encoded by PRDM1 gene, Blimp-1 acts as a repressor of beta-interferon (β-IFN) gene expression.
BME	Basement membrane extracts
bp	Base pair
BSA	Bovine Serum Albumin
CAPRI	Ca^{+2} promoted Ras inactivator
CCND1	Gene (protein coding) cyclin D1belongs a cyclin family protein specific for controlling G1/S point cell cycle.
CCND2	Gene (protein coding) cyclin D2 belongs a cyclin family protein specific for controlling

	G1/S cell cycle transition
CCND3	Gene (protein coding) cyclin D3 belongs a cyclin family protein specific for controlling G1/S cell cycle transition
CD14 (+)	Cluster of differentiation 14 also known as CD14 is a human gene. The protein encoded by this gene is a component of the innate immune system.
CD4 (+)	CD4 T cells are generally treated as having a pre-defined role as helper T cells within the immune system.
CD34 (+)	CD34 molecule is a cluster of differentiation molecule present on certain cells within the human body. It is a cell surface glycoprotein and functions as a cell-cell adhesion factor. It may also mediate the attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells.
CD8 (+)	These cells are also known as CD8+ T cells since they express the CD8 glycoprotein at their surface
CDC25	Cdc25 is a dual-specificity phosphatase first isolated from the yeast Schizosaccharomyces pombe as a cell cycle defective mutant. As with other cell cycle proteins such as Cdc2 and Cdc4, the "cdc" in its name refers to "cell division cycle"
Cdc42	Cell division control protein 42 homolog is a protein involved in regulation of cell cycle.
Cdc43	The gene CAL1 (also known as CDC43) of *S. cerevisiae*.
CDC44	A putative nucleotide-binding protein required for cell cycle
CDK	Cyclin-dependent kinase
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
cDNA	Complementary DNA
c-JNK	c-Jun N- terminal kinases (JNK´s), were originally identified as kinases that bind and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain
c-JNK	c- Jun N-terminal kinase
CLL	Chronic lymphocytic leukaemia
cm^2	Square centimeters
c-Met	Protooncogene that promotes tumor cell migration
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
c-Myc	c-Myc is a regulator gene that codes for a transcription factor.
CO_2	Dioxide carbon
Cox-2	Is an enzyme responsible for inflammation and pain.
CRK	CRK gene (or v-Crk) encodes a member of an adapter protein family that binds to several tyrosine phosphorylated proteins.
CRSP3	Mediator of RNA polymerase II transcription subunit 23, is an enzyme that in humans is encoded by the MED23.
CS	Costello syndrome
Ct	Fold change of miRNA expression
DAAM1	Disheveled-associated activator of morphogenesis 1 is a protein that in humans is encoded by the DAAM1 gene. DAAM1 is involved in cell motility, adhesion and cytokinesis and other functions of the cell cortex are mediated by the reorganization of the actin cytoskeleton.
DAB2IP	Ras GTPase activating-like protein RASAL DAG dyacylglycerol
DAF-12	The nuclear receptor DAF-12 has roles in normal development and determination of adult in C. elegans.
DAG	Dyacyl glycerol
DCF	Also known as DCF-1 gene is related to the differentiation of neural stem cells in knock-out mouse model.
DCF-DA	Dichlorodihydrofluorescein diacetate
DCFH	Dichlorodihydrofluorescein
DCK	Deoxycytidine kinase (DCK) is required for the phosphorylation of several deoxyribonucleosides and their nucleoside analogs. Deficiency of DCK is associated

	with resistance to antiviral and anticancer chemotherapeutic agents
DEPC	Diethylpyrocarbonate
DICER	an endoribonuclease in the RNase III family that cleaves double- stranded RNA (dsRNA) and pre-microRNA (miRNA) into short double-stranded RNA
dir	Direct
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxirribodonucleic acid
DNMT1	DNA cytosine-5 methyl transferase
dNTP's	Plural of dinucleotide triphosphate
dsDNA	Double strand DNA
E. coli	Escherichia coli
E2F1	Is a transcription factor encoded by the E2F1 gene, it plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses.
E-cadherin	E-cadherin is a Ca^{+2}-dependent, transmembrane cell adhesion molecule. It plays an important role in the growth, development and in the intercellular adhesion of epithelial cells.
EDTA	Ethylenediaminetetraacetic acid
Egfl7	EGF-like domain-containing protein 7 is a protein that in humans is encoded by the EGFL7 gene. Intron 7 of EGFL7 hosts the miR-126 microRNA gene.
EGFR	Epidermal growth factor receptor
EMT	Epidermal mesenchymal transition
ER	Estrogen receptor
ERbB	The epidermal growth factor receptor (EGFR) is the founding member of the ErbB family of four structurally related receptor tyrosine kinases.
ERK	Extracellular signal-regulated kinases, a kind of protein molecule.
ESCC	Epithelial squamous cell carcinoma
ESR1	ESR1 gene codifies for ER (α)
EVL	Ena/VASP proteins are actin-associated proteins involved in a range of processes dependent on cytoskeleton remodeling and cell polarity such as axon guidance and lamellipodial and filopodial dynamics in migrating cells. EVL enhances actin nucleation and polymerization. Required to transform actin polymerization into active movement for the propulsive force of *Listeria monocytogenes*
EZH2	Enhancer of zeste homologue 2
FAK	Focal adhesion kinase transcription
FBS	Fetal bovine serum
FGF	Fibroblasts growth factor
Fig.	Figure
FLOT1	FLOT1 gene codifies for a Flot1 protein.
FLOT2	A protein codified for FLOT2 gene, is a highly conserved protein isolated from caveolae/lipid raft domains that together growth factor receptors linked to signal transduction pathways.
FOSL-1	The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation.
Foxp3 (+)	Foxp3 gene codified in humans for a protein called FOXP3 (forkhead box P3) which is a transcriptional regulator.
FSH	Follicle-stimulating hormone
GATA-3	This gene encodes a protein which belongs to the GATA family of transcription factors. The protein contains two GATA-type zinc fingers and is an important regulator of T-cell development and plays an important role in endothelial cell biology.
G0/G1	Gap0/Gap1 cell cycle cell phase

G1/S	Gap1/S is the synthesis of DNA cell cycle phase
G418	Geneticine 418
GAPs	Ras GTPase activating protein
GATA-1	GATA1 gene (protein-coding), GATA binding protein 1 (globin transcription factor1). This gene encodes a protein which belongs to the GATA family of transcription factors. . The protein plays an important role in erythroid development by regulating the switch of fetal hemoglobin to adult hemoglobin.
GDP	Guanine-dinucleotide diphosphate of Ras oncoproteins
GEFs	Guanine-nucleotide exchange of Ras oncoproteins
GNAI1	Guanine nucleotide binding proteins are heterotrimeric signal-transducing molecules consisting of alpha, beta, and gamma subunits. The alpha subunit binds guanine nucleotide, can hydrolyze GTP, and can interact with other proteins. The protein encoded by this gene represents the alpha subunit of an inhibitory complex. The encoded protein is part of a complex that responds to beta-adrenergic signals by inhibiting adenylate cyclase. Two transcript variants encoding different isoforms have been found for this gene.
GNAI2	The protein encoded by this gene is an alpha subunit of guanine nucleotide binding proteins (G proteins). The encodedprotein contains the guanine nucleotide binding site and is involved in the hormonal regulation of adenylate cyclase. Several transcript variants encoding different isoforms have been detected for this gene, but the full-length nature of only two are known so far.
GSK	Glycogen sunthase kinase 3
GTP	Guanosine-5'-triphosphate (GTP) is a purine nucleoside triphosphate. It can act as a substrate for the synthesis of RNA during the translation process.
h	Hour
H_2O	Water
HBEGF	Heparin binding EGF-like growth factor
HDAC4	Histone deacetylase 4
HER2	HER2 (Human Epidermal Growth Factor Receptor 2) also known as Neu, ErbB-2, CD340 (cluster of differentiation 340) or p185 is a protein that in humans is encoded by the ERBB2 gene. HER2 is a member of the epidermal growth factor receptor (EGFR/ErbB) family.
HER3	Receptor tyrosine-protein kinase erbB-3 is an enzyme that in humans is encoded by the ERBB3 gene. This gene encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. This membrane-bound protein has a neuregulin binding domain but not an active kinase domain. It therefore can bind this ligand but not convey the signal into the cell through protein phosphorylation
HIF-1α	Hypoxia inducible factor 1α
HMGA2	HMGA2 (high mobility group AT-hook 2) is a transcriptional regulator that it functions in cell cycle regulation through CCNDA2.
Hmsh1/2	Tumour suppressor gene involved in DNA mismatch repair
HnRNP	Heterogeneoud nuclear ribonucleoproteins are predominantly nuclear RNA binding proteins that form complexes with RNA polymerase II transcripts.
H-Ras	H-Ras oncoprotein encoded by H-Ras gene
hTERT	Telomerase reverse transcriptase is a catalytic subunit of the enzyme telomerase, which together with the telomerase RNA component (TERC), are the most important components of the telomerase complex.
HPV	Human papiloma viruses
HVP	Hipervariable region of RAS proteins
HVP18	Human Papilloma virus, serotype 18
IDH	Isocitrate dehydrogenase 1 (NADP+) soluble
IFN's	Interferons
IL-2	Interleukin 2 (IL-2) is an interleukin, a type of cytokine signaling molecule in the immune system.

INEGI	Instituto Nacional de Estadìstica Geografía e Informatica de Mèxico
IRS-1	Insulin receptor substrate-1
JAG	Jagged a gene that encodes a putative zinc finger transcription
JAK	Janus protein tyrosine kinase
JMML	Juvenile myelomonocytic myeloid leukemia
KAI1	Kangai 1L suppression of tumorigenecity 6
KDa	Kilodaltons
Ki ^{67}	Protein characteristic of G1, M and S cell cycle but not of G0
Kiss1	Kiss peptin receptor is a metastatic suppressor
Klf4	Kruppel like factor 4
K-Ras	Oncogene Ras
KSR	Kinase suppesor of Ras
KSR1	Kinase suppressor of Ras
l	Liter
MAKP1	Tumor suppressor regulated by RAas functions as a growth inhibitor
MAPK	Mitogen activated protein kinase
MAXP1	Tumor suppressor regulated by Ras
Mdm2	Mouse double minute 2 homolog also known as E3 ubiquitin-protein ligase
MEFs	Murine embryonic fibroblasts
MEF2C	Myocite enhancer factor 2C transcription
Men1	Tumor suppressor gene which codifies for Ref receptor
MERTK	c-Met tyrosine kinase
MEST	Mesoderm specific transcription homolog gene
mg/ml	milligram/milliliter
$MgCl_2$	Magnesium chloride
min	Minute
miRISC	Protein complex that inhibits translation elongation
miRNA/miRNAs	microRNA/ miRNAs
miRtrons	miRNAs
MKK4	Mitogen activated protein kinase, kinase 4
MKK7	Mitogen activated protein kinase, kinase 7
Mkp1	A non-receptor dual specific phosphoprotein phosphatase
ml	Mililiters
ml/well	mililiters per well
mM	Milimolar
MMP-2	Metalloproteinase 2
MMP-9	Metalloproteinase 9
mos	Protooncogene serine/threonine protein kinase mos in an enzyme that is codified by mos gene
mRNA	Messenger Ribodonucleic acid
MRP1	Multidrug resistance associated protein-1 axis
MSC's	Mesenchymal stem cells
MSG	Metastasis suppressor gene
mTOR	Mammalian target of rapamycin
MW	Molecular weight
myc	c-Myc oncogenic transcription factor
MYCN	v-myc myelocytomatosis viral related oncogene
myomiR	MicroRNAs involved in myoblast and skeletal muscle differentiation
N	Normal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDPK's	Nicotin diphosphate kinases
NF1	Neurofibrimin

NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
ng	Nanograme
NGAL	Neutrophil gelatinase-associated lipocalin
NIRF	A ring finger protein involved in cell-cycle regulation
nM	Nanomolar
NOTCH3	NOTCH proteins (1-4) are a family of transmembrane receptor that play important roles in determination of cell fate
NP-40	A commercially detergent used for treating whole protein extracts
N-Ras	Oncogene Ras
NSCLC	Non small cell lung cancer
NSE	Neuron specific enolase
nt	Nucleotide
^{o}C	Centigrade temperatura scale
Oct4	Octamer binding transcription factor 4
OG	Oncogene
OncomiR	miRNA that acts as oncogene
ORF	Open Reading frame
p	P value
P120GAP	A GTPase activating protein
P21	P21 c-H-Ras protein
P21WAF1	Cyclin dependent kinase inhibitor 1
p38	Mitogen activated protein kinase
p53	Tumor suppressor protein
p63	Tumour suppressor protein
p68/DDX	A dead box family of RNA helicases
P70S6K	Serine threonine kinase
p73	Tumour suppressor
PA	Protective antigen
PAK1	Serine threonine p21 activating kinases
p-AKT	phosphorylated AKT
PAX7	Paired box transcription factor 7
PBS	Phosphate buffer solution
PBS-Glu	Phosphate buffer solution-glucose
PCR	Polymerase chain reaction
PDGFRA	Plateled derived growth factor receptor alpha polypeptide
PDK1	3-phosphoinositide dependent kinase1
PEP	Phosphoenolpyruvate
p-ERK	Phosphorylated extracellular signal regulated kinase
PFU taq polymerase	High fidelity Taq polymerase
PGA	2-phospho-D-glycerate
pH	Hydrogen potential
PhD	Philosophy Doctor
PI3K	Phosphoinositol 3 kinase
PKCβII	Protein kinase isoform β II
PLCγ	Phospholipase protein
PPI2	Phosphoinositide 2-kinase
PPI3	Phosphoinositide 3-kinase
PR	Progesterone receptor
Pre-miRNA	Precursor miRNA
Pri-miRNA	miRNA precursor
pRK5-C1	C terminal empty vector plasmid (used as control transfection)
pRK5-p19	Plasmid pRK5 designed for overexpressing p19 protein

pRK5-p19 (G12S)	Plasmid pRK5 designed for overexpressing p19 (G12S) Costello mutant protein
pRK5-p21	Plasmid pRK5 designed for overexpressing p21 protein
pRK5-p21 (G12S)	Plasmid pRK5 designed for overexpressing p21 (G12S) mutant Costello protein
pRK5-p21 (Q61L)	Plasmid pRK5 designed for overexpressing p21 (Q61L) mutant Costello protein
psi	Presion unit
PTEN	Phosphatase and tensin homolog protein
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
Rac-1	Ras related C3 botulinum toxin substrate 1
RACGAP	Rac-GTPase activating protein
Rack-1	The receptor for activated c kinase 1
Raf1	GEF factor
RafGEF	Raf guanidine Exchange factor
Ral	Ral GTP binding protein
Ras	Oncogene ras
RASA1	Also known as p120 Ras GAP
RASSF1	Ras association Ral GDS/AF-6 domain family member 1
Rb	Retinoblastoma
RB1	Retinoblastoma protein1
RbCl	Rubidium chloride
rev	Reverse
RFU	Relative fluorescence units
RGS19	A regulator of G protein signaling
Rho	Rho GTPase protein
RHOC	Ras homolog gene family member C
Rin	A neuron specific and calmodulin binding small G protein
Rip1	Receptor interating protein 1
RISC	RNA induced silencing complex
RNA	Ribodonucleic acid
RNAs	Plural of RNA
RNAse	Enzyme which degradates RNA
RNHU6	Control of miRNA PCR REAL TIME
ROCK2	Rho associated coiled-coiled containing protein
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RT-qPCR	Real Time quantitative PCR
RRM2	Ribonucleoprotein reductase subunit
RT-PCR	Real time PCR
RUNX2	Run related transcription factor 2
s	Second
Sc35	A splicing factor protein
Scr	Tyrosine kinase oncogene
Scr-1	Steroid receptor activator 1
Scr-3	Steroid receptor activator 3
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl sulphate Protein Acrilamyde Gel Electrophoresis
SHP2	Src homology 2 domain containing phosphate 2
SIN-1	Positive control for ROS assay
SNAIL	Snail zinc transcription factor
SNPs	Single nucleotide polymorphism

SOC medium	Super optimal broth with catabolic repression
SOS	An enzymatic factor interacting with RAS
SOX-2/4	A transcription factor family that regulates SEMA3 and plexin transcription
SOX4	SRY-box containing transcription factor
SP1	A human transcription factor implicated in embryogenesis
Spread1	Sprouty related EVH1 domain containing 1
Src	Proto-oncogene tyrosine protein kinase
SREBP1	Sterol regulatory element-binding transcription
STAT1/3	A signal transducer and activator of transcription
TBS	Triphosphate Buffer solution
TCTP	Translationally controlled tumor protein
TGF-β	Tumour growth factor β
TIMP-3	A matrix metalloproteinase tissue inhibitor 3
TNC	Tenascin
Traf2	TNF receptor-associated factor 2
TRBP	A double strand RNA binding protein required for the recruitment of Ago2
TRE	Temporal regulatory element
TREGS	Foxp3 (+) regulatory T cells
TSC	Tumorous sclerosis complex
TSG	Tumour suppressor gene
TUTase	Uridylyl transferase
U18	Internal control for miRNA PCR REAL TIME assay
UHRF2	E3 ubiquitin protein ligase
UTR	Untranslated terminal región
V	Volume
VDUP1	Vitamin D3 upregulated protein 1
VEGF	Vascular endothelial growth factor
Vim	Vimectine
vs	Versus
w/v	Weigh/ volumen
Wnt	A family of proteins which regulate cell-cell adhesions
Wt-1	Wilms tumour suppressor gen
Zcchc11	A RNA uridynyltransferase
ZEB2	Zinc finger E-boxing homeobox 2
ZNF238	A transcription factor downregulated in rhabdomyosarcoma
β-gal	B-galactosidase
µl	Microliters
µM	Micromolar

SUMMARY

c-H-Ras gene renders two proteins by alternative splicing called p19 and p21, nevertheless both proteins have the same origin, they differ in their size and function. In this work we provide evidence that p19 c-H-Ras protein promotes an anticancerigen state in the cell whereas p21 c-H-Ras promotes some aspects of metastasis.

Even though p19 c-H-Ras lacks of a typical GTPase domain (152-165 residues), at difference of p21; it can activate several signalling pathways binding with other proteins as such as RACK1, p73, and PKCβII; the protein complexes formed had been localized in the nucleus which suggests that p19 orchestrates multiple cell functions from the nucleus (Camats et al., 2009).

For the experimental development of this phD thesis we decided to overexpress p19, p21, or their mutant protein variants transient and ectopically in *HeLa* cells in order to analyze their role in some aspects of cancer processes as such as proliferation, capacity of invasion, reactive oxygen species production, capacity of forming tumours, and their contribution to miRNAs expression profile. Three mutations were analyzed in these assays: Q61L, G12S, and G12A, the first of them, has a high oncogenic capacity in a constitutive manner blocking GAP's function, and in other hand G12S and G12A mutations are the most frequent DNA changes detected in at codon 12 of H-Ras gene in Costello Syndrome patients' samples of fibroblasts; moreover G12S and G12A mutations produce an aminoacid change of Gly/Ser and Gly/Ala respectively, that activate constitutively GTP-bound conformation and activate downstream effectors such as MAPK, PI3K kinase and RasGDS.

We also analyzed the contribution of p19, or p21 proteins in knock-out H-*Ras* (-/-) and double knock-out for *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts (MEF's); these cell lines have the advantage that they do not show gene redundancy in their expression, so in other words this means that the absence of one member of Ras proteins does not cause that other Ras protein assume its functions.

Our results showed that a general upregulation of miRNAs expression profile were detected when p19, p21 or their mutant variants were overexpressed in *HeLa* cells.

Further experiments were performance in knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21, in these assays we explored the role of p19 and p21 protein in our miRNAs previously selected, and we found a differential expression and was accompanied by an exceptional increase in miR-206 (96.16 fold change value) when pEGFP-p19 was expressed and miR-342 (12.20 fold change value) when pEGFP-p21 was expressed. In addition, miR-206 was consistently downregulated in fibroblasts obtained from tumours of Costello Syndrome patients, and recent reports have correlated its misregulation with the development of rhabdomyosarcoma in these patients.

In other hand, overexpression of p21 (G12S) protein in *HeLa* cells showed the highest rate of invasion, followed by p21, p19 (G12S) and p19 proteins; in further invasion assays cotransfecting p19/p21 (G12S) proteins we could observed that p19 was able to reduce the high invasion environment conferred of p21 (G12S) protein. In the case of G12A or G12S fibroblasts of Costello patients, the highest invasion capacity rate was conferred by G12A mutation.

p19 also showed a low rate of proliferation in double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19, further cytometry analyses revealed that p19 induces a quiescent arrest in G0/G1 phase cell cycle, these results were also confirmed with the low capacity of forming colonies from a single cell of p19 protein, at the contrary the expression of those proteins contained (G12S) mutation showed bigger and more number of colonies. Additionally, overexpression of p19 protein (pRK5-p19 vector) in *HeLa* cells also conferred protection against reactive oxygen species emission overexpressing NM23H1 protein, this effect was also detected in high ROS emission environment.

I. INTRODUCTION

1.1 CANCER

Cancer is a major human health problem worldwide, an estimated of 12.66 million of people were diagnosed and 7.56 million died for this disease in 2008. The most commonly diagnosed cancers in the world are lung, breast, colorectal, stomach and prostate, and the incidence and the risk factors of this disease are different between men and women; for example lung cancer is still the most common cancer diagnosed in men worldwide and breast cancer is the most common cancer diagnosed in women (World Health Organization, 2011). In Spain, colorectal cancer is the second cause of mortality although lung cancer showed the highest incidence and mortality in men followed by prostate, colorectal and bladder cancer and in women breast cancer is the most frequent followed by colorectal and uterus cancer (Sociedad Española de Oncología Médica, http://www.seom.org; Miñarro et al., 2000; Sanchez et al., 2010). In North America, specifically in México and United States, the highest mortality rates are cervical and breast cancer, in women and prostate cancer in men (Malvezzi et al., 2004; Ahmedin et al., 2010; Bruni et al., 2010; Jemal et al., 2010, INEGI, 2011).

Since cancer is increasing in all countries, is mandatory the emerging necessity of finding new treatments that can stop this disease. Even though a significant progress have been achieved in understanding the molecular basis of cancer, it has not been possible to find a cure because is a complex disease in which many genes are involved. In the next chapter, we will review the literature in order to better understand the transformation processes that cancer involves.

1.1.2 Carcinogenesis

The origin of a malignant transformation is a complex process that can take many years in which a normal cell converts into a cancer cell. This long process of transformation involves complex interactions between environmental and endogenous factors, and it had been divided in three different stages: initiation, promotion and progression (fig.1); (Martinez et al., 2003; Lopéz-Otín and Matrisian, 2007).

Initiation is the first step of carcinogenesis and starts with an irreversible genetic change in one or more cancer related genes (see 1.1.4 section); these changes are induced by chemical or physical agents that can damage DNA structure. DNA lesions include single or double-strand breaks, chromosomal translocations, amplifications and different types of mutations in the DNA bases (point mutations, deletions, and insertions). Since initiation is a permanent genetic change, any daughter cell produced from the division of the mutated cell will also carry the mutation (Yoshikawa et al., 1990).

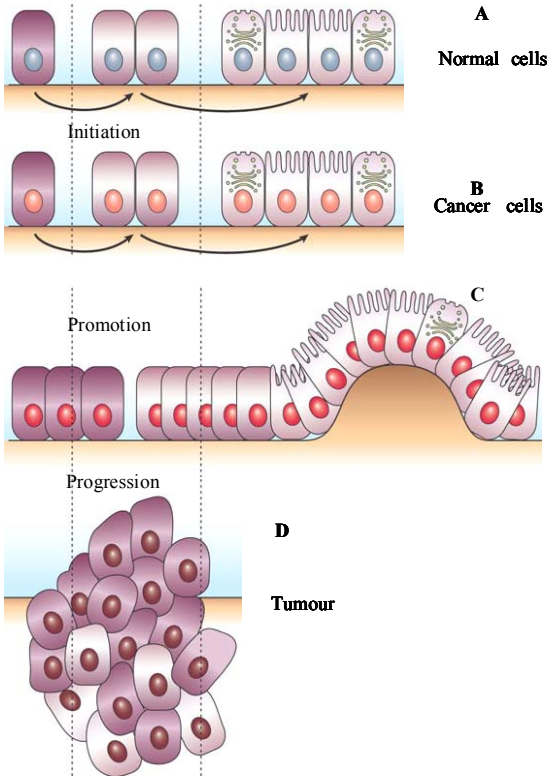


Fig. 1 Stages of cancer. A) normal cells, **B)** initiation of normal cell into mutated cells, **C)** promotion and **D)** progresion of cancer cells which promotes tumorigenesis, (Taipale and Beach, 2001).

In the promotion stage, the population of initiated cells increased rapidly and proliferation leads to eventual formation of non-invasive tumour called benign tumour, which can be removed from the body by surgery.

The third staged called progression is the most dangerous stage of carcinogenesis, and it refers to accumulative mutations in initiated cells which permit to acquire new additional abilities and skills to cancer cells, in order to compete with normal cells for nutrients and space. The most important acquired characteristics are: 1) an increase in growing rate, 2) capacity of invasion, and 3) alteration in biochemistry and morphology and metastasis (Martinez et al., 2003).

1.1.3 Molecular basis of cancer phenotypes

Even though each cancer is different due to the high range of mutagenesis, eight essential alterations had been detected in cell physiology that collectively drives into malignant growth (table 1). Each of these physiological changes represents a novel capabilities acquired during tumour development and are present in all types of human tumours, (Hannagan and Weinberg, 2000; Abbott et al., 2006; Colotta et al., 2009; Hanagan and Weinberg, 2011).

Cancer cell characteristics
• Self sufficient in growth signals
• Insensivity to growth inhibitory (antigrowth) signals
• Evasion of programmed cell death (Apoptosis)
• Limitless replicative potential
• Sustained angiogenesis
• Activating invasion and metastasis
• Genomic instability
• Inmmunological misregulation

Table 1. The hallmarks of cancer. (Hanagan and Weinberg, 2000; Collota et al., 2009; Hanagan and Weinberg, 2011).

Normal cells divide only when they receive growth signals from other cells in their transmembrane receptors. In cancer cells, these receptors are overexpressed and extracellular inhibitory signals which should be maintained them in a post-mitotic stage are ignored; moreover they produce their own growth signals, meanwhile other signals are released for avoiding their programmed cell death (apoptosis), all this process contributes to a continuously replicative state (limitless replicative potential). In each division, DNA can be mutated, and with the time, cancer cells acquire more malignant characteristics due to the continuous mutations, the most dangerous is the capacity of producing metastasis,

which involves detachment of cells from the original tumour, invasion through the basement membrane, intravasation into the blood stream, and eventual extravasations from the blood stream at a distant site where implantation (tissue invasion), and tumour cell proliferation give rise to cancer metastases (fig. 2), (Hannagan and Weinberg, 2000; Abbott et al., 2006; Colotta et al., 2009; Hanagan and Weinberg, 2011).

In order for this process successfully occurred, extracellular matrix has to be remodelling, due to the rearrangement of cytoskeleton producing new structures called podosomes that are composed of an actin-bundle core, which confers motility through extracellular matrix, and they form clusters and rosettes during cell migration (Dessai et al., 2008). Angiogenesis is other fundamental acquired characteristic for metastasis progression and is the capacity of inducing the growth of new capillaries thought a great variety of proteins, principally inducers and inhibitors that provide nutrients for growing tumours.

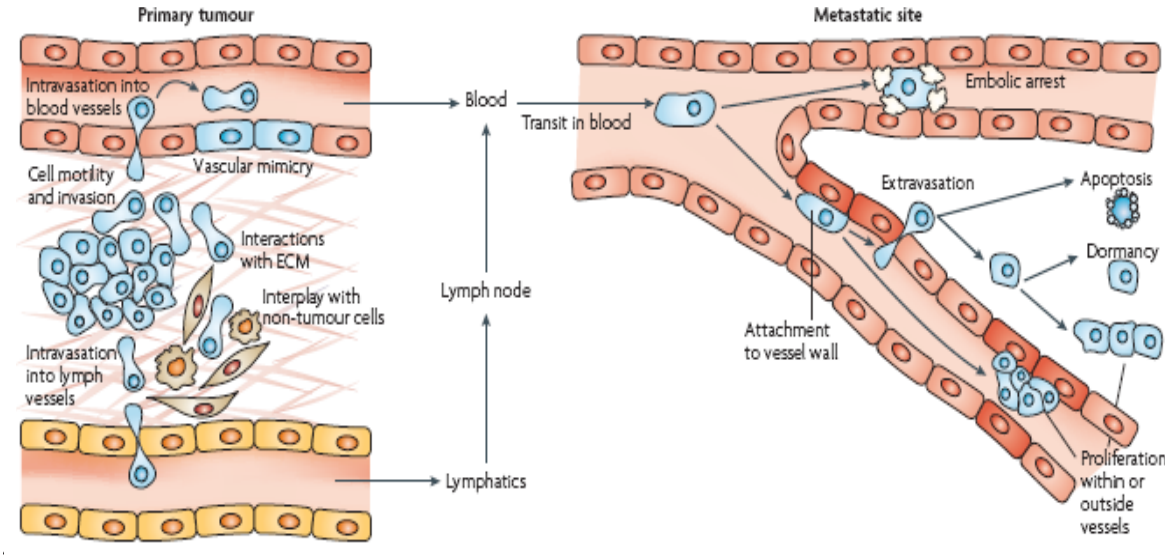


Fig. 2 The metastatic process. Cancer cells are in blue, non tumour cells in beige and endothelial cells in red, (Sahai, 2007).

Tumour microenvironment also contributes to genetic instability which represents the sixth hallmark of cancer described by Hanagan and Weinberg, in 2011. Mutations can alter the metabolism of cells, and can produce the "Warburg effect", in which malignant cells producing a glucose dependent metabolism which utilize glycolysis instead of mitochondrial oxidative phosphorylation for glucose metabolism, and secrete lactate; these

effect had been correlated with the expression of certain oncogenes principally p53, which activates NF-κB transcription factor, and HIF-1α (hypoxia inducible factor 1α), (Hanagan and Weinberg, 2011; Haigis et al., 2012; Johnson and Perkins, 2012).

An immunological misregulation also can increase inflammation that contributes to DNA damage, proliferation, metastasis, invasion, apoptosis evasion, and resistance to hormones and chemotherapeutic agents (Aggarwal et al., 2009; Hanagan and Weingberg, 2011). The principal molecules responsible of inflammation are ROS (Reactive Oxygen Species), and they are enough to induce genomic instability and to transform normal fibroblasts into cancer fibroblasts with more aggressive behaviour, an *in vitro* effect known as "field effect" (Martinez-Outschoorn et al., 2010).

1.1.4 Cancer related genes

Carcinogenesis can be initiated by DNA mutations in key regions known as oncogenes (OG), tumour suppressor genes (TSG), and metastasis suppressor genes (MSG). These alterations are usually somatic events although germ-line mutations can predispose a person to heritable or familiar cancer, a single genetic change is rarely sufficient for the development of a malignant tumour and cancer usually requires alterations in a number of cancer-related genes.

A) Oncogenes (OG)

Oncogenes proceed from normal genes called proto-oncogenes which are dominant mutated genes that encode for proteins that help to regulate signalling pathways which control the cell cycle, apoptosis, genetic stability, cell differentiation and morphogenic reactions. Oncoproteins can be *growth factors, growth factor receptors, membrane-associated guanine nucleotide- binding proteins, serine-threonine protein kinases, cytoplasm tyrosine kinases, nuclear proteins and cytoplasmic proteins* (table 2); (Martinez et al., 200; 3Hernandez-Menéndez and Ríos-Hernández, 1999; Kopnin, 2000; Croce, 2008).

Oncogenes	Protein name	Function	Neoplasm(s)
Growth Factors			
c-sis	Platelet derived growth factor precursor	Induces cell proliferation	Fibrosarcoma, glioblastoma, osteosarcomas, breast cancer.
Receptor tyrosine kinases			
EGFR	Epidermal growth factor receptor	To transduce signals for cell growth and differentiation	Squamous cell carcinoma, breast cancer
Tyrosine kinases			
Scr	Tyrosine kinase	Mediate the activation receptor, cell proliferation, migration, differentiation and survival	Chronic myelogenous leukaemia
Serine-Theronine protein kinases			
raf	Serine-threonine kinase	Involved in organism development, cell cycle regulation, cell proliferation, differentiation, cell survival and apoptosis	Malignant melanoma, sarcoma
mos	Serine-threonine kinase		
Nuclear proteins			
myc	Transcription factor	They regulate transcription of genes that induce cell proliferation	Burkitt's lymphoma

Table 2. Oncogenes. (Martínez et al., 2003)

B) Tumour suppressor genes (TSG)

Tumour suppressor genes are also called anti-oncogenes, they codify for proteins that can direct or indirectly protect the cell against carcinogenesis. The TSG generally follow the "two hit-hypothesis" which implies that both alleles that code for a particular protein must be affected before an effect is manifested, this is because if only one allele had been damage the other one can produce the protein in a normal way, so the mutation is recessive. Even though, they are some exceptions with the "two hit" rule for TSG, as the p53 mutation, (Sherr, 2004). In cancer cells, TSG are inactivated and the control of normal cell cycle is missed which produces a permanent proliferation stage.

They are two different types of TSG: "gatekeepers" and "caretakers" (table 3). Gatekeepers inhibit directly cell growth or promote cell death, so their activity regulates

directly tumour cell proliferation; and caretakers do not directly suppresses proliferation, but function to promote stability because they function in DNA repair pathways , so their missregulation can increase mutation rates (Martinez et al., 2003).

TSG	Protein function	Neoplasm (s)
APC	Cell adhesion	Colon
BCRA 1	Transcription factor, DNA repair	Breast and ovary
BCRA 2	DNA repair	Breast and ovary
CDK4	Cyclin D kinase	Melanoma
hMLH1	DNA mismatch repair	HNPCC
hMSH2	DNA mismatch repair	HNPCC
hPMS1	DNA mismatch repair	HNPCC
hPMS2	DNA mismatch repair	HNPCC
Men 1	Ret receptor	Thyroid
NF1	GTPase	Neuroblastoma
p53	Transcription factor	Colon, lung, breast
Rb	Cell cycle checkpoint, transcriptional corepression	Retinoblastoma
WT-1	Transcription factor	Childhood kidney

Table 3. Tumour suppressor genes, (Martínez et al., 2003).

C) Metastasis suppressor genes (MSG)

Metastasis suppressor genes are able to inhibit metastasis in a secondary organ without affecting tumour growth at the primary site; this is possible because MSGs promote dormancy in disseminated tumour cells. They affect many aspects of signal transduction pathways that are involved in invasion, proliferation (growth-factor-receptor signalling; mitogen-activated protein kinase pathway), cell-cell communication and transcription (table 4), (Steeg, 2003; Horak et al., 2008).

Gene	Cancer cell type	Function
NM23H	Melanoma Breast Colon Oral squamous cell	Histidine kinase; phosphorylates KSR, which might reduce ERK1/ERK2 activation
MKK4	Prostate Ovarian	MAPKK, phosphorylates and activates p38 and JNK kinases
KAI1	Prostate Breast	Integrin interaction EGFR desensitization
BRMS1	Breast Melanoma	GAP-junctional communication
KiSS1	Melanoma Breast	G- protein-coupled-receptor ligand
RHOGDI2	Bladder	Regulates RHO and RAC function
CRSP3	Melanoma	Transcriptional co-activador
VDUP1	Melanoma	Thioredoxin inhibitor

Table 4. Metastasis suppressor genes. (Steeg, 2003).

The human NM23 family is the most studied MSG family, which consists of eight related genes that encode widely expressed proteins known as NM23H1 through NM23H8. Nucleoside diphosphate kinases (NDPKs) were originally identified as essential housekeeping enzymes that are required for the synthesis of nucleoside triphosphates, and they play a role in maintaining the intracellullar nucleotide concentration (Mi-Jin et al., 2012). Altered NDPK expression (up and downregulation) has been reported to be involved in many cellular processes, including oncogenesis, cellular proliferation, differentiation, motility, development, DNA repair and apoptosis. A pathological role of extracellular NM23H1 was suggested in multiple studies of patient's serum in which NM23H1was overexpressed as in extracellular fluid from cancer tissues (table 5).

Type of cancer	Reference
Head and neck squamous cell carcinoma	Haeng Ran et al., 2011
Melanomagenesis	Jarret et al., 2012a and b
Human high metastatic large cell lung cancer cell lines	Gao et al., 2010
Nasopharyngeal carcinoma	Li et al., 2012a
Breast cancer	Buxton and Yokdang, 2011; Rustamadji et al., 2012
Non Hodgkin lymphoma	Mi-Jin et al., 2012
Hepatocellular carcinoma	Dong et al., 2011
Colorectal carcinoma	Alves de Oliviera et al., 2010
Myeloid leukemia K562 cell line	Jin et al., 2009

Table 5. Misregulation of NM23H1 protein detected in several types of cancers

Misregulation of NM23H1 induce to DNA damage promoting genetic instability and loss of adhesion, upregulating metalloproteinases expression (Boissan et al., 2009; Li et al., 2010b; Conery et al., 2010; She et al., 2010; Lilly et al., 2011); therefore downregulation of NM23H1 had been correlated with a poor diagnostic, radiation resistance and survival in patients with various haematological malignancies (Li et al., 2010; Niitsu et al., 2011); and laryngeal carcinoma (Lionello et al., 2012 and Marioni et al., 2012).

All of these oncogenic hallmarks have been associated with the activation of several oncogenic pathways as such as Ras/BRAF/MAPK (Qin et al., 2011b), PI3K/AKT signalling pathways including Rac-1 and Scr kinase expression (Boissan et al., 2009);

besides NM23H1 is a positive regulator of cytokine production and forms a protein complex with p53 and when these proteins are downregulated, NM23H1 protects the cell against oxidative stress (An et al., 2008; Okabe-Kado et al., 2012).

1.1.5 Tumour microenvironment

A) Reactive oxygen species (ROS)

Every day our body produces huge amounts of dangerous oxidants that are product of normal aerobic metabolism, but our organism can regulate them by a variety of antioxidant systems, even though an imbalance in this antioxidant buffering capacity produces oxidative stress, which produces several types of oxidants, and the most important are called reactive oxygen species (ROS), (table 6). ROS reactivity is produced for one or more unpaired electrons in their outer orbital, which makes them very unstable and tending to react with other molecules to pair this electron and thereby generate more stable molecules. ROS can damage lipids, proteins and principally DNA in which can cause structural alterations as such as nicking, base-pair mutations, rearrangement, deletions, insertions and sequence amplifications. Degradation of the bases will produce numerous products including 8-OH-guanidine, hydroxymethylurea, urea, thymine glycol; thymine and adenine ring opened and saturated products (Poulsen et al., 1998; Adly, 2010).

Radicals	Non Radicals
Superoxide: O^{-2}	Hydrogen peroxide: H_2O_2
Hydroxyl: OH-	Hypochlorus acid: HOCl
Peroxyl: RO_2-	Hypobromus acid:HOBr
Alkoxyl : RO-	Ozone: O_2
Hydroxyperoxyl: HO_2-	Singlet Oxygen: $^1O_2$

Table 6. Reactive oxygen species (ROS). (Adly, 2010).

Accumulation of reactive oxygen species (ROS) coupled with an increase in oxidative stress has been implicated in the pathogenesis of several disease states. In cancer, ROS are involved in the initiation and progression processes, they have the capacity of missregulate TSG, and OG involving in signal transduction pathways that regulate the mitosis, cell proliferation, motility, metabolism and apoptosis through the activation of transcription factors and cellular related pathways (Waris and Absan, 2006; Dan et al., 2011; Rossana and Salvarote, 2012).

ROS production activate ERK and MAP kinase signalling patways which switching a vital role from autophagy to apoptosis cell in human induced colon cancer cells (Hsieh et al., 2010; Mendez-Samperio et al., 2010); besides ROS also regulate VEGF expression in ovarian cancer cells and they are required for inducing angiogenesis and tumour growth (Xia et al., 2007).

1.1 *RAS* MAMMALIAN GENES FAMILY

Ras is an evolutionary and conserved family of genes present in many organisms. In mammals are three members called *N-Ras*, *H-Ras* and *K-Ras,* and they are located on human chromosomes 1, 11 and 12 respectively (Lowy and Willumsen, 1993, Wennerber et al., 2005). *H-Ras* and *K-Ras* were originally described as transforming oncogenes of rat sarcoma Harvey and Kirsten viruses, respectively (DeFeo et al., 1981; Ellis et al., 1981), and *N-Ras* was found in a human neuroblastoma cell line (Shimiku et al., 1983).

H-Ras, K-Ras and N-Ras genes encode five proteins: p19 H-Ras, p21 H-Ras, K-Ras 4A, K-Ras 4B and N-Ras (Cohen et al., 1989; Shubbert et al., 2007). All of them present high homology (85%) in the first 165 amino acids, even though they show differences at "HVR" hypervariable region (which is located in 25 amino acids of the carboxy-terminal), and contains two signal sequences that cooperate in targeting Ras to the plasma membrane. The first signal sequence is the CAAX box. All Ras proteins, with the exception of p19 H-Ras, contain a terminal CAAX box in the 186-189 position (extreme C-terminus). In this box the "C" represents a cystein, "A" represents an aliphatic amino acid (leucine, isoleucine or valine) and "X" or metionine, serine, leucine or glutamine (fig. 3), (Prior and Hancok, 2001).

The CAAX box is sequentially post-translationally modified to become it more hydrophobic: the cysteine is farnesylated, the AAX sequence is removed by proteolysis, and then the C-terminal cysteine is carboxylmethylated. The second signal sequence consists of a polybasic stretch of six lysine residues (175-180) in K-Ras, or palmitoylation of cysteine 181 in N-Ras, or cysteines 181-184 in p21 H-Ras (Prior and Hancock, 2001; and Hancock, 2003). For Ras activity are essential five continuous domains (5-63, 72-92,

109-123, 139-165 and 186-189); and a CAAX box which contributes also for the correct subcellular localization.

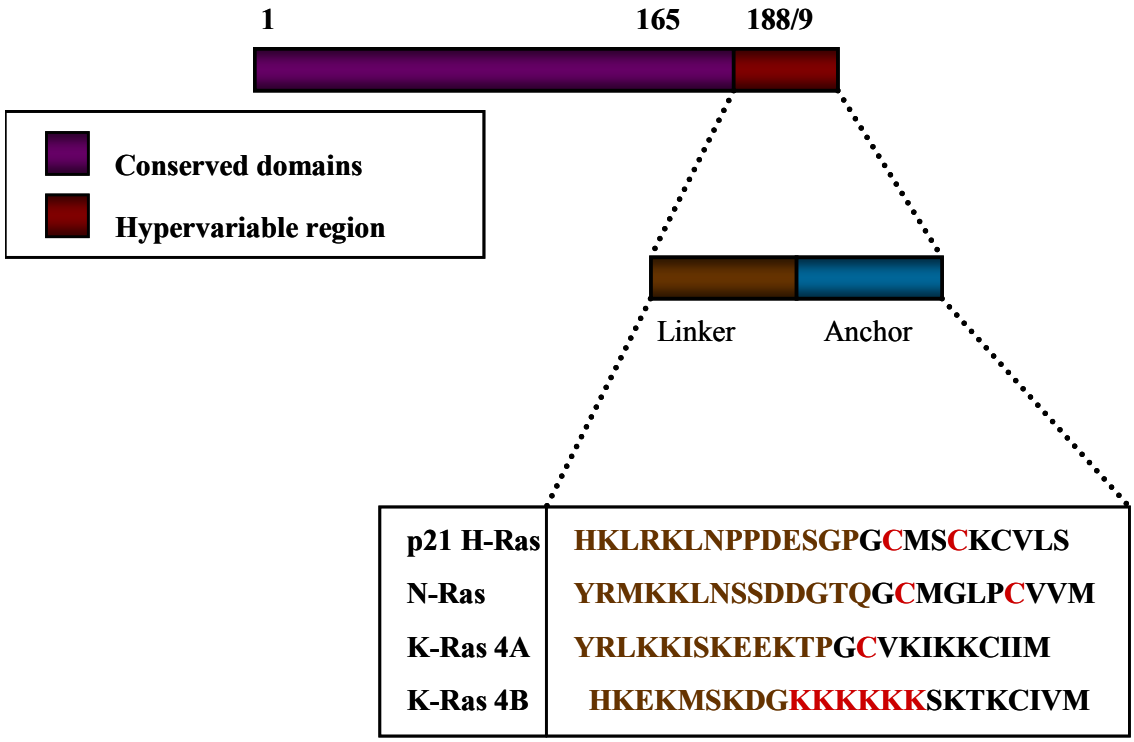


Fig. 3. Ras hypervariable region. Ras conserved domain is represented in purple bar and hypervariable region in red bar, this region is conformed by a linker and anchor region different in all Ras proteins (Prior and Hancock, 2001).

1.2.1 Localization of Ras proteins in the cell

P21 H-Ras traffics through the Golgi to caveolae and lipid rafts, and it exists in a dynamic equilibrium between cytoplasmic organelles (mitochondria), lipid rafts (caveolar and non caveolar nature) and the disordered plasma membrane. By other hand, p19 H-Ras splice variant which lacks of the C-terminal HVR, is localized in nucleus and peripherical nuclear. N-Ras protein traffics to cytoplasmic organelles (mitochondria), and raft domains (positive and negative caveolin), in a similar manner in p21 H-Ras. In contrast to H-Ras and N-Ras, K-Ras is largely excluded from the Golgi due polybasic C-terminal HVR, and is transported to the plasma membrane, specifically K-Ras 4B is located also in cytoplasmic organelles (mitochondria), but is more frequent detected in non raft disordered plasma membrane, and K-Ras 4A spatial distribution remains largely unstudied (Arozarena et al., 2011; Castellano and Santos, 2011). Plasma membrane localization of Ras is crucial for its

function. This is where Ras proteins interact with its upstream activators, all of them with the exception of p19 c-H-Ras (fig. 4).

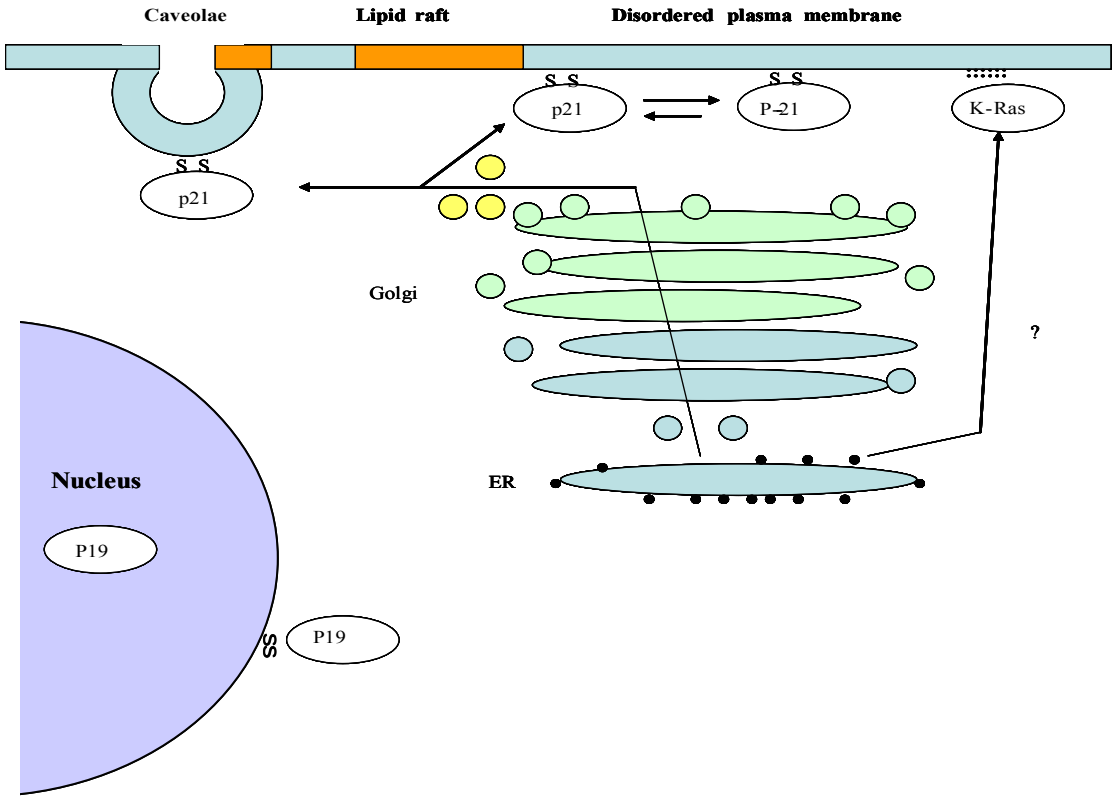


Fig. 4 Localization of H-Ras proteins in the cell. P21 c-H-Ras proteins traffics through the Golgi to caveolae and lipid rafts; in other hand, p19 c-H-Ras is located in nucleus and peripherical nuclear, (Prior and Handcock 2001, Camats Malet, 2008a, phD thesis).

1.2.2 Ras signal transduction

These proteins are part of a broader class known as G proteins, and they act in essence like simple ON/OFF switches (fig. 5). Whether they are "on" or "off" is determined by the small molecules to which they bind: "ON" binding to guanine nucleotide triphosphate (GTP), and "OFF" when binding to guanine nucleotide diphosphate (GDP). The switch is flicked "ON" by guanine- nucleotide exchange factors (GEFs), which exchange bound to GDP for GTP, this interchange requires Mg^{+2} (Mori et al., 2002; Cullen and Lockyer, 2006), and "OFF" by GTPase activating proteins (GAPs), which accelerates the ability of Ras proteins to hydrolyse GTP into GDP (Rehman and Bos, 2004). The GTP hydrolysis returns from active Ras protein conformation to inactive state. The hydrolysis can be done by GTPase intrinsic activity of Ras, however this activity is lower than the hydrolysis

catalysed by other molecules. The exchange of GDP to GTP induces a conformational change in the proteins that allows them to interact with their downstream effectors and carry out their multiple functions (Campbell et al., 1998; Malumbres and Barbacid, 2003; Rehmann and Bos, 2004; Rodriguez- Viciana, 2004; Mitin et al., 2005).

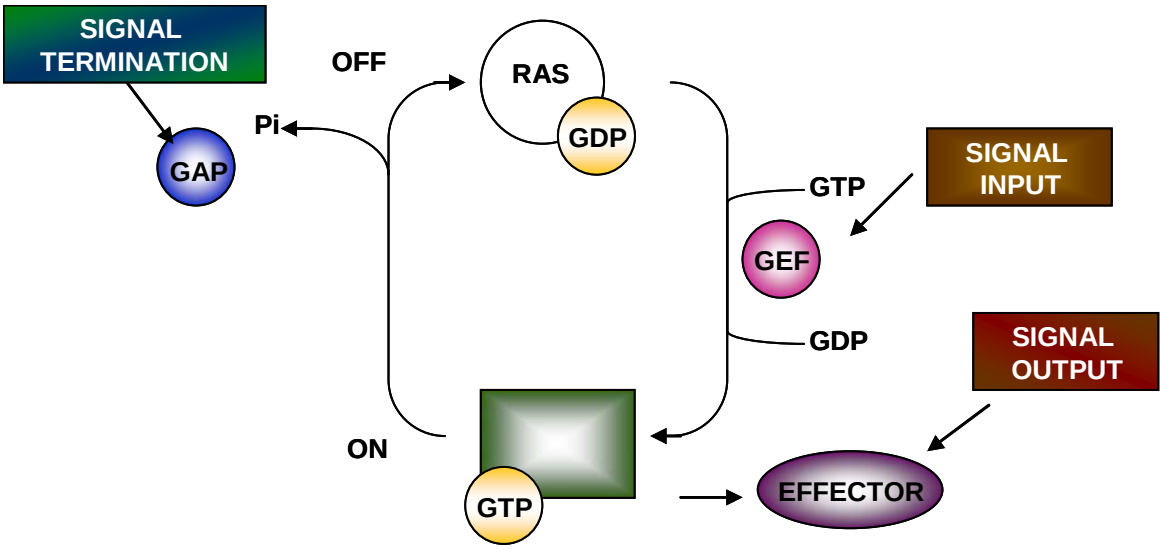


Fig. 5 The GTPase cycle of Ras proteins. The switch is "ON", when Ras proteins bind to GTP and "OFF" when they binding GDP. The switch is fliked "ON" by GEF's, which exchange bound to GDP to GTP (Donovan et al., 2002; Cullen and Lockyer et al., 2006).

Mammalian proteins capable of functioning as GAP's for H-, N- and K-Ras, represent a large an divergent protein family and include p120GAP, neurofibrimin (NF1), the GAP1 family, including GAP1 ^{IP4BP}, Ca^{+2}-promoted Ras inactivator (CAPRI), Ras GTPase activating-like protein RASAL as well as the SynGAP family (DAB2IP), some of those are expressed ubiquitously, while others show very restricted expression profiles, making it difficult to assign a member of the GAP family with Ras inactivation in a given cell/tissue. NF1 and p120GAP bind Ras isoforms without any preference (Grewal et al., 2011). Four principal Ras pathways have been recognized: Ral-GEF pathway, PI3K/AKT pathway, Ras/Raf/MAPK pathway and ERK 1/2 pathway (fig. 6).

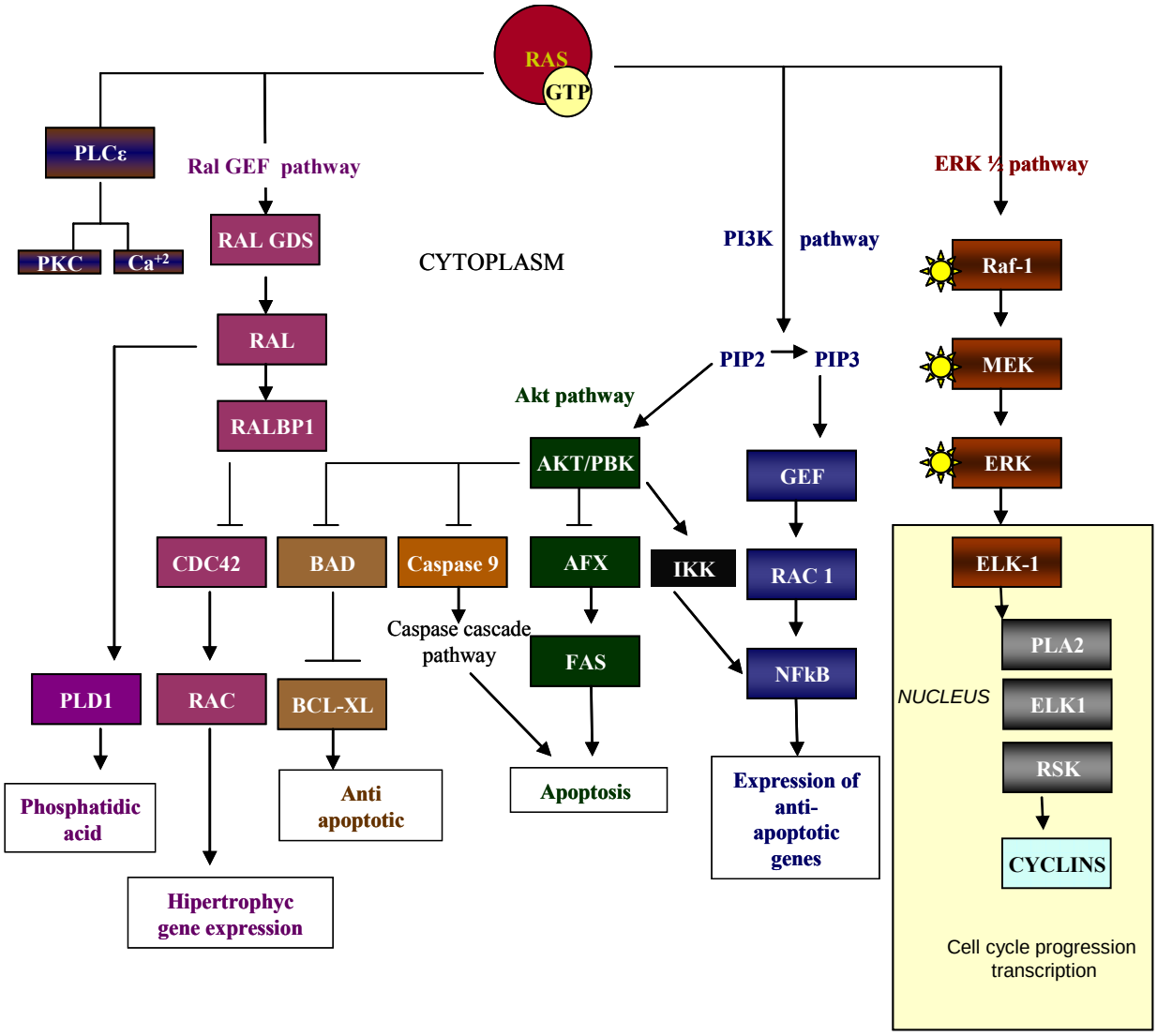


Fig. 6 Ras signalling transduction pathways. Ras proteins belong to the superfamily of monomeric GTPases, which are involved in receptor-mediated signal transduction pathways Ras proteins transmit extracellular signals that promote the growth, proliferation, differentiation and survival of cells. The signaling cascade starts from the plasma membrane where the growth factor (e.g. epidermal growth factor (EGF), or fibroblasts growth factor (FGF)), binds to its enzyme-linked receptor causing receptor dimerization. This leads to phosphorylation of the intracellular parts of the receptors, which activates guanine exchange factors (GEF), such as Sos. GEFs are attached to the receptor by the adaptor proteins shc and grb-2. GEFs promote the exchange of GDP attached to inactive Ras to GTP, which leads to activated Ras. Ras-GTP is constantly inactivated by Ras-GAP proteins (e.g., NF1 protein, p120GAP), which promote the intrinsic GTPase of Ras. This leads to the hydrolyzation of active Ras-GTP into inactive Ras-GDP. The major downstream target of Ras-GTP is mitogen-activated protein kinase (MAPK), but it is also known to activate other targets as such as PI3K, Ras-related guanine nucleotide dissociation stimulator (RalGDS) and phospholipase Cε (PLCε).Activation of MAPK occurs through specific phosphorylation of both a threonine and a tyrosine separated by a single amino acid. The first component of MAPK is called Raf, which is activated on the plasma membrane by Ras-GTP. Raf phosphorylates mitogen-activated kinase 1/2 (MEK1/2 kinase), which activates the extracellular regulated kinase 1/2 (ERK1/2 kinase or p44/42 MAPK) by phosphorylation. ERK1/2 kinase phosphorylates a variety of downstream targets, which results in changes in gene expression and the catalytic activities of enzymes. There are two other signaling pathways related to MAPK. These are called p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and they are mainly involved in transmitting inflammatory signals, (Downward, 2003).

A) Ras/Raf/MAPK pathway

This pathway regulates a great variety of cellular functions as growth, division, differentiation, wound healing, repair, adhesion and migration; and also stimulates angiogenesis through changes in expression of genes directly involved in the formation of new blood vessels.

Pathway activation begins when c-RAF binds to Ras, phosphorylated RAF activates MAPK1 which further activates and phosphorylates MEK2, which subsequently phosphorylates and activates ERK1 and ERK2, these proteins subsequently are translocated to the nucleus where interact with some transcriptional factors as members of Ets family. Then transcriptional factors influence the expression of specific genes (for example c-fos, HBEGF) codifying for proteins implicated on the control of cellular proliferation and/or differentiation (Donward, 2002; Johnson and Rasvan, 2002; Domingo-Villanueva, 2006; Molina and Adjei, 2006).

Recently Baljuls et al., 2012, reported that the Ras-related tumor suppressor gene DiRas3 (which is lost or down-regulated in more than 60% of ovarian and breast cancers) forms a complex with H-Ras and c-Raf, the protein complex DiRas3/H-Ras and c-Raf regulates localization, dimerization and kinase activity of c-Raf; and recruitment and anchoragec-RAF two components of the membrane skeleton, suppression of C-Raf/B-Braf heterodimerization and inhibition of c-Raf kinase activity.

B) JNK/SAPK pathway

This pathway is activated by ultraviolet light, cytokines, osmotic shock, RNA/DNA inhibitors and protein synthesis and to a lesser extent by growth factors. This spectrum of regulators suggests that enzymes are transducers of a variety of stress responses. In contrast to Ras/Raf/MAPK pathway, upstream signal transduction mechanisms for the JNK cascade are less understood (Cobb and Schaefer, 1996; Bode and Dong, 2003).

The JNKs bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity. C-Jun is a component of the AP-1 transcription complex, which is

an important regulator of gene expression. AP-1 contributes to the control of many cytokine genes and is activated in response to environmental stress radiation, and growth factors (similar to JNK stimuli activation). Regulation of JNK pathway is complicated and is influenced by many mitogen kinases, which allows a wide range of stimuli to activate this MAPK pathway (Johnson and Rasvan, 2002).

Daniluk et al., 2012; had been reported the presence of oncogenic Ras inflammatory stimuli which initiated a positive feedback loop involving NF-κB via Cox-2 that further amplifies Ras activity to pathological levels (Daniluk et al., 2012).

B) PI3K/AKT pathway

Phosphoinositides are lipid kinases able to phosphorylate other molecules, the pathway initiates with the conversion of phosphatidylinositol-3, 4-biphosphate (PPI2, inactive form) to phosphatidylinositol-3, 4, 5-triphosphate (PPI3, active form). PPI3 acts directly as a second messenger, binding several cytoskeleton kinase proteins and modulating the activity either by conformational changes and/or their membrane translocation. The principal kinase activated by PI3K is AKT (also known as PKB), one of the major effectors downstream of AKT is mTOR complex 1 (mTORC1), which integrates many inputs, including growth factor signalling, the energy state of the cell and nutrient and O_2 availability; moreover AKT also activates several cellular proteins, including GSK3α (glycogen synthase kinase 3α), FoxO factors, MDM2, BCL-2 interacting mediator of cell death (BIM) and BCL-2 associated agonist of cell death (BAD) to facilitate cell survival and cell cycle entry. In addition, AKT phosphorylates and inactivates tuberous sclerosis 2 (TSC2), a GTPase-activating protein for Ras homologue enriched in brain (RHEB). Inactivation of TSC2 allows RHEB to accumulate in the GTP-bound state and thereby activate mTORC1. In turn mTORC1 phosphorylates p70S6 kinase and 4E-binding protein 1 (4EBP1), 4EBP2 and 4EBP3. These phosphorylation events lead to the increased translation of mRNAs that encode many cell cycle regulators (such as MYC and cyclin D1), as well as certain ribosomal proteins and elongation factors (Engelman, 2009). This signalling cascade can be antagonized by the action of the phosphatase and tensin homolog (PTEN), a widely recognized tumor suppressor which dephosphorylates PIP3.

The PI3K/AKT signalling pathway regulates the activities of a broad range of downstream molecular effectors, which in turn act synergistically to mediate a number of cell behaviours and properties in both normal and pathological conditions including proliferation, adhesion, migration, invasion, metabolism, and survival, angiogenesis (Macaluso et al., 2002; Paez et al., 2002; Hernández-Aya and González-Angulo, 2011; Dituri et al., 2011; Karar and Maity, 2011; Kang et al., 2013). Elevated PI3K signalling drives cancers, especially different breast cancer subtypes and autoinmmune diseases such as lupus erythematosus and rheumatoid arthritis (Rommel et al., 2007; Castaneda et al., 2010; Miller et al., 2010; Kassi and Moutsatsou, 2010; Adams et al., 2011; Hernandez-Aya and González-Angulo, 2011).

D) Ral GEF pathway

Four Raf GEF proteins that have been identified as Ras effectors: Ras GDS, RGL, RGL1 and RGL3. RAL-GTP activates the phospholipase D that, by hydrolysis of phosphatidilcolines, generates satured and monoinsatured phosphatides, which are putative activator Rho activator molecules. RAL also seems to interact with Cdc42 and RAC GAP (Urano et al., 1996; Macaluso et al., 2002; Rodriguez-Viciana et al., 2004).

1.2.3 Oncogenic activation of Ras

As stated before, *Ras* are important proto-oncogenes in humans that have been implicated in up to 30% of all tested tumours. A punctual mutation in *Ras* codons converts these genes into active oncogenes. The mutations have been found in premalignal tissues also, which indicates that *Ras* also has an important role at the transformation state. All the *Ras* mutations can effect two biochemical changes: a misfold GTPase activity and a downregulated interchange of GDP/GTP. The first change is associated with mutations in the positions 12, 13, 59, 61 and 63; 12 and 61 positions are two hot spots for Ras oncogenic mutations (Catalogue of somatic mutations in cancer: http://sanger.ac.uk.uk/cosmic). The second mutation is related at the positions 16, 17, 116, 117, 119, 144. Consequently, these oncogenic Ras mutant proteins are locked in the active, GTP bound state, leading to constitutive, misregulated activation of Ras function (Bos, 1989; Lowy and Willumsen,

1993; Campbell et al., 1998; Shubbert et al., 2007; Castellano and Santos, 2011), furthermore, *Ras* also can be activated in tumours by lost of GAPs (Downward, 2002).

Ras proteins have been involved indirectly in metastatic phenotype development, because these proteins can promote the acquisition of accumulative alterations in cellular pathways which produce cytoskeleton rearrangements, lost of cell adhesions (metalloproteases overexpression), invasion tissues, extravasation into lymphatic and blood vessels, and finally apoptosis evasion (Hernández-Alcoceba et al., 2000), (fig. 7).

In 2011, Janardhan and their colleagues reported that Ras oncoproteins can activate T-cells (especially CD28+ and CD4+) and they increase subsequently AKT/JNK/ERK downstream signalling pathway, which induces to activate Warburg effect and apoptosis evasion.

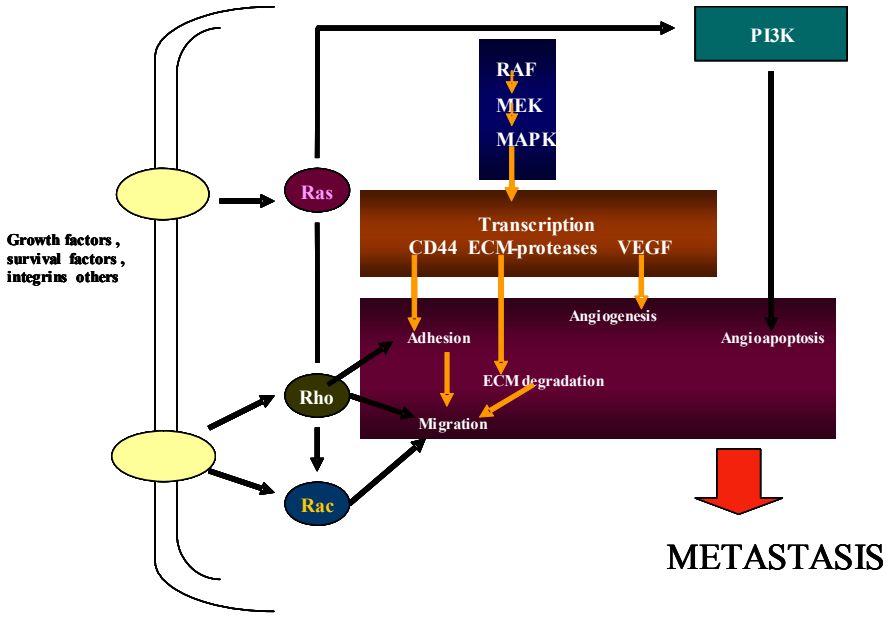


Fig. 7 Ras molecular mechanism related to metastasis, (Hernandéz-Alcoceba, et al., 2000).

Even though the high grade of homology of Ras proteins had been established, new findings suggest that Ras isoforms may not be completely redundant in their functions, this can be clearly envisioned from their distinctive implication in human carcinogenesis in which *Ras* gene mutations are different and depend of the tissues for example: *K-Ras* mutations predominating in pancreatic, colon and lung cancers; and *N-Ras* mutations

characterizing skin neoplasias and myeloid leukemias (15%), and *H-Ras* in melanoma, oral, thyroid, mammary, bladder, kidney, seminomas or Hurthle cell carcinomas (10 %), (table 7), (Malaney and Daly, 2001; Downward, 2002; Colicelli, 2004; Castro et al., 2006; Castellano and Santos, 2011; Fernandez-Medarde and Santos, 2011). In transformed fibroblasts *in vitro* cultures, N-Ras regulates adhesion, whereas K-Ras 4B coordinates motility, and colonic epithelium it stimulates hyperproliferation whereas N-Ras prevents apoptosis (Castellano and Santos, 2011); furthermore N-Ras and H-Ras control the differentiation program triggered by the TCR (T Cell antigen receptor) at distinct stages of T-cell maturation, this effect contributes principally to apoptosis evasion and infiltration metastasis of damaged T-cells in cancer (Iborra et al., 2012).

In *in vivo* studies, GEF Ras GRF1 is more active on H-Ras and R-Ras than N-Ras and K-Ras; H-Ras also activates more Raf-1/ERK1 and PI3K signalling pathways than the other Ras proteins, in other hand GAP NF1 is four times more efficient in downregulating H-Ras than N-Ras; and SOS GEF facilitates the interchange of interchange of GDP/GTP in preferentially in H-Ras proteins followed by N- and K-Ras.

Besides K-Ras is specifically regulated by RASSF2, and the K-Ras 4B activates more Raf-1/ERK and NF-κB signalling pathways than H- and N-Ras proteins, their downregulation is preferentially catalyzes nucleotide exchange by Smg21GDS, N-Ras is more downregulated by GEF Ras GRF2, (Arozarena et al., 2011, Castellano and Santos., 2011).

Moreover, some transcription factors activates Ras protein expression and this effect also upregulates the expression of some genes (Atg5, NoXa, BNIP3), which induces autophagy in several human cancers (colorectal, breast, cervical) activating several signalling pathways: RACK1/MKK/JNK; MEK/ERK; PI3K (class I), that induce dramatic cell proliferation in tumour development (Shang-Ying, 2011, Kumar et al., 2012).

Organ /Tissue	Tumour type	H-Ras	N-Ras	K-Ras
Biliary tract	Adenocarcinoma	0 (151)	2 (194)	35 (934)
Bladder	Transitional cell carcinoma	12 (1166)	2 (322)	4 (427)
Breast	Carcinoma	1 (542)	2 (330)	4 (544)
Cervix	Adenocarcinoma	9 (249)	3 (64)	8 (611)
Colon	Adenocarcinoma Adenoma	0 (76) 0 (3)	2 (55) 1 (11)	36 (4310) 22 (3545)
Ganglia	Neuroblastoma Other	0 (64) N/A	8 (103) N/A	3 (63) 27 (298)
Leukemias	AML CML CMML JMML	0 (1216) 0 (265) 1 (118) 0 (41)	12 (3404) 3 (532) 15 (157) 19 (165)	4 (1778) 2 (313) 11 (84) 7 (143)
Lymphomas	ALL Burkitt's lymphoma Hodgin's lymphoma Plasma cell myeloma	0 (284) 0 (30) 2 (44) 2 (185)	10 (703) 10 (30) 16 (45) 20 (484)	7 (549) 3 (30) 0 (44) 6 (403)
Liver	Hepatocellular carcinoma	0 (163)	4 (202)	4 (307)
Lung	Large cell carcinoma Non small cell carcinoma Squamous cell carcinoma Other (neoplasia)	4 (50) 0 (683) 1 (261) N/A	4 (49) 1 (695) 0 (360) N/A	21 (189) 16 (3575) 6 (1407) 22 (563)
Pancreas	Ductal adenocarcinoma Endocrine tumour	0 (110) 0 (2)	1 (138) 75 (4)	69 (3483) 1 (68)
Prostate	Adenocarcinoma	6 (489)	2 (509)	8 (1002)
Skin	Basal cell carcinoma Squamous cell carcinoma Malignant melanoma	7 (180) 9 (236) 1 (904)	1(147) 7 (107) 20 (3466)	4 (147) 5 (107) 2 (924)
Soft tissue	Angiosarcoma Leiomyosarcoma Liposarcoma Rhabdomyosarcoma Myxoma Malignant fibrous histiocytomapleomorphic sarcoma	0 (6) 3 (30) 6 (72) 4 (158) 0 (19) 15 (117)	0 (6) 0 (13) 0 (21) 11 (151) 0 (19) 2 (57)	49 (53) 8 (173) 4 (45) 4 (162) 11 (19) 16 (131)
Stomach	Adenocarcinoma Other	4 (218) 11 (9)	2 (205) 0 (1)	6 (2054) 6 (241)
Testis	Germinoma Seminoma	0 (56) 17 (30)	7 (115) 0 (30)	7 (190) 0 (23)
Thyroid	Anaplastic carcinoma Follicular carcinoma Papillary carcinoma Hurtle´s cell carcinoma	4 (440) 5 (381) 2 (1525) 16 (44)	17 (436) 17 (392) 4 (1941) 4 (26)	9 (433) 4 (372) 2 (1654) 0 (41)

.

Table 7. Distribution of frequency of Ras mutations in human tumours. Values presented as the total percentage of clinical samples analyzed (n shown in parentheses) for that particular tumor type. Boldface corresponds to tumors presenting significance high rates (>10) of mutation in ras genes. ALL=acute lymphoblastic leukemia; AML= acute myelogenous leukemia; CLM=chronic myeloid leukemia; CMML= chronic myeolomonocytic leukemia; JMML; juvenile myelomonocytic myeloid leukemia; N/A= not available, (Fernandez-Medarde and Santos, 2011).

1.3 *H-RAS* ALTERNATIVE SPLICING

1.3.1 Alternative splicing

The alternative splicing reaction is an eukaryotic mechanism that permits to separate and reconnect the exons of a mRNA to produce alternative exon arrangements. These different combinations are translating later and generate a unique and different sequence of amino acids, producing isoforms of proteins that partially differ in their peptide sequence and chemical and biological activity (Li et al., 2007).

More than 60% of human gene products are processed by alternative splicing, and even some of these proteins have multiple or thousand of splicing patterns. The most pronounced differences between tissues were seen for the frequencies of alternative 3' splice site and alternative 5' site, which were about 50 to 100 higher in the liver than in any other human tissue (Yeo et al., 2004).

In a typical multiexon mRNA, the splicing pattern can be altered by different ways (fig. 8). The alternative splicing can be performed at 5'-terminal exons through the use of alternative promoters and at 3'-terminal exons can be switched by combining alternative splicing with alternative poliadenylation sites. They are different types of exons, the constitutive exons and the cassette exons. In the first case, the exons always are included in the mRNA, and in the second case only one of the exons can be included because they are mutually exclusive. The exons can also be lengthened or shortened by altering the position of one of their splice sites. Another spliced pattern is the failure to remove an intron, a splicing pattern called intron retention (Breitbart et al., 1987; Black, 2003).

The minor cassette exons are less conserved than the major alternative and constitutive exons. This observation is not only a consequence of the exon birth but also by lineage-specific loss of alternative exons and sites, depending on exon functionality (Nurtdinov et al., 2007).

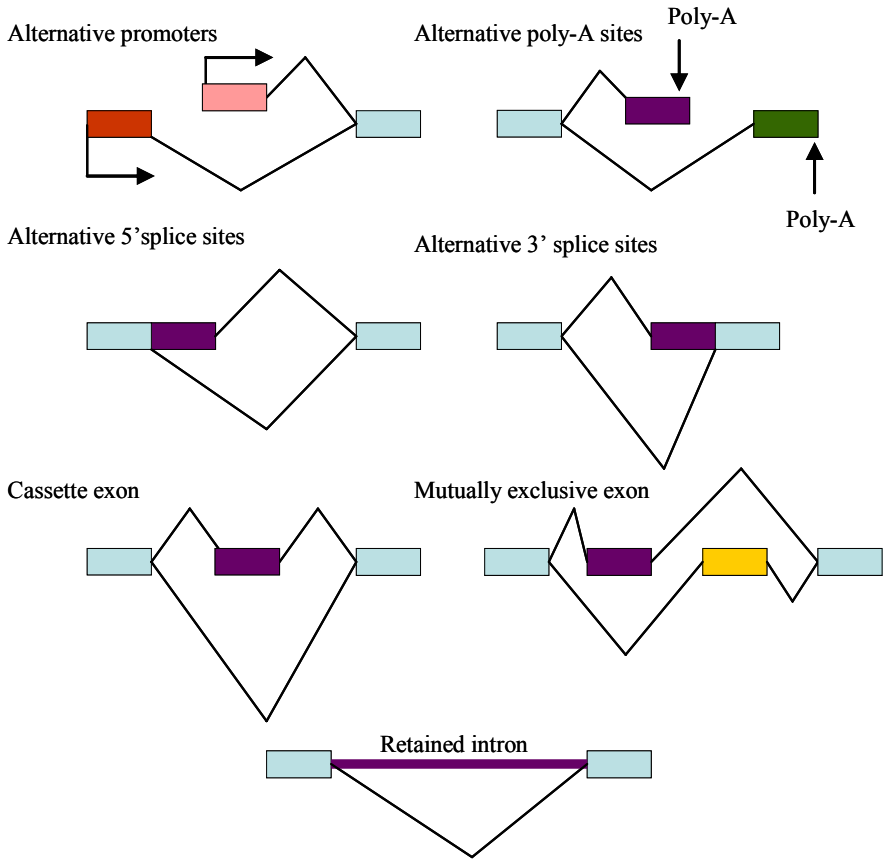


Fig. 8 Alternative splicing patterns. Splicing pattern can be altered by different ways at 5'-terminal exons through the use of alternative promoters and at 3' terminal exons can be switched by combining alternative splicing with alternative poliadenylation sites, (Li et al., 2007).

1.3.2 Alternative splicing of *c-H-Ras* gene renders two proteins: p21 and p19

In 1989, Cohen et al., reported that H-Ras pre-mRNA has an alternative splicing of the last encoding exon. The *c-H-Ras* gene then could renders two mRNAs, each of them codifying for a different proteins called p19 and p21. They suggested also that one of the products of this alternative splicing, p19 protein, lacked transforming potential and showed that the transforming activity of the *H-Ras* gene is inversely proportional to the efficiency of the alternative splicing of the H-Ras pre mRNA toward p19 mRNA, and it had been suggested that p19 protein could act as a negative regulator of the p21 protein. The mRNA structure of both alternative sequences are identical in their first coding exons (called 1, 2, 3 and 4a), they have two non-codifying exons (0 and 4b located at 5'UTR and 3'UTR regions respectively), these exons are separated by intronic regions designed as A-E. The alternative exon is called IDX (82 nucleotides) and is located between 3 and 4a exons. The pre-mRNA H-Ras can be processed into two mRNAs, p21 mRNA (which exclude IDX exon) and p19 mRNA (that includes IDX exon), (fig. 9). So p19 mRNA codifies for a

shorter protein than p21 mRNA, since IDX exon contains a premature stop codon, therefore p19 does not contain CAAX motif (Guil et al., 2003a).

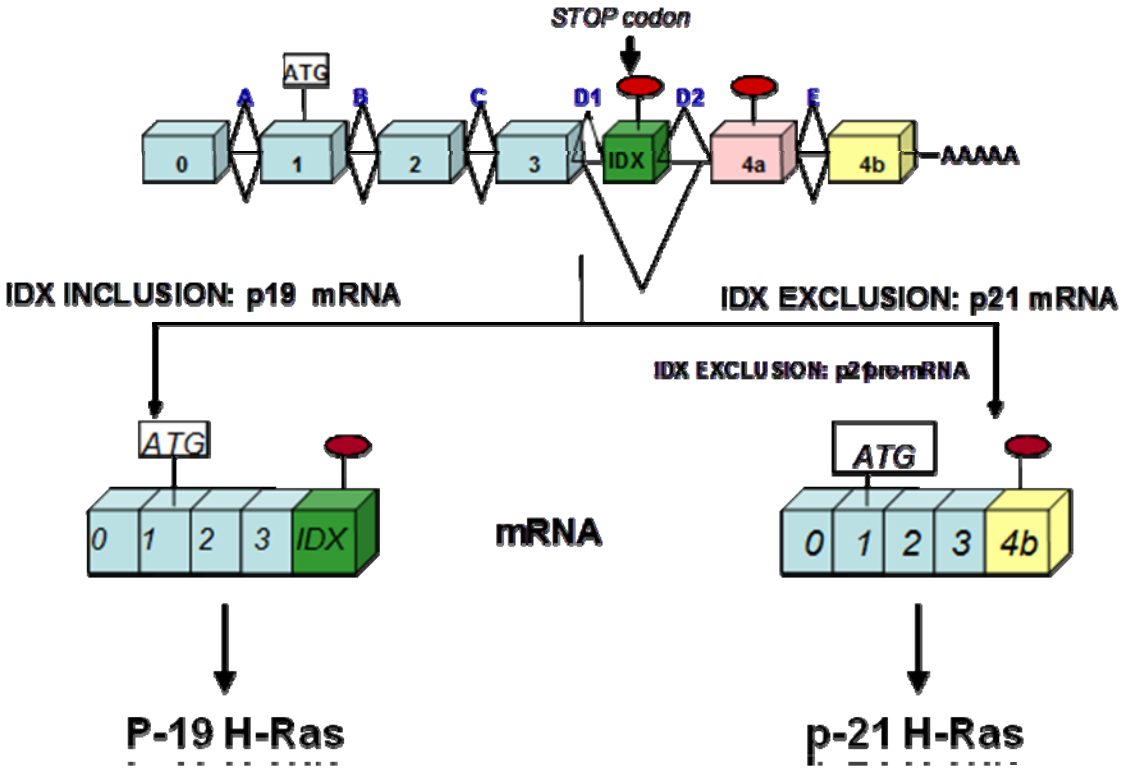


Fig. 9 Alternative splicing of *c-H-Ras* gene renders two proteins: p21 and p19, (Guil et al., 2003a).

Is important to notice that p68 RNA helicase (DDX5) alters activity of cis and –trans acting factors in the alternative splicing of *c-H-Ras*. P68 RNA helicase unwinds the stem-loop of IDX-ras ISS1 form, which includes IDX exon structure and prevents binding of hnRNPH to IDX-ras ISS1, moreover p68 RNA helicase alters the dinamyc localization of SC35, a splicing factor that promotes IDX inclusion (Camats-Malet et al., 2008c).

1.3.3 P19 c-H-Ras protein

P19 mRNA is stable and as abundant as p21 mRNA. The two proteins are similar at their first 150 amino acids, but they differ in the C-terminal amino acid sequences. P19 lacks residues 152-165 of p21, so p19 is not able to bind GTP and it localizes in the nucleus/cytosol in contrast by p21 which localizes at membrane plasmatic and can bind GTP.

During yeast two hybrids assays realized by Camats-Malet, 2008a; p19 did not bind p21 typical effectors and activators as Raf1, Rin1, MAXP1, AFG, PI3 and SOS, CDC25 and p120GAP, therefore p19 do not interferes with p21 activity, by kidnapping or down modulating these effectors and activators, these results also compared the oncogenic activity of p19 (W164A) mutant variant vs., p19 protein and this mutant acted as a negative dominant of p19.

By other hand, p19 can interact also with RACK1, and p73α/β (Guil et al., 2003a; Jeong et al., 2006; Camats-Malet et al, 2008b); the protein complex formed by p19/p73β activates the transcription of several target genes, and the complex p19/p73α modulates the expression of Mdm2 which in turn regulates the activity of p53, p63 and p73 proteins through feedback loops. Mdm2 is the major negative regulator of p53; it represses their transcriptional activation and also ubiquitinates it promoting their degradation fot the proteosome (Jeong et al., 2006). This mechanisms is regulated by NFAT1 a transcription factor which is responsible of activating MDM2 oncogene independent of p53 (Zhang et al., 2012).

Another important function of p19 H-Ras, was detected in lung cancer H1299 cells, in which p19 inhibited proliferation mostly increasing Neuron Specific Enolase (NSE) a truncate form of the classic enolase which converts 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) in the glycolisis pathway and also plays an important role in neuronal regeneration and reinnervation (Kirino, 1983). The interaction between p19 ras/C-terminal and NSE is enough to inhibit the enzymatic activity α and γ enolases, and p19 Ras enhanced transcriptional activity of p53 which leads to p53-mediated apoptosis (Sang-Ming et al., 2009).

1.3.3.1 Intracellular signalling pathways of p19 protein

A) RACK1 and pKC pathway.

RACK1 is an scaffolding protein that has been linked to a variety of signalling systems including protein kinase C, growth factors, and IFNs, STAT1, tyrosine kinases and phosphatases, IL-2, IL-4, and erythropoietin (Usacheva et al., 2003, Lopéz-Bergami et al., 2005). P19 is able to interact with RACK1 and as observed by two yeast hybrid assays and FRET assays. Colocalization of p19 and pKCβII was observed at perinuclear region which can suggest that three proteins would coexist as a multimeric protein complex. The total pKC activity increased 4.5- times when p19 was overexpressed. p19 produces a signalling cascade which involves the phosphorylation of ERK1, but not the ERK2, MAPK 1, 2 and 3 or JKN 1, 2, 3; perhaps the increase of PKC driven by p19 overexpressed does not lead to phosphorylation of JNK and consequently the c-Jun transcription factor was not also phosphorylated (Camats-Malet, 2008 PhD thesis UAB). Lopéz-Bergami in 2005, indicated that activation of PKC and its binding to RACK1 was not enough to activate JNK, and then a second stimulus was required for activating MAPK/ERK pathway.

B) MEK 1/2 pathway

P19 can regulate the phosphorylation of ERK1 even though p19 does not bind RAF1. This effect can be possible as a consequence of the increasing level of PKCβII that may phosphorylate Raf1, and also other signals acting on ERK1, ERK2, MEK1 and MEK2 pathway. Activation of PKCβII, Raf and their downstream signalling are only promoted by p19 and not by p21 (Camats-Malet, 2008a).

C) c-Jun pathway

Overexpression of p19 protein in *HeLa* cells upregulated c-Jun levels; in contrast p19 (W164A) mutant variant protein produced a downregulation of c-Jun, which indicates that p19 regulates the AP1 transcription complex, but not the phosphorylation of c-Jun (Camats-Malet, 2008a).

D) TSC pathway

Overexpression of p19 in *HeLa* cells produces a hypophosphorylation of AKT which indicates inhibition of the PI3K/insuling signalling pathway due to a lack of p-AKT. Moreover upregulation of TCTP was also detected with overexpression of p19 and inactivation of p70S6K (Camats-Malet, 2008 PhD thesis, and Camats et al., 2008b).

1.3.3.2 P19 induces an irreversible quiescence state in cell cycle

Hypophosphorylation of AKT had been correlated with a quiescent arrest in G1/S cell cycle. In this context our colleague Camats-Malet, found that p19 overexpression induces an upregulation of FOXO1 in *HeLa cells*, which is a transcription factor that shows transcriptional changes when AKT was hypophosphorylated, and this was enough to induce a G1/S phase delay in cell cycle which is a reversible quiescence state, and prevents to entry into apoptosis (Camats et al., 2009).

1.3.3.3 The role of p19 c-H-Ras protein in cancer and metastasis processes

MiRNAs are small RNA molecules that can silence the genetic expression. The miRNA alterations are involved in the initiation and progression of human cancers. MiRNA-expression profiling of human tumours has identified signatures associated with diagnosis, tagging progression, prognosis and response to cancer treatment, so profiling can identify miRNA genes that might represent downstream targets of activated oncogenic pathways or that target protein

Due the importance of miRNAs in cancer, in our laboratory miRNA expression was analyzed in *HeLa* cells overexpressing p19 protein in microarrays. In these findings two new important insights were found, firstly that *H-Ras* is not only regulated by let-7 miRNA family, and second that *H-Ras* alternative splicing can promote the transcription of p19 over p21 protein that in turn might modulate the expression of certain miRNAs. These results were revalidated performing PCR Real Time assays for miR-342, miR-206, miR-330, miR-138, and miR-99b, all of them were upregulated when p19 was overexpressed but not a similar effect was detected when p19 (W164A) was overexpressed.

In these experiments it was also observed that 21 miRNAs were upregulated by the increasing concentration of p19 mutant some of them are miR-145, miR-377, miR-294, miR-302, miR-196-b and miR-185, miR-342, miR-516, miR-213, miR-147, miR-219 (Camats-Malet 2008 PhD thesis, UAB and García-Cruz et al., submitted article and this PhD thesis).

1.4 COSTELLO SYNDROME (CS)

1.4.1 Definition and disease characteristics

Costello Syndrome [OMIM No. 218040]* was described for the first time by Costello, in 1977. Is a rare congenital disorder typically characterized by coarse face (full lips, large mouth); fine, curly or sparse hair, short stature, neonatal feeding problems, mental retardation, loose integuments of the back of the hands (a typical hand posture, finger shape); deep and soft skin palmar and plantar creases, tight Achilles tendons, (fig. 10);

*OMIM= Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/OMIM

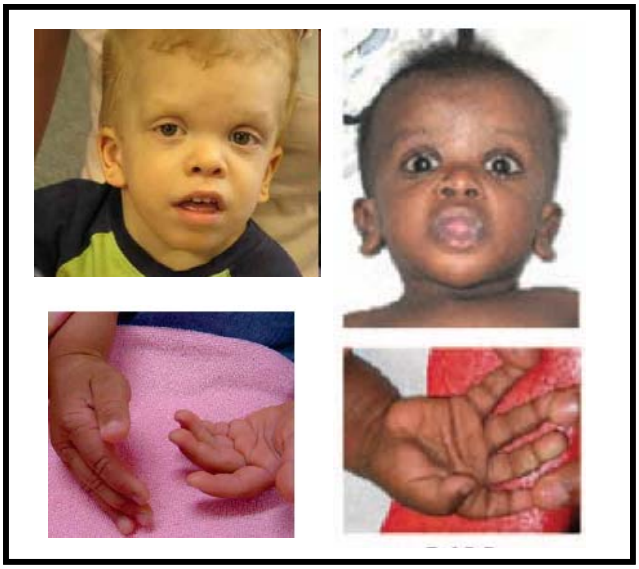


Fig. 10 Costello Syndrome phenotype, (Gripp et al., 2006)

cardiac hypertrophy, cerebral ventricular dilatation, developmental disability, is frequent that patients show papillomata or they develop other benign tumours including papilloma which is frequently found in nose, they also present several cancers including rhabdomyosarcoma, gangloneuroblastoma and bladder carcinoma, they have a delayed puberty, mental retardation, hypogonadism, osteoporosis and the development of

gastroesophageal reflux (Madhukara and Kumaran, 2007; Kerr et al., 2006 and 2008; Lin et al., 2008; Rauen, et al., 2008; Schulz et al., 2008a; Tidyman and Rauen, 2009; Tajir et al., 2012).

1.4.*2 H-Ras* mutations in Costello Syndrome

The genetic cause of Costello Syndrome (CS) was detected by sequencing the entire coding region of the Ras of CS patients (Aoki et al., 2008). The majority of CS patients (80%) present a heterozygous misense dominant mutation in the *H-Ras* gene on chromosome 11p.13.3 mutation (Gripp et al., 2006); the most frequent changes affect one of the glycine residues in position 12 or 13, (G12S or G12A) and this result in a typical phenotype (table 8). These mutations induce a constitutive activation of *H-Ras*, in turning causing increased activation of downstream effectors in signalling pathways controlling cell proliferation and differentiation (Aoki et al., 2005). Other mutations have been detected in CS patients, resulting in G12V, G12C, G12E, G13C and G13D; in the case of G12C and G12D both mutations were lethal in severe Costello Syndrome patients (Lorenz et al 2012). Only one individual with a different mutation affecting an aminoacid G12/13 (K117R) has been identified (Kerr et al., 2006). Malignant solid tumours of adulthood, such as bladder carcinoma or lung carcinoma are often associated with somatic *H-Ras* mutations. Mutation hotspots are the glycines in positions 12 and 13 and the glutamine in position 61, misense mutations at these positions lead to increased activity of the gene product in a severe way (Kerr et al., 2006; Gripp et al., 2006; Aoki et al., 2008; Denayer et al., 2008; Meineke et al., 2008a; Rauen et al., 2008; Sol-Church and Gripp, 2009).

Mutation	Nucleotide substitution	Number of cases	Frequency in tumours (%)
G12S	34G→A	80%	6.5
G12A	35G→C	8.8%	0.4
G13D	38G→A	4.4%	4.4
G12Vb	35GC→TT* 35G→T	2.2%	44.2
G13C	37G→T	2.2%	0.6
T58I	173C→ exon 3	<1%	
A146v	437C→n 4	< 1%	

Table. 8 *H-Ras* mutations reported in Costello Syndrome * The G12V mutations is typically due to a G to T transversion at position 35 in tumours; however, in one individual with Costello Syndrome, a double mutation occurred resulting in the same predicted amino acid change, (Gripp et al., 2006).

1.4.3 Diagnosis/testing

The diagnosis of CS is based on clinical findings and is confirmed by molecular genetic testing. The genomic DNA is obtained from each patient or parent and then the samples are sequenced. Sequence analysis of exon 2 (the first translated exon) detects missense mutations in 80-90% of individuals tested. If no mutation is identified in exon 2, all other coding exons need to be sequenced (Gripp et al., 2006).

1.4.4 Costello solid tumours

CS patients have a greater risk of developing malignant tumours than in general population. The overall tumor incidence is approximately 15% over the lifetime of individuals with a *H-Ras* mutation. The most frequent neoplasias are rhabdomyosarcoma and neuroblastoma in early childhood, in contrast transitional cell carcinoma of the bladder occurs in older adults (70% age > 65 years) in the general population, whereas it occurs in adolescent with CS (Gripp et al., 2001; Gripp et al., 2008). In rhabdomyosarcoma of Costello Syndrome patients could have an overexpression of RasGRF1 mRNA, which can induces cell filopodia, and suggest the role of RasGRF1 in cell migration vía p42/44, MAPK and AKT (Tarnowski et al 2012).

1.4.5 *In vivo* systems for studying Costello Syndrome

A) Mouse model

A mouse model for CS was generated by Schuhmacher et al., 2008; in order to obtain this model, they targeted ES cells by knocking in an oncogenic G > T missense mutation in the second base of the twelfth codon of the *H-Ras* locus, this change allowed the replacement of a normal GGA normal sequence which generates a glycine to GTA which codifies for a valine, a mutation detected in a number of a CS patients that leads to expression of a constitutive *H-Ras* (G12V) protein. For monitoring *H-Ras* expression, at the single-cell level, an internal ribosomal entry site –β-gal-neomycin resistance fusion protein (IRES-β-geo) cassette, was included in the 3' UTR region, the expression of these sequences generates a chimeric protein with β-gal activity under the regulation of the endogenous *H-Ras* proto-oncogene promoter, thereby the histological detection of gene expression patterns was possible due using X-gal staining methods.

Mutant mice *H-Ras* (+/G12V), were born as the expected mendelian ratio, were fertile, and survived at rates comparables to those of their wild-type counterparts form more than a year. The oncogenic H-Ras (G12V) protein expression were similar to those of the endogenous wild type H-Ras protein, which indicates that the cassette IRES-β-geo cassette does not affect expression of the targeted l *H-Ras* (G12V) locus. The H-Ras (G12V) protein was able to bound to GTP, and the H-Ras (612V-GTP) was twice as abundant in H-Ras (G12V/G12V) mice as in H-Ras (+/G12V) animals. The activation levels of ERK1, ERK2, and MEK 1 were similar between *H-Ras* (G12V) and *H-Ras* (*-/-)* mice, only AKT level was slightly decrease in the brain of H-Ras (G12V).

In section 1.4.1 was mentioned that majority of CS patients have certain predisposition to tumour development tumours (primary neuroblastomas, rhabdomyosarcomas, and bladder carcinomas); even though at the contrary as they expected with *H-Ras* (G12V), these mice did not prone tumor development, and the (G12V) mutation could contribute to papilloma development, but is not sufficient to initiate neoplastic process. *H-Ras* (G12V) mutant mice display facial dysmorphia, caused by depression of the nasal bridge and premaxillar bone, and chroanal atresia, they have showed shortened maxillary, molar process, and xygomatic bone (fig. 11). All these defects induced a prominent forehead and blunt nose that clearly distinguished *H-Ras* (G12V), mice form their wild-type littermates. Mutant mice also displayed engrossed lips, more sebaceous glands, and cardiomyopathies than in control animals. In other hand, both *H-Ras* (+/G12V) and *H-Ras* (G12V/G12V) mice had developed systemic hypertension, characterized by high systolic and diastolic arterial pressures under anesthetized and conscious conditions. Angiotensin II levels in these mutant models were significantly elevated, especially in *H-Ras* (G12V/G12V) mice, whose Ang II levels were 2.5 fold that of wild type animals.

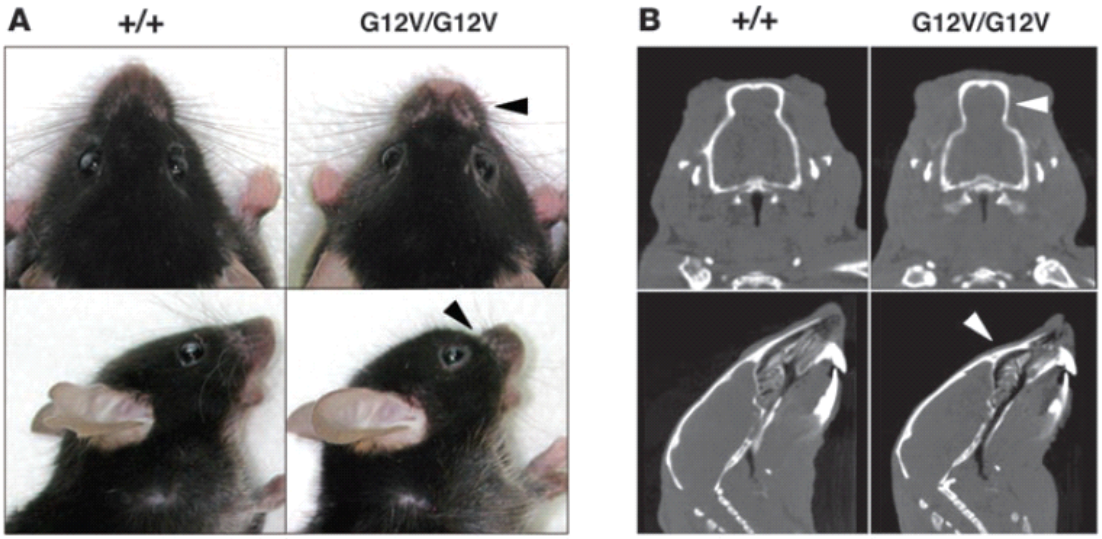


Fig. 11 Facial dysmorphia of mutant costello mice. **A)** Top and side of heads of *H-Ras* wild type (+/+) and *H-Ras* (G12V/G12V). The prominent forhead and blunt nose of mutant animals (arrowheads) can be observed. **B)** CT section of *H-Ras* (+/+) wild type and *H-Ras* (G12V/G12V) litlemattes. Top coronal projection, Arrowhead indicates chroanal atresia. Bottom: sagital projection. Arrowhead indicates that shortened and depressed nasal bridge and premaxilar bone (Schuhmacher et al., 2008).

C) Zebrafish model

This is the most recent transgenic animal Costello model developed by Santoriello et al., 2009; and contains a germline integration which generates zebrafish that constitutively express low levels or can be induced to express high levels of oncogenic *H-Ras* (G12V), and found that these fish display several developmental defects that are hallmarks of the Costello Syndrome.

The germline line was developed by Tol2 gene trap system, which codifies for a chimeric H-Ras (v12) protein tagged with GFP at the N-terminous. The GFP-tagged did not affect the function of *H-Ras* (V12) *in vitro* and *in vivo*. Two transgenic lines were obtained: Gt (GFP-H-Ras V12)^{io1} and Gt (GFP-H-Ras V12)^{io2}, were called P1 and P2 respectively. Both proteins were expressed ubiquitously, mainly confined to the cell membrane and Golgi apparatus, as visualized in fibroblasts cultures derived from fluorescent embryos at 24 h post-fertilization. A 25% of the progeny of hemizygous crosses, showed abnormal morphology that was visible by 5 weeks of age. The morphological phenotype includes: shorter body length, flattened head with increased interocular distance, small heart, and

scoliotic spine (fig.12). Other visible phenotypes include an enlarged gill area and swimming near the water surface, both characteristics indicated that fishes had reduced blood oxygenation, and sterility.

Consistently with Costello Syndrome patients, the phenotype was worsened with increasing age, in older fish (5-12 months of age), was detected a higher frequency of tumour formation compared with wild type populations (1-500) and that tumours developed at an earlier stage that in wild-type fish (5 months vs >2 years). Half of the tumours (n=6/12) were lymphatic infiltrations of the gut, liver or interstitial tissue; however, two transgenic fish developed metastatic melanomas, one had gut carcinomas, two had hepatocarcinoma and one developed rhabdomyosarcoma.

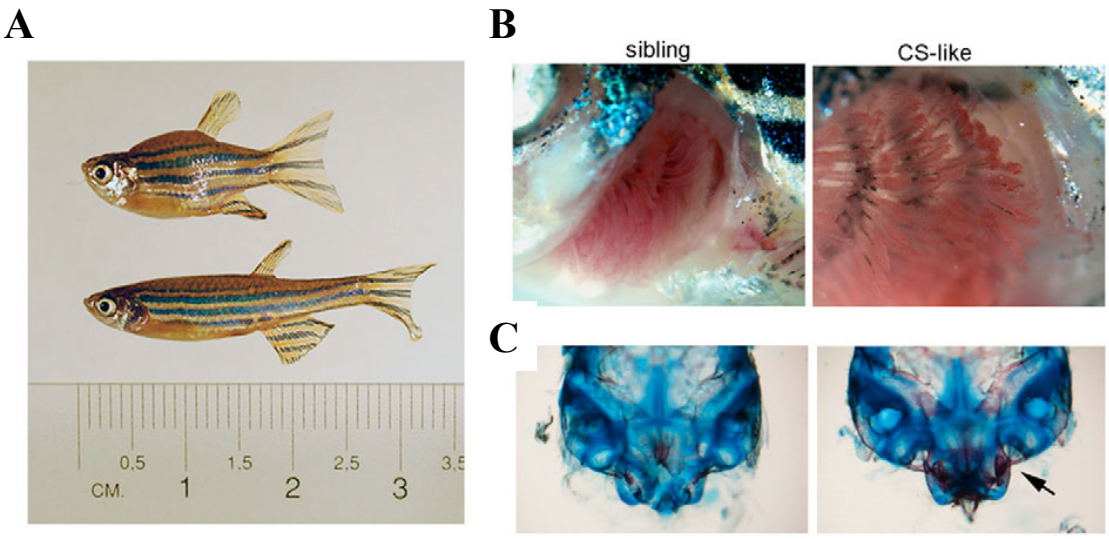


Fig. 12 Costello zebrafish model. A) CS-like fish have reduced body size compared with normal sibling fish; **B)** they also have a smaller heard, and enlarged gill area and **C**); craniofacial dysmorphogenesis with increased ossification of the Weberian complex (arrow), (Santoriello et al., 2009).

In zebrafish Costello model, the GFP-H-RasV12 expression was slightly higher (approximately 1.4 times) than the level of endogenous Ras expression, but an increased in the phosphorylation of ERK1, ERK2 or AKT was not detected. The low levels of oncogenic H-Ras expression were associated with both reduce proliferation and an increase in senescence markers in adult progenitor cell compartments in the brain and hearth, together with activated DNA damage responses. Overexpression of H-Ras through a heat-

shock-induced promoter in larvae led to hyperproliferation, activation of the DNA damage response and TP53-dependent cell cycle arrest. Thus, oncogene-induced senescence of adult proliferating cells contributes to the development of Costello Syndrome and provides an alternative pathway to transformation in the presence of widespread constitutively active *H-Ras* expression.

1.5 MIRNAs

MiRNAs or MicroRNAs are a family of small (~22 nucleotides, nt) endogenous, noncoding RNAs molecules that can mediate the expression of target genes with complementary sequences. MiRNAs recognize their target mRNAs based on sequence complementary and act on them to cause the inhibition of protein translation by degradation of miRNA (Díaz-Prado y Antón-Aparicio, 2010). It is estimated that one-third of the genes are regulated by miRNAs so they have shown important regulatory roles in various developmental, differentiation, cell proliferation, stress response, metabolism and apoptosis pathways of different organisms. Indeed, aberrant miRNA expression has been documented in human disease and in animal models as well, with evidence for a causative role in tumorigenesis. Nowadays, the number of miRNAs identified in humans is constantly increasing, and currently more than 1500 miRNAs are known.

1.5.1 The history of miRNAs

In 1993, Lee and their colleagues discovered the first miRNA called lin-4 in the nematode *Caenorhabditis elegans*. The lin-4 gene was transcribed into a 22 nt RNA molecule and found to inhibit protein synthesis. This molecule recognizes complementary sites in the 3' untranslated region (3' UTR) of the lin-14 messenger, so, it downregulates the translation of lin-14 during the transition from the first to the second larval stage of development. Since 1993, thousands of miRNAs have been discovered in 58 different species.

1.5.2 MiRNAs nomenclature

The rapid growth of the miRNA field has been facilitated by the adoption of a consistent naming scheme; names are assigned by the Registry based on guidelines agreed by a number of prominent miRNA researchers and discuss elsewhere. MiRNAs are assigned sequential numerical identifiers. The database uses abbreviated 3 or 4 letter prefixes to designate the species, **hsa** for *Homo sapiens*, **mmu** for mouse followed by miR for matures sequences or mir for the precursors sequences called hairpins, so for orthologous sequences the names are hsa-miR-101 (*Homo sapiens*) and mmu-miR-101 (mouse). Paralogous sequences whose mature miRNAs differ at only one or two positions are given lettered suffixes for example, mmu-miR-10a and mmu-miR-10b in mouse. Distinct hairping loci that give rise to identical mature miRNAs have numbered suffixes (e.g. dme-mir-281-1 and dme-mir-281-2 in *Drosophila melanogaster*). It had been observed that target recognition most often involve a short 6-8 nt "seed" fragment at the miRNA 5' end pairing to an exact complementary sequence in the 3' UTR. The miRNA duplex is excise from opposite arms and produces two different miRNAs sequences from the same hairpin precursor, such mature sequences are currently named of the form miR-5' arm and miR-3' arm (Griffiths-Jones et al., 2006).

1.5.3 MiRNAs biogenesis

Formation of mature miRNA follows four-step process, described in (fig. 13):

1) miRNA genes are transcribed into primary miRNA called pri-miRNA;
2) the pri-miRNA is cleaved into pre-miRNA, which is then transported into the cytoplasm;
3) finally, the pre-miRNA is cleaved and unwound to form nature miRNA (Zeng, 2006; Winter et al., 2009)
4) The majority of miRNAs genes are transcribed by RNA polymerase II as mono- or polycistronic primary transcripts (pri-miRNAs), they can be found as exonic or intronic regions of codifying or non codifying genes. Some miRNAs have their own promoters and they are expressed independently, or in some cases they are located in clusters and share the same transcriptional regulation. Pri-miRNAs that are 5' capped and polyadenylated (poly A tail), and they are stem-loop structures

containing the sequences of mature miRNAs embedded in the arm of a stem (Diaz-Prado y Antón-Aparicio, 2010). It had been reported that polymerase III can transcript also some miRNAs (miRNAs located in 19 miR cluster, miRNAs with upstream rRNA, repeat or transposable elements), (Erson and Petty, 2008).

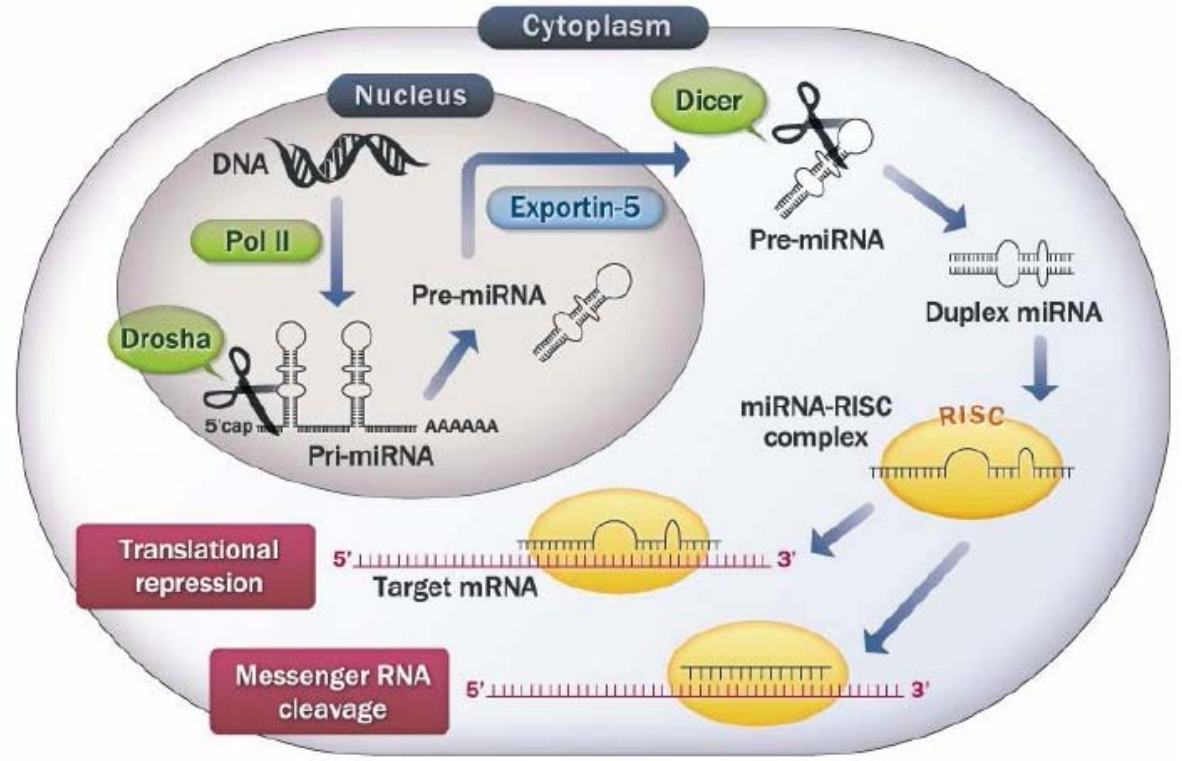


Fig. 13 Biogenesis of miRNAs. MiRNA genes are transcribed by RNA polymerase II (Pol II). The resulting long transcript is capped with a specially-modified nucleotide at the 5′end, polyadenilated with multiple adenosines and spliced. The product is called primary miRNA (Pri-miRNA). Drosha crops Pri-miRNA into precursor-miRNA (Pre-miRNA). Pre-miRNA hairpins are exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, the pre-miRNA hairpin is cleaved by the RNAse III enzyme Dicer. One strand is taken into the RNA-induced silencing complex (RISC), where the miRNA and its target interact. miRNAs that bind to mRNA targets with perfect matching induce mRNA cleavage, whereas translational repression is induced when matching is imperfect (Nohata et al., 2012).

These hairping-like structures will be cleaved by two distintic complexes: Drosha and its partner DGCR8 (DiGeorge syndrome critical region gene 8) in the nucleus, and Dicer in the cytoplasm, both are members of the RNAse II enzyme family. Drosha cleaves the pri-miRNA, releasing a ~65 nt precursor miRNA (pre-miRNA) hairpin, Wu et al., 2009, reported that Drosha showed a shift in their processing of pri-miRNAs and generates multiple pre-miRNAs of miR-142 in T cells which reveals that the mature miRNA

sequence is not absolutely fixed and it can inhibits a high frequency of 5' end polymorphism.

Pre-miRNA is transported out to the nucleus via a nuclear membrane transporter Exportin 5, together with Ran-guanosine triphosphate. In some cases Drosha is not necessary for cleaving the pri-miRNA and an intron-derived miRNAs are released from their host transcripsts after splicing alternative, these miRNAs received the name of miRtrons (Shomron and Levy, 2009). Subsequently, Dicer cleaves the pre-miRNAs and the miRtrons into double-stranded RNA in the cytoplasm miRNA duplex is then assembled into a multiprotein complex, RNA-induced silencing complex (RISC), which is composed of Dicer, TRBP, and Argonaute 2 (Ago2), (miRNP complex). Only one strand of the miRNA duplex, designated as the "guide" strand (5'- 3') is loaded into a large multi-protein miRNA ribonucleoprotein complex (miRNP, also referred to as the miRISC complex) with the assistance of RNA helicase proteins (RNA helicase p68/DDX5) and is now poised to modulate target gene expression. The other excluded strand of the miRNA-miRNA* duplex is subsequently degradated (3'-5'). They are instances that both sides of the miRNA-miRNA* duplex are equally retained and can associate with the miRNP complex to target distinct subsets of mRNAs for down-regulation (Zeng, 2006; Breving and Esquela-Kerscher, 2009).

1.5.4 Mechanisms of action of miRNAs

The repression of miRNA:mRNA it seems to occur in 3' UTR region of the targets mRNA or the open reading frame (ORF) of target mRNA, the complexity of miRNA dependent gene expression is further extended by the fact that more than one miRNA can cooperatively bind to the same 3' UTR and that each miRNA can regulate hundreds of targets. The binding position depends on how complementary the miRNA complex is to the ORF of mRNA, they are two different routes for silencing mRNAs. In the first case, a high miRNA-target complementary produces a cleavage or degradation of the mRNA target by Argonaute 2 (Ago 2), in the second case; a low miRNA-target complementary produces a translational repression (Legendre et al., 2006; Diaz-Prado y Antón-Aparicio, 2010). It is important to point out that some miRNAs bind several mRNAs, and some mRNAs have

several binding sites for different miRNAs. Three types of interactions between miRNAs and mRNAs were identified, which differ according to the region of the miRNA bound to the mRNA: 1) binding occurs predominantly via the 3' UTR region of the miRNA; 2) binding occurs predominantly through the central region of the miRNAs; and 3) via the 5' UTR region of the miRNA.

1.5.5 MiRNAs have a pleyotropic role in cancer development

Human miRNAs play a pleytropic role in cancer development because they are located at fragile sites in genomic regions, their amplification, upregulation or downregulationg can missregulate genes involved in cell differentiation thereby contributing to tumour formation by stimulating proliferation, angiogenesis, and/or invasion, so they can show dual functions as both TSG and OG, depending of the context (table 9).

miRNA	Role	Cancer types	Reference
hsa-miR-135	TS	Renal cell carcinoma	Yamada et al 2012
hsa-miR-29c	TS	Gastric cancer	Saito et al., 2012.
hsa-miR-29	TS	Osteosarcoma pathogenesis and progression	Zhang et al., 2012e
hsa-miR-218	TS	Neck squamous cell carcinoma	Kinoshita et al., 2012
hsa-miR-23	TS	Prostate cancer	Majid et al., 2012
hsa-miR-17-92	OG	Lung cancer Lymphoma	Zhang et al., 2007; Finoux and Chartrand, 2008; Mott, 2009; Ventura and Jacks, 2009.
hsa-miR-372 **hsa-miR-373**	OG	Testicular germ cell tumours	Voorhoeve et al., 2007.
Has-miR-let-7a	MSG		Zhang et al., 2007; Finoux and Chartrand, 2008; Manikandan et al., 2008.
hsa-miR-335 **has-miR-206** **hsa-miR-126**	OG	Breast cancer	Png et al., 2011a
hsa-miR-10b **hsa-miR-520c** **hsa-miR-122a** **hsa-miR-199a**	OG	Breast cáncer	Lopez-Camarillo et al., 2012

Table 9. MiRNAs as tumor suppressors and oncogenes, (Esquela-Kerscher and Slack, 2006).

The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation. A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein, which presents a oncogenic role

would also result in tumour formation. In both cases, (miRNAs acting as TSG or OG) the overall outcome is the same, and it might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation (Esquela-Kerscher and Slack, 2006).

A) OncomiRs

MiRNAs that are amplified or overexpressed in cancer could act as oncogenes (frequently called "oncomiRs"), and they negatively inhibit tumour suppressor genes and/or genes that control cell differentiation or apoptosis. The most representative oncomiR is miR-17-92 cluster which is an polycistronic oncomiR located at chromosome 13q31, a genomic locus that is amplified in lung cancer and several kinds of lymphoma, including diffuse large B-cell lymphomas, MiR-17-92 cluster function is related with the expression of c-Myc gene which regulates also the expression of cell cycle transcription factor gene E2F1 (Zhang et al., 2007; Finoux and Chartrand, 2008; Mott, 2009; Ventura and Jacks, 2009). Other known oncomiRs are miR-372 and miR-373, both promote cell proliferation and tumour development by neutralizing p53 mediated CDK inhibition, in human testicular germ cell tumours, thought direct inhibition of expression of the tumour suppressor gene LATS2 (Cho, 2007; Zhang et al., 2007).

B) MiRNAs as tumour suppressors

Several miRNAs have been implicated as tumour suppressors based on their physical deletion or reduce expression in human cancer. Tumour suppressor miRNAs expression indicates that their normal expression can limit cancer cell growth or induce apoptosis in cell culture or upon transplantation in suitable host animals. Let-7 is the most studied tumour suppressor gene, it is located in a chromosome region that is usually deleted in human cancers and their missregulation is associated with poor prognostic; *RAS* oncogene is negatively regulated by miRNA let-7, (Zhang et al., 2007; Finoux and Chartrand, 2008; Manikandan et al., 2008).

C) MiRNAs as metastatic gene suppressors and their role in angiogenesis

MetasmiR is a new term that identifies miRNAs involves in cell migration and invasion processes. The first evidences of metasmiRs were reported by Png et al., 2011a and 2011b; they found that miRNA-335, miR-206 and miR-126 expression were significantly reduced in primary breast neoplasm of patients who went to on to develop metastases. Two of these miRNAs, miR-335 and miR-206, were found to suppress migration, invasion, and metastatic colonization without inhibiting overall tumuor growth; while miR-126 was found to suppress metastasis in part through the inhibition of growth thought diminishing angiogenesis development. Since then, more metasmiRs have been reported, for example, in breast cancer miR-10b suppresses a homeobox which subsequently activates RHOC gene; miR-373, miR-520c both regulate CDC44 expression, and miR-335, miR-126, miR-206, miR-122a, miR-199a and miR-489 suppress metastasis and angiogenesis (Lopéz-Camarillo et al., 2012).

D) MiRNAs involved in other cell functions

MiRNAs can impair cell proliferation or induce apoptosis through oncogene targeting, and they are frequently called apopmiRs. The most known examples are miR-15a-16-1 and let-7 and miR-21 which regulate PTEN in PI3K/AKT/mTor signalling pathway. Due of miRNAs are able to affect practically all biological processes, including proliferation and apoptosis, is not surprising that miRNAs can impact response to specific drugs including chemotherapy, for example miR-21 exert its antiapoptotic effect by targeting the tumor suppressor phosphatase and tensing homolog and programmed cell death, this miRNA and miR-200b were overexpressed in cholangiocarcinoma cell lines, where their inhibition increased sensitivity to gemcitabine. In other hand, in breast cancer, overexpression of miR-221 and miR-222 are responsible for resistance to anti-estrogenic therapies, as tamoxifen, and fulvestran, whereas ectopic expression of oncosuppressor miR-205 improves the responsiveness to tyrosine kinase inhibitors through direct targeting of HER3 (Iorio and Croce, 2012).

1.5.6 MiRNAs selected

Compelling evidence reveals that there is a causative link between miRNAs deregulation and cancer development and progression. For this reason, we selected a miRNAs group from literature for developing the experimental phase of this PhD thesis. We chose these set of miRNAs because some of them acting as a tumours suppresors, protooncogenes or metasmiRs. Camats et al., 2008a; chose miR-206 and miR-335 because in 2008, these miRNAs showed an important role in regulation in breast cancer. Other miRNAs chosen are related with the expression of some important aspects of metastasis as such as loss of adhesion, local invasion, apoptosis evasion, colonization and angiogenesis in several types of cancers, (table 10).

MiRNA	Function in cancer	Chromosomal localization	Mature Sequence
hsa miR-126	MetasmiR, angiomiR	9	hsa-miR-126-3p
hsa miR-138	Tumor suppressor gene	3	hsa-miR-138-5p
hsa miR-206	MetasmiR, angiomiR	6	hsa-miR-206
hsa miR-330	Tumor suppressor gene	7	hsa-miR-330-3p
hsa miR-342	Tumor suppressor gene	14	hsa-miR-342-3p
hsa miR-335	MetasmiR, tumor suppressor gene	7	hsa-miR-335-5p
hsa miR-374	Tumor suppressor gene	X	hsa-miR-374-3p
hsa miR- let-7a	Tumor suppressor gene	9	hsa-miR-let-7a-5p

Table 10. MiRNAs selected for developing this PhD thesis. Source: http://atlas.dmi.unict.it/mirandola/index.html

A) MiR-126

MiR-126 and miR-126* (complement of miR-126, and called miR-123 in the past) are encoded by intron 7 of the *egfl7* gene which codifies for the novel endothelial cell-derived secreted protein epidermal growth factor-like domain 7 (EGFL7) that had been suggested to control vascular tubulogenesis, and both miRNAs are involved in developmental inflammation, metastasis and angiogenesis via CD34(+) and CD14(+) peripheral blood mononuclear cells, in highly vascularized tissues (Wang et al., 2008a; Chen et al., 2011c; Mocharla et al., 2012), due of these characteristics miR-126 is emerging as a novel and potential serum biomarker for risk of myocardial infarction (Long et al., 2012;Zhu et al., 2011a and 2012b; Zampetaki et al., 2012).

Changes in the expression levels of miR-126 and miR-126* have been reported in various types of diseases, including cardiovascular diseases, diabetes type 1, cholesterol metabolism, obesity, ulcerative colitis, Alzheimer and asthma (Wang et al., 2008a; Mattes et al., 2009; Zampetaki et al., 2010; Feng et al., 2012). Misregulation of miR-126 had been reported also in cancer and in the majority of the cases, a downregulation had been detected, (table 11).

MiR-126 regulates loss of adhesion and tyrosine phosphorylated signalling, pathways that promote tumour proliferation and invasion (Liu et al., 2009a, 2011a; Felli et al., 2013). MiR-126 is inhibited by Src, and subsequently miR-126 negatively regulates several proteins as such as Crk (a protein which is a component of the focal adhesion network),; ADAM9 (disintegrin and metalloproteinase domain containing protein 9), that regulates cellular migration, invasion modulating the expression of E-cadherin in pancreatic cancer cells (Hamada et al., 2012); RhoA/ROCK signalling pathway which controls proliferation in colon cancer cells (Li et al., 2013); and angiogenic factors which confers resistance against hypoxia which results in an increase of cell survival in mesenchymal stem cells (MSCs), (Huang et al., 2012). MiR-126 plays a fundamental strategy in the body for detecting and eliminating cancer cells, because miR-126 activates CD4(+) and Foxp3(+) regulatory T cells, (called TREGs), which eliminates cancer cells and they subsequently

endow effectively antitumour effect of CD8(+) T cells in murine breast cancer model (Qin et al., 2013).

Type of cancer	Reference
Breast cancer Breast cancer metastasis to lung	Negrini et al., 2008 ; Tavazoie et al., 2008; Png et al., 2011a; Yang et al., 2011; Zhu et al., 2011b ; and Hafez et al., 2012
Pancreatic cancer	Musiyenko et al., 2008 ; Frampton et al., 2012; Hamada et al., 2012; Jiao et al., 2012
Non small cell lung cancer Lung cancer (miR-126-3p and miR-126-5p)	Liu et al., 2009a; Sun et al. ; 2010 ; Gao et al., 2010 ; Miko et al., 2009 and 2011; Donnem et al.,2011 and 2012; Jusufovic et al., 2012; Tomasetti et al., 2012; Zhou et al., 2012 ; Zhu et al., 2012 ; Guan et al., 2012 ; Vosa et al ., 2012
Squamous carcinoma and adenocarcinoma	Guan et al., 2012
Cervix cancer cell lines	Wang et al.,2008a and 2012,
Glioma	Yu et al., 2012
Prostate cancer	Musiyenko et al., 2008 ; Meister and Schmidt, 2010, Png et al., 2010 ; Watahiri et al., 2011.
Bladder cancer	Watahiki et al., 2011; Zaravinos et al., 2012; Snowdon et al., 2012 ; Li et al., 2012 b and c.
Parathyroid and thyroid carcinoma	Li et al., 2012b and 2012c, Snowdown et al., 2012
Rectal cell carcinoma	Slaby et al., 2012; Li et al., 2012b
Colorectal cancer Colorectal with brain metastasis	Li et al., 2012b, and 2012c ; Guo et al ., 2008
Hepatocellular carcinoma Metastatic recurrance of hepatocellular carcinoma	Han et al., 2012 ; Chen et al., 2013
Mesothelioma	Santoriello et al., 2009 ; Santorelli et al., 2011; Tomasetti et al., 2012 ;
Gastric cancer	Feng et al., 2010 ; Otsubo et al., 2011
Leukemia Pediatric acute myeloid leukemia	Li and Chen, 2011; Luo et al., 2011; Zhu et al., 2012; Min et al., 2012; Ishihana et al., 2012. Daschkey et al., 2013
Urothelial cancer	Snowdown et al., 2012
Esophageal squamous carcinoma	Cai et al., 2011; Hamada et al., 2011
Multiple myeloma	Min et al., 2012
Human melanoma	Felli et al., 2013
Oral squamous cell carcinoma	Sasahira et al., 2012

Table 11. Misregulation of miR-126 in cancer

Downregulation of miR-126 had been implicated in misregulation Ras/PI3K/AKT/VEGF-A cell signalling pathway, in which miR-126 inhibits directly PI3K (subunit p85β) AKT, ERK and subsequently VEGF-A (Vascular endothelial growth factor A) expression and their downstream signals (Guo et al., 2008; Chen et al., 2011c; and Zhu et al., 2012). In this via, miR-126 contributes with four different aspects to cancer process 1) confers resistance to anticancer agents (adriamycin and viscritine) mediated by MRP1 (a multidrug resistance-associated protein-1 axis); 2) contributes to increase ROS production (Guo et al., 2008;

Kuhnert et al., 2008; Liu et al., 2009a; Donnem et al., 2011; Meng et al., 2012 and Zhu et al., 2012b); 3) misregulates other proteins involved in tyrosine kinase-induced signaling via the MAPK and PI3K pathways, included Sprouty-related EVH1 domain-containing protein 1 (SPREAD1), (Meng et al., 2012); and 4) downregulation of VEGF-A induce to a cell cycle arrest in the G1 phase in lung cancer cell lines (A549, Y-90, and SPC-A1) and reduced tumour growth (Liu et al., 2009a; Donnem et al., 2011; Zhu et al., 2011b).

Felli et al., 2013, found that miR-126 and miR-126* downregulation promotes a upregulation in ADAM9 and MMP-7 expression (both proteins involved in loss of adhesion and metastasis), which subsequently activate HB –EGF a soluble ligand of the EGFR (Epidermal Growth Factor Receptor) and HB-EGF-C and intracellular carboxy-terminal fragment which translocates to the nucleus. HB-EGF activates several intracellular cascades of signalling downstream: 1) Ras/RAF/MEK/ERK; 2) AKT/PI3K; 3) EGFR/MAPK and 4) CCND1, CCNDE1 and CCNDE3 which contribute to regulate cell cycle.

In prostate cancer cells derived from prostate cancer xenograft models, re-establish of the ectopic expression of miR-126 and miR-126* regulate directly growth inhibition though cell cycle arrest and apoptosis in two different vias: 1) regulating SOX-2 (a transcription factor) by targeting their 3' UTR region in gastric cancer cells (Otsubo et al., 2011); or 2) producing a cell cycle arrest in phase G0/G1 to S, via IRS-1 (insulin receptor substrate-1) in MCF-7 breast cancer cell line (Zhang et al., 2008); besides the miR-126 restablished expression inhibited the migration and invasiveness (Meister and Schimidt, 2010; Png et al., 2010); the molecular mechanism of inhibiting metastasis had been reported recently by Zhang et al., 2013, in which miR-126/miR-126* direct inhibit stromal cell-derived factor-1 alpha (SDF-1α) expression and indirectly suppress the expression of chemokine (C-C motif) ligand 2 (Ccl2) of cancer cells in an SDF-1α-dependent manner.

In zebrafish, p21 activated was identified as a novel target of miR-126a/b and pak1 3'UTR region was differently regulated by these miRNAs (Zou et al., 2011).

B) MiR-138

MiR-138 regulates genomic stability, metastasis and resistance to treatments in cancer cells so their misregulation has been detected in several types of cancers (table 12).

Type of cancer	Reference
Head and neck carcinoma Head and neck squamous cell carcinoma	Liu et al., 2009c and 2009d. Jin et al., 2013
Squamous cell carcinoma	Liu et al., 2011b
Tongue squamous cell carcinoma	Jin et al., 2011b
Papillary thyroid carcinoma Anaplastic thyroid carcinoma cell lines Thyroid carcinoma	Yip et al., 2011 Mitomo et al., 2008 Vriens et al., 2012
Hepatocellular carcinoma	Wang et al., 2012b and c
Lung carcinoma	Seike et al., 2009; Qin et al., 2011a.
Nasopharyngeal carcinoma	Liu et al., 2012a
Melanoma	Poliseno et al., 2012
Bladder cancer resistant to cisplatin Agressive muscle invasive bladder cancer	Nordenfoft et al., 2012; Pignot et al., 2012.
Malignant glioma	Chan et al., 2012
Leukemia cells Chronic myeloid leukemia	Vriens et al., 2011; Zhao et al., 2010 Xu et al., 2012
Human gastric cancer	Yao et al., 2009
Clear cell renal carcinoma Clear cell renal carcinoma (786 cells)	Girgis et al., 2012; Song et al., 2011 Liu et al., 2011b
Human esophageal squamous cell carcinoma	Yuan et al., 2012
Astrocytoma (Grade I to III)	Li et al., 2011b
Colon cancer	Paun et al., 2009
Non small cell lung cancer cells Lung adenocarcinoma cell lines Lung cancer	Wang et al., 2011b Quin et al., 2011a Seike et al., 2009
Bladder cancer	Pignot et al., 2012
Breast, endometrial and pancreatic carcinoma cell lines	Lee et al 2012.
Osteosarcoma	Namlos et al., 2012
Esophageal squamous cell carcinoma	Gong et al., 2013
Cholangiocarcinoma	Wang et al., 2013

Table 12. Misregulation of miR-138 in cancer

MiR-138 is an important regulator of genomic stability, and this action can be exerted by different pathways 1) by direct repression of histone HZAX formation and expression, HZAX histone induces chromosomal instability after DNA damage; 2) targeting human telomerase reverse transcriptase gene (hTERT) in an inverse correlation, 3) inhibiting

homologous recombination, 4) enhancing sensitivity to multiple DNA damaging agents and (Mitomo et al., 2008, Wang et al., 2011b); and 4) regulating p53 signalling via Oct4, SOX-2 and KLF4 in pluripotent 3- stem generation (Ye et al., 2012).

Silencing of miR-138 activates NF-KB (nuclear factor KB) which is a key signalling pathway for developing esophageal squamous cell carcinoma (ESCC) activating multiple components of lipid rafts as such as FLOT1, FLOT2 and caveolin (Gong et al., 2013).

Moreover miR-138 expression, downregulates some proto-oncogenes expression which are involved in the initiation and progression of several different types of tumours, for example: GNAI2, FOSL-1, HIF-1α (Hypoxia-inducible factor-1 alpha), and GATA1. Downregulation of GNAI2 (G protein α inhibiting activity polypeptide), reduces proliferation, and induces to a cell cycle arrest and apoptosis (Jiang et al., 2011); in other hand, FOSL-1 (a member a Fos gene family) inhibition, produces a reduction of metastasis induced by E-cad (E-cadherin) expression and enhanced Vim (vimectin) expression, (Liu et al., 2011).

MiR-138 inhibited cell migration and invasion in ovarian cancer mouse model targeting EGFR/SOX4/ and HIF-1α (Ye et al., 2013), and increased apoptosis and reduced migration in clear cell renal cell carcinoma cells (Song et al., 2011);

Besides in a recent report, miR-138 had been implicated in a novel circuitry in chronic myeloid leukemia (CML) cell lines resistant to imatinib treatment which is associated with the enhancement of GATA1 activity, which binds to the miR-138 promoter and in turn is repressed by ACR-ABL; in this circuit, miR-138 downregulates BCR-ABL (c-abl oncogene 1, non-receptor tirosyne kinase) and CCND3 cyclin expresion. Therefore, BCR-ABL/GATA1/miR-138 circuitry is implicated in pathogenesis of CML and its clinical response to imatinib treatment (Xu et al., 2012).

MiR-138 regulates three key different aspects in cancer development 1) the epithelial mesenchyimal transition (EMT); 2) metastasis; 3) cell cycle progression and 4) it might

reverse multidrug resistance (Zhao et al., 2010). EMT is stimulated by downregulation of miR-138, and some direct targets of this circuit includes vimectin (Vim), ZEB2 (Zinc finger E-boxing homeobox 2), and EZH2 (enhancer of zeste homologue), which had been reported as direct target of miR-138 that inhibits proliferation and tumor growth in NSCLC (non small cell lung cancer cells and tissues), (Zhang et al., 2013a). Moreover miR-138 regulates EMT by three different pathways: 1) controlling the expression of Vim, (Liu et al 2011a); 2) targeting ZEB2 which is overexpressed in various human cancers and acts as an important regulator of cell growth and 3) controlling invasion targeting the expression of E-cadherin gene (Zhang et al., 2013).

Four proteins are direct and confirmed targets of miR-138 and they are: RhoC, ROCK2 and NGAL and FAK. Downregulating of miR-138 induces a increment in the expression of RhoC and ROCK2 proteins which promotes reorganization in morphology cytoskeleton initiating cell migration and invasion. Inhibition of RhoC expression induces to G1/S arrest of cell cycle and a hypophosphorylation of ERK and it also diminishes MMP-2 and MMP-9 metalloproteinases expression (Wang et al., 2013). NGAL (neutrophil gelatinase-associated lipocalin) has been detected upregulated in many cancers in which promotes cell migration and tumorigenesis (Lee et al., 2012). Another target of miR-138 is FAK (Focal adhesion kinase) which is a 125 kDa non-receptor kinase overexpressed in many types of tumours (Golubovskaya et al., 2013). FAK confers resistance to cancer treatments and abolition in their expression affect invasion, drug sensitivity and tumor growth in cancer cells. As we mention above, miR-138 expression restoration contributes to induce G1/S cell cycle arrest that is also promoted by inhibition of several cyclins as such as CCND1 and CCND3 (Wang et al., 2012a and Liu et al., 2012b).

C) MiR-206

MiR-206 belongs to a miR-206/miR-1 cluster which is located in 6p12.2 chromosomal region, both miRNAs are similar in terms of expression and function, but miR-206 differs from miR-1 by four nucleotides. This cluster is a pivotal key for regulation of multiple target genes including those involved in myoblast and skeletal muscle differentiation, functions that confer a novel term known as “myomiR” (Gagan et al., 2012, Goljanek et al.,

2012; Nohata et al., 2012). MiR-206/miR-1 cluster change their expression quickly in response of exercice and it regulates pulmonary artery smooth muscle cell proliferation in hypertensive mouse, which suggests that miR-206 serum concentration could be used as molecular biomarker in failure hearth (Jalali et al., 2012; Oliveira-Carvalho et al., 2012). Downregulation of miR-206 is frequently detected in several types of cancers, (table 13).

Type of cancer	Reference
Colorectal cancer	Vickers et al., 2012
Laryngeal carcinoma	Wang et al., 2010c; Zhang et al., 2011a.
Breast cancer (ER + α receptor)	Adams et al., 2007; Kondo et al., 2008; Negrini et al., 2008; Adams et al., 2009; Leivonen et al., 2009; Di Leva et al., 2010; O'Day, 2010; Yoshimoto et al., 2011; Chen et al., 2012a; Nohata et al., 2012.
Clear cell renal cell carcinoma	Zhou et al., 2010
Ovarian carcinoma	Guo et al., 2011
Lung cancer Lung carcinogenesis induced	Wang et al., 2011a; Nohata et al., 2012. Wu et al., 2013
Rhabdomyosarcoma	Taulli et al., 2009: Yan et al., 2009; Missiaglia et al., 2010; Miyachi et al., 2010 ; Macquarrie et al., 2012 ; Nohata et al., 2012.
Osteosarcoma	Namlos et al., 2012.
Endometrioid adenocarcinoma	Nohata et al., 2012
Papillary thyroid carcinoma	Liu et al., 2013
Gastric cancer	Zhang et al., 2013

Table 13. Misregulation of miR-206 in cancer

Breast cancer (BC) is a heterogeneous disease, which comprising multiple entities associates with distinctive histological and biological features, clinical presentations and behaviours and responses to therapy. Microarray-based technologies have unraveled four molecular subtypes of breast cancer: luminal, normal like, HER2 and basal like (Perou et al., 2000; Weigelt et al., 2010). In luminal breast cancer, estrogen receptor (ER) α is essential for estrogen-dependent growth, and its level of expression is a crucial determinant of response to endocrine therapy and prognosis, in this cancer miR-206 is downregulated and their re-established expression suppressed filopodia formation and regulates several proteins involved in cell adhesion a such as Cdc42, Cdc44 (two transmembrane glycoproteins) and MMP-2, and MMP-9 (both metalloproteases, involved in matrix degradation and loss of adhesion), which diminished VEGF-A expression but not VEGF-β (Liu et al., 2010). These proteins are required for remodelling cytoskeleton, conferring

filophodia, transformation, migration, and invasion capacity in cancer cells (Anderson et al., 2006; Liu et al., 2010; Limana et al., 2011; Zhang et al., 2011a).

Even in ERα (-) BC subtypes (normal like, and HER2), which are highly aggressive and non responsive to hormonal therapy as such as Luminal A and Basal like molecular subtypes, re-established of miR-206 expression showed some differential changes in cancer progression as such as 1) modulation of expression of *ESR1* (Estrogen receptor gene) in two binding *EST1* 3'UTR sites, but only in ERα not in ERβ, which suggested the existence of a feedback loop (Adams et al., 2007; Kondo et al., 2008; Leivonen et al., 2009; O'Day et al., 2010); and 2) regulation of steroid receptor activators (Src-1 and Src-3), and the transcription factor GATA-3, all of them required to mediate estrogenic responses. It is interesting to note that EGFR/MAPK signalling pathway enhances miR-206 activity and can induce the transition from an ERα (+) (Luminal-A phenotype), into a ERα (-), Basal-like phenotype, (Adams et al., 2009).

Besides miR-206 restoration of their expression promotes a myogenic differentiation and blocked tumor growth in retinoblastoma in *in vivo* xenografted mice by switching the global mRNA expression profile by two different pathways: 1) controlling directly the c-Met expression, a cell surface receptor tyrosine kinase that is up-regulated in a variety of tumours, and leads to cellular activation and it contributes to tumour growth, invasiveness, and metastasis (Dongsheng et al., 2009; Taulli et al., 2009; Yan et al., 2009) or 2) acting as a genetic switch to a transition for the cell from a proliferative growth phase to differentiation, in which miR-206 increases their expression that affects downstream a great variety of transcription factors, signalling pathways, including those involved in differentiation of skeletal muscle, chromatin remodelling and apoptosis evasion (McQuarrine et al., 2012; Goljanek et al., 2012), as such as NOTCH, KLF4 and PAX7.

NOTCH3 is a transcription factor that regulates skeletal muscle stem cells differentiation and apoptotic cell death, its misregulation promotes undifferentiation in myocites and increases phosphorylation of p38 phosphatase, Mkp1 and Mef2c. In turn Mef2c also

induces miR-2/miR206 cluster, which directly downregulate NOTCH3 and allow differentiation to proceed (Song et al., 2009; Gagan et al., 2012).

MiR-206 also regulates KLF4 (Kruppel-like factor 4) which modulates cell fate in primaty human colon cancer cells (Lin et al., 2011a; Parasramka et al., 2012) and PAX7 a transcription factor requires for muscle cell biogenesis (Dey et al., 2011).

MiR-206 also induces a G0/G1 cell cycle arrest through modulation of CCD2 in gastric cancer (Zhang et al., 2013; Zhou et al., 2013) and inhibited estrogen-induced proliferation in breast cancer cells (Chen et al., 2012).

D) MiR-330

MiR-330 is a potential tumour suppressor; the re-established in their normal expression inhibits some aspects of metastasis as such as cell proliferation, colony formation, tumour growth, apoptosis evasion and increases sensibilization in cancer cells to some anticancerigen drugs. MiR-330 oncogenic effects were related with the regulation of CD42, CDC43 and CDC44 transmembrane glycoproteins, which are direct targets of miR-330, and are related with multiple cellular functions including cell-cell and cell-matrix interactions, lymphocyte activation and homing, haematopoiesis, tumour metastasis an cell migration in colorectal and breast cancer (Jeyapalan et al., 2010; Li et al., 2013).

MiR-330 upregulation expression was detected in non-small cell lung cancer brain metastasis (NSCLC), and this effect used to differentiated NSCL patients with positive brain metastasis (BM +; miR-330 overexpressed); even thought, upregulation of miR-330 was not correlated with age, gender, stage at diagnosis or histology in 15 brain metastasis patients, (Arora et al., 2011). In colon and cancer cell lines an upregulation of miR-330 was detected and correlated with *in vitro* gentamicine resistance, which was acquired for the posttranscriptional regulation of dCK in cancer cells (Hodzic et al., 2011). Moreover in gliomablastoma (U87 and U251 cell lines) an overexpression of miR-330 had effects upon proliferation, migration, invasion, cell cycle and apoptosis (Qu et al., 2012).

Downregulation of miR-330 had been also reported in human prostate cancer cell lines in which also promotes cell proliferation and apoptosis evasion through E2F1 transcription factor, mediated suppression of AKT phosphorylation signalling (Lee et al., 2009).

E) MiR-335

MiR-335 resides on chromosome 7q32 and its misregulation had been reported in tumour different types of cancers, (table 14).

Type of cancer	Reference
Meningioma	Shi et al., 2012
Breast cancer stem-like cell lines Breast cancer patients' samples Breast cancer lung metastasis Breast cancer development Breast cancer (genetic variation)	Negrini et al., 2008; Tavazoie et al., 2008; Yang et al., 2011 ; Heyn et al., 2011 ; Png et al., 2011b ; Polytarchou et al., 2012 ; Hafez et al., 2012 ; Zhang et al., 2012 ; Zu et al., 2012.
Gastric cancer	Xu et al., 2011 and 2012 ; Yang et al., 2012
Malignant astrocytoma Human glioma	Shu et al., 2011a; Jiang et al., 2012.
Neuroblastoma	Lynch et al., 2012
Acute granulocytic leukemia Acute Myeloid Leukaemia	Luo et al., 2011; Bryant et al., 2012
Colorectal cancer	Vickers et al., 2012
Clear cell renal cell carcinoma	White et al., 2011
Malignant astrocytoma cells	Shu et al., 2011a
Hepatic stellate cell migration Hepatocellular carcinoma (hypermethylation)	Chen et al., 2011b ; Dohi et al., 2012
Malignant adernocortical tumours	Schmitz et al., 2011
Cologenic cancer	Wang et al., 2010
Multiple myeloma	Ronchetti et al., 2008
Prostate cancer	Xiong et al .,2013
Osteosarcoma	Hu et al., 2012

Table 14. Misregulation of miR-335 in cancer

Genetic alterations of miR-335 can alter susceptibility to several types of cancer, including 1) deletions in miR-335 which are a common event in human breast cancer and they have been correlated with ovarian cancer recurrence; 2) hypermethylation which produces a downregulation in miR-335 and its host gene MEST in hepatocellular carcinoma tumours with distant metastasis, (Dohi et al., 2012); 3) single nucleotide polymorphisms (SNPs) in 3' UTR region of miR-335 targets, for example in BIRC5 gene (baculoviral IAP repeat

containing 5), which may increase individual susceptibility to lung cancer probably by attenuating the interaction between miR-335 and BIRC5.

Furthermore, upregulation of miR-335 expression had been related with misregulation of tumour suppressor gene BRCA1 mRNA expression which suggesting a functional dominance of IDH (repressor) signalling and led to decreased cell viability and an increase in apoptosis, (Heyn et al., 2011); it also promoted cell proliferation in meningiomas, and astrocytomas (both are brain cancers); and inhibited cell cycle in the G0/G1 cell arrest targeting Rb1 (Retinoblastoma protein1) signalling pathway via pRB/p105 protein complex level (Shi et al 2012); apoptosis evasion and metastasis (Shu et al., 2011a).

Activation of miR-335 plays an important role in the induction of p53 dependent cell cycle arrest in order to limit proliferation and neoplastic cell transformation. After DNA damage or altering pRB/p105 protein activation of miR-335/p53 axis induces hyperproliferation and increases transformation *in vitro* in the absence of the p53 tumor suppressor pathway (Scarola et al., 2010).

In *in vitro* human glioma cell line,, miR-335 upregulation is an independent marker for predicting the clinical outcome of patients and it had been detected in corresponding non neoplastic brain tissues, and this effect was associated with a high grade of metastasis (type III-IV), (Jiang et al., 2012). In a recent work, Yan et al., 2012; identified miR-335 upregulation as a signature to recognize high frequency recurrence and non-recurrence cases, and poor survival ($p<0.01$) in the gastric cancer patients samples of chinese people.

In contrast, miR-335 was dramatically downregulated in gastric cancer cells, adrenocortical carcinomas (ACCs) in which miR-335 was taken as diagnostic biomarker for discriminating between ACCs from ACAs (adrenocortical adenomas), which helps to detect early malignant behaviour in cases with indeterminate malignant potential (Schmitz et al., 2011); and in MG-63 osteosarcoma cell line (Hu et al., 2012).

The miR-335 expression restoration suppresses invasion and metastasis via Bcl-w- induced phosphoinositide 3-kinase-AKT-SP1 pathway (SP1); (Xu et al., 2011; Yan et al., 2012).

Other putative targets of miR-335 are MEST, RASA1, DAAM1, ARPC5L, TNC, JAG, MAP2, RUNX2 (Wang and Ruan, 2010a; Chen et al., 2011b; Tome et al., 2011; Ronchetti et al., 2008); RASA1 is a known gene related to cell proliferation and anti-apoptosis, and its expression is inversely proportional to miR-335 (Wang et al., 2010a); TNC (tenascin-C) enhanced migration *in vitro* hepatic stellate cells (HSCs), in which miR-335 is downregulated; and TNC increases migration which is inhibited by miR-335 expression restoration (Chen et al., 2011b). MiR-335 is a key regulator in the regulatory axis miR-335/SOX4/Semaphorin-plexin in tumorigenesis of pancreatic cancer, in this circuit miR-335 regulates the expression of SOX4, a transcription factor which activates the Semaphoring/Plexin family genes that are involved in tumorigenesis of pancreatic cancer cells (Huang et al., 2012).

F) MiR-342

Hsa miR-342 is a miRNA encoded as intron of the gene EVL, so their expression is coordinated with that of EVL gene. In some cancers, a downregulation in miR-342 had been reported (table 15).

Type of cancer	Reference
Multiple myeloma	Ronchetti et al., 2008
Lung adenocarcinoma	Dacic et al., 2010
Breast cancer (triple negative subtype) Breast cancer tamoxifen resistance Cancer associated fibroblast in breast cancer (hsa-miR-342-3p) ER-positive breast cancer Ki67 low tumours (miR-342-3p)	Savad et al., 2012 Citelly et al., 2010 Zhao et al., 2012 Van der Auwera et al., 2010 Zhao et al., 2012 Endo et al., 2012
Chronic lymphocytic leukaemia Acute promyelocytic leukaemia	Demarchis et al., 2008 ; Wu et al., 2012 ; Fayyad-Kazan et al., 2013.
Colorectal adenocarcinoma and adenomas	Grady et al., 2008; Wang et al., 2011b
Glioma	Wang et al., 2012
Malignant peripheral nerve sheath tumours	Presneau et al., 2012

Table 15. Misregulation of miR-342 in cancer

In colorectal adenocarcimona and adenomas miR-342 downregulation had been also associated with EVL-hsa miR-342 locus silenced gene which produces a high grade of methylation and indicates that is an early event or carcinogenesis. Interestingly miR-342 expression reconstitution in HT-29 colorectal cancer cell line induces apoptosis, suggesting that miR-342 could function as a proapoptotic tumor suppressor, (Grady et al., 2008).

In multiple myeloma, EVL mRNA is a direct target of miR-342-3p and plays a role in plasma cell homing and/or interactions with the bone marrow environment, (Ronchetti et al., 2008); in gliomas, miR-342 downregulation had been positively correlated with histopatological grades, and for that reason it might be used as serum biomarker (Wang et al., 2012); in lung adenocarcinoma, (genotype tumours: EGFR (+), K-Ras (+); and EGFR/K-Ras (-)), (Dacic et al., 2010).

In breast cancer (BC), miR-342 downregulation had been reported in several molecular subtypes: 1) in triple negative [ER α (-); PR (-); HER2 (-)], is a novel inmunohistochemical biomarker; 2) in ER (+) , and HER2 (+) BC subtypes, miR-342 downregulation was associated with tamoxifen resistance in MCF7 breast cancer cell lines (MCF-7/HER2Δ16 in HER2 (+); and MCF-7/ TAMR1 and LCC2 variants in HER2 (-)), (Savad et al., 2012); surprisingly, miR-342 expression restoration in both cell lines sensitized them to tamoxifen-induced apoptosis with a dramatic reduction in cell growth (Citelly et al., 2010). Moreover miR-342 downregulation had been associated with inflammatory subtypes of BC with a high grade of proliferation and undifferentiation (Van der Auwera et al., 2010; Zhao et al., 2012).

In other hand, miR-342 upregulation had been reported in leukemias, specifically in chronic lymphocytic leukaemia (CLL), in which miR-342 might activate mature B cells and it may participate in B cell transformation processes (Li et al., 2011a); and in acute promyelocytic leukaemia (APL) during APL differentiation (De Marchis et al., 2009).

G) MiR-374

Currently only a few reports exist for miR-374 function, in one report miR-374 downregulation was detected in primary small cell lung cancer tumours (Miko et al., 2009); and it also contributes to promote breast cancer metastasis activating Wnt/β-canenin signalling (Cai et al., 2013a); besides it had been reported that miR-374 expression is modulated by dietary polyphenols at nutritional doses via apoE gene (apoE gene, modulates expression of genes in the liver), this effect was the same even if structurally different all polyphenols induced a similar miRNA expression profile, (Milenkovik et al., 2012).

Other recent reports of miR-374 are related with Ca^{+2} transport (Gong et al., 2012), and metabolism and development of neural cells (Chang et al., 2012). In the first case, miR-374 suppressed claudin 14 protein expression, through physical interaction, claudin-14 blocks the paracellular cation channel critical for Ca^{+2} reabsortion in the thick ascending limb (TAL) in the kidney, besides miR-374 transcript levels are regulated by extracellular Ca^{+2} in a reciprocal manner as a claudin-14.

H) MiR-let-7

Let-7 is a family of ten mature subtypes of miRNAs in humans, including let-7a, let-7b let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, miR-98 and miR-202, in which mature let-7a and let-7f were produced by precursor sequences (let-7a-1, let-7a-2, let-7a-3, let-7f-1, let-7f-2). All of these miRNAs are distributed over eight genomic clusters that have widespread effects in development, muscle formation, cell adhesion and gene regulation. These 10 family members represent 9 distinct let-7 sequences with identical seed sequences, and very likely overlapping sets of targets (Wang et al., 2012f).

A number of studies have reported that let-7 is down or up-regulated in numerous types of cancers, (table 16) and they are considered as tumor suppressors, which suggests that they have different and specific functions in the same cell, or tissue-specific regulation, although downregulation is the most frequently misregulation associated with poor outcome in patients with cancer (Nair et al., 2012).

Type of cancer	Reference
Serous ovarian cancer Ovarian cancer with por prognosis Ovarian cancer (let-7a, and b)	Mahajan et al., 2010; Helland et al., 2011; Bayani et al., 2011 ; Yang et al., 2012b Zaman et al., 2012.
Neuroblastoma	Moleenaar et al., 2012
Breast cancer (let-7a-2) Breast cancer (triple negative type) Breast cancer (Let-7a/b/c) Breast cancer ERα, PR and Ki-67 status inversely correlated with HER2 status (let-7a, c, and d) RAD52 binding site Cancer associated fibroblasts (let-7g) in breast cancer Breast cancer with lymph node metastasis	Chen et al., 2011a ; Zhao et al., 2011 Sun et al., 2012 ; Sakurai et al., 2012 ; Lv et al., 2012 ; Cerne et al., 2012 ; Jiang et al., 2012 ; Sohn et al., 2012 Hu et al., 2013.
Medulloblastoma (let-7a-2; let-7e), glioblastoma	Lee et al., 2010Wang et al., 2012a
Acute Myeloid leukemia (let-7c)	Pelosi et al., 2012
Pancreatic adenocarcinoma (Let-7 c, and f)	Ali et al., 2012
Ewing Sarcoma (let-7g)	De vito et al., 2011 ;Sohn et al., 2012.
Esophageal squamous cell carcinoma (let-7b and let-7c)	Sugimura et al., 2012
Myeloproliferative neoplasms Philadelphia chromosome-negative myeloproliferative neoplasms	Martin et al., 2012 ; Ikeda et al., 2012.
LNCaP Prostate cancer cell lines and PC3	John-Aryankalayil et al., 2012
Prostate cancer stem cells (let-7b) Prostate cancer growth (let-7c); Prostate cancer cells (PC3 and LNCaP cell lines)	Liu et al., 2012b Nadiminty et al., 2012 ; Kong et al., 2012 ;
Colon cancer induced in rats	Parasramka et al., 2012
Colorectal carcinoma (let-7a) Chemorefractory metastatic colorectal cancer Colorectal cancer (K-Ras 3'UTR polymorphism)	Ruzzo et al., 2012; Mosakhani et al., 2012 Kjersem et al., 2012; Wang et al., 2012g
Oral cancer cells	Jakymiw et al., 2010
Oesophagus cancer	Hamano et al., 2012
Non small cell lung cancer (stage I patients) Lung cancer	Lu et al., 2012. : Miao et al., 2012; Jusufovic et al., 2012 ; Kang et al., 2013
Hepatobiliary and pancreatic cancer Hepatocellular carcinoma (Let-7c)	Saito et al., 2011 ; Jin et al., 2011b ;Huang et al., 2011 ; Zhu et al., 2011c .
Gastric cancer cell line	Oshima et al., 2010
Juvenile myelomonocytic leukemia Acute myeloid leukemia	Steineman et al., 2010 ; Pelosi et al., 2012
Nasopharyngeal carcinoma cells	Wong et al., 2011
Papillary thyroid cancer (let-7f)	Ricarte-Filho et al., 2009
Endometrial adenocarcinoma Endometrial carcinogenesis	Zhang et al., 2012b Romero-Pérez et al., 2012
Osteosarcoma	Di Fiore et al., 2012
Ovarian cancer	Yang et al., 2012b; Zaman et al., 2012
Liver cancer progression	Chen et al., 2012
Head and neck carcinoma	Tu et al., 2013
Retinoblastoma	Marzi et al., 2012

Table 16. Misregulation of miR-let-7 in cancer

Hu et al., 2013, found a significant correlation in some let-7 member's family downregulation and a poor clinical outcome in patients with breast cancer (let-7a, let-7b, and let-7g) with lymph node metastasis compared to those without lymph node metastasis.

Enforced expression of let-7b significantly inhibited breast cancer cells motility and affected also the dynamics, in other hand restoration of let-7 expression induced tamoxifen sensitivity by downregulation of ER α (+) signaling in BC cell lines, (Zhao et al., 2011).

A downregulation of let-7 family members expression (7a-2, -7c, and -7g) have been detected in lung cancer and it was correlated with a resection recurrence, high aggressive stage and poor survival diagnostic (Lu et al., 2012). Another important mechanism which promotes downregulation of let-7 expression can be due to diminish in their somatic copy numbers, loss of let-7 focal deletion was detected principally in four let-7 family members, in three types cancer types: medulloblastoma (let-7a-2 and let-7e), breast cancer (let-7a-2), and ovarian cancer (let-7a-3/let-7b), (Wang et al., 2012f; Zaman et al., 2012). In pancreatic cancer cell lines, let-7 has an inverse correlation to RRM2 expression (ribonucleoprotein reductase subunit) involved in deoxyribonucleotide synthesis, which confers chemoresistance of pancreatic cancer to nucleoside analogues for example gemcitabine, (Bhutia et al., 2013), and in pancreatic cancer, let-7 and other miRNAs detected may regulate lymphatic metastasis in this type of cancer modulating cancer stem cells, (Luo et al., 2013). In other hand, upregulation of let-7 (-a, -b, -c, -f, -g) and miR-98 have been detected in non-small cell lung cancer patients and it was useful to differentiate adenocarcinoma and squamous cell carcinoma ($p<0.05$, T test), (Fassina et al., 2011).

Regulation of let-7 expression

The first point of regulation of let-7 expression is located in their promoter, which contains two different start sites and is regulated by two different cis elements, the temporal regulatory element (TRE), and the transcription element, both elements play different roles in regulating let-7 expression in specific tissues in developing of *Caernohabditis elegans* and it might be possible that could occur also in mammals including human beings (Kai et al., 2013).

Other point of regulation of let-7, is driving by their principal repressor Lin28 (which is a homologue of the *Caernohabditis elegans,* lin-28 gene), an evolutionary conserved RNA-binding protein, two different molecules of lin28 (A and B), are involved in a double negative feedback loop regulation, which it means that both molecules regulate each other. Let-7 suppresses the expression of Lin28 through let-7 binding sites in the Lin28 3' UTR, and Lin28 suppresses the production of mature let-7 inducing a 3'-terminal uridinylation of let-7 precursor by uridylyl transferase (TUTase, TUT4) with a long extension of uridine, leading to the failure of Dicer processing and finally to degradation (Yang et al., 2010; Piskounova et al., 2011).

Lin28 and Lin28B are overexpressed in primary human tumours and human cancer cell lines (with an overall frequency approximately 15%) and it had been linked to repression of let-7 family miRNAs and derepression of let-7 targets. Lin28 and Lin28B facilitate cellular transformation *in vitro*, and their overexpression are associated with advanced disease across multiple tumour types and poor clinical prognosis of breast cancer tumours (Ji and Wang, 2009; Chen et al., 2011a; Helland et al., 2011; Sakurai et al., 2011, Piskounova et al., 2011; Van Wynsberghe et al., 2011; Cai et al., 2013b); and neuroblatoma (No authors listed, 2012) even more LIN28 mediates paclitaxel resistance by modulating p21, Rb and let-7a miRNA in BC cells (Lv et al 2012).

Another proteins and RNA-binding proteins are able to regulate let-7, for example hnRBPA1 which recognize preferentially precursors of the let-7 family, (Towbin et al., 2012), and Zcchc11 a enzyme that regulates miRNAs, it can uridylate let-7 precursors to decrease quantities of the mature miRNA in embryogenic stem cell lines, suggesting to mediate cytokine and growth factor expression (Jones et al., 2012). P53 also regulates direct and negatively the expression of let-7 (a, and b sequences), when the cell is exposure to agents that induce stress including ionizing radiation. P53 binding to a let-7 region upstream of the let-7 gene following radiation exposure and this promotes pro-apoptotic signalling pathways via Ras/MAPK kinase signalling pathway and PTEN, Raf suppressor genes (Boominathan, 2010; Rutkowski et al., 2011; Saleh et al., 2011); recently findings

have provided evidence that p53 stimulated by the DNA-damaging agent doxorubicin (DOX) induced the expression of TTP (tritetraprolin), which in turn increased let-7 levels through downregulation of Lin28a; this minicircuit shows a new mechanism for the widespread decrease in TTP and let-7 and chemoresistance observed in human cancers and let-7 which exhibit suppressive effects on cell growth through down-regulation of protooncogenes (Lee et al., 2013).

Besides, let-7 is a novel indirect downstream target of Wnt/β-catenin pathway which maintains stem cell properties in both normal and malignant tissues; this signalling pathway induces Lin-28 upregulation and let-7 downregulation in BC stem cell phenotype and mouse tumor models (Cai et al., 2013b).

Indirect points of let-7 regulation in their expression have been reported recently, Chen et al., 2011d, describes that miR-107 binds directly to let-7 and the internal loop of the let-7/ miR-107 duplex is critical for repression of let-7 expression. Overexpression of miR-107 was found to be highly expressed in malignant tissue with advanced breast cancer, and its expression was inversely correlated with let-7 expression in tumours and cancer cell lines. Ectopic overexpression of miR-107 in human cancer cell lines lead desestabilization of mature let-7, increased expression of let-7 targets, and increased malignant phenotypes. Even more, some steroid receptors (as such as DAF-12), can upregulate the expression of some members of let-7 miRNA family, which means that gonad primary functions are not only confined to hormone production (Ikeda et al., 2012); some treatments as such as follicle stimulate hormone (FSH), used very often for female infertility can regulate indirectly let-7 expression, modulating the HMGA2 expression in normal fimbrial epithelial, treatment with not occurrence of p53 mutation (Zhang et al., 2012g).

In BC genotypes HER2 (+) and triple negative (ER (-), PR (-), HER2 (-)), SHP2 (Src-homology 2 domain-containing phosphatase 2) showed to play a fundamental role in the processes in human epidermal growth factor receptor. Activation of SHP2 activates also stemness-associated transcription factors, including C-myc (v-myc myelocytomatosis viral oncogene homolog) and ZEB1 (zinc finger E-box binding homeobox 1), which resulted in

the repression of let-7 miRNA and the expression of a set of SHP2 signature genes, activated simultaneously in a large subset of human primary breast tumours that are associated with invasive behaviour and poor prognosis. Silencing or knocking down SHP2 eradicated breast tumour-initiating cells in xenograft models and prevents invasion, tumour growth and metastasis (Aceto et al., 2012).

Mechanisms of action

Let-7 can regulate the expression of mRNAS binding in the 3', or 5' UTR region of mRNAs which containing internal ribosome entry sites, and even more let-7a may represses the translation of target mRNAs by binding to and inhibiting translating polyribosomes. Deadenylation is another route which let-7 may indirectly contribute to decay of some mRNAs (Wang et al., 2012f).

Target oncogenic signalling pathways of let-7

Oncogenes regulated by let-7

C-myc, Ras, High mobility group A (HMGA2), Janus protein tyrosine kinase (JAK), signal transducer and activator of transcription 3 (STAT3) and *NIRF* are oncogenes that are critical in tumorigenesis, proliferation and invasion; and are targeted by let-7. These oncogenes activate the expression of their downstream target proteins that indirectly regulate the cell cycle, apoptosis and cell adhesion.

C-myc protooncogene is frequently activated in tumours; it increases cell growth, division and survival by increasing the synthesis of its target proteins, some of which are involved in the regulation of cell cycle and apoptosis. Overexpression of let-7 family members (-a, -b, -d, -e, -g, and –i), downregulates Myc mRNA and proteins, downregulating directly their 3' UTR binding region (He et al., 2010; Wong et al., 2011); in neuroblastoma, suppression of let-7 is mediated by the increase of MYCN levels (No authors listed, 2012).

RAS oncogene

Ras oncoproteins are key point for developing tumorigenesis and metastasis, because they controlling different signalling pathways including Ras/Raf/mitogen activated protein

kinase, Ras/PI3K/AKT/MTor, Ras/Rho/Ral following activation by complex signalling cascades. Ras is also able to bind numerous GAPs and GEFs effectors (Section 1.2.2 of this PhD thesis). The three human *Ras* genes (*H-, N-, and K-Ras*) are negatively regulated by let-7 and they contain multiple complementary sites in their 3' UTRs (Johnson et al., 2005).

Recent reports have shown that a single nucleotide polymorphisms (SNPs) in the let-7 complementary sites in the 3' UTR of *K-Ras* resulted in their upregulation which had been correlated with a poor prognosis of patients of lung, colorectal and BC (Chin et al., 2008; Zhang et al., 2011 b; Jiang et al., 2012). In metastatic colorectal cancer patients a SNP in *K-Ras* LCS6 had been correlated with resistance to therapy involving the EGFR (Endothelial Growth Factor Receptor) in metastatic colorectal cancer patients, (Sebio et al., 2012); and resistance to wit 5-fluorouracil and oxaliplatin +/- cetuximab treatments (Kjersem et al., 2012); even though they are not evidences for genetic association with the *K-Ras* gene single nucleotide polymorphisms variants in risk of endometriosis and in ovarian cancer (Caiola et al 2012; Luong et al 2012).

In pancreatic cancer cells (MIAPaca-2 cell line) a downregulation of let-7i , which leads a misregulation of Ras, increase GAPs effectors activity and GTPase activity (Ali et al., 2012), and an upregulation of let-7 resulted in a lower expression of Ras protein, a reduction in cell proliferation in glioma and lung cancer cells and a decrease in the size of gliomas and lung tumour in nude mice (Lee et al., 2010; and He et al., 2010).

Another major let-7 target identified is *HMGA2* (High mobility group 2A), which is a chromatin-associated non-histone protein capable of modulating chromatin architecture and thus affecting transcription. The maintenance of stemness and proliferation were dependent on HMGA2 negative regulation of p16 ^{Ink} ad p19 ^{Arf}, two important regulators of cell cycle progression. HMGA2 is expressed in both benign and malignant tumours mostly due to deregulation via chromosomal aberrations on 12q13-15 HMGA2 a down and overexpression of let-7 have been detected in lung, prostate (Nadiminty et al., 2012), ovarian (Yang et al., 2012), pancreatic, pituitary, thyroid (Ricarte-Filho et al., 2009); Philadelphia chromosome-negative myeloproliferative neoplasms, in which let-7

downregulation could contribute to the proliferative hematopoiesis with conferring a growth advantage at the level of a hematopoietic stem cell in some cases (Ikeda et al., 2012); and uterine cancers. In some cases this effect was correlated again with higher tumour grade and tumour invasiveness, which suggesting that loss of let-7 expression contributes to the aggressive phenotype of these cancers via re-expression of HMGA2. This metastatic effect can be modulated via RAS/MEK pathway, which promotes an epithelial transition in which a decrease of HMGA2 alters the function of transcriptional repressors like SNAIL which subsequently diminishes the expression of E-cadherin. Even though in cancer cell lines assays, the restoration of let-7 expression of gain and loss-of –function did not influence HMGA2 expression and neither on the prevention of the transformed phenotype (Watanabe et al., 2009; Helland et al., 2011).

JAK-STAT3 pathway.

JAK is a member of the protein tyrosine kinase family, activated JAK activates STAT3 downstream, STAT3 activation is involved in genesis, differentiation, apoptosis, inflammation, cancer development and immune response. STAT3 is a direct target of let-7a, mediating cell proliferation in HepG2 cells (Wang et al., 2012f).

NIRF oncogene and let-7

NIRF protein is the product of *UHRF2* gene, and it regulates DNA methylation**.** DNA damage may activate a p53-dependent checkpoint pathway resulting in the induction of p21WAF1 expression and subsequent cell cycle arrest at the G1/S phase, though the inhibition of CDK activity and DNA replication. A reduction in the levels of UHRF family members have been reported to correlate with p21WAF1 expression. Let-7a suppressed NIRF expression via binding to the 3' UTR of NIRF mRNA and this effect enhances p21WAF1 expression in lung cancer cells, indicating that let-7a may contribute to the regulation of cell cycle NIRF/p53/p21/CDK signalling pathway (He et al., 2009; Wang et al., 2012f).

Other direct targets of let-7 are eIF4F (which is an initiation factor responsible for recruiting mRNA to a ribosome); E2F2 (a transcription factor which plays a crucial role in

the control of cell cycle and action of tumour suppressor proteins and is also a target of the transforming proteins of small DNA tumour viruses), (Donk et al., 2010); caspase-3 (which is a pivotal protease activated during apoptosis); and Blimp-1(which is an important factor for terminal differentiation of lymphocytes and epidermal cells and is considered a crucial determinant in plasma cell differentiation). Loss of Blimp-1 expression has been described as a characteristic event in Hodgkin lymphoma and other human lymphomas. The reduced expression of Blimp-1 was determined to be the result of upregulation of two miRNAs able to engage the Blimp-1 mRNA: the endogenous miR-9 and let-7a, (Nie et al., 2010).

However let-7 also affects directly the cell cycle mediating certain downstream proteins as such as CCD1 (cyclin 1), CCND2, CDK6 (cyclin-dependent kinase 6), CDK4 (cyclin-dependent kinase 4); D3 and p21 cell cycle inhibitor mRNA that were downregulated in malignant melanomas by let-7b (Schultz et al., 2008b; Dong et al., 2010). A recent report had showed that let-7 and miR-125 regulate quiescence in human fibroblast, and it seems that both miRNAs are required for this quiescent effect, even more these miRNAs also regulate gene expression profiles, in particular, the induction of extracellular matrix proteins (Suh et al., 2012), this finding leads to think in let-7 as a potential tool treatment for different types of cancer, but the mechanism by which cancer cells become to a quiescent state in cell cycle is still unknown and further experiments are needed.

Using bioinformatics and experimental approaches, four genes were identified as let-7 direct targets including PAK1, DIAPH2, RDX and IRGB8 all of them involved in the actin cytoskeleton pathway, so reinforced expression of let-7b significantly inhibits breast cancer motility and affects actin dynamics (Hu et al., 2013).

IV. MATERIAL AND METHODS

4.1 Biologic Material

4.1.1 Cell cultures

A) *HeLa* cells

HeLa cell line was the first human epithelial cancer cell line established in long term culture in 1952. It was derived from adenocarcinoma of the cervix. *HeLa* cells are hypermetriploid (3n+) containing a total number of 76-80 chromosomes and 22-25 abnormal chromosomes per cell. *HeLa* proliferate abnormal and rapidly and contein multiple copies of integrated HPV 18, which contributes to p53 degradation and apoptosis evasion (MacVille et al., 1999).

Adherent *HeLa* cells were used in some experiments and cultured in standard conditions as detail in Guil et al., 2003a; and Guil et al., 2003b. This cell line was bought to European Collection of cell cultures (Catalogue No. 93021013; fig. 14).

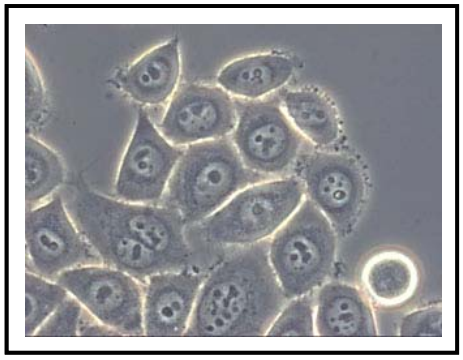


Fig. 14 *HeLa* cells.

B) Knock-out *H-Ras*, (-/-) and double knock-out *H-Ras* (-/-), *N-Ras*, (-/-); murine embryonic fibroblasts

We obtained as a gift from Dr. Eugenio Santos' group research [1]; knock-out *H-Ras, (-/-)* and double knock-out *H-Ras (-/-), N-Ras (-/-)* murine embryonic fibroblasts as such as their control wild type murine embryonic fibroblasts *H-Ras (+/+), N-Ras (+/+)*.

1. Dr. Santos current adscription: Centro de Investigación del Cáncer –IBMCC (CSIC-USAL) University of Salamanca, Campus Unamuno 37007, Salamanca, Spain.

C) Mutant fibroblasts obtained from tumours of Costello Syndrome patients

We obtained the cells from Dr. Sol-Church[2] group research, the following cells showing the typical mutations (G12S or G12A) found in Costello Syndrome patients, table 17.

2. Dr. Sol-Church current adscription: Biomolecular Core Laboratory. Hospital Nemours I Alfred Dupont for children. 1600 Rockland Road Wilmington, Delaware 19803.

066	Skin fibroblasts	G12S
187	Tumour fibroblasts	G12S
181	Tumour prostate fibroblasts	G12S
242	Skin fibroblasts	G12A
2529 CRL	Control fibroblasts	None

Table 17. Mutant fibroblasts analyzed of Costello Syndrome patients. Two fibroblasts cell lines which contained (G12S) mutation (designed as 066 and 187 cell lines) and one Costello fibroblast contained (G12A) mutation (242 cell line), were analyzed in our assays. Control cell line was obtained from a "normal" subject (2529 CRL cell line).

4.1.2 Bacteria

We employed two different strains for transforming bacteria, DH5α and XL-Blue.

4.1.3 Plasmids

We used two different plasmids for performing our experiments:

- pRK5 vector, was used for overexpressing transient and ectopically the mammalian proteins of our interest in *HeLa* cells
- pEGFP-C1 vector was transfected for expressing pEGFP-p19 or pEGFP-p21 proteins separately in knock-out *H-Ras* (-/-) or double knock–out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblast.

The maps of each plasmid are describe in appendix 8.1

4.1.4 Oligonucleotides

We used different oligonucleotides for performing our experiments, the first group was used for overexpressing H-Ras proteins and their mutant variants in *HeLa* cells, in these experiments the sequences are shown in table 18 and also were employeed for cloning our pRK5 vectors and for confirming the sequence of each plasmid.

A) Oligonucleotides sequences designed for overexpressing H-Ras proteins and their mutant variants in *HeLa* cells.
pRK5-p19: XBAI-P19 dir: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' p19-ECO RV-rev: 5'- CTG ACC GAT ATC TCA CAT GGG TCC CGG-3'
pRK5-p19 (G12S): XBAI-P19 dir 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' p19-ECO RV-rev : 5'- CTG ACC GAT ATC TCA CAT GGG TCC CGG-3'
pRK5-p21: XBAI-p19 dir: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' 5' CTG ACC GAT ATC TCA GGA GAG CAC ACA CTT GC-3'
pRK5-p21 (Q61L): XBAI-P21 dir:: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' 5' CTG ACC GAT ATC TCA GGA GAG CAC ACA CTT GC-3'
pRK5-p21 (G12S): XBAI-P21 forward: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' p19-ECO RV reverse : 5'- CTG ACC GAT ATC TCA CAT GGG TCC CGG-3'

Table 18. Oligonucleotides sequences designed for expressing H-Ras proteins and their mutant variants in *HeLa* cells In this table the full sequences used for overexpressing H-Ras proteins and their mutant variants in a transient and ectopically manner in *HeLa* cells are shown.

The second group of oligonucleotides was employeed for cloning our pEGFP vectors which expressed pEGFP-p19 or pEGFP-p21 in murine embryonic fibroblasts knock-out *H-Ras* (-/-) or double knock-out *H-Ras* (-/-), *N-Ras* (-/-) in a stable manner (table 19).

B) Oligonucleotides sequences used for expressing pEGFP-p19 or pEGFP-p21 proteins in knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-) , *N-Ras* (-/-) murine embryonic fibroblasts
pEGFP-p19 kpn2Ip19 forward : (5'-AAG TCC GGA ATG ACG GAA TAT AAG CTG-3') E3(p19) BamHI reverse : (5'-GGT GGA TCC TCA CG CCG GGT CTT GGC-3')
pEGFP-p21 pGFP-p21 forward : (5'-AAGTCCGGAATGACGGAATAT AAG CTG-3') pEGFP-p21 reverse : (5'CGAGGATCCTCAGGAGAGCACACACTTGCAGCT-3'

Table 19. Oligonucleotides sequences used for expressing pEGFP-p19 or pEGFP-p21 proteins in knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts.

The third set of oligonucleotides employeed for our experiments were used for detecting the G12S or G12AS mutation in the second exon of *H-Ras* gene of DNA isolated from tumours of Costello Syndrome patients table 20.

C) For detecting Costello mutations :
H-Ras Int1F2 (forward) : ACCTGTTCTGGAGGACGGTAA **H-Ras Int2R (reverse) :** CCTTAGAGGAAGCAGGAGACA

Table 20. Oligonucleotides used for amplifying exon 2 of *H-Ras*. These oligonucleotides were used in order for detecting typical Costello mutations in exon 2 of *H-R*as gene on DNA isolated from tumours of Costello Syndrome patients.

4.1.5 Antibodies

Primary antibodies used for performing western blots are shown in table 21, these antibodies were diluted in 1% BSA solution and they were detected after incubation with the appropriate HRP-conjugated secondary antibodies (table 22).

Antibody	MW (kDa)	No. catalog	Provider	Dilution	Solvent	Source
H-Ras (C20)	25	SC-520	Santa Cruz Biotechnology	1/200	milk (5%)/TBS 1X	rabbit
NM23H1 (C20)	23	SC-343	Santa Cruz Biotechnology	1/100	BSA (5%)/TBS 1X	rabbit

Table 21. List of primary antibodies used for performing western blots.

2nd antibody	source	Provider	No. Catalog	Dilution	Solvent
Antirrabit HRP	goat	Dako P0448	P0448	1/10,000	Milk (5%)/TBS 1X

Table 22. List of secondary antibodies used for performing western blots.

4.2 Methods

4.2.1 DNA methods

4.2.1.1 DNA extraction

A) Minipreps

In order to obtain the DNA insert for cloning our plasmids we used the NucleoSpin ® kit (Macherey- Nagel).

B) Maxipreps

For purifying DNA from plasmids in a high scale, we inoculated 250 ml of bacteria culture and then we used the NucleoBond ® PC 500 kit (Macherey-Nagel) optimizing the method using ghe NucleoBond ® Finalizer kit (Macherey- Nagel).

4.2.1.2 Transformation of competent cells.

Competent cells were generated starting from the strain E. coli DH5α or XL-Blue by using the RbCl transformation protocol. Competent cells were transformed using the selected vector which contained the sequence with a selection marker (Ampiciline Amp, or Kanamicine, Kn). Transformation was carried out by thermic shock: competent bacteria were incubated with the vector 10 min on ice, followed by incubation at 42º C

for 2 min and a final step at 4º C. The transformants were resuspended in 500 µl of SOC medium, and spread on a plate containing antibiotics and incubated at 37º C.

4.2.1.3 PCR (polymerase chain reaction) and general conditions

In order to obtain our PCR products, we followed the general protocol described by Ausubel in 1999, only if was necessary we changed the protocol adjusting the conditions for improving the amplification of DNA fragments the components of the reaction are shown in table 23.

Component	Volume (µl)
DNA template	**20 ng**
PCR 10x Buffer ($MgCl_2$ 2 mM)	**2.5**
dNTP's 10 mM	**0.5**
Primer dir 10 µM	**0.5**
Primer rev 10 µM	**0.5**
DNA Taq polymerase (PFU 2,5 U/µl)	**1.0**
H_2O Add volume until	**25**

Table 23. General conditions for PCR reaction

All the components were mixed and thaw them in ice meanwhile the termocycler was programmed. Table 24, shows the programme commonly used in our laboratory.

Conditions PCR cycles program	
A.Initial denaturalization: 3 min to 93º C (A) 1 min to 94º C (B)	
B. Denaturalization: 2 min to 94º C	
C.Annealing: 40 s, to specific temperature* (50º C)	
D.Extension: n min to 72º C n= 1 min	Go to B and repeat C, D, and E steps 30 times
E. Final extension: 5 min to 72º C	
F. Maintain the samples: time= infinitum 4ºC	

Table 24. PCR cycles program. (*) means that the specific temperature is specific for each pair of oligonucleotides and *n* it is the extension time (1' for each 1000 bp).

4. 2.1.4 Cloning of DNA fragments obtained by PCR

The DNA fragments obtained by PCR were purified in agarose gel (1,7%) electrophoresis, then the gel was stained with the band ethidium bromide, then the band was cutted and finally was purified using GFX ™ PCR DNA and gel purification kit (GE HEALTHCARE)®, following the instructions of the provider.

4.2.1.5 Mutant plasmids

Mutations in p19 or p21 protein sequences were performed using the QuickChange ® kit (Stratagene) following the manufacturers' advices. The mutation inserted in p19 was (G12S) and for p21 were (Q61L) or (G12S). All of these mutant sequences were cloning in pRK5 vectors which induce a strong overexpression of proteins of our interest.

4.2.1.6 DNA sequencing of fibroblasts obtained from tumours of Costello Syndrome patients

The Genomic DNA purification of cells from Costello Syndrome Patients was performanced following the protocol of Puregene, Genomic DNA purification kit protocol of Gentra Systems, catalog No. D-5002, Minneapolis, USA. For the G12S mutation screen, a discrete 575 bp region of *H-Ras* containing exon 2 was amplified using primers F1 and R1 (table 25).

Component	Volume (µl)
Q-solution	5.00
Orange Dye	2.50
10x Quiagen Buffer	2.50
10 µM Primer reverse (Int 2R) 10 µM Primer forward (1F2)	2.50 2.50
25 mM $MgCl_2$	1.50
25 mM dNTP mix	0.50
Quiagen Taq polymerase	0.15
H_2O to (25 µl)	

Table 25. PCR components for screening mutations in *H-Ras* gene of mutated fibroblasts from Costello Syndrome patients

In table 26, the PCR cycling parameters for detecting Costello mutations are shown.

WINDOW 1					
Temperature (°C)	96	59	72	6	
Time (min)	5:00	0:00	0:00	0:00	1x cycle
WINDOW 2					
Temperature (°C)	96	59	72	6	
Time (min)	0:30	0:30	1:00	0:00	40 x cycles
WINDOW 3					
Temperature (°C)	96	59	72	6	
Time (min)	0:00	0:00	10:00	0:00	1x cycle
WINDOW 4					
Temperature (°C)	96	59	72	6	
Time (min)	0:00	0:00	000	0:00	0x cycles

Table 26. *H-Ras* gene, PCR cycling parameters introduced in the robot termocycler. The robot termocycler used for these reactions was a very useful mechanic device that permitted to change the reaction plate from one reaction platform to another and this permitted to continue with the PCR reaction eliminating the time necessary for waiting until the platform reached the desire temperature for initiating the next amplification cycle. The PCR conditions of each reaction platform are represented in each window, so the robot thermocycler have 5 reactions platforms instead of only one which is the case of a traditional PCR termocyclers.

A) PCR products gel electrophoresis of Costello Syndrome patients

PCR products obtained of the PCR reaction for detecting mutations in samples of Costello Syndrome patients were purified by electrophoresis. The samples were loaded in 2% agarosa gel and running (1 h, 100 V), besides 2.5 µl of Track LT ™, 100 bp DNA ladder of Invitrogen ®, catalog No. 10488-058, was also loaded for molecular weight reference.

B) PCR purification of Costello Syndrome patients

After the electrophoresis, a band of DNA of 600 Kb approximately was detected in our agarosa gel, and then was butted and purified following the Quick purification protocol.

C) PCR of exon 2 of *H-Ras* gene

Before sequencing the exon 2 of *H-Ras* gene of samples of Costello Syndrome patients was necessary to amplify the isolated fragment by a PCR reaction using a terminator (ABI BigDye Terminator V.3.1 Chemistries). PCR components are shown in table 27 and cycling parameters are shown in table 28.

Component	Volume (µl)
Terminator chemistry (2.5 x)	1.0
5x Dilution buffer	1.5
(IF2, 3.2 M) dir primer	1.0
(2R, 3.2 M) rev primer	1.0
DNA	1.5
H_2O	5.0

Table 27. PCR components reaction

Temperature	Time	Cycles
96°C	1:00 min	
96°C 50°C 60°C	0:10 s 0:05 s 04:00 min	25
4 °C	infinite	

Table 28. Sequencing PCR cycling parameters

D) SDS treatment

The next step for detecting *H-Ras* mutations from samples of Costello Syndrome patients was to purify the Sequencing Reaction PCR, following the SDS protocol of the BigDye Terminator v 3.1 cycle sequencing kit. Briefly, we adjusted the volume of each sample until 20 µl, and then 2.2 µl of 2.2% SDS solution was added to each sample, we mixed thoroughly and then we heated to 98° C for 5 min. After this treatment the samples were cooled to room temperature before proceeding to the next step.

E) Dye Terminator Removal

In order to remove the dyes terminators utilized in the PCR reaction, we employeed the DTR Gel Filtration Cartriges, Edge Biosystems Catalog No. 42453, following the manufacturer's advice.

F) Sequencing

All the samples were analyzed on an ABI 3130XL Genetic Analyzer. All sequences were analyzed by direct observation of the electropherogram and using Sequencer 4.8 (Gene Codes Corporation, Ann Harbor MI). Costello mutations were detected in the electropherogram by directly observation.

4.2.2 RNA Methods

4.2.2.1 Isolation of small RNAs

The first method used to obtain miRNAs was the Trizol® Reagent of Invitrogen, we followed the instructions provided by the kit. MiRNA extraction was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware), miRNAs amounts recovered were not enough, and for that reason we decided to change our miRNAs extraction protocol for optimizing the method, so instead of Trizol we used miRVANA miRNA isolation protocol.

4.2.2.2 Isolation and enrichment of miRNAs

MiRNAs were extracted using miRVANA^{TM} miRNA isolation kit from Ambion Inc. (Austin, Tx). Isolation was performed as per the manufacturer's protocol. Briefly, the pellet collected was washed twice with DEPC/PBS 1x solution and then the pellet was homogenized in 400 µl in lysis/binding buffer and 1/10 miRNA homogenate additive and thawed in ice for 10 min. Small miRNAs were then isolated using the enrichment protocol for small RNAs provided with the kit. MiRNA samples were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware).

4.2.2.3 DNase treatment and removal reagents

MiRNAs samples were treated using DNAse Free ^{TM} digestion kit from Ambion in order to minimize genomic contamination.

4.2.2.4 MiRNAs quality control for Taqman PCR Real Time analyses of Costello Syndrome patients samples

The quality of miRNAs samples was analysed using an Agilent RNA 600 Nano kit Quick Start Guide, the protocol was followed as the manufacturer's advice.

4.2.2.5 Retrotranscription

cDNAs were obtained from our miRNAs samples following the protocol of reverse transcriptase Kit (AB Applied Biosystems), 100 ng of each sample was used according to the manufacturer's instructions. Conditions for RT-PCR reaction are showed in table 29.

Step type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	00	4

Table 29. RT-PCR conditions for performing cDNA for miRNAs assays.

4.2.2.6. MiRNAs Taqman PCR Real Time Assay.

A) Assay principle

The quantitative PCR Real Time (qPCR) is nowadays a powerfull DNA technique which permits not only amplified to thousand to millions fold of DNA sequence or a cDNA template, as a traditional PCR, besides this technique can measure the amount of PCR in each cycle. The number of cycles and the amount of PCR end product can theorically be used to calculated the initial quantity of genetic material because the technique use fluorescent markers that are incorporated into the PCR product. The increase in fluoresce signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reports used include double- double-stranded DNA (dsDNA) binding dyes, or dye molecules attached to PCR primers or probes that are incorporated into the product during amplification. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with scanning capability. By plotting fluorescence against the cycle number, the real time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction (fig. 15)

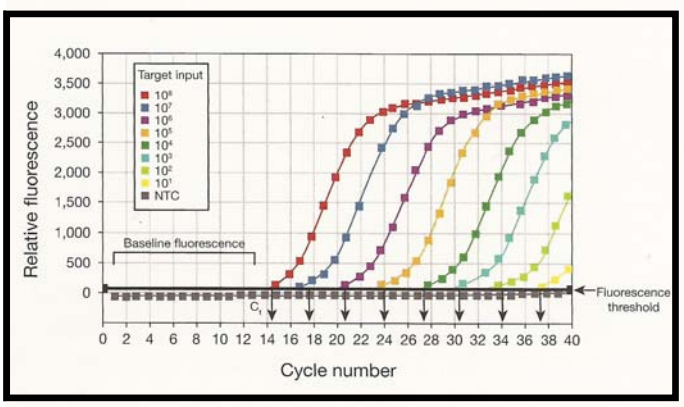


Fig. 15 Amplification plots created in miRNA PCR REAL TIME assay. Amplification plots are created when the fluorescence signal from each sample is plotted against cycle number; therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR experiment.

B) Assay protocol

MiRNAs Taqman PCR Real Time were performed using 1,33 µl of the resultant cDNA in a total volume of 20 µl; miRNAs amplifications were analyzed in a thermocycler from AB Applied ABI 7000 Real Time PCR system. All PCR reactions were run by triplicate and fold change value of each sample was calculated by ΔΔ Ct method, (Livak and Schmittgen, 2001; Pfaffl, 2001); using a gene expression control, relative to U18 or let-7 in *HeLa* cells transfections and RNHU6 for transfected murine embryonic fibroblasts lines.

The efficiency of amplification of each miRNAs provided by AB Applied Biosystem was tested by the commercial provider so it was not necessary to perform a standard amplification curve for each miRNA. The conditions for Taqman PCR Real Time Assay are shown in table 30.

Parameter	Value			
Run mode	9600 emulation (default)			
Sample volume	20 µl			
Thermal Cycling parameters	Step	AmpliTaq Gold Enzyme Activation	PCR	
		HOLD	Cycle (40 cycles)	
			Denature	Anneal/ Extend
	Time	10 min	15 s	60 s
	Temp (^{o}C)	95 min	95 s	60 s

Table 30. MiRNA Taqman thermal cycling parameters.

C) MiRNAs expression profile calculation

The method that we used to calculate the number of copies is the comparative quantification algorithms ΔΔ Ct this technique is very popular and is an improvement method on the ΔΔ Ct method. Relative mRNA abundance values were calculated according to the equation shows in fig.16, using threshold cycle (Ct) values from cuadruplicate assays as previously described (Pfaffl, 2001).

$$\frac{\text{miRNA target}}{\text{miRNA end control}} = \frac{E_{\text{target}}^{\text{Ct target}}}{E_{\text{end control}}^{\text{Ct, end control}}} \times 1000$$

Fig. 16 Formula used for calculating relative miRNA target abundance values.

PCR efficiency values for miRNA endogenous control and the miRNA target gene, *E* end control (endogenous control) and *E* target, were calculated as described (Pfaffl, 2001). Results are given in number of copies of miRNA target 1000 copies of miRNA endogenous control. The sequence of amplified PCR products was confirmed by DNA sequencing in Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). The fold change was calculated dividing the number of copies obtained of miRNA target between the number of copies obtained of miRNA transfection control (empty vector); (Navarro et al., 2009).

We have selected a group of miRNAs which have been reported that they showed low expression in different tumours, and when their normal expression is re-established in different cancer cell lines, tumorigenesis, metastasis and invasion are suppressed (see 1.5.6 section).

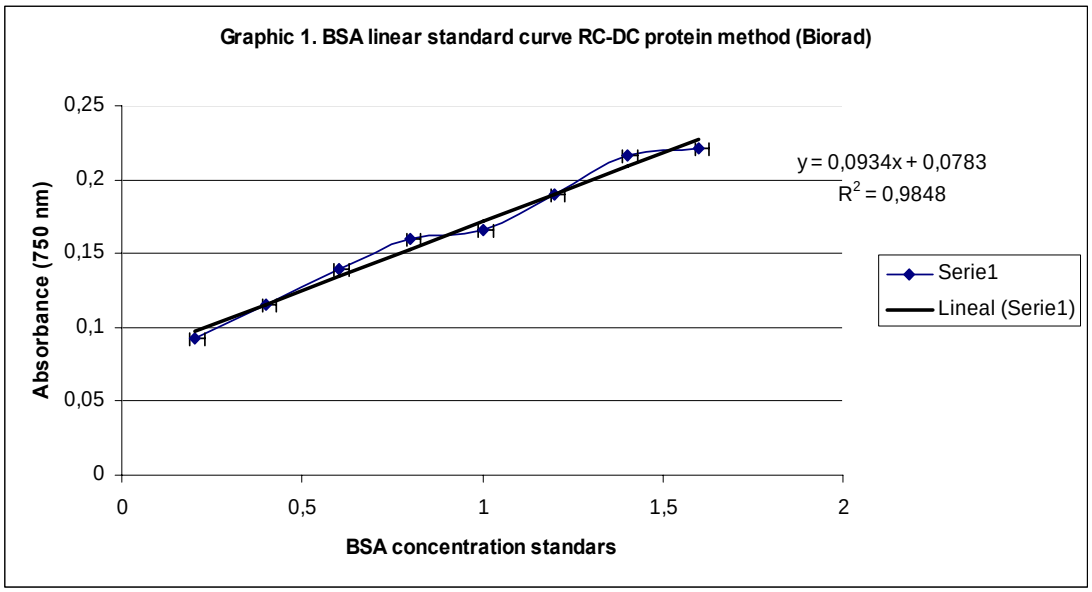
4.2.3 Protein methods

4.2.3.1 Whole cell extract

In order to performance western blots, we obtained the whole protein extract as follows, transfected cells were harvested from 25 cm^2 bottle and the pellet was resuspended in 100 µl of a NP-40 lysis buffer, then the samples were vortexed and incubated them in rotating overnight at 4^o C, 24 h later, the samples were lysated using a syringe, vortexed and centrifuged 5 min to 10, 000 rpm. The supernatant was diluted 1:1 with SDS 2X electrophoresis buffer (2%) and then immediately frozen and stored at -80^o C.

4.2.3.2 Protein determination

Protein concentrations were determined using RC DC Protein Assay kit (Bio-Rad). We followed the Microfuge Tube Assay Protocol (1.5 ml). Graphic 1 shows BSA linear standard curve, obtained for calculating the protein amount of each sample.



Graphic 1. BSA standard line RC DC protein method (Biorad).

4.2.3.3 SDS/PAGE

SDS-PAGE (polyacrylamide gel electrophoresis) is used to separate proteins based on their size. SDS-PAGE, coupled with Western blotting (immunoblotting) is typically used to determine the presence and/or relative abundance of a given protein. The gel is a cross linked polymer matrix used to support and separate the molecules its density can be controlled varying the monomer concentration. After cross linking has taken place, the samples are loaded in small wells in the gel and the assembly undergoes electrophoresis. Electrophoresis involves applying an electric current to the gel and allowing the proteins to migrate though the matrix. In order for the proteins to migrate through the gel (fig., 17), they are firstly denatured and negatively charged by exposure to a detergent such as sodium dodecyl sulphate (SDS). The amount of bound SDS is relative to the size of the protein, and proteins have a similar charge to mass ratio. Bands in different lanes separate based on the individual components sizes. A molecular weight marker that produces bands of known size is used to help identify proteins of interest.

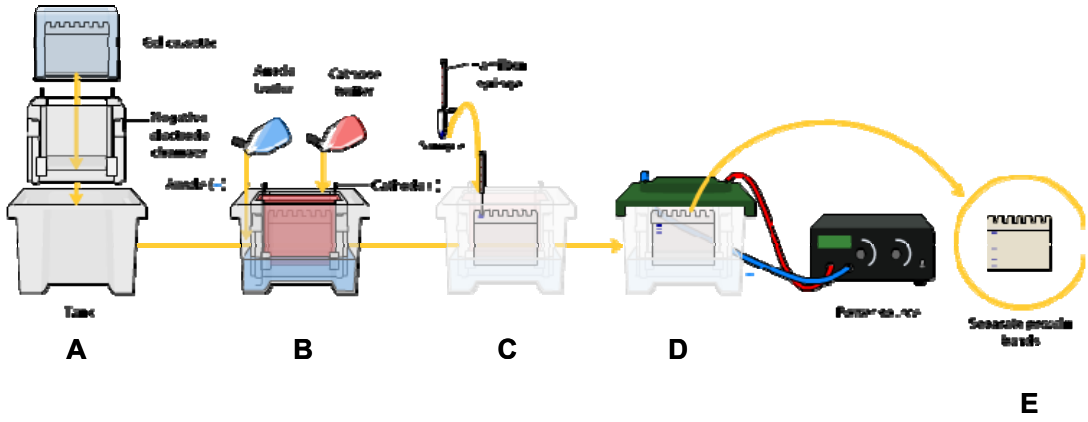


Fig. 17 SDS-PAGE. A) Gel cassette contains the acrylamide gel stocked into two glasses, then the gel cassette is put it into the electrophoresis chamber and then into the tank. **B)** The tank is filled with the anode and catode buffer. **C)** The samples and the weight molecular markers are loaded into the gel with a Hamilton syringe. **D)** An electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive (+) electrode (anode). **E)** Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty (they encounter more resistance farther down the gel, while larger ones will have remained closer to the point of origin).

For performing electrophoresis assays, 100 µg of each protein extract was running into a 12.5% SDS PAGE. In stocking phase of the gel the samples were running at 50 V, then when samples reached the separating gel, voltage was increased to 100 v. After electrophoresis, the samples were transferred into a nitrocellulose membrane, for performing western blot analysis.

4.2.3.4 Western blot analysis

A) Assay principle

Western blot assay is also called protein immunoblot, and is a widely analytical technique used for detecting specific proteins in a sample of tissue homogenate or extract. The first step of this technique is using a gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), which is placed on top of the gel, and a stack of filter papers placed on top of that (fig. 18) The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.

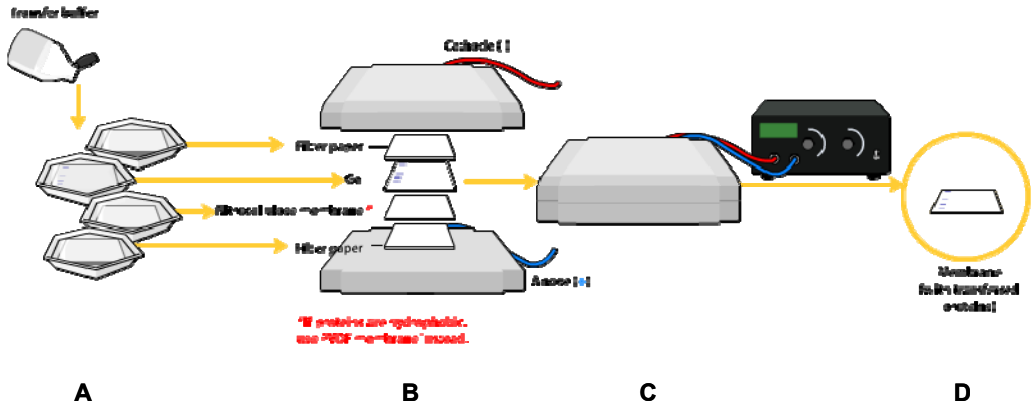


Fig. 18. Western blot transfer. A) Filter paper, gel, and nitrocellulose membrane are damped with transfer buffer. **B)** The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. **C)** The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. **D)** As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Ponceau S dye.

Once the proteins are transferred into the membrane, blocking is the next step and its necessary for avoying non-specific binding, and it can be achived by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS) 1X, with some percentage of detergent such as Tween 20 or Triton X-100.

Membrane is incubated overnight with the primary antibody, when the period of incubation had finished the membrane is rinsed to remove unbound primary antibody, then the membrane is exposed to a second antibody, directed at a species-specific porcion of the primary antibody. This second antibody is usually linked to biotin or other reporter enzyme such as alkaline phosphatase or horseradish peroxidase. In our experiments, we used a horseradish peroxidase-linked secondary antibody to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. Chemiluminiscence can be detected using a photographic film or and more recently by CCD cameras which capture a digital image of the Western blot. The image is then analyzed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density.

B) Assay protocol

The SDS-PAGE gel was transferred onto Protan ® Nitrocellulose Transfer Membrane (Whatman). Membranes were blocked with 5% non fat milk or 1% BSA then incubated

overnight with primary antibodies (table 21). Membranes were washed three times with 5 ml of TBS/ 10% tween and then incubated with secondary antibodies (table 22).

B) Chemiluminiscence detection

Finally the chemiluminescences of the bands were revealed using the Super Signal ® West Femto Mazimun Sensivity Substrate (Thermo Scientific). Band densities were measured using LAS-3000 luminescent image analyzer with Multi Gauge software version 3.0 (Fuji Photo Film, Japan).

4.2.4 Cell culture methods

4.2.4.1 Cell culture

All the cell lines evaluated in this PhD thesis were seeded in the desire density for each assay with DMEM complete (DMEM+ Foetal Bovine Serum (FBS 1%) + antibiotics (Penicillin+ Streptomycin, 0.1%). When the cell lines were transfected DMEM without FBS and antibiotics was employed. For stable transfections we used DMEM + FBS (1%)+ antibiotics+ selective antibiotic (for stable transfection pEGFP-C1 vector was used and cells were transfected with pEGFP-C1 used as a control transfection; pEGFP-p19 or pEGFP-p21. All the cell lines transfected with pEGFP vectors were previously selected adding geneticine G418 (1000 μg/ml DMEM complete) to the medium cell culture per a month.

4.2.4.2 Tripsinization of adherent cells

For detaching our transfected cells, first old media was removed, and the monolayer cells were rinse one time with PBS 1X , later 1.0 ml of 0.25% of trypsin /EDTA (w/v) solution was added to cell culture plates and then they were incubated for ~3 min, after that period of time flask was checked under inverted microscope to make sure that cells were lifting off of the plate. When a single cell suspension had been obtained, 10 times volume of complete media was added into the trypsin solution. The cell suspension was transferred to a sterile 15 ml conical centrifuge tube. Cells were centrifuged at 1,500 rpm for 3 min in the tabletop centrifuge, the supernatant was discarded carefully. The cell pellet was resuspended in 10 ml of fresh complete media and collects 50 μl to count cell number. Once cells/ml solution was counted, seed cells into new flask or cell dishes cultures to get a final concentration that you desire (table 23).

	Surface Area (mm^2)	Seeding Density X10 ^{6}	Cells at confluence	Versene (ml, of 0.53 mM, EDTA)	Trypsin (ml of 0.05% trypsin, 0.53 mM EDTA)	Growth medium (ml)
Dishes						
35 mm	962	0.3	1.2	1	1	2
60mm	2827	0.8	3.2	3	2	3
100 mm	7854	2.2	8.8	5	3	10
150 mm	17671	5.0	20.0	10	8	20
Cluster Plates						
6-well	962	0.3	1.2	2	2	3-5
12-well	401	0.1	0.4	1	1	1-2
24-well	200	0.05	0.2	0.5	0.5	0.5-1.0
Flasks						
T-25	2500	0.7	2.8	3	3	3-5
T-75	7500	2.1	8.4	5	5	8-15
T-160	16000	4.6	18.4	10	10	15-30

Table 31. Useful number for cell culture. The number of cells on a confluent plate, dish, or flask will vary with cell type. For this table, *HeLa* cells were used. Reference: http://tools.invitrogen.com/content/sfs/appendix/Cell_Culture/cell%20culture%20useful%20numbers.pdf

4.2.4.3 Cells cryopreservation

The confluent cells were harvested and rinsed with PBS 1x twice, then the pellet was resuspended in 10% DMSO/90 % FBS cold solution to obtain a suspension of $1x10^6$ cells/ml, dissolve the pellet and transfer 1 ml of cell suspension in cryovials immediately and simultaneously keep the vials in mini-cooler unit or ice bath. Finally transfer cryovials into -80^{o}C freezer for four days and then transfer the cryovials in an appropriate rack of liquid nitrogen and record the position of samples in your register.

4.2.4.4 Thawing cells

Cryovials were kept in 37^{o}C waterbath or warmed them between the hands, until just small ice-clumps remains. Cell suspension was transfer in a labelled 10 cm culture dishes using a sterile pipette. Here cell suspension should be mixed well by sucking and dispensing by pipette to breakdown the clumps of the cells. The suspension was diluted in a 10 ml of DMEM media. Then allow to growth in CO_2 incubator/37^{o}C.

4.2.4.5 Counting cells (Neubauer chamber method)

50 µl of the cell suspension were transferred into a 1.5 ml centrifuge tube, 50 µl of trypan blue ($C_{34}H_{28}N_6O_{14}S_4$) solution was added to the cell suspension (1:2 dilution factor) in the centrifuge tube and the cell suspension was resuspending using micropipette. With a cover-slip in place, use 200 µl micropipette and transfer 25 µl of the trypan blue-cell suspension to a chamber on the hemocytometer. Only viable cells

were counted in the neubauer chamber (4 squares of the chamber). The total number of cells will be determined using the following calculations:

Cells per ml = (the average count per square) (the dilution factor) ($1x10^4$)

Total cell number = cells per ml x the original volume of fluid from which cell sample was removed.

Reference: Sigma Catalog, 1989, commonly used tissue culture techniques, p. 1492.

4.2.4.6 Cell transfections

A) *In vitro* DNA transient transfection using Lipofectamine™ and PLUS ™ reagent of Invitrogen.

Day 01. One day before the transfection, 10 x 10^5 murine embryonic fibroblast knock-out *H-Ras* (-/-) or double knock-out *H-Ras* (-/-) and *N-Ras* (-/-) were seeded in a 25 cm^2 t-flask.

Day 02. Two micrograms of DNA were diluted in 250 µl of DMEM without antibiotics, then this dilution was mixed with 8 µl of plus reagent and 12 µl of lipofectamine. The total volume was added to the cultures and they were incubated during 5 h at 37°C; 5% CO_2 incubator. When the time of incubation had finished, the medium was replaced with DMEM (FBS 1% + antibiotics), 24 h postransfection, other protocols can be followed.

B) *In vitro* DNA transient and stable transfections using Jet Pei ™ of Polyplus ®

Day 01. One day before the transfection, 10 x 10^5 murine embryonic fibroblasts knock-out *H-Ras* (-/-), or double knock-out *H-Ras* (-/-), *N-Ras* (-/-) were seeded in a 25 cm^2 T-flask.

Day 02. Two micrograms of DNA were diluted in 90 µl of 150 mM NaCl and 5 µl of Jet Pei, following the instructions of the provider. Transfections were incubated for 1 h at 37°C and 5%/ CO_2. When the transfection time had finished, the cell culture media which contained the complexes was removed and replaced with complete DMEM (FBS 1%+ antibiotics). After 24 h, selection process was initiated adding to the media geneticine (G418), (1000 µg/ml), per a month.

4.2.5 Methods for analyzing transfected cell lines

4.2.5.1 Proliferation cell assays

A) Assay principle

Cancer cells acquired abilities for proliferating without limits and this hallmark is necessary for tumour progression and metastasis development, for this reason we were interested in knowing the proliferation rate of our stable double knock-out *H-Ras* (-/-) and *N-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-p19 or pEGFP-p21.

B) Assay protocols

Neubauer chamber proliferation assay

Cells from each transfection or lineage were seeded ($1x10^4$ cells per well) in 6 well plates with 10% FBS, DMEM. After 24 h, medium was removed by harvesting cells treated with 0.4 ml of 0.25% trypsin/EDTA for 3 min, and followed by addition of 0.6 ml of DMEM complete. Each cell line was resuspended and counted with trypan blue each day during 6 days and for each cell line. All treatments were performed in triplicate in at least three independent experiments.

Cyquant proliferation assay

Cell proliferation rate was also evaluated using Cyquant assay. This assay measures the DNA content of cells using fluorescent indicators. Then $5x10^2$ cells of each transfected cell line (pRK5-p19, pRK5 p19 (G12S), pRK5- p21, pRK5-p21 (QL61) and pRK5-p21 (G12S)), were seeded in 96 well plates. Six wells per cell line were seeded into 96 well plates; proliferation assays were repeated three times in a independent way. Cyquant working solution (200 µl) was added to each well, then the wells were incubated at 37°C 1 h, and finally the absorbance of each well was measure in an Victor ^{3} *(PerkinElmer Wallac, Turku, Finland)* spectrophotometer or in a spectra Max5 spectrophotometer from Bionova Cientifica, Molecular Advices (excitation 485 nm, emission 528 nm). Fluorescence was expressed as relative fluorescent units (RFU).

4.2.5.2 Clonogenic anchorage agar assays

A) Assay principle

Clonogenic anchorage assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is formed for a least 50 cells. This assay evaluates the potential of each cell in the population for its ability to undergo "unlimited" division, (Franken et al., 2006).

B) Preparation of reagents and material

To initiate the colony growth in soft agar assays of each transfected cell line, firstly was necessary to prepare an agar stock of 5% that will be used for preparing the lower and the upper phases as follows: 5 g of agar (catalog No. 18300012, Invitrogen) were resuspended in 100 ml of miliQ water, and then suspension was sterilized and stored at room temperature in canonical flasks.

C) Assay protocol

The day of the experiment, aliquots of agar were melted and kept in water bath at 46^{o}C, when agar aliquots were diluted with a mixture of one part of the melted 5% agar (3 ml) mixed with 27 ml of DMEM complete was prepared. Then, for preparing the base layer, 600 µl of diluted agar was added to each well giving a final concentration of (0.5%) agar, and then plates were kept at room temperature under sterile conditions in a hook until the gel became solid. The upper layer was prepared diluting 5 ml of the base layer with 3 ml of a suspension cell containing 1x10^{4} cells/ml; 600 µl of this mix was added in each well upon the base layer; then the plates were incubated in a humidified CO_2 incubator at 37^{o}C, and supplemented with 300µl of serum-supplemented media on a top of base layer. After 4 weeks, the cells were fixed with 0.4 ml of glutaraldehyde 0.5% /PBS 1X, during 10 min and stained with 0.025% crystal violet in 20% methanol/PBS 1X, the samples were washed several times with PBS 1X in order to eliminate the crystal violet residues.

D) Evaluation of clonogenic anchorage agar assay

The number of colonies formed in each assay was calculated by direct observation in a DP71 Olympus Magnifying glass (10X objective). Each experiment was done at least twice in triplicates

4.2.5.3 Invasion assays

A) Assay principle

This assay determinates the capacity of invasion of the cell; this means their capacity of metastasis. The malignant cell has the capacity of detachment, migrating and forming distant colonies in new tissues. We can measure the capacity of invasion *in vitro* using disposable permeable supports, called inserts which provides a relatively simple *in vitro* approach to performing chemotaxis and cell invasion assays. Common barriers employed for invasion assays include collagen, fibronectin and lamining coatings as well as more complex extracellular or basement membrane extracts (BME). More elaborative invasion assays establish a monolayer of endothelial cells on the permeable support in place of or in addition to, the protein coatings or BME listed above.

Cell suspension was diluted in DMEM without FBS, then placed in the top of chamber, and incubated in the presence of test media containing specific chemo attractants in the bottom chamber (in this assay we used DMEM+ 10% FBS). Cells migrate from the top chamber through the coated filter pores to the bottom of the filter. Cell dissociation/Calcein AM solution was placed in the bottom chamber to dissociate the migrating cells from the filter and add a fluorescent label. Fluorescence in the bottom chamber is proportional to the number of migrating cells, (fig. 19).

B) Assay protocol

Invasion assays were done using InnoCyte TM Cell Invasion Assay Kit (24 well) of Calbiochem (No. catalog QIA-129) following the protocol of the provider. Briefly, 0.5 $x10^6$ cells/ml cell suspension was prepared for each cell line in serum-free medium, 350 µl of cell suspension was added to the upper chamber of each insert, then the cells were incubated at 37°C, 5% CO_2 during 48 h. After that time the cells were stained with 500 µl of cell staining solution. The fluorescence was measure at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence was expressed as relative fluorescent units (RFU).

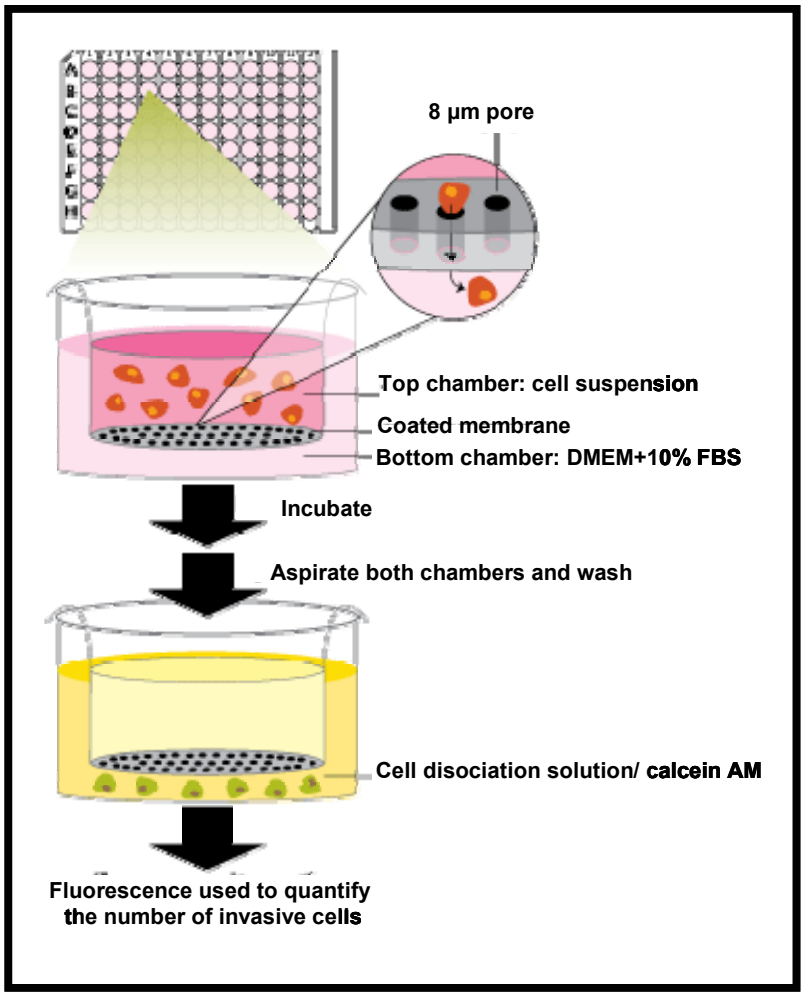


Fig. 19 Cell Invasion Assay. The invasion chamber consists of two chambers separated by a filter coated with BME or different ECM components. Cells were seeded on the top chamber, and then those cells that have the ability to migrate can pass through the filter of the insert and will be settled in the bottom chamber.

4.2.5.4 Reactive Oxygen Species Assay (ROS)

A) Assay Principle

The assay employs the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA). In brief, DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2', 7'-Dichlorodihydrofluorescein (DCF) by ROS (fig. 20). The fluorescence intensity is proportional to the ROS levels within the cell cytosol. The effect of antioxidant or free radical compounds on DCF-DA can be measured against the fluorescence of the provided DCF standard (Xia et al., 2007).

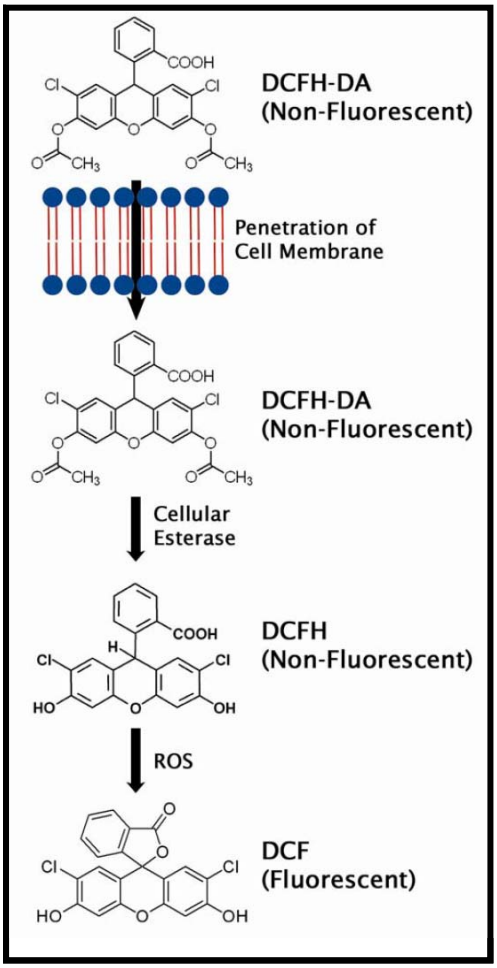


Fig. 20 Mechanism of DCF for detecting ROS species

B) Preparation of reagents

Three solutions are necessary for performing the protocol of ROS assay and they are prepared as follows:

PBS-Glucose (10 Mm) buffer

Dilute 1:10 glucose (Ca^{+2}, Mg^{+2}) stock 10x, add 1.98 g of glucose to 1 l of PBS-Buffer fill up to 950 ml and adjust pH to 7.1 using NaOH (10 N) solution to fill up to 1 l.

Preparation of dye (H2DCF-DA)

Prepare a 1 mM stock solution with ethanol (100%); 4.87 mg/10 ml ethanol (store at -20^{o}C for a month). Dilute stock solution 1:1000 in PBS-Glu (Mg^{+2}, Ca^{+2}) to 1 µM final concentration, e.g. 10 µl dye + 9990 µl PBS-Glu. Approximately 10 ml of this reagent is necessary for each 96-well plate.

Positive control (SIN 1)

SIN 1 working solution always has to be prepared fresh at a 5 µM final concentration. Firstly, SIN 1 stock has to be prepared as follows: 1.0 mg of SIN 1 reagent was diluted in 5 ml of miliQ water, then working solution is prepared diluting 5 µl of 1 mM stock of SIN 1 in 995 ml of PBS- Glucose 1x buffer.

C) Assay protocol

Day 01

Transient transfected *HeLa* cells were seeded (1x10 ^{5} cells per well) in a 96 wells plate (5 wells per cell line), then plates were incubated 24 h at 37^{o}C, 5% CO_2.

Day 02

Transfected cells, were loading with dye (H2DCF-DA; 100 µl /well (1 µM final) dilute it with PBS with Ca^{+2} and Mg^{+2} and 10 mM Glucose; PBS-Glucose; p.H. 7.1) for 30 min. Thereafter the cells were rinsing once with PBS 1x and add PBS-Glucose (100 µl), for performing the positive control instead of PBS-glucose add SIN 1 solution (5 µM, 100 µl /well). The plates then were incubated at 37^{o}C, 5% CO_2. The fluorescence was measured after 30 and 90 min at excitation 485 nm and emission 528 nm. The spectrophotometer used was spectra Max model 5 of Bionova Cientific, Molecular Advices (Scherz-Shouval et al., 2007).

D) Calculation of percentage of ROS production

Fluorescence was expressed as relative fluorescent units; the percentage or ROS emission was calculated using as a reference of 100% the ROS emission detected in our *Hela* cells without transfection.

4.2.5.5 Cytometry analysis

A) Assay principle

Flow cytometry is a laser-based technology that is used to measure characteristics of biological materials. It offers a rapid, objective, and quantitative method for analysis and purification of cells in suspension. Cells interact with a light beam as they pass by single file in a liquid stream; interaction with light is generally measured as light scatter and fluorescence according to staining of the cells. If a fluorochrome is specifically and stoichiometrically bound to a cellular component, the fluorescence intensity will ideally represent the amount of that particular component, (Jaroszeski and Radcliff, 1999).

B) Assay protocols

Propidium iodide cell cycle analyses.

The cells were seeded into 10 cm cell culture dishes to avoid the inhibition of their cell cycle when they are confluent. Each cell line was seeded ($1x10^{6}$ cells) per triplicate. Then 24 h later cell cultures were transfected with Lipofectamine TM method using the plasmids of our interest. After 24 h postransfection we collected the medium of each sample and centrifuged them (this procedure avoids to loose the cells in mitosis), later cells were harvested, and the pellet was collected in the same tube and washed twice with 5 ml of 1% BSA/ EDTA 10 mM/PBS 1X cold, then the pelled was resuspended in 900 µl of PBS 1X cold and 2.1 ml of absolute ethanol were added drop by drop and vortexing at the same time in order to obtain a final concentration of ethanol 70%. The ethanol cellular suspension was fixed at least two hours. The day of cytometry analysis the fixed samples were centrifugated, and washed twice with 5 ml with 1% BSA/ EDTA 10 mM/PBS 1X, then we added 2.5 µl de RNAse (1mg/ml) and 25 µl of propidium iodide (1 mg/ml) were added, the samples were incubated 30 min at room temperature before doing the cytometry analysis. The samples were analysed in an Epics XL Beckman Coulter Cytometer.

Analysis of DNA content and green fluorescence protein expression

$1x10^{6}$ *HeLa* cells were seeded in 10 mm cell dish and they were transfected following the Jet pei transfection protocol described previously in section 3.2.4.6. Stable cell lines were just counted and seeded, 24 h later, the medium of each sample was collected and and centrifuged (this procedure avoids to loose the cells in mitosis), later we trypsinized the cells, and the pellet was collected in the same tube and washed twice with 1% BSA/

EDTA 10 mM/PBS 1X in cold, then the pellet was resuspended in 500 µl of cold paraphormaldehide 2%, pH 7.2 and incubated at 4°C during 1 h. When the incubation was finished the samples were washed one time with cold 1% BSA/EDTA 10 mM/ PBS 1X, then the samples were centrifuged and resuspended in 900 ml of cold PBS 1X and 2.1 mL of ethanol absolute were added, drop by drop and vortexing to obtain a final concentration of ethanol 70%. The ethanol cellular suspension was fixed at least 2 h. The day of cytometry analysis the fixed samples were centrifuged, and washed twice with 5 ml with 1% BSA/ EDTA 10 mM/PBS 1X, then 2.5 µl of RNAse (1 mg/ml) plus, and 25 µl of propidium iodide (1 mg/ml) were added; later samples were incubated 30 min at room temperature before performing the cytometry analysis. The cells were analyzed into XL cytometer.

Iodide propidium cell cycle analyses of green fluorescent protein (GFP) previous separation by Moflo Dako cytometer.

$5x10^6$ *HeLa* cells were cotransfected using pEGFP-C1/pRK5-C1, pEGFP-C1/pRK5-p19, or pEGFP-C1/pRK5-p19 (G12S) vectors with Jet Pei reagent as described recently in section 4.2.4.6. 24 h postransfection transfected cells were separated using a Moflo Dako cytometer (laser of 488 nm excitation and 530 nm emission; 6 psi, 70 micras Tip), in two different pools: GFP (+) and GFP (-); both fractions were fixed immediately in 5 ml of 70% ethanol and rinse twice in PBS 1X, then the fractions were stained with working solution iodide propidium (IP) and incubated for 24 h, 4°C; the next day stained GFP fractions were analyzed in an Epics XL Beckman Coulter cytometer.

C) Cytometry analyses results

The results of these analyses showed a graphic which represents the percent of cells in each phase of the cell cycle.

4.2.6 Statistical Analyses

Statistical analysis was performed using Mann-Whitney U test (non parametric test, two tailed), $p<0.05$ was considered statistically significant. Each experiment was performed at least 3 times and individual samples were run by triplicate.

In some cases, we employeed an ANOVA (one way analysis of variance) and a Bartlett's test for equal variances; in both cases $p<0.05$ was considered statistically significant.

III. AIMS

3.1 GENERAL AIM

To determinate if p19 c-H-Ras protein has an antiproliferative and antimetastatic role in cancer process.

3.2 SPECIFIC AIMS

1. To obtain transient *HeLa* cell lines, which overexpress p19, p21 or their mutant variant proteins separately.

2. To obtain stable knock-out *H-Ras (-/-)* and double knock-out *H-Ras (-/-), N-Ras,(-/-)* murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21 proteins.

3. To analyse miRNAs global expression profile in knock-out *H-Ras (-/-)* murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21.

4. To analyze miRNAs expression profile in transfected *HeLa* cells overexpressing p19, p21 or their mutant variant proteins.

5. To evaluate the invasion capacity of p19, p21 and their mutant proteins in transient and stable transfected cell lines and in mutant fibroblasts obtained from tumours of Costello Syndrome patients.

6. To determinate proliferation rates of p19, and p21 proteins in stable pEGFP-p19 and pEGFP-p21 double knock-out *H-Ras (-/-), N-Ras,(-/-)* murine embryonic fibroblasts.

7. To analyse the cell cycle induced by p19, p21 or their mutant variants proteins in transient transfected *HeLa* cell lines and in stable knock-out *H-Ras (-/-), N-Ras,(-/-)* murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21.

8. To evaluate the capacity of forming colonies of p19, p21 or their mutant variants proteins in our transient transfected *HeLa* cell lines or in stable knock-out *H-Ras (-/-)* and *double knock-out H-Ras (-/-), N-Ras,(-/-)* murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21.

9. To evaluate the Reactive Oxygen Species (ROS assays) production, when p19, p21 and their mutant proteins are overexpressed in transient transfected *HeLa* cell lines

10. To determinate if NM23H1 protein expression confers protection against ROS emission when p19 H-Ras is overexpressed.

IV. MATERIAL AND METHODS

4.1 Biologic Material

4.1.1 Cell cultures

A) *HeLa* cells

HeLa cell line was the first human epithelial cancer cell line established in long term culture in 1952. It was derived from adenocarcinoma of the cervix. *HeLa* cells are hypermetriploid (3n+) containing a total number of 76-80 chromosomes and 22-25 abnormal chromosomes per cell. *HeLa* proliferate abnormal and rapidly and contein multiple copies of integrated HPV 18, which contributes to p53 degradation and apoptosis evasion (MacVille et al., 1999).

Adherent *HeLa* cells were used in some experiments and cultured in standard conditions as detail in Guil et al., 2003a; and Guil et al., 2003b. This cell line was bought to European Collection of cell cultures (Catalogue No. 93021013; fig. 14).

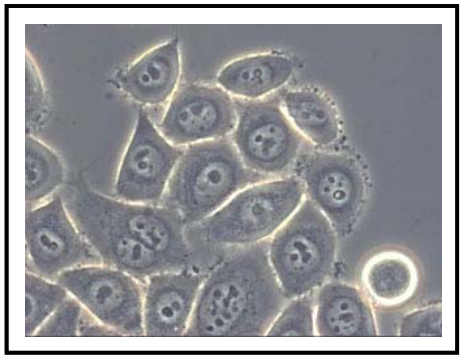


Fig. 14 *HeLa* cells.

B) Knock-out *H-Ras*, (-/-) and double knock-out *H-Ras* (-/-), *N-Ras*, (-/-); murine embryonic fibroblasts

We obtained as a gift from Dr. Eugenio Santos' group research [1]; knock-out *H-Ras, (-/-)* and double knock-out *H-Ras (-/-), N-Ras (-/-)* murine embryonic fibroblasts as such as their control wild type murine embryonic fibroblasts *H-Ras (+/+), N-Ras (+/+)*.

1. Dr. Santos current adscription: Centro de Investigación del Cáncer –IBMCC (CSIC-USAL) University of Salamanca, Campus Unamuno 37007, Salamanca, Spain.

C) Mutant fibroblasts obtained from tumours of Costello Syndrome patients

We obtained the cells from Dr. Sol-Church[2] group research, the following cells showing the typical mutations (G12S or G12A) found in Costello Syndrome patients, table 17.

2. Dr. Sol-Church current adscription: Biomolecular Core Laboratory. Hospital Nemours I Alfred Dupont for children. 1600 Rockland Road Wilmington, Delaware 19803.

066	Skin fibroblasts	G12S
187	Tumour fibroblasts	G12S
181	Tumour prostate fibroblasts	G12S
242	Skin fibroblasts	G12A
2529 CRL	Control fibroblasts	None

Table 17. Mutant fibroblasts analyzed of Costello Syndrome patients. Two fibroblasts cell lines which contained (G12S) mutation (designed as 066 and 187 cell lines) and one Costello fibroblast contained (G12A) mutation (242 cell line), were analyzed in our assays. Control cell line was obtained from a "normal" subject (2529 CRL cell line).

4.1.2 Bacteria

We employed two different strains for transforming bacteria, DH5α and XL-Blue.

4.1.3 Plasmids

We used two different plasmids for performing our experiments:

- pRK5 vector, was used for overexpressing transient and ectopically the mammalian proteins of our interest in *HeLa* cells
- pEGFP-C1 vector was transfected for expressing pEGFP-p19 or pEGFP-p21 proteins separately in knock-out *H-Ras* (-/-) or double knock–out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblast.

The maps of each plasmid are describe in appendix 8.1

4.1.4 Oligonucleotides

We used different oligonucleotides for performing our experiments, the first group was used for overexpressing H-Ras proteins and their mutant variants in *HeLa* cells, in these experiments the sequences are shown in table 18 and also were employeed for cloning our pRK5 vectors and for confirming the sequence of each plasmid.

A) Oligonucleotides sequences designed for overexpressing H-Ras proteins and their mutant variants in *HeLa* cells.
pRK5-p19: XBAI-P19 dir: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' p19-ECO RV-rev: 5'- CTG ACC GAT ATC TCA CAT GGG TCC CGG-3'
pRK5-p19 (G12S): XBAI-P19 dir 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' p19-ECO RV-rev : 5'- CTG ACC GAT ATC TCA CAT GGG TCC CGG-3'
pRK5-p21: XBAI-p19 dir: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' 5' CTG ACC GAT ATC TCA GGA GAG CAC ACA CTT GC-3'
pRK5-p21 (Q61L): XBAI-P21 dir:: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' 5' CTG ACC GAT ATC TCA GGA GAG CAC ACA CTT GC-3'
pRK5-p21 (G12S): XBAI-P21 forward: 5-TCC TAG TCT AGA ATC ACG GAA TAT AAG GTG GTG -3' p19-ECO RV reverse : 5'- CTG ACC GAT ATC TCA CAT GGG TCC CGG-3'

Table 18. Oligonucleotides sequences designed for expressing H-Ras proteins and their mutant variants in *HeLa* cells In this table the full sequences used for overexpressing H-Ras proteins and their mutant variants in a transient and ectopically manner in *HeLa* cells are shown.

The second group of oligonucleotides was employeed for cloning our pEGFP vectors which expressed pEGFP-p19 or pEGFP-p21 in murine embryonic fibroblasts knock-out *H-Ras* (-/-) or double knock-out *H-Ras* (-/-), *N-Ras* (-/-) in a stable manner (table 19).

B) Oligonucleotides sequences used for expressing pEGFP-p19 or pEGFP-p21 proteins in knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-) , *N-Ras* (-/-) murine embryonic fibroblasts
pEGFP-p19 kpn2Ip19 forward : (5'-AAG TCC GGA ATG ACG GAA TAT AAG CTG-3') E3(p19) BamHI reverse : (5'-GGT GGA TCC TCA CG CCG GGT CTT GGC-3')
pEGFP-p21 pGFP-p21 forward : (5'-AAGTCCGGAATGACGGAATAT AAG CTG-3') pEGFP-p21 reverse : (5'CGAGGATCCTCAGGAGAGCACACACTTGCAGCT-3'

Table 19. Oligonucleotides sequences used for expressing pEGFP-p19 or pEGFP-p21 proteins in knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts.

The third set of oligonucleotides employeed for our experiments were used for detecting the G12S or G12AS mutation in the second exon of *H-Ras* gene of DNA isolated from tumours of Costello Syndrome patients table 20.

C) For detecting Costello mutations :
H-Ras Int1F2 (forward) : ACCTGTTCTGGAGGACGGTAA **H-Ras Int2R (reverse) :** CCTTAGAGGAAGCAGGAGACA

Table 20. Oligonucleotides used for amplifying exon 2 of *H-Ras*. These oligonucleotides were used in order for detecting typical Costello mutations in exon 2 of *H-R*as gene on DNA isolated from tumours of Costello Syndrome patients.

4.1.5 Antibodies

Primary antibodies used for performing western blots are shown in table 21, these antibodies were diluted in 1% BSA solution and they were detected after incubation with the appropriate HRP-conjugated secondary antibodies (table 22).

Antibody	MW (kDa)	No. catalog	Provider	Dilution	Solvent	Source
H-Ras (C20)	25	SC-520	Santa Cruz Biotechnology	1/200	milk (5%)/TBS 1X	rabbit
NM23H1 (C20)	23	SC-343	Santa Cruz Biotechnology	1/100	BSA (5%)/TBS 1X	rabbit

Table 21. List of primary antibodies used for performing western blots.

2nd antibody	source	Provider	No. Catalog	Dilution	Solvent
Antirrabit HRP	goat	Dako P0448	P0448	1/10,000	Milk (5%)/TBS 1X

Table 22. List of secondary antibodies used for performing western blots.

4.2 Methods

4.2.1 DNA methods

4.2.1.1 DNA extraction

A) Minipreps

In order to obtain the DNA insert for cloning our plasmids we used the NucleoSpin ® kit (Macherey- Nagel).

B) Maxipreps

For purifying DNA from plasmids in a high scale, we inoculated 250 ml of bacteria culture and then we used the NucleoBond ® PC 500 kit (Macherey-Nagel) optimizing the method using ghe NucleoBond ® Finalizer kit (Macherey- Nagel).

4.2.1.2 Transformation of competent cells.

Competent cells were generated starting from the strain E. coli DH5α or XL-Blue by using the RbCl transformation protocol. Competent cells were transformed using the selected vector which contained the sequence with a selection marker (Ampiciline Amp, or Kanamicine, Kn). Transformation was carried out by thermic shock: competent bacteria were incubated with the vector 10 min on ice, followed by incubation at 42º C

for 2 min and a final step at 4º C. The transformants were resuspended in 500 µl of SOC medium, and spread on a plate containing antibiotics and incubated at 37º C.

4.2.1.3 PCR (polymerase chain reaction) and general conditions

In order to obtain our PCR products, we followed the general protocol described by Ausubel in 1999, only if was necessary we changed the protocol adjusting the conditions for improving the amplification of DNA fragments the components of the reaction are shown in table 23.

Component	Volume (µl)
DNA template	**20 ng**
PCR 10x Buffer ($MgCl_2$ 2 mM)	**2.5**
dNTP's 10 mM	**0.5**
Primer dir 10 µM	**0.5**
Primer rev 10 µM	**0.5**
DNA Taq polymerase (PFU 2,5 U/µl)	**1.0**
H_2O Add volume until	**25**

Table 23. General conditions for PCR reaction

All the components were mixed and thaw them in ice meanwhile the termocycler was programmed. Table 24, shows the programme commonly used in our laboratory.

Conditions PCR cycles program	
A.Initial denaturalization: 3 min to 93º C (A) 1 min to 94º C (B)	
B. Denaturalization: 2 min to 94º C	
C.Annealing: 40 s, to specific temperature* (50º C)	
D.Extension: n min to 72º C n= 1 min	Go to B and repeat C, D, and E steps 30 times
E. Final extension: 5 min to 72º C	
F. Maintain the samples: time= infinitum 4ºC	

Table 24. PCR cycles program. (*) means that the specific temperature is specific for each pair of oligonucleotides and *n* it is the extension time (1' for each 1000 bp).

4. 2.1.4 Cloning of DNA fragments obtained by PCR

The DNA fragments obtained by PCR were purified in agarose gel (1,7%) electrophoresis, then the gel was stained with the band ethidium bromide, then the band was cutted and finally was purified using GFX ™ PCR DNA and gel purification kit (GE HEALTHCARE)®, following the instructions of the provider.

4.2.1.5 Mutant plasmids

Mutations in p19 or p21 protein sequences were performed using the QuickChange ® kit (Stratagene) following the manufacturers' advices. The mutation inserted in p19 was (G12S) and for p21 were (Q61L) or (G12S). All of these mutant sequences were cloning in pRK5 vectors which induce a strong overexpression of proteins of our interest.

4.2.1.6 DNA sequencing of fibroblasts obtained from tumours of Costello Syndrome patients

The Genomic DNA purification of cells from Costello Syndrome Patients was performanced following the protocol of Puregene, Genomic DNA purification kit protocol of Gentra Systems, catalog No. D-5002, Minneapolis, USA. For the G12S mutation screen, a discrete 575 bp region of *H-Ras* containing exon 2 was amplified using primers F1 and R1 (table 25).

Component	Volume (µl)
Q-solution	5.00
Orange Dye	2.50
10x Quiagen Buffer	2.50
10 µM Primer reverse (Int 2R) 10 µM Primer forward (1F2)	2.50 2.50
25 mM $MgCl_2$	1.50
25 mM dNTP mix	0.50
Quiagen Taq polymerase	0.15
H_2O to (25 µl)	

Table 25. PCR components for screening mutations in *H-Ras* gene of mutated fibroblasts from Costello Syndrome patients

In table 26, the PCR cycling parameters for detecting Costello mutations are shown.

WINDOW 1					
Temperature (°C)	96	59	72	6	
Time (min)	5:00	0:00	0:00	0:00	1x cycle
WINDOW 2					
Temperature (°C)	96	59	72	6	
Time (min)	0:30	0:30	1:00	0:00	40 x cycles
WINDOW 3					
Temperature (°C)	96	59	72	6	
Time (min)	0:00	0:00	10:00	0:00	1x cycle
WINDOW 4					
Temperature (°C)	96	59	72	6	
Time (min)	0:00	0:00	000	0:00	0x cycles

Table 26. *H-Ras* gene, PCR cycling parameters introduced in the robot termocycler. The robot termocycler used for these reactions was a very useful mechanic device that permitted to change the reaction plate from one reaction platform to another and this permitted to continue with the PCR reaction eliminating the time necessary for waiting until the platform reached the desire temperature for initiating the next amplification cycle. The PCR conditions of each reaction platform are represented in each window, so the robot thermocycler have 5 reactions platforms instead of only one which is the case of a traditional PCR termocyclers.

A) PCR products gel electrophoresis of Costello Syndrome patients

PCR products obtained of the PCR reaction for detecting mutations in samples of Costello Syndrome patients were purified by electrophoresis. The samples were loaded in 2% agarosa gel and running (1 h, 100 V), besides 2.5 µl of Track LT ™, 100 bp DNA ladder of Invitrogen ®, catalog No. 10488-058, was also loaded for molecular weight reference.

B) PCR purification of Costello Syndrome patients

After the electrophoresis, a band of DNA of 600 Kb approximately was detected in our agarosa gel, and then was butted and purified following the Quick purification protocol.

C) PCR of exon 2 of *H-Ras* gene

Before sequencing the exon 2 of *H-Ras* gene of samples of Costello Syndrome patients was necessary to amplify the isolated fragment by a PCR reaction using a terminator (ABI BigDye Terminator V.3.1 Chemistries). PCR components are shown in table 27 and cycling parameters are shown in table 28.

Component	Volume (µl)
Terminator chemistry (2.5 x)	1.0
5x Dilution buffer	1.5
(IF2, 3.2 M) dir primer	1.0
(2R, 3.2 M) rev primer	1.0
DNA	1.5
H_2O	5.0

Table 27. PCR components reaction

Temperature	Time	Cycles
96°C	1:00 min	
96°C 50°C 60°C	0:10 s 0:05 s 04:00 min	25
4 °C	infinite	

Table 28. Sequencing PCR cycling parameters

D) SDS treatment

The next step for detecting *H-Ras* mutations from samples of Costello Syndrome patients was to purify the Sequencing Reaction PCR, following the SDS protocol of the BigDye Terminator v 3.1 cycle sequencing kit. Briefly, we adjusted the volume of each sample until 20 µl, and then 2.2 µl of 2.2% SDS solution was added to each sample, we mixed thoroughly and then we heated to 98° C for 5 min. After this treatment the samples were cooled to room temperature before proceeding to the next step.

E) Dye Terminator Removal

In order to remove the dyes terminators utilized in the PCR reaction, we employeed the DTR Gel Filtration Cartriges, Edge Biosystems Catalog No. 42453, following the manufacturer's advice.

F) Sequencing

All the samples were analyzed on an ABI 3130XL Genetic Analyzer. All sequences were analyzed by direct observation of the electropherogram and using Sequencer 4.8 (Gene Codes Corporation, Ann Harbor MI). Costello mutations were detected in the electropherogram by directly observation.

4.2.2 RNA Methods

4.2.2.1 Isolation of small RNAs

The first method used to obtain miRNAs was the Trizol® Reagent of Invitrogen, we followed the instructions provided by the kit. MiRNA extraction was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware), miRNAs amounts recovered were not enough, and for that reason we decided to change our miRNAs extraction protocol for optimizing the method, so instead of Trizol we used miRVANA miRNA isolation protocol.

4.2.2.2 Isolation and enrichment of miRNAs

MiRNAs were extracted using miRVANA^{TM} miRNA isolation kit from Ambion Inc. (Austin, Tx). Isolation was performed as per the manufacturer's protocol. Briefly, the pellet collected was washed twice with DEPC/PBS 1x solution and then the pellet was homogenized in 400 µl in lysis/binding buffer and 1/10 miRNA homogenate additive and thawed in ice for 10 min. Small miRNAs were then isolated using the enrichment protocol for small RNAs provided with the kit. MiRNA samples were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware).

4.2.2.3 DNase treatment and removal reagents

MiRNAs samples were treated using DNAse Free ^{TM} digestion kit from Ambion in order to minimize genomic contamination.

4.2.2.4 MiRNAs quality control for Taqman PCR Real Time analyses of Costello Syndrome patients samples

The quality of miRNAs samples was analysed using an Agilent RNA 600 Nano kit Quick Start Guide, the protocol was followed as the manufacturer's advice.

4.2.2.5 Retrotranscription

cDNAs were obtained from our miRNAs samples following the protocol of reverse transcriptase Kit (AB Applied Biosystems), 100 ng of each sample was used according to the manufacturer's instructions. Conditions for RT-PCR reaction are showed in table 29.

Step type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	00	4

Table 29. RT-PCR conditions for performing cDNA for miRNAs assays.

4.2.2.6. MiRNAs Taqman PCR Real Time Assay.

A) Assay principle

The quantitative PCR Real Time (qPCR) is nowadays a powerfull DNA technique which permits not only amplified to thousand to millions fold of DNA sequence or a cDNA template, as a traditional PCR, besides this technique can measure the amount of PCR in each cycle. The number of cycles and the amount of PCR end product can theorically be used to calculated the initial quantity of genetic material because the technique use fluorescent markers that are incorporated into the PCR product. The increase in fluoresce signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reports used include double- double-stranded DNA (dsDNA) binding dyes, or dye molecules attached to PCR primers or probes that are incorporated into the product during amplification. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with scanning capability. By plotting fluorescence against the cycle number, the real time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction (fig. 15)

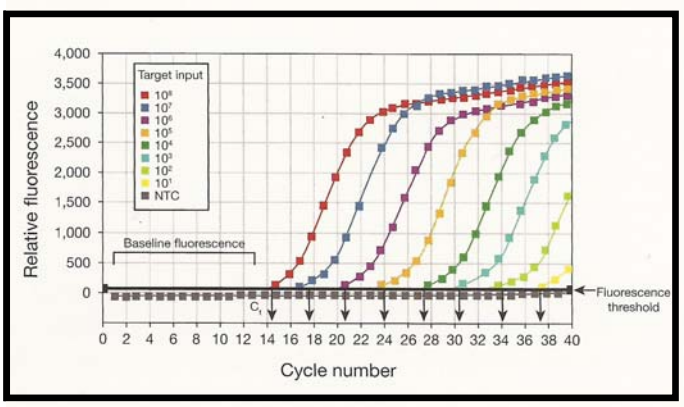


Fig. 15 Amplification plots created in miRNA PCR REAL TIME assay. Amplification plots are created when the fluorescence signal from each sample is plotted against cycle number; therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR experiment.

B) Assay protocol

MiRNAs Taqman PCR Real Time were performed using 1,33 µl of the resultant cDNA in a total volume of 20 µl; miRNAs amplifications were analyzed in a thermocycler from AB Applied ABI 7000 Real Time PCR system. All PCR reactions were run by triplicate and fold change value of each sample was calculated by ΔΔ Ct method, (Livak and Schmittgen, 2001; Pfaffl, 2001); using a gene expression control, relative to U18 or let-7 in *HeLa* cells transfections and RNHU6 for transfected murine embryonic fibroblasts lines.

The efficiency of amplification of each miRNAs provided by AB Applied Biosystem was tested by the commercial provider so it was not necessary to perform a standard amplification curve for each miRNA. The conditions for Taqman PCR Real Time Assay are shown in table 30.

Parameter	Value			
Run mode	9600 emulation (default)			
Sample volume	20 µl			
Thermal Cycling parameters	Step	AmpliTaq Gold Enzyme Activation	PCR	
		HOLD	Cycle (40 cycles)	
			Denature	Anneal/ Extend
	Time	10 min	15 s	60 s
	Temp (^{o}C)	95 min	95 s	60 s

Table 30. MiRNA Taqman thermal cycling parameters.

C) MiRNAs expression profile calculation

The method that we used to calculate the number of copies is the comparative quantification algorithms ΔΔ Ct this technique is very popular and is an improvement method on the ΔΔ Ct method. Relative mRNA abundance values were calculated according to the equation shows in fig.16, using threshold cycle (Ct) values from cuadruplicate assays as previously described (Pfaffl, 2001).

$$\frac{\text{miRNA target}}{\text{miRNA end control}} = \frac{E_{\text{target}}^{\text{Ct target}}}{E_{\text{end control}}^{\text{Ct, end control}}} \times 1000$$

Fig. 16 Formula used for calculating relative miRNA target abundance values.

PCR efficiency values for miRNA endogenous control and the miRNA target gene, *E* end control (endogenous control) and *E* target, were calculated as described (Pfaffl, 2001). Results are given in number of copies of miRNA target 1000 copies of miRNA endogenous control. The sequence of amplified PCR products was confirmed by DNA sequencing in Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). The fold change was calculated dividing the number of copies obtained of miRNA target between the number of copies obtained of miRNA transfection control (empty vector); (Navarro et al., 2009).

We have selected a group of miRNAs which have been reported that they showed low expression in different tumours, and when their normal expression is re-established in different cancer cell lines, tumorigenesis, metastasis and invasion are suppressed (see 1.5.6 section).

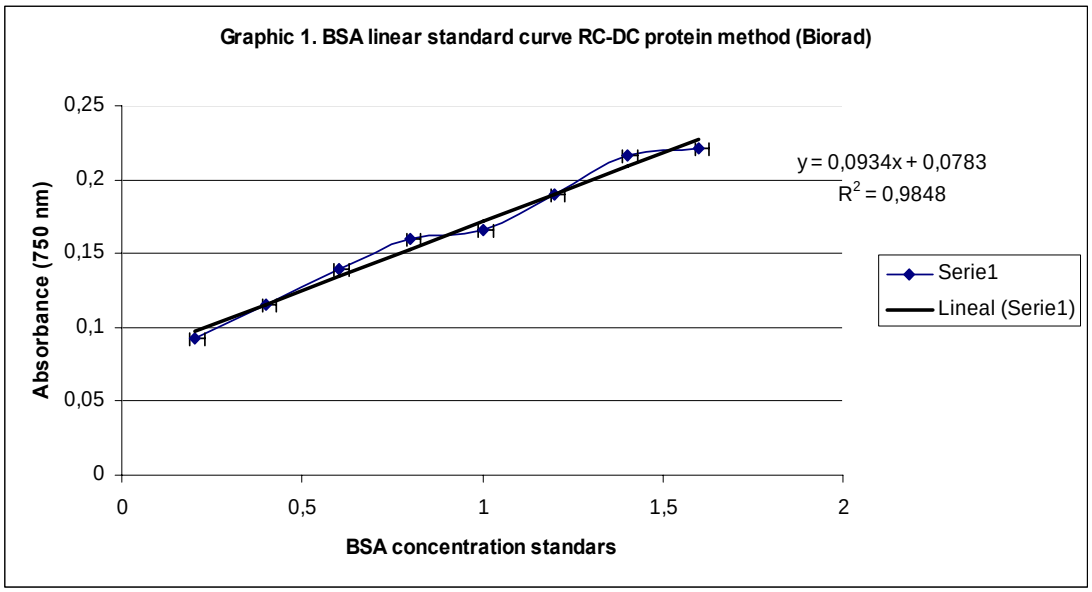
4.2.3 Protein methods

4.2.3.1 Whole cell extract

In order to performance western blots, we obtained the whole protein extract as follows, transfected cells were harvested from 25 cm^2 bottle and the pellet was resuspended in 100 µl of a NP-40 lysis buffer, then the samples were vortexed and incubated them in rotating overnight at 4^o C, 24 h later, the samples were lysated using a syringe, vortexed and centrifuged 5 min to 10, 000 rpm. The supernatant was diluted 1:1 with SDS 2X electrophoresis buffer (2%) and then immediately frozen and stored at -80^o C.

4.2.3.2 Protein determination

Protein concentrations were determined using RC DC Protein Assay kit (Bio-Rad). We followed the Microfuge Tube Assay Protocol (1.5 ml). Graphic 1 shows BSA linear standard curve, obtained for calculating the protein amount of each sample.



Graphic 1. BSA standard line RC DC protein method (Biorad).

4.2.3.3 SDS/PAGE

SDS-PAGE (polyacrylamide gel electrophoresis) is used to separate proteins based on their size. SDS-PAGE, coupled with Western blotting (immunoblotting) is typically used to determine the presence and/or relative abundance of a given protein. The gel is a cross linked polymer matrix used to support and separate the molecules its density can be controlled varying the monomer concentration. After cross linking has taken place, the samples are loaded in small wells in the gel and the assembly undergoes electrophoresis. Electrophoresis involves applying an electric current to the gel and allowing the proteins to migrate though the matrix. In order for the proteins to migrate through the gel (fig., 17), they are firstly denatured and negatively charged by exposure to a detergent such as sodium dodecyl sulphate (SDS). The amount of bound SDS is relative to the size of the protein, and proteins have a similar charge to mass ratio. Bands in different lanes separate based on the individual components sizes. A molecular weight marker that produces bands of known size is used to help identify proteins of interest.

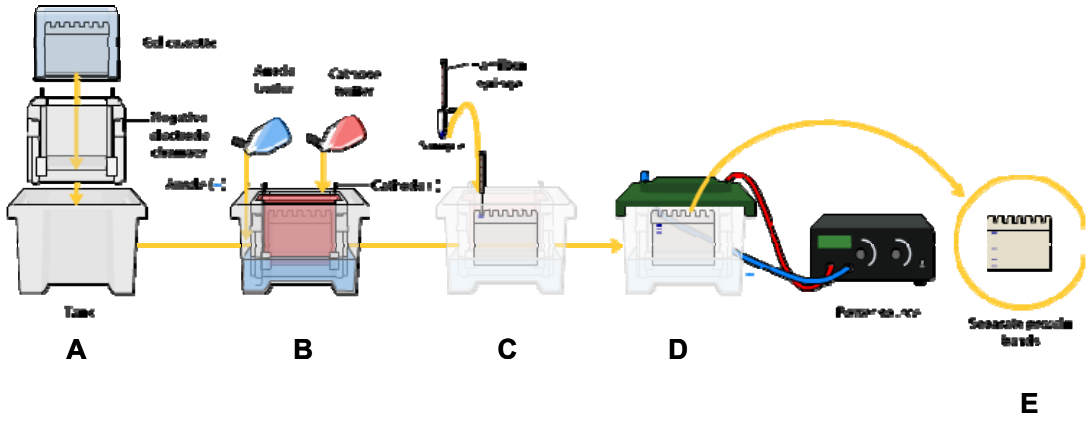


Fig. 17 SDS-PAGE. A) Gel cassette contains the acrylamide gel stocked into two glasses, then the gel cassette is put it into the electrophoresis chamber and then into the tank. **B)** The tank is filled with the anode and catode buffer. **C)** The samples and the weight molecular markers are loaded into the gel with a Hamilton syringe. **D)** An electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive (+) electrode (anode). **E)** Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty (they encounter more resistance farther down the gel, while larger ones will have remained closer to the point of origin).

For performing electrophoresis assays, 100 µg of each protein extract was running into a 12.5% SDS PAGE. In stocking phase of the gel the samples were running at 50 V, then when samples reached the separating gel, voltage was increased to 100 v. After electrophoresis, the samples were transferred into a nitrocellulose membrane, for performing western blot analysis.

4.2.3.4 Western blot analysis

A) Assay principle

Western blot assay is also called protein immunoblot, and is a widely analytical technique used for detecting specific proteins in a sample of tissue homogenate or extract. The first step of this technique is using a gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), which is placed on top of the gel, and a stack of filter papers placed on top of that (fig. 18) The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.

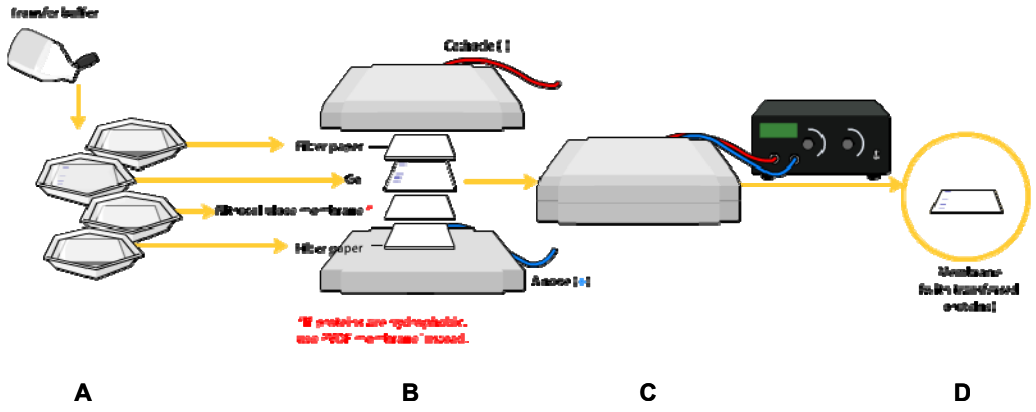


Fig. 18. Western blot transfer. A) Filter paper, gel, and nitrocellulose membrane are damped with transfer buffer. **B)** The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. **C)** The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. **D)** As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Ponceau S dye.

Once the proteins are transferred into the membrane, blocking is the next step and its necessary for avoying non-specific binding, and it can be achived by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive) in Tris-Buffered Saline (TBS) 1X, with some percentage of detergent such as Tween 20 or Triton X-100.

Membrane is incubated overnight with the primary antibody, when the period of incubation had finished the membrane is rinsed to remove unbound primary antibody, then the membrane is exposed to a second antibody, directed at a species-specific porcion of the primary antibody. This second antibody is usually linked to biotin or other reporter enzyme such as alkaline phosphatase or horseradish peroxidase. In our experiments, we used a horseradish peroxidase-linked secondary antibody to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. Chemiluminiscence can be detected using a photographic film or and more recently by CCD cameras which capture a digital image of the Western blot. The image is then analyzed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density.

B) Assay protocol

The SDS-PAGE gel was transferred onto Protan ® Nitrocellulose Transfer Membrane (Whatman). Membranes were blocked with 5% non fat milk or 1% BSA then incubated

overnight with primary antibodies (table 21). Membranes were washed three times with 5 ml of TBS/ 10% tween and then incubated with secondary antibodies (table 22).

B) Chemiluminiscence detection

Finally the chemiluminescences of the bands were revealed using the Super Signal ® West Femto Mazimun Sensivity Substrate (Thermo Scientific). Band densities were measured using LAS-3000 luminescent image analyzer with Multi Gauge software version 3.0 (Fuji Photo Film, Japan).

4.2.4 Cell culture methods

4.2.4.1 Cell culture

All the cell lines evaluated in this PhD thesis were seeded in the desire density for each assay with DMEM complete (DMEM+ Foetal Bovine Serum (FBS 1%) + antibiotics (Penicillin+ Streptomycin, 0.1%). When the cell lines were transfected DMEM without FBS and antibiotics was employed. For stable transfections we used DMEM + FBS (1%)+ antibiotics+ selective antibiotic (for stable transfection pEGFP-C1 vector was used and cells were transfected with pEGFP-C1 used as a control transfection; pEGFP-p19 or pEGFP-p21. All the cell lines transfected with pEGFP vectors were previously selected adding geneticine G418 (1000 μg/ml DMEM complete) to the medium cell culture per a month.

4.2.4.2 Tripsinization of adherent cells

For detaching our transfected cells, first old media was removed, and the monolayer cells were rinse one time with PBS 1X , later 1.0 ml of 0.25% of trypsin /EDTA (w/v) solution was added to cell culture plates and then they were incubated for ~3 min, after that period of time flask was checked under inverted microscope to make sure that cells were lifting off of the plate. When a single cell suspension had been obtained, 10 times volume of complete media was added into the trypsin solution. The cell suspension was transferred to a sterile 15 ml conical centrifuge tube. Cells were centrifuged at 1,500 rpm for 3 min in the tabletop centrifuge, the supernatant was discarded carefully. The cell pellet was resuspended in 10 ml of fresh complete media and collects 50 μl to count cell number. Once cells/ml solution was counted, seed cells into new flask or cell dishes cultures to get a final concentration that you desire (table 23).

	Surface Area (mm^2)	Seeding Density X10 ^{6}	Cells at confluence	Versene (ml, of 0.53 mM, EDTA)	Trypsin (ml of 0.05% trypsin, 0.53 mM EDTA)	Growth medium (ml)
Dishes						
35 mm	962	0.3	1.2	1	1	2
60mm	2827	0.8	3.2	3	2	3
100 mm	7854	2.2	8.8	5	3	10
150 mm	17671	5.0	20.0	10	8	20
Cluster Plates						
6-well	962	0.3	1.2	2	2	3-5
12-well	401	0.1	0.4	1	1	1-2
24-well	200	0.05	0.2	0.5	0.5	0.5-1.0
Flasks						
T-25	2500	0.7	2.8	3	3	3-5
T-75	7500	2.1	8.4	5	5	8-15
T-160	16000	4.6	18.4	10	10	15-30

Table 31. Useful number for cell culture. The number of cells on a confluent plate, dish, or flask will vary with cell type. For this table, *HeLa* cells were used. Reference: http://tools.invitrogen.com/content/sfs/appendix/Cell_Culture/cell%20culture%20useful%20numbers.pdf

4.2.4.3 Cells cryopreservation

The confluent cells were harvested and rinsed with PBS 1x twice, then the pellet was resuspended in 10% DMSO/90 % FBS cold solution to obtain a suspension of $1x10^6$ cells/ml, dissolve the pellet and transfer 1 ml of cell suspension in cryovials immediately and simultaneously keep the vials in mini-cooler unit or ice bath. Finally transfer cryovials into -80^{o}C freezer for four days and then transfer the cryovials in an appropriate rack of liquid nitrogen and record the position of samples in your register.

4.2.4.4 Thawing cells

Cryovials were kept in 37^{o}C waterbath or warmed them between the hands, until just small ice-clumps remains. Cell suspension was transfer in a labelled 10 cm culture dishes using a sterile pipette. Here cell suspension should be mixed well by sucking and dispensing by pipette to breakdown the clumps of the cells. The suspension was diluted in a 10 ml of DMEM media. Then allow to growth in CO_2 incubator/37^{o}C.

4.2.4.5 Counting cells (Neubauer chamber method)

50 µl of the cell suspension were transferred into a 1.5 ml centrifuge tube, 50 µl of trypan blue ($C_{34}H_{28}N_6O_{14}S_4$) solution was added to the cell suspension (1:2 dilution factor) in the centrifuge tube and the cell suspension was resuspending using micropipette. With a cover-slip in place, use 200 µl micropipette and transfer 25 µl of the trypan blue-cell suspension to a chamber on the hemocytometer. Only viable cells

were counted in the neubauer chamber (4 squares of the chamber). The total number of cells will be determined using the following calculations:

Cells per ml = (the average count per square) (the dilution factor) ($1x10^4$)

Total cell number = cells per ml x the original volume of fluid from which cell sample was removed.

Reference: Sigma Catalog, 1989, commonly used tissue culture techniques, p. 1492.

4.2.4.6 Cell transfections

A) *In vitro* DNA transient transfection using Lipofectamine™ and PLUS ™ reagent of Invitrogen.

Day 01. One day before the transfection, 10 x 10^5 murine embryonic fibroblast knock-out *H-Ras* (-/-) or double knock-out *H-Ras* (-/-) and *N-Ras* (-/-) were seeded in a 25 cm^2 t-flask.

Day 02. Two micrograms of DNA were diluted in 250 µl of DMEM without antibiotics, then this dilution was mixed with 8 µl of plus reagent and 12 µl of lipofectamine. The total volume was added to the cultures and they were incubated during 5 h at 37°C; 5% CO_2 incubator. When the time of incubation had finished, the medium was replaced with DMEM (FBS 1% + antibiotics), 24 h postransfection, other protocols can be followed.

B) *In vitro* DNA transient and stable transfections using Jet Pei ™ of Polyplus ®

Day 01. One day before the transfection, 10 x 10^5 murine embryonic fibroblasts knock-out *H-Ras* (-/-), or double knock-out *H-Ras* (-/-), *N-Ras* (-/-) were seeded in a 25 cm^2 T-flask.

Day 02. Two micrograms of DNA were diluted in 90 µl of 150 mM NaCl and 5 µl of Jet Pei, following the instructions of the provider. Transfections were incubated for 1 h at 37°C and 5%/ CO_2. When the transfection time had finished, the cell culture media which contained the complexes was removed and replaced with complete DMEM (FBS 1%+ antibiotics). After 24 h, selection process was initiated adding to the media geneticine (G418), (1000 µg/ml), per a month.

4.2.5 Methods for analyzing transfected cell lines

4.2.5.1 Proliferation cell assays

A) Assay principle

Cancer cells acquired abilities for proliferating without limits and this hallmark is necessary for tumour progression and metastasis development, for this reason we were interested in knowing the proliferation rate of our stable double knock-out *H-Ras* (-/-) and *N-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-p19 or pEGFP-p21.

B) Assay protocols

Neubauer chamber proliferation assay

Cells from each transfection or lineage were seeded ($1x10^4$ cells per well) in 6 well plates with 10% FBS, DMEM. After 24 h, medium was removed by harvesting cells treated with 0.4 ml of 0.25% trypsin/EDTA for 3 min, and followed by addition of 0.6 ml of DMEM complete. Each cell line was resuspended and counted with trypan blue each day during 6 days and for each cell line. All treatments were performed in triplicate in at least three independent experiments.

Cyquant proliferation assay

Cell proliferation rate was also evaluated using Cyquant assay. This assay measures the DNA content of cells using fluorescent indicators. Then $5x10^2$ cells of each transfected cell line (pRK5-p19, pRK5 p19 (G12S), pRK5- p21, pRK5-p21 (QL61) and pRK5-p21 (G12S)), were seeded in 96 well plates. Six wells per cell line were seeded into 96 well plates; proliferation assays were repeated three times in a independent way. Cyquant working solution (200 µl) was added to each well, then the wells were incubated at 37°C 1 h, and finally the absorbance of each well was measure in an Victor ^{3} *(PerkinElmer Wallac, Turku, Finland)* spectrophotometer or in a spectra Max5 spectrophotometer from Bionova Cientifica, Molecular Advices (excitation 485 nm, emission 528 nm). Fluorescence was expressed as relative fluorescent units (RFU).

4.2.5.2 Clonogenic anchorage agar assays

A) Assay principle

Clonogenic anchorage assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is formed for a least 50 cells. This assay evaluates the potential of each cell in the population for its ability to undergo "unlimited" division, (Franken et al., 2006).

B) Preparation of reagents and material

To initiate the colony growth in soft agar assays of each transfected cell line, firstly was necessary to prepare an agar stock of 5% that will be used for preparing the lower and the upper phases as follows: 5 g of agar (catalog No. 18300012, Invitrogen) were resuspended in 100 ml of miliQ water, and then suspension was sterilized and stored at room temperature in canonical flasks.

C) Assay protocol

The day of the experiment, aliquots of agar were melted and kept in water bath at 46^{o}C, when agar aliquots were diluted with a mixture of one part of the melted 5% agar (3 ml) mixed with 27 ml of DMEM complete was prepared. Then, for preparing the base layer, 600 µl of diluted agar was added to each well giving a final concentration of (0.5%) agar, and then plates were kept at room temperature under sterile conditions in a hook until the gel became solid. The upper layer was prepared diluting 5 ml of the base layer with 3 ml of a suspension cell containing 1x10^{4} cells/ml; 600 µl of this mix was added in each well upon the base layer; then the plates were incubated in a humidified CO_2 incubator at 37^{o}C, and supplemented with 300µl of serum-supplemented media on a top of base layer. After 4 weeks, the cells were fixed with 0.4 ml of glutaraldehyde 0.5% /PBS 1X, during 10 min and stained with 0.025% crystal violet in 20% methanol/PBS 1X, the samples were washed several times with PBS 1X in order to eliminate the crystal violet residues.

D) Evaluation of clonogenic anchorage agar assay

The number of colonies formed in each assay was calculated by direct observation in a DP71 Olympus Magnifying glass (10X objective). Each experiment was done at least twice in triplicates

4.2.5.3 Invasion assays

A) Assay principle

This assay determinates the capacity of invasion of the cell; this means their capacity of metastasis. The malignant cell has the capacity of detachment, migrating and forming distant colonies in new tissues. We can measure the capacity of invasion *in vitro* using disposable permeable supports, called inserts which provides a relatively simple *in vitro* approach to performing chemotaxis and cell invasion assays. Common barriers employed for invasion assays include collagen, fibronectin and lamining coatings as well as more complex extracellular or basement membrane extracts (BME). More elaborative invasion assays establish a monolayer of endothelial cells on the permeable support in place of or in addition to, the protein coatings or BME listed above.

Cell suspension was diluted in DMEM without FBS, then placed in the top of chamber, and incubated in the presence of test media containing specific chemo attractants in the bottom chamber (in this assay we used DMEM+ 10% FBS). Cells migrate from the top chamber through the coated filter pores to the bottom of the filter. Cell dissociation/Calcein AM solution was placed in the bottom chamber to dissociate the migrating cells from the filter and add a fluorescent label. Fluorescence in the bottom chamber is proportional to the number of migrating cells, (fig. 19).

B) Assay protocol

Invasion assays were done using InnoCyte TM Cell Invasion Assay Kit (24 well) of Calbiochem (No. catalog QIA-129) following the protocol of the provider. Briefly, 0.5 $x10^6$ cells/ml cell suspension was prepared for each cell line in serum-free medium, 350 µl of cell suspension was added to the upper chamber of each insert, then the cells were incubated at 37°C, 5% CO_2 during 48 h. After that time the cells were stained with 500 µl of cell staining solution. The fluorescence was measure at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence was expressed as relative fluorescent units (RFU).

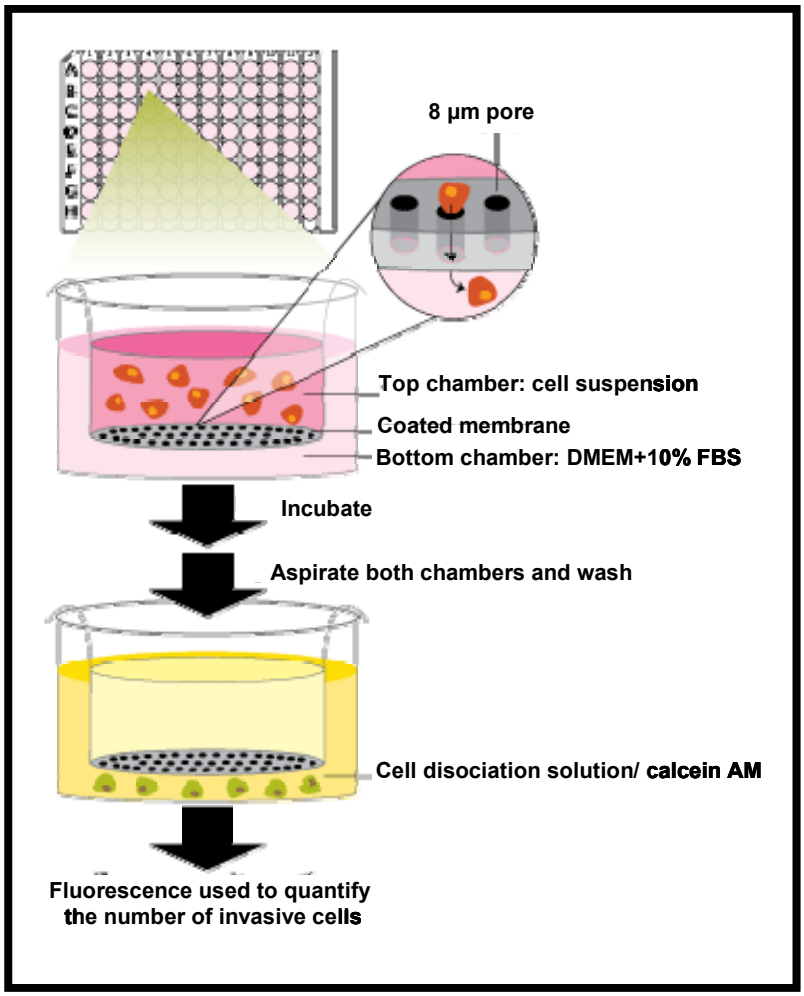


Fig. 19 Cell Invasion Assay. The invasion chamber consists of two chambers separated by a filter coated with BME or different ECM components. Cells were seeded on the top chamber, and then those cells that have the ability to migrate can pass through the filter of the insert and will be settled in the bottom chamber.

4.2.5.4 Reactive Oxygen Species Assay (ROS)

A) Assay Principle

The assay employs the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA). In brief, DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2', 7'-Dichlorodihydrofluorescein (DCF) by ROS (fig. 20). The fluorescence intensity is proportional to the ROS levels within the cell cytosol. The effect of antioxidant or free radical compounds on DCF-DA can be measured against the fluorescence of the provided DCF standard (Xia et al., 2007).

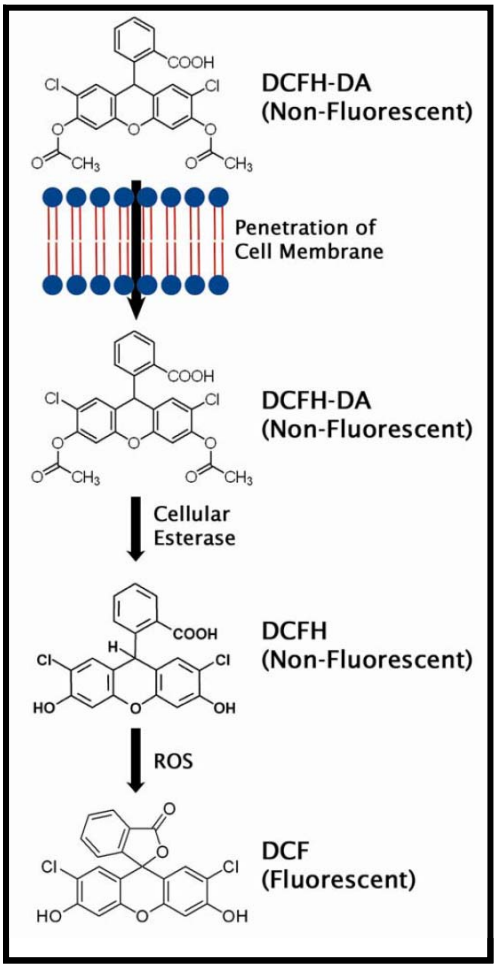


Fig. 20 Mechanism of DCF for detecting ROS species

B) Preparation of reagents

Three solutions are necessary for performing the protocol of ROS assay and they are prepared as follows:

PBS-Glucose (10 Mm) buffer

Dilute 1:10 glucose (Ca^{+2}, Mg^{+2}) stock 10x, add 1.98 g of glucose to 1 l of PBS-Buffer fill up to 950 ml and adjust pH to 7.1 using NaOH (10 N) solution to fill up to 1 l.

Preparation of dye (H2DCF-DA)

Prepare a 1 mM stock solution with ethanol (100%); 4.87 mg/10 ml ethanol (store at -20^{o}C for a month). Dilute stock solution 1:1000 in PBS-Glu (Mg^{+2}, Ca^{+2}) to 1 µM final concentration, e.g. 10 µl dye + 9990 µl PBS-Glu. Approximately 10 ml of this reagent is necessary for each 96-well plate.

Positive control (SIN 1)

SIN 1 working solution always has to be prepared fresh at a 5 µM final concentration. Firstly, SIN 1 stock has to be prepared as follows: 1.0 mg of SIN 1 reagent was diluted in 5 ml of miliQ water, then working solution is prepared diluting 5 µl of 1 mM stock of SIN 1 in 995 ml of PBS- Glucose 1x buffer.

C) Assay protocol

Day 01

Transient transfected *HeLa* cells were seeded (1x10 ^{5} cells per well) in a 96 wells plate (5 wells per cell line), then plates were incubated 24 h at 37^{o}C, 5% CO_2.

Day 02

Transfected cells, were loading with dye (H2DCF-DA; 100 µl /well (1 µM final) dilute it with PBS with Ca^{+2} and Mg^{+2} and 10 mM Glucose; PBS-Glucose; p.H. 7.1) for 30 min. Thereafter the cells were rinsing once with PBS 1x and add PBS-Glucose (100 µl), for performing the positive control instead of PBS-glucose add SIN 1 solution (5 µM, 100 µl /well). The plates then were incubated at 37^{o}C, 5% CO_2. The fluorescence was measured after 30 and 90 min at excitation 485 nm and emission 528 nm. The spectrophotometer used was spectra Max model 5 of Bionova Cientific, Molecular Advices (Scherz-Shouval et al., 2007).

D) Calculation of percentage of ROS production

Fluorescence was expressed as relative fluorescent units; the percentage or ROS emission was calculated using as a reference of 100% the ROS emission detected in our *Hela* cells without transfection.

4.2.5.5 Cytometry analysis

A) Assay principle

Flow cytometry is a laser-based technology that is used to measure characteristics of biological materials. It offers a rapid, objective, and quantitative method for analysis and purification of cells in suspension. Cells interact with a light beam as they pass by single file in a liquid stream; interaction with light is generally measured as light scatter and fluorescence according to staining of the cells. If a fluorochrome is specifically and stoichiometrically bound to a cellular component, the fluorescence intensity will ideally represent the amount of that particular component, (Jaroszeski and Radcliff, 1999).

B) Assay protocols

Propidium iodide cell cycle analyses.

The cells were seeded into 10 cm cell culture dishes to avoid the inhibition of their cell cycle when they are confluent. Each cell line was seeded ($1x10^{6}$ cells) per triplicate. Then 24 h later cell cultures were transfected with Lipofectamine TM method using the plasmids of our interest. After 24 h postransfection we collected the medium of each sample and centrifuged them (this procedure avoids to loose the cells in mitosis), later cells were harvested, and the pellet was collected in the same tube and washed twice with 5 ml of 1% BSA/ EDTA 10 mM/PBS 1X cold, then the pelled was resuspended in 900 µl of PBS 1X cold and 2.1 ml of absolute ethanol were added drop by drop and vortexing at the same time in order to obtain a final concentration of ethanol 70%. The ethanol cellular suspension was fixed at least two hours. The day of cytometry analysis the fixed samples were centrifugated, and washed twice with 5 ml with 1% BSA/ EDTA 10 mM/PBS 1X, then we added 2.5 µl de RNAse (1mg/ml) and 25 µl of propidium iodide (1 mg/ml) were added, the samples were incubated 30 min at room temperature before doing the cytometry analysis. The samples were analysed in an Epics XL Beckman Coulter Cytometer.

Analysis of DNA content and green fluorescence protein expression

$1x10^{6}$ *HeLa* cells were seeded in 10 mm cell dish and they were transfected following the Jet pei transfection protocol described previously in section 3.2.4.6. Stable cell lines were just counted and seeded, 24 h later, the medium of each sample was collected and and centrifuged (this procedure avoids to loose the cells in mitosis), later we trypsinized the cells, and the pellet was collected in the same tube and washed twice with 1% BSA/

EDTA 10 mM/PBS 1X in cold, then the pellet was resuspended in 500 µl of cold paraphormaldehide 2%, pH 7.2 and incubated at 4°C during 1 h. When the incubation was finished the samples were washed one time with cold 1% BSA/EDTA 10 mM/ PBS 1X, then the samples were centrifuged and resuspended in 900 ml of cold PBS 1X and 2.1 mL of ethanol absolute were added, drop by drop and vortexing to obtain a final concentration of ethanol 70%. The ethanol cellular suspension was fixed at least 2 h. The day of cytometry analysis the fixed samples were centrifuged, and washed twice with 5 ml with 1% BSA/ EDTA 10 mM/PBS 1X, then 2.5 µl of RNAse (1 mg/ml) plus, and 25 µl of propidium iodide (1 mg/ml) were added; later samples were incubated 30 min at room temperature before performing the cytometry analysis. The cells were analyzed into XL cytometer.

Iodide propidium cell cycle analyses of green fluorescent protein (GFP) previous separation by Moflo Dako cytometer.

$5x10^6$ *HeLa* cells were cotransfected using pEGFP-C1/pRK5-C1, pEGFP-C1/pRK5-p19, or pEGFP-C1/pRK5-p19 (G12S) vectors with Jet Pei reagent as described recently in section 4.2.4.6. 24 h postransfection transfected cells were separated using a Moflo Dako cytometer (laser of 488 nm excitation and 530 nm emission; 6 psi, 70 micras Tip), in two different pools: GFP (+) and GFP (-); both fractions were fixed immediately in 5 ml of 70% ethanol and rinse twice in PBS 1X, then the fractions were stained with working solution iodide propidium (IP) and incubated for 24 h, 4°C; the next day stained GFP fractions were analyzed in an Epics XL Beckman Coulter cytometer.

C) Cytometry analyses results

The results of these analyses showed a graphic which represents the percent of cells in each phase of the cell cycle.

4.2.6 Statistical Analyses

Statistical analysis was performed using Mann-Whitney U test (non parametric test, two tailed), $p<0.05$ was considered statistically significant. Each experiment was performed at least 3 times and individual samples were run by triplicate.

In some cases, we employeed an ANOVA (one way analysis of variance) and a Bartlett's test for equal variances; in both cases $p<0.05$ was considered statistically significant.

V. RESULTS

5.1 MiRNAs Taqman PCR Real Time

5.1.1 Ectopically overexpression of p19, p21 and their mutant proteins regulate a set of miRNAs in *HeLa* cells.

We previously showed in microarray assays that cell growth and metastatic genes were regulated by p19 protein, in that previous work, p19 protein was compared to these p19 (W164A) mutant protein which prevent p19/RACK1 and p19/p73 binding. Additional studies of miRNA arrays were performance in order to know if p19 can control miRNA expression, and we observed that p19 but not p19 (W164A) increase the expression of a set of miRNAs included mR-342, miR-138, miR-206, miR-330 and miR-99b, (Camats-Malet PhD thesis 2008, UAB and García-Cruz et al., submitted)

For this PhD thesis we also were interested in determinate if p19 and p21 H-Ras proteins which contained Q61L or G12S mutant variants can increases also the miRNAs expression profile. Q61L mutation has a high oncogenic capacity of activating proteins in a constitutive manner blocking GAPs function. In other hand, G12S is the most frequent mutation detected in Costello Syndrome at codon 12 of H-Ras with aminoacid change Gly/Gly found in "normal" individuals into Gly/Ser; this change activates constitutively GTP-bound conformation and activates downstream effectors such as MAPK, PI3K kinase and RasGDS (Aoki et al., 2005; Lin et al, 2008). All human cells contained both p19 and p21 H-Ras proteins at different levels as a consequence of their alternative splicing.

For our experiments, we transfected transiently *HeLa* cells with pRK5 vector which induces a strong overexpression of the proteins of our interest. The cell lines obtained were:

- pRK5-C1 (which was used as control transfection and contained the vector alone)
- pRK5-p19 (which contained the sequence for p19 H-Ras protein),
- pRK5-p19 (G12S); (which contains coding sequence for overexpressing p19 mutant Costello Syndrome protein),
- pRK5-p21 (containing p21 H-Ras sequence)
- pRK5-p21 (Q61L) containing the aminoacid change Gly/Lys,

- pRK5-p21 (G12S) containing p21 Costello mutant sequence.

The miRNAs set selected for these assays included a) miR-342, miR-206 and miR-330 as miRNAs that we already known to be upregulated by p19 expression, (Camats-Malet PhD thesis 2008, UAB and Garcia-Cruz et al., submitted); b) miR-206, miR-126 and miR-335 as miRNAs that, significantly reduced the ability of CN34-LM1 and CN34-BoM1 cells to metastasize to the lung (Tavazoie et al., 2008); c) miR-374 as miRNA, which is known to be associated with the aggressive small cell lung cancer (Miko et al., 2009); d) let-7 as miRNA that targets *Ras* genes (Bussing et al., 2008), and e) miR-138, which regulates proliferation, (Mitomo et al., 2008), cell cycle (Wang et al., 2012; Liu et al 2012b), and epithelial mesenchymal transition in metastasis development (Guo et al., 2013), (table 27).

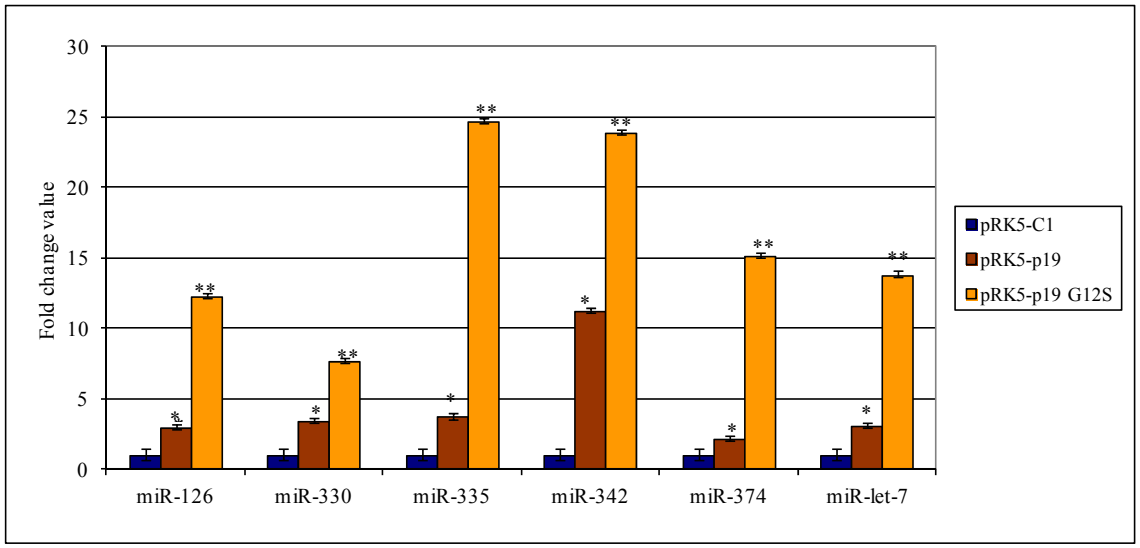
Cellular transfections were performed using Lipofectamine (Invitrogen). After 24 h postransfection miRNAs extraction was carried out with miRVANA kit (Ambion). MicroRNA Reverse Transcriptase Kit (AB Applied Biosystems) was used to according to the manufacturer's instructions. For doing the PCR real time we employed an ABI 7000 termocycler. All the transfections were performed at least in three independent assays. All the PCR REAL TIME assays were quadruplicate analyzed for each sample. MiRNAs expression profiles were calculated using the method ΔΔCt (endogenous internal control U18, U6 and let-7). The results were calculated using the number of copies of each miRNA (experiment and control) and then the fold change was calculated dividing the number of copies (experiment)/ number of copies (control); (Plaff, 2001).

miRNA	Related with	Reference
miR-126	MiR-126 regulates loss of adhesion, tumour proliferation, angiogenesis and invasion. It regulates RAS/PI3K/AKT /VEGF-A and MAPK signalling pathways and CCND1, CCNDE1, CCNDE3 cyclins.	Felli et al., 2013; Hamada el al., 2012; Zhu et al., 2012b; Huang et al., 2012a; Liu et al., 2011b and 2009a; Chen et al., 2011c; Guo et al., 2008.
miR-138	MiR-138 regulates genomic instability, inhibits metastasis and resistance in cancer cells vía EGFR/SOX4/HIF1 and it regulates proto-oncogenes expression which control proliferation, cell cycle arrest and apoptosis	Golubovskaya et al., 2013; Wang et al.,2013; Ye et al., 2013; Zhang et al., 2013a; Ye et al., 2012; Xu et al., 2012a; Wang et al 2011b; Liu et al., 2011a; Song et al., 2011; ; Jiang et al., 2010; Mitomo et al., 2008
miR-206	MiR-206 is a pivotal myomiR, it regulates also filopodia formation and several proteins involved in adhesion, matrix degradation, and cell cycle arrest via CCND2 cyclin. It regulates also the expression of some transcription factors involved in tumour growth, invasiveness and metastasis.	Zhou et al., 2013; Zhang et al., 2013b; Gagan et al., 2012, Goljanel et al., 2012; Nohata et al., 2012; Stahlhut et al., 2012; Liu et al., 2010a; Chen et al 2012a; Dongsheng et al., 2009; Taulli et al., 2009; Yan et al., 2009; Song et al., 2009.
miR-330	miR-330 is a potential tumour suppressor and inhibits several aspects of metastasis as such as proliferation, colony formation, tumour growth, apoptosis evasion and increases sensitivity in cancer cells to some anticarcinogenic treatments.	Li et al., 2013 : Qu et al., 2012; Hodzic et al., 2011; Jeyapalan et al., 2010; Lee et al., 2009
miR-335	MiR-335 regulates invasion, metastasis apoptosis, cell proliferation and cell cycle. It suppresses also activation of PI3K/AKT/SP1 and miR-335/SOX4/Semaphorin-plexin signalling pathways	Dohi et al., 2012; Jiang et al.,2012; Yan et al 2012a; Huang et al., 2012b; Chen et al, 2011b; Shu et al., 2011a; Tome et al., 2011; Heyn et al., 2011; Xu et al., 2011; Scarola et al., 2010; Wang and Ruan et al., 2010; Ronchetti et al., 2008;
miR-342	MiR-342 regulates apoptosis and tamoxifen resistance resistance in breast cancer	He et al., 2013; Fayyad-Kazan et al., 2013; Wang et al 2012b; Savad et al., 2012; Citelly et al., 2010; Grady et al., 2008; Ronchetti et al., 2008.
miR-374	MiR-374 is related with Ca^{++2} transport and metabolism and development in neural cells	Gong et al., 2012; Chang et al., 2011
miR let-7	Let-7 regulates MAPK and AKT signaling pathways and several transcription factors (MY, E2F2, EIF4F); oncogenes (all RAS genes and their proteins); and other proteins involved in apoptosis activation (Caspase-3); loss of adhesion (E-caderin, PAK1 DIAPH2, RDX AND IRGB8); cell cycle progression (CCND1, CCND3 and CDK4); HMGA2 and estrogen receptors levels (ER-α isoforms).	Hu et al., 2013; Wong et al., 2011; Helland et al., 2011; Zhao et al., 2011; Dong et al., 2010; Nie et al., 2010; Watanabe et al., 2009; Schultz et al., 2008b.

Table 32 MiRNAs that were selected for evaluating their expression profile in cell cultures established *in vitro* for this PhD thesis.

A) Overexpression of p19 and p19 (G12S) proteins increase the miRNAs expression profile in *Hela* cells.

When p19 and p19 (G12S) protein were overexpressed in *HeLa* cells separately, we observed a general increase in all the miRNAs expression profiles tested, especially in miR-342 and miR-335 (graphic 2). Nevertheless, p19 (G12S) protein increased dramatically the expression of these miRNAs (24.62- fold change value for miR-342 and 23.85- fold change value for miR-335 respectively, (graphic 2 and table 33). It is important to notice that two miRNAs did not amplify in all assays performance and they were miR-206 and miR-138, and this is the reason of their fold change values are not shown.



Graphic 2. MiRNAs Taqman PCR Real Time expression profile obtained in *HeLa* cells overexpressing pRK5-p19 or pRK5-P19 (G12S) proteins. *HeLa* cells were transiently transfected with pRK5 vectors separately: pRK5-C1 (used as transfection control); pRK5-p19 which contains the sequence for overexpressing p19 c-H-Ras protein; and pRK5-p19 (G12S) contained the sequence for overexpressing p19 (G12S) Costello mutant protein. Overexpression of p19 and p19 (G12S) proteins showed a upregulation in all miRNAs evaluated, but in p19 (G12S) protein the increment was dramatic, especially in miR-342 and miR-335 fold change value.Three independent experiments were done, each of them per quadruplicate (n=12, * and ** means a significant statistic value of $p<0.05$, calculated by Mann Whitnney U-T test). GraphPad Prism version 4.0 Software.

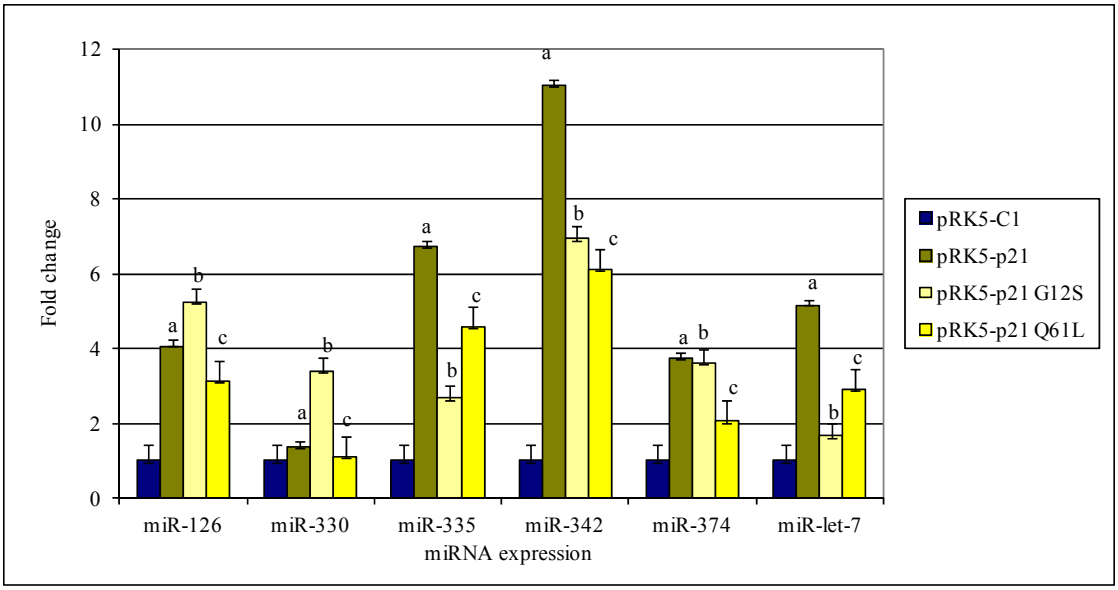
FOLD CHANGE VALUES

miRNA	pRK5-C1	pRK5-p19	pRK5-p19 (G12S) Costello
miR-126	1	2.92	12.21
miR-206	N/A	N/A	N/A
miR-138	N/A	N/A	N/A
miR-330	1	3.41	7.59
miR-335	1	3.72	24.64
miR-342	1	11.20	23.85
miR-374	1	2.12	15.06
miR-let-7	1	3.05	13.76

Table 33. Fold change values of miRNAs detected in *Hela* cells overexpressing pRK5-p19 or pRK5-p19 (G12S) proteins separately. MiR-342 showed a dramatic upregulation induced by p19 protein overexpression, and miR-335 increased by overexpression of p19 (G12S) protein expression. Three independent experiments were done, each of them per quadruplicate (n=12), N/A means that miR-206 and miR-138 did not amplify (N/A) in the experiments. In these assays pRK5-C1 was the empty vector used as a control for transfection; pRK5-p19 vector contained the sequence for overexpressing p19 c-H-Ras protein and pRK5-p19 (G12S) vector, had the sequence for overexpressing the p19 (G12S) Costello mutant protein.

B) Overexpression of p21 and their mutant variant proteins increase the miRNAs expression profile in *HeLa* cells

In these assays, we also observed a general increase in miRNAs expression profile similar to the data obtained with overexpression of p19 and p19 (G12S) proteins. In these assays miR-342 and miR-335 were also upregulated for p21 and p21 (Q61L) proteins, but the presence of G12S mutation in p21 induces a upregulation of miR-126 not detected in the other p21 cell lines (graphic 3 and table 34)



Graphic 3. MiRNAs Taqman PCR Real Time expression profile obtained in *Hela* cells overexpressing pRK5-p21, pRK5-p21 (Q61L) or pRK5-p21 (G12S) proteins. *HeLa* cells were transiently transfected

with pRK5 vectors: pRK5-C1 (empty vector used as transfection control), pRK5-p21, pRK5-p21 (Q61L), or pRK5-p21 (G12S) vectors. After 24 h postransfection, miRNAs and cDNA were obtained for performing miRNA Taqman PCR Real Time. Three independent experiments were done, each of them per quadruplicate (n=12, a, b and c, means a significant difference calculated by Anova, one way analysis of variance, $p < 0.05$, p value= 0.036), GraphPad Prism version 4.0

The highest increment in these assays, was detected in miR-342 (11.02- fold change value) when pRK5-p21 was overexpressed, followed of miR-335 upregulation (6.72-fold change value); similar results of miRNAs expression values were obtained in pRK5-p21 (Q61L) cell line (6.11- fold change value for miR-342 and 4.57- fold change value for miR-335, respectively). Moreover it was possible to see the contribution of G12S mutation to miRNA expression profile because this mutation upregulated miR-342 (again, 6.94- fold change value) and miR-126 (5.24-fold change value). These results suggests that p21 and p21 (Q61L) proteins drive molecular mechanisms for overexpressing miR-342 and miR-335; and p21 (G12S) protein, modulates the expression of miR-126.

In table 34, fold change values of each miRNA expression evaluated are reported when p21 proteins are overexpressed (pRK5-p21, pRK5-p21 (Q61L) and pRK5-p21 (G12S) transfected *Hela* cell lines). Fold change values of miR-206 and miR-138 are reported as N/A which means that these miRNA PCR Real Time assay did not amplified, for that reason these results are not shown.

FOLD CHANGE VALUES

miRNA	pRK5-C1	pRK5-p21	pRK5-p21 (Q61L)	pRK5-p21 (G12S) Costello mutant
miR-126	1	4.07	3.13	5.24
miR-206	N/A	N/A	N/A	N/A
miR-138	N/A	N/A	N/A	N/A
miR-330	1	1.38	1.12	3.41
miR-335	1	6.72	4.57	2.69
miR-342	1	11.02	6.11	6.94
miR-374	1	3.73	2.06	3.63
miR let-7	1	5.16	2.92	1.68

Table 34. Fold change values of miRNAs detected in *HeLa* cells overexpressing pRK5-p21, pRK5-p21 (Q61L) or pRK5-p21 (G12S) proteins separately After 24 h postransfection, miRNAs and cDNA were obtained for performing miRNA Taqman PCR Real Time, results showed that it might exist a correlation between the miRNAs expression profile and the grade of oncogenic transformation of cancer cells. N/A means that miRNA did not amplify in the assay**.** Three independent experiments were done, each of them per quadruplicate.

5.1.2 MiRNAs Taqman PCR Real Time expression profile of knock-out *H-Ras, (-/-)* murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21.

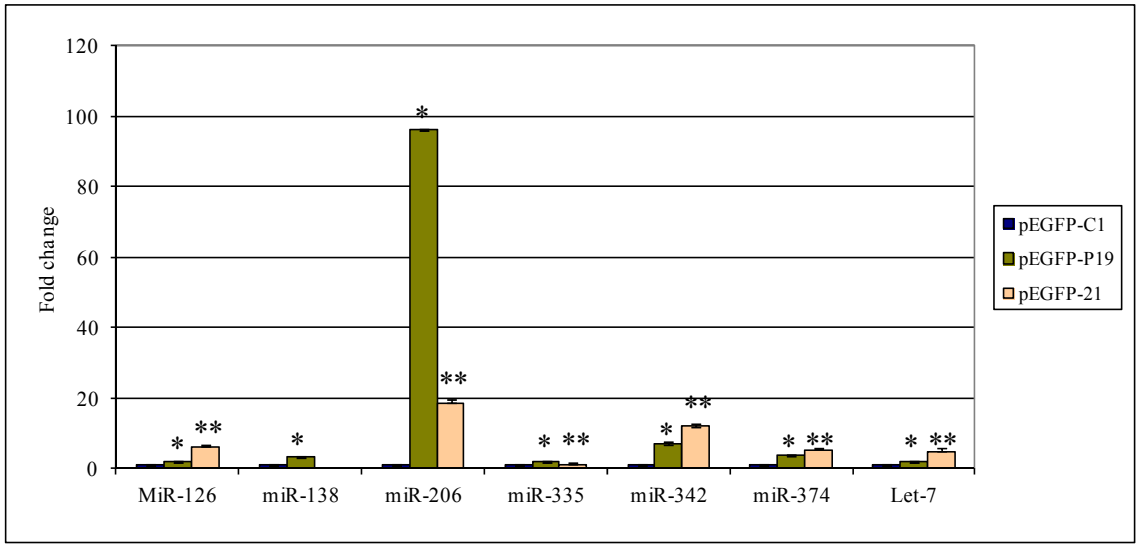
A) P19 and p21 proteins showed differential miRNAs expression profile when are expressed in knock-out *H-Ras,* (-/-) murine embryonic fibroblasts

They are evidences that H-Ras proteins do not have genetic redundancy (Castellano and Santos, 2011; Castellano et al., 2009) that could cover up the specific contribution of p19 or p21 proteins in miRNAs expression profile, and due of this reason we wanted to evaluate the miRNA expression profile in knock-out *H-Ras* (-/-) murine embryonic fibroblast transfected with pEGFP-p19 or pEGFP-p21. These transfected cell lines were previously selected using kanamycine (500 μg/μl) during a month, in order to eliminate those cells that did not contain the plasmid of our interest, then those transfected and stable cells were harvested and in further experiments miRNAs were obtained for evaluating miRNAs Taqman PCR Real Time.

Interestingly, our miRNAs expression data showed a differential upregulation in two miRNAs depending of the H-Ras protein expressed, (Graphic 4). For example, miR-206 was upregulated in the presence of pEGFP-p19. It is important to point out that miR-206 amplified very well in these assays at difference of our transient transfected *HeLa* cell lines with pRK5 vectors, due of this it was possible to calculate miR-206 fold change values and at the contrary, miR-330 did not amplified in knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19, or pEGFP-p21.

As we can see in graphic 4, a dramatic increase of miR-206 was observed when pEGFP-p19 was expressed in our knock-out *H-Ras* (-/-) murine embryonic fibroblasts (96.16- fold change); this effect was not seen in pEGFP-p21 knock-out *H-Ras* (-/-) murine embryonic fibroblasts (18.80-fold change value); these results are accordingly with those obtained by Camats-Malet, 2008 PhD thesis, UAB, in which it was reported that overexpression of p19 protein leads an upregulation of miR-206 expression and also induced G1/G0 quiescent state in the cell. Besides miR-342 also showed differential expression in knock-out *H-Ras*

(-/-) murine embryonic fibroblasts when pEGFP-p21 was expressed (12.21- fold change) at difference of pEGFP-p19 expression in the same cell line (7.11-fold change value).



Graphic 4. MiRNAs Taqman PCR Real Time expression profile obtained in knock-out *H-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-p19 or pEGFP-p21 proteins. In these cell lines, miR-206, miR-342, miR-374 and miR-let-7 increased their expression profile, even though differential expression were detected, in miR-206 and miR-342 which depending of the type of H-Ras protein expressed in knock-out *H-Ras* (-/-) murine embryonic fibroblasts; miR-206 was upregulated when pEGFP-p19 was expressed (96.16-fold change) and miR-342 (12.20- fold change) was upregulated when pEGFP-p21 was expressed. MiR-138 and miR-330 did not amplify. Three independent experiments were done, each of them per quadruplicate (n=12; * and ** means a statistic difference analyzed by ANOVA (one way analysis of variance), *P* value= 0.036, $p < 0.05$). PEGFP-C1 was used as transfection control, it contains the sequence for the green fluorescent protein (GFP); pEGFP-p19 vector contains the sequence for pEGFP-p19, and pEGFP-p21 vector contains the sequence for expressing pEGFP-p21 protein. GraphPad Prism version 4.0 Software.

The fold change values of miRNAs expression profiles of knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21 are shown in table 30.

FOLD CHANGE VALUES

miRNA	pEGFP-C1	pEGFP-p19	pEGFP-p21
miR-126	1.00	2.00	6.3
miR-138	1.00	3.41	N/A
miR-206	1.00	96.16	18.80
miR-330	1.00	N/A	N/A
miR-335	1.00	1.96	1.26
miR-342	1.00	7.11	12.21
miR-374	1.00	3.69	5.38
miR let-7	1.00	1.87	4.96

Table 35. Fold change values of miRNAs detected in knock-out *H-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-p19 or pEGFP-p21 proteins separately. PEGFP-C1 is the empty vector used as a control for transfection and contains the sequence for the green fluorescent protein (GFP); pEGFP-p19 vector contains the sequence for pEGFP-p19, and pEGFP-p21 vector contains the sequence for pEGFP-p21.

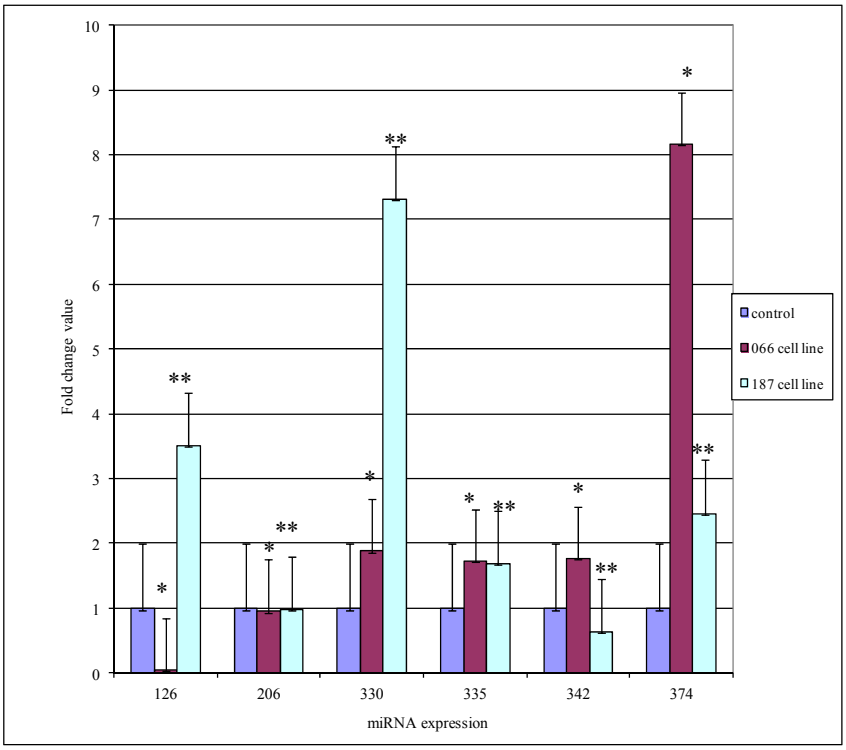
5.1.3 MiRNAs expression profile of mutant fibroblasts obtained from tumours of Costello Syndrome patients

A) MiRNAs expression profile in (G12S) fibroblasts of Costello Syndrome patients

In these experiments we decided to analyze the contribution of two of the most frequent mutations detected in Costello Syndrome (G12S or G12A), in terms of regulating the expression profile of our selected set of miRNAs. For these experiments, we analyzed G12A or G12S fibroblasts cell lines obtained from tumours of Costello Syndrome patients that were removed by surgery. Two of these cell lines contained the mutation G12S (fibroblasts cell lines designed as "187" and "66") and one fibroblast line which contained the mutation G12A (designed as "067" fibroblasts line). These cell lines were evaluated and compared with fibroblasts obtained from "normal" subjects fibroblasts (called "control"). For calculating the fold change values, was necessary to use let-7, because the other two controls used in other miRNAs Taqman PCR Real Time assays (RNHU6 and U18); did not amplify in these assays, and for this reason this data is not shown; besides miR-138 also did not amplified.

In all Costello fibroblasts cell lines evaluated, we found a general upregulation in miR-330, miR-335 and miR-374; and a downregulation in miR-206 expression.

In graphic 5, is evident that G12S mutation in Costello fibroblasts, contribute to a upregulation of miR-374 and a downregulation of miR-206 (8.17- and 0.95- fold changes respectively (table 36).



Graphic 5 MiRNAs Taqman PCR Real Time expression profile detected in (G12S) fibroblasts obtained from tumours of Costello Syndrome patients. The mutation G12S increased the miRNA expression profile in a general way in miR-330, miR-335 and miR-374, and a downregulation of miR-206. Three independent experiments were done, each of them per quadruplicate (n=3, * and ** means a statistic difference calculated by anova (one-way analysis of variance), Bartlett's test for equal variances $p<0.05$. For these experiments, let-7 was used as amplification control for calculating fold change values. GraphPad Prism version 4.0

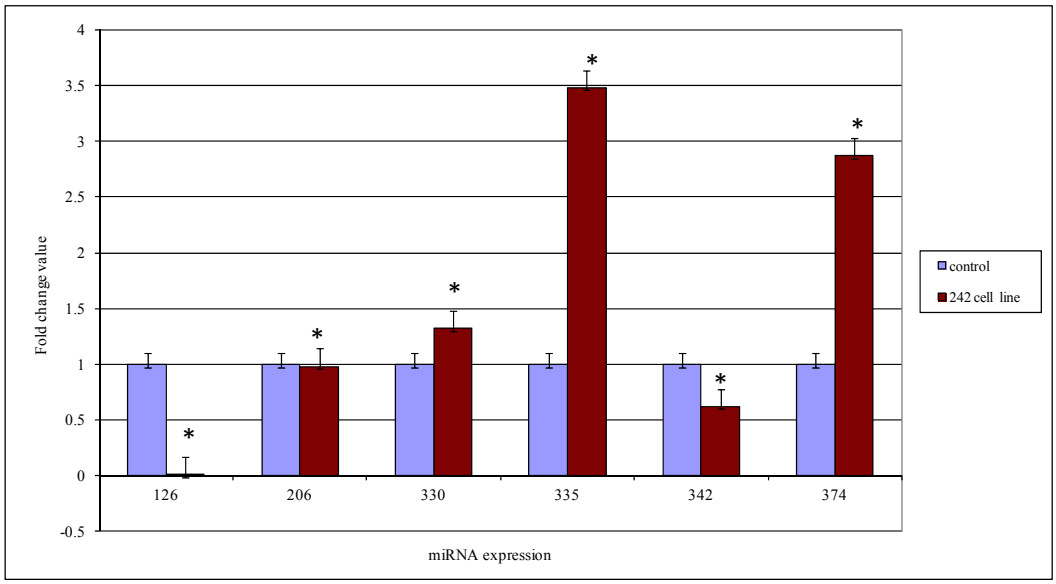
FOLD CHANGE VALUES

miRNA	Control	066	187
miR-126	1	0,05	3.5
miR-206	1	0.95	0.97
miR-330	1	1.88	7.31
miR-335	1	1.73	1.68
miR-342	1	1.77	0.63
miR-374	1	8.17	2.46

Table 36. Fold changes values of miRNAs detected in (G12S) mutant fibroblasts obtained from tumours of Costello Syndrome patients. A general upregulation in miR-330, miR-335 and miR-374 expression and a downregulation of miR-206 were detected in fibroblasts of Costello Syndrome patients which contained G12S mutation. In these assays let-7 was used as amplification control for calculating fold change values.

B) MiRNAs expression profile in (G12A) fibroblasts of Costello Syndrome patients

In other hand, G12A mutation in Costello fibroblasts, induced an upregulation in miR-335, miR-374 and miR-330 and a downregulation of miR-206, similar data was obtained in G12S Costello Syndrome fibroblasts of patients (Graphic 6 and table 37).



Graphic 6. MiRNAs Taqman PCR Real Time expression profile of (G12A) fibroblasts obtained from tumours of Costello Syndrome patients. The mutation G12A promoted an increase in the expression of miR-330, miR-335, miR-342 and miR-374, and miR-206 was again downregulated (similar results obtained were obtained in miRNAs assays of G12S, see graphic 6). Three independent experiments were done, each of them per cuadruplicate. A significative statistical difference was represented by (*), (n=3); calculated by anova (one-way analysis of variance), Bartlett's test for equal variances $p<0.05$. GraphPad Prism version 4.0

FOLD CHANGE VALUES		
miRNA	**Control**	**242**
miR-126	1	0.01
miR-206	1	0.98
miR-374	1	1.32
miR-335	1	3.48
miR-342	1	0.62
miR-330	1	2.87

Table 37. Fold changes values of miRNAs detected in (G12A) mutant fibroblasts obtained from tumours of Costello Syndrome patients.

5.2 Invasion assays

The first acquired characteristic of cancer cells is their ability of detaching from surrounding cells; this step is followed by the development of invasion which is the beginning of metastasis. To further analyze the capacity invasion of our *Hela* cell lines transfected transiently with pRK5-C1, pRK5-p19, pRK5-p19 (G12S), pRK5-p21, pRK5-p21 (G12S) and pRK5-p21 (G12S); we used Innocyte TM de Calbiochem kit for invasion assays. Briefly 0.5 $x10^6$ cells/ml or each transfected *Hela* cell line were seeded in DMEM without FBS; 350 µl of cell suspension was added to each insert, later the cells were incubated at 37°C/5%, CO_2 during 48 h. Each well contains an insert in which the cells are seeded, during the period of incubation cancer cells that have developed abilities for detaching and showed invasion capacity can pass through the insert and they settled in the inner chamber. After a period of incubation those cells contained in the inner chamber were stained and fluorescence was measured (485 nm, excitation, 520 nm emission). Previous results were obtained in relative fluorescent units (RFU), and then calculated number of cells ($1x10^4$ cells).

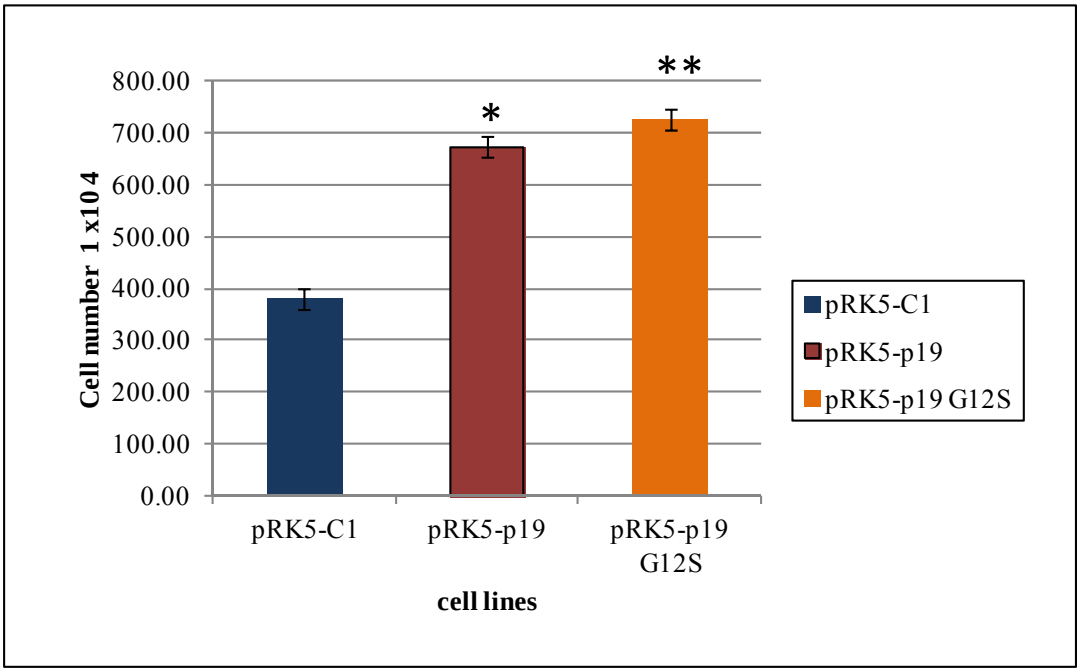
5.2.1 Overexpression of p19 (G12S) protein increased invasion capacity more than p19 protein

We also decided to evaluate the capacity of invasion in *HeLa* cells acquired by overexpression of p19 or p19 (G12S) proteins. For these experiments, we overexpressed transient and ectopically pRK5 vectors: pRK5-C1 (empty vector used as a transfection control), pRK5-p19 (which contained the sequence for overexpressing p19 c-H-Ras

protein) and pRK5-p19 (G12S), (which contained the sequence for overexpressing p19 G12S protein mutant of Costello Syndrome).

All our transfected cell lines were seeded and evaluated the same day, but for a better understanding, results were divided in two graphics (graphic 7 and graphic 8). In graphic 7, the data of capacity of invasion assays of pRK5-p19 and pRK5-p19 (G12S) cell lines are shown; and in graphic 8, the results of pRK5-p21 and their mutant variants are shown.

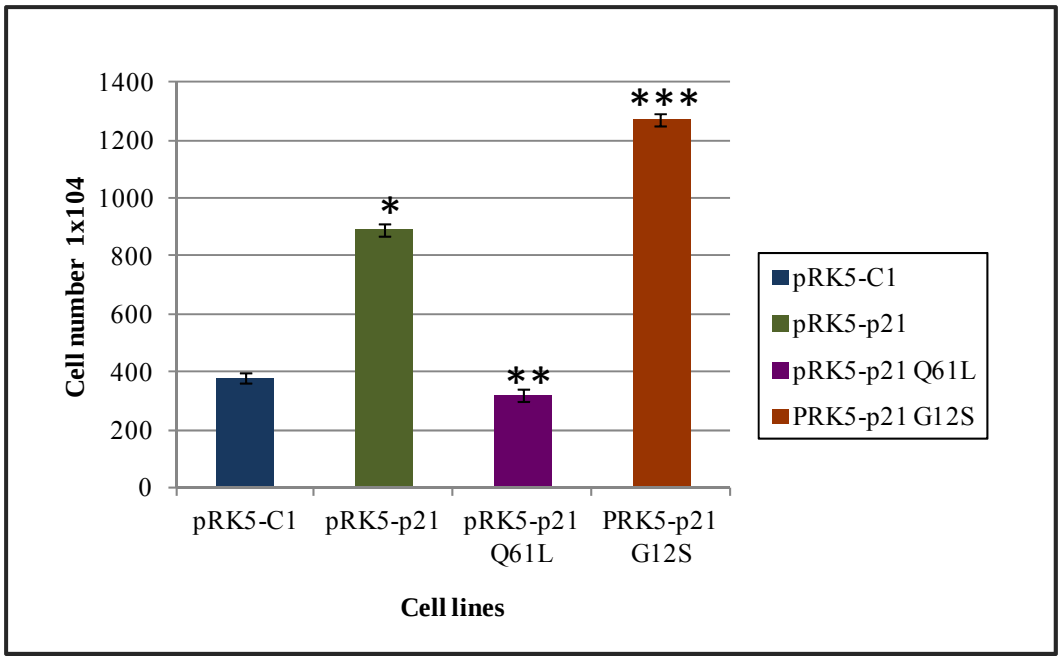
In graphic 7, overexpression of pRK5-p19 (G12S) protein induced an increase of invasion capacity of the cells, which was also detected in pRK5-p19 cell line, even though this increment was minor than the increment detected in pRK5-p19 (G12S) cell line, is probably that this increment in pRK5-p19 cell line was due of the transfection process that induces a stress state in the cell.



Graphic 7 Invasion assays on *HeLa* cells overexpressing pRK5-p19 or pRK5-p19 (G12S) proteins. Invasion capacity is similar in pRK5-p19 and pRK5-p19 (G12S) Costello mutant proteins overexpressed in *HeLa* cells. Three independent experiments were done, each of them per quadruplicate (n=12; (*) and (**) means a significant statistic value of *p*, of Mann Whitnney U-T test, $p < 0.05$), GraphPad Prism version 4.0.

These results indicate that high level overexpression of p19 and p19 (G12S) Costello mutant proteins, resulted in important transformed phenotypes *in vitro* assays.

We also performance invasion assays overexpressing ectopically in *HeLa* cells, pRK5-p21 and their mutant proteins pRK5-p21 (Q61L) and pRK5-p21 (G12S). In the case of pRK5-p21 protein overexpression produces a higher capacity of invasion than pRK5-p19 protein and pRK5-p19 (G12S) Costello mutant overexpression. Surprisingly, overexpression of pRK5-p21 (Q61L), showed a lower invasion capacity similar to the invasion observed in the control. Besides pRK5-p21 (G12S) *HeLa* cell line exhibited the highest level of invasion compared with others transfected *HeLa* cell lines (graphic 8).

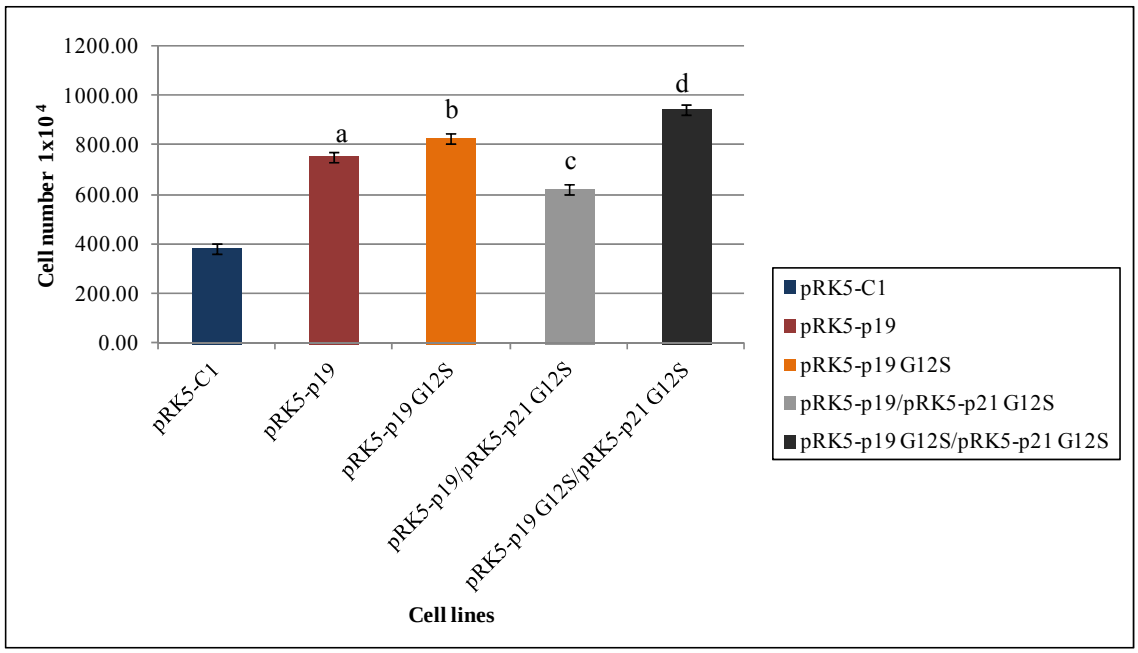


Graphic 8. Invassion assays on *HeLa* cells overexpressing pRK5-p21, pRK5-p21 (Q61L) or pRK5-P21 (G12S) proteins (Innocyte TM Cell Invasion Assay Kit of Calbiochem). Invasion capacity was evaluated in the following transfected *HeLa* cell lines: pRK5-C1 (used as a control), pRK5-p21, pRK5-p21 (Q61L) and pRK5-p21 (G12S). In the graphic pRK5-p21 (G12S) cell line showed the highest invasion followed by pRK5-p21. The experiments were done at two times in an independent way and for triplicate each time (*), (**) and (***), mean a statistical significance difference, Mann Whitney-U T test, $p \leq 0.0001$). Notice that pRK5-p21 (Q61L) cell line, *p* value did not show statistical significance difference. GraphPad Prism version 4.0 software.

5.2.2 In higher invasion capacity conditions, p19 is able to limitate invasion capacity

In our previous results of invasion assays, we detected that pRK5-p21 (G12S) cell line exhibited the highest level of invasion, and this environment condition was used for

evaluating the behaviour of p19 protein, therefore we cotransfected our *HeLa* cells with pRK5-p19/pRK5-p21 (G12S) or pRK5-p21 (G12S)/ pRK5-p19 (G12S) vectors. In effect, as we expected, p19 was able to decrease the invasion conferred by pRK5-p21, in the cotransfection pRK5-p21 (G12S), is important to notice that in pRK5-p19 (G12S)/pRK5-p21 (G12S) cotransfection this effect was not seen (Graphic 9).

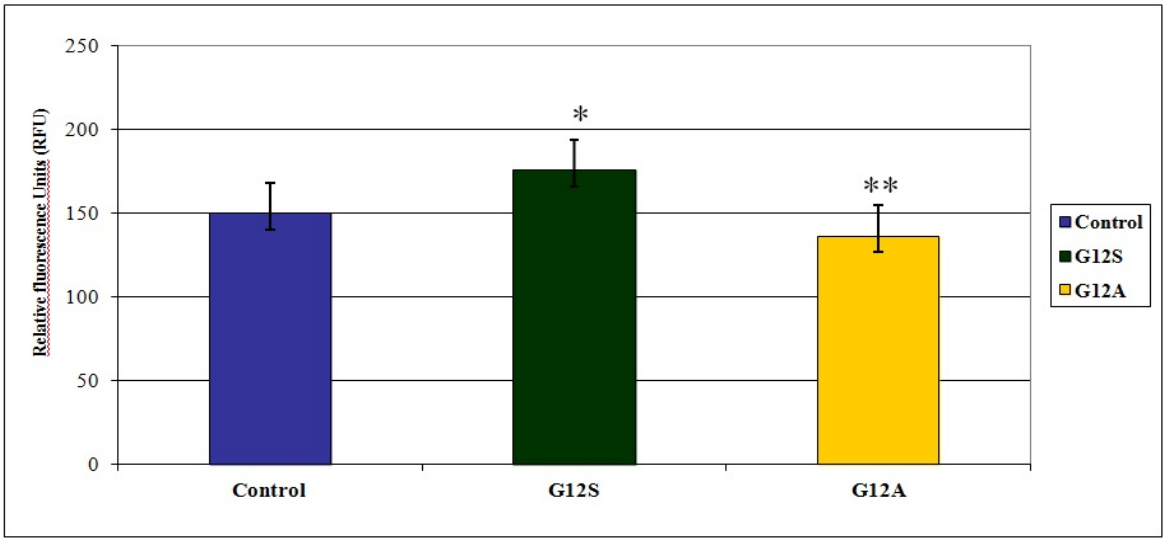


Graphic 9. P19 protein decreased the high capacity of invasion promoted by overexpression of pRK5-p21 (G12S) protein in *HeLa* cells. In previous invasion assays performanced we detected that pRK5-p21 (G12S) cell line had the higher invasion capacity and this effect was used to evaluate the behaviour of p19 when we overexpressed both proteins in our cotransfected *HeLa* cell lines with pRK5-p19/pRK5-p21 (G12S) and pRK5-p19 (G12S)/pRK5-p21 (G12S). In effect, as we expected, p19 protein was able to limitate the high invasion conditions promoted by p21 (G12S); is important to notice that this effect was not detected in the cotransfection with pRK5-p19 (G12S)/pRK5-p21 (G12S).Three independent experiments were done, each of them per triplicate (n=9, a, b, c, and d mean a statistic significant value of $p < 0.00001$; Mann Whitnney U-T test). The experiments were done at two times in an independent way and for triplicate each time. GraphPad Prism version 4.0 Software.

5.2.3 G12S mutation in Costello Syndrome is more invasive than G12A mutation

Due the most frequent mutations detected in patients of Costello Syndrome are G12S or G12A, and these substitutions disrupt guanine nucleotide binding and cause a constitutive activation of the Ras/MAPK pathway and several transcriptional factors that have influence in the expression of specific genes which codifying for proteins implicated on the control of cellular proliferation and/ or differentiation, (Denayer et al., 2008; Tydyman et al., 2009; Gremer et al., 2010). We decided to determinate the functional role of G12S or G12A

mutations in Costello Syndrome in *in vitro assays*. For these experiments, we defrost G12S and G12A mutated fibroblast cell lines obtained from tumours of Costello patients. Our results indicated that G12S fibroblasts showed a higher capacity of invasion than those fibroblasts that contained G12A mutation (Graphic 10); and these results are disagree with the report of Hinek et al., 2000, in which they established that G12A mutation is more invasive than G12S.



Graphic 10. Invasion assays of G12S mutation vs., G12A mutation in fibroblasts obtained from tumours of Costello Syndrome patients. A suspension of 0.5x 10^6 cells/ ml suspension was prepared and 350 µl of the suspension was seeded in each insert with DMEM without FBS, then cell cultures were incubated at 37°C/ 5% CO_2 for 48 h. When the time of incubation had finished, those cells that passed through the insert were harvested and stained with calcein cell suspension. Fluorescence was measured (480 nm excitation and 520 nm emission). The G12S fibroblasts were more invasive than G12A fibroblasts and normal control; (*) and (**) means a statistical significance difference, calculated by Mann Whitney-U T test, $p \leq 0.0001$). The experiments were done at two times in an independent way and for triplicate each time). GraphPad Prism version 4.0.

Our results suggest that G12S mutation confers a higher invasion capacity than G12A mutation in fibroblasts of Costello Syndrome, and is probably that they have more capacity activating Ras/Raf/MEK/ERK and PI3K/AKT signaling pathways that lead cell growth and invasion *in vitro*.

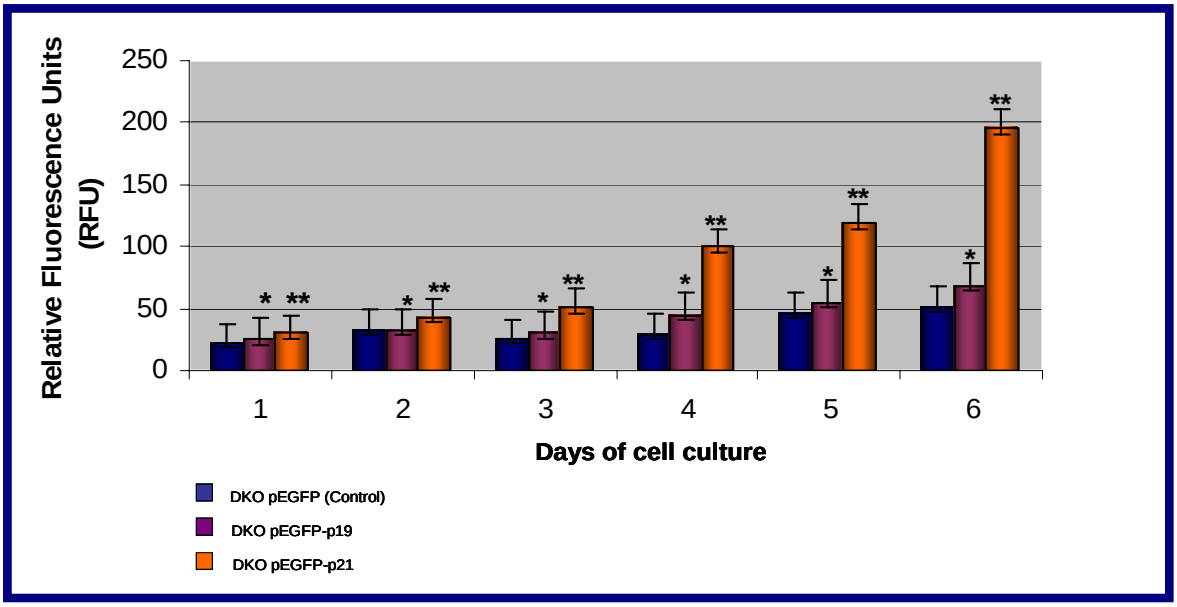
5.3 Proliferation Assays

Another important ability that cancer cells have to develop is the capacity to escape from normal regulatory mechanisms that control how many times a cell can divide, for this

reason we decided to performance proliferation assays using Cyquant proliferation assay of Invitrogen. Briefly, 500 cells of each transfected cell line were seeded in each well in a plate of 96 wells; six plates were prepared and they were incubated at 37°C/5% CO_2, 24 h later one plate was frozen at -80°C, every day this procedure was repeated, until all the plates were frozen. The seventh day of the experiments, all the plates were defrozen, washed with PBS 1X solution, stained with working solution and incubated them in dark conditions at 37°C, - 1 h. Then fluorescence was detected (485 nm, excitation, and 528 nm emission). Fluorescence was expressed as relative fluorescent units (RFU).

5.3.1 Overexpression of p19 protein presents a lower proliferation than p21 protein

At the beginning of our proliferation experiments, we evaluated our pRK5 transfected *HeLa* cell lines which overexpressed H-Ras proteins and their mutant variants (pRK5-p19, pRK5-p19 (G12S), pRK5-p21, pRK5-p21(Q61L) and pRK5-p21 (G12S)), but results were not conclusive (data not shown), for that reason we decided to repeat our proliferation experiments using double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 and pEGFP-p21 because these cell lines did not show redundancy of *Ras* genes (Castellano et al., 2007); and they should clearly the contribution of each protein to proliferation. In these assays, (graphic 11), we found that pEGFP-p19 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts proliferation was lower than in pEGFP-p21 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts.



Graphic 11 Proliferation assay of double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with PEGFP-p19 or pEGFP-p21. pEGFP-p21 double knock-out *H-Ras (-/-), N-Ras* cell line showed the highest proliferation rate in this experiment, and pEGFP-p19 double knock-out *H-Ras (-/-), N-Ras* (-/-) cell line the proliferation rate was similar to the control. In the graphic (*) and (*) means a statistical significance difference calculated by anova (one way analysis of variance), p(0.05≤). The experiments were done at four times in an independent way and for triplicate each time. GraphPad Prism version 4.0.

Is probably that p19 H-Ras protein can induce a low proliferation stage in the cell by several mechanisms as such as modulation of telomerase activity via p19/p73 complex (Camats et al., 2009); or binding neuron specific enolase (Sang-Ming et al., 2009).

5.4 Cytometry analyses

To asses if the stage cell cycle progression was modified by p19 or p21 proteins we studied the cell phase distribution of *HeLa* cells overexpressing transiently pRK5- p19; pRK5-p19 (G12S), or pRK5-p21 proteins, using iodide propidium (IP) but the results were not clear (data not shown); then we modified our experiment cotransfecting pEGFP-C1/pRK5-p19 or pEGFP-C1/pRK5-p19 (G12S) proteins. After 24 hrs postransfection GFP(+) and GFP(-) cells were selected using a MOFLO cytometer, and then were fixed in 70% ethanol solution for 24 h at 4^{o}C, at the end of that period of time cells were stained with IP and incubated in dark conditions 1 h at 37^{o}C then cells re-analyzed in a XL-cytometer.

5.4.1 P19 induces a quiescent state in G0/G1 cell cycle phase

In these experiments, overexpression of pRK5-p19 protein was observed to increase the number of cells in the G1 phase by 78.47% and prolong G1 phase length (fig. 22B and 22D), which indicates that plays a role in regulating the switch from the G2 to S phase, thereby inducing a G1/S phase delay and also a decrease in the G2 phase. Moreover, G1/S quiescent state was also promoted by pRK5-p19 (G12S) cell line; and the number of cells found in the G1 phase was 83.93%, (fig. 22C and D) this finding suggest that the mutation G12S (which induces a constitutive activation of RAS/MAPK pathway) has not influence in the activation of proliferation, and the only presence of p19 H-Ras protein can promote a quiescent arrest in G1 cell cycle phase.

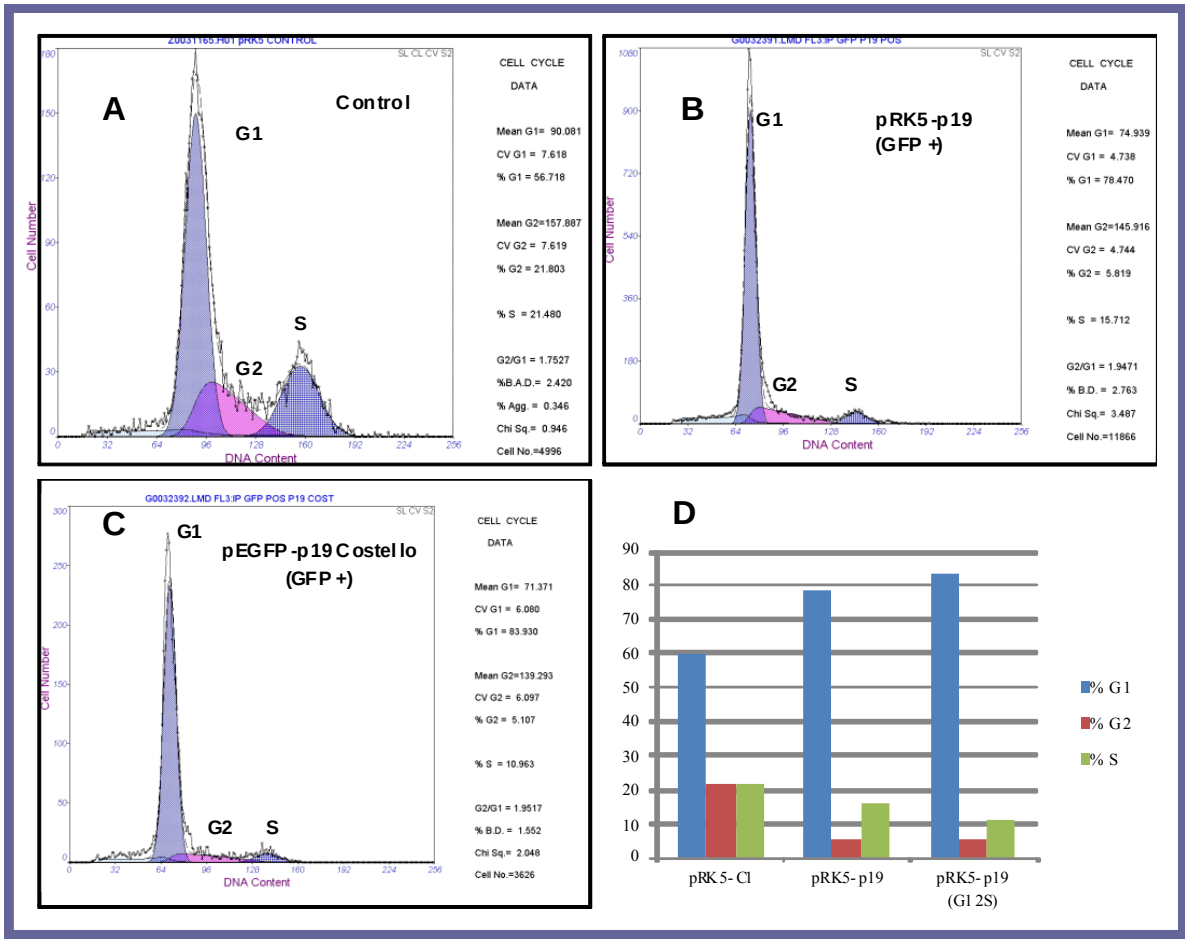


Fig. 21 Cell cycle analyses of *HeLa* cells overexpressing p19 or p19 (G12S) proteins. A) Cell cycle phases of pRK5-C1/ pEGFP-C1 cotransfection (control); **B)** Cell cycle phases of pRK5-p19/ pEGFP-C1 cotransfection; **C)** Cell cycle phases of pRK5-p19 (G12S) Costello mutant/ pEGFP-C1 cotransfection. These experiments showed that both cotransfections pRK5-p19/pEGFP-C1 (Panel B) and pRK5-P19 (G12S) Costello mutant/pEGFP-C1 (Panel C), induces a quiescent state in G0/G1 cell cycle comparing with the control Panel (A).

The percentages of each phase detected in the cell cycle of each of our analyzed cell lines are shown in table 38.

	% CELL CYCLE PHASE			
Cell line	**% G1**	**%G2**	**%S**	**G2/G1**
pRK5	59.71	21.8	21.48	1.75
pRK5-p19	78.47	5.81	15.71	1.94
pRK5-p19 (G12S) Costello mutant	83.93	5.10	10.96	1.95

Table 38. Analyses of cell cycle in *HeLa* cells overexpressing pEGFP-p19 or pEGFP-p21 (G12S) proteins.

Further analyses of cytometry performanced in knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p19 (G12S) proteins confirmed the quiescent cell cycle state in G1 phase promoted by p19.

In the figure 23A, knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-C1 (which contains the empty vector and was used as transfection control) showed that the majority of the cells were in S phase (83.94%), and only a small percent of the cells was in G1 phase (1.68%); in knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 the cells were arrest in G1 phase (57.45%), and a minor percent was in S phase (38.21%), (fig. 23B); and the majority of knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p21 were in S phase (50.49%), (fig. 23C). These findings are agreed with the results reported by Castellano et al., 2009; in which they found that the absence of *H-Ras* loci affected the profile of transcriptional wave detected during G1 progression more strongly than did the absence of *N-Ras* loci. H-Ras was predominantly functionally associated with growth and proliferation. Moreover p19 can be regulating the cell cycle progression by several pathways, 1) by phosphorylation of ERK1, that could be acting as negative modulator (Camats et al., 2009), 2) binding p73β via MDM2 protein (Jeong et al., 2006) or 3) binding neuron specific enolase via p73, which repressed lung cancer cell proliferation (Jang et al.,2010).

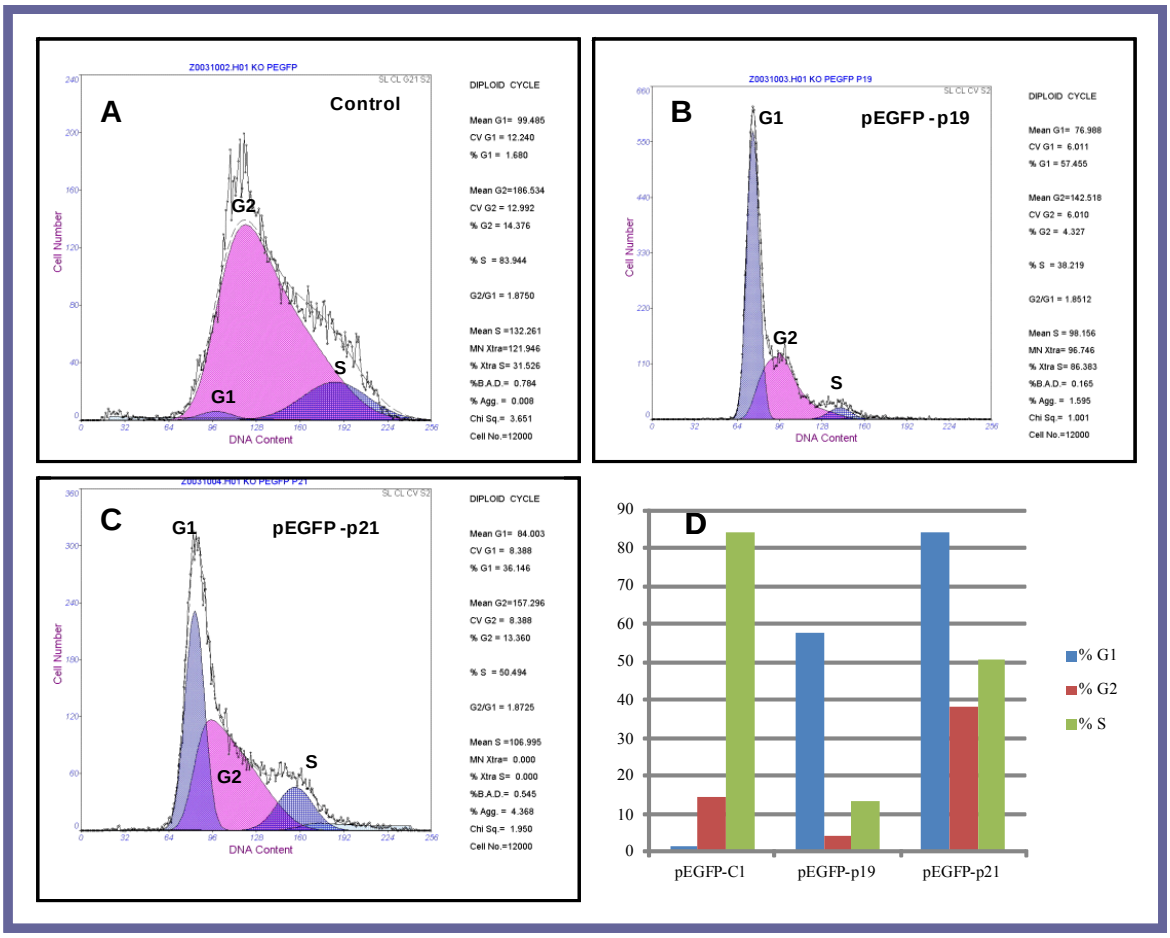


Fig. 22 Cell cycle analyses of knock-out *H-Ras* (-/-) murine embryonic fibroblasts, expressing pEGPF-p19 or pEGFP-p21 proteins. A) Cell cycle of knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-C1 (control) **B**) Cell cycle of knock-out *H-Ras* (-/-) murine embryonic fibroblasts pEGFP-p19 and **C)** Cell cycle of knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p21.

	% CELL CYCLE PHASE			
Knock-out H-Ras (-/-) cell lines	**G1%**	**G2%**	**S%**	**G1/G2**
pEGFP-C1 (control)	1.68	14.37	83.94	1.87
pEGFP-p19	57.45	4.32	38.21	1.85
pEGFP-p21	36.14	13.36	50.49	1.87

Table 39. Cell cycle analyses of knock-out *H-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-p19 or pEGFP- p21 proteins separately.

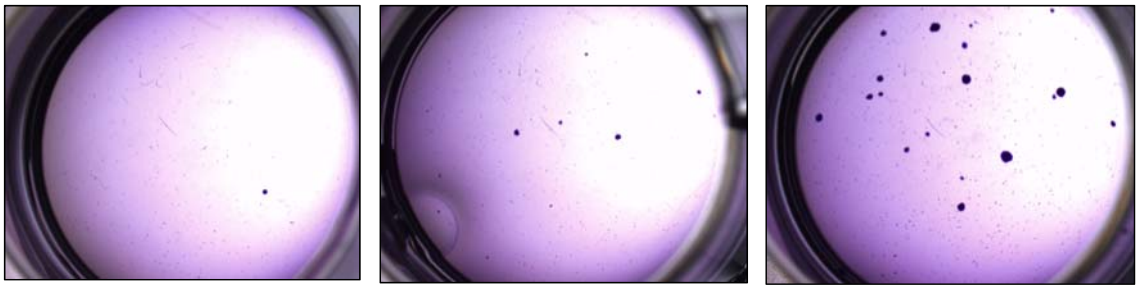
5.5 Capacity of forming colonies assay (clonogenic anchorage agar assay)

The acquisition of migratory and invasive capabilities by tumour cells is a crucial stage in the progression of solid tumours from a benign to a malignant state and is a process that could take many years. Capacity of forming tumours of cancer cells is a key point for developing metastasis and only a fraction of them retains the capacity of growing without limits and produce colonies in distant tissues.

Fortunately, the capacity of forming colonies can be evaluated *in vitro and the* assay essentially tests every cell in the population for its ability to undergo "unlimited" division (Franken et al., 2006). In these assays we analyzed the clonogenic ability of our transient transfected *HeLa* cell lines that overexpressed p19, p21 or their mutant variants (pRK5 plasmids: pRK5-p19, pRK5-p19 (G12S) pRK5-p21, pRK5-p21 (Q61L), pRK5-p21 (G12S)); and in our knock-out *H-Ras* (-/-), and double knock-out *H-Ras* (-/-) , *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21.

5.5.1 P19 (G12S) showed the highest capacity of forming colonies in agar than p19 protein

In clonogenic anchorage agar assays overexpressing pRK5-p19 or pRK5-p19 (G12S) proteins in *HeLa* cells more colonies were detected in the pRK5-p19 (G12S) cell line than in pRK5-p19 (fig. 23).



A **B** **C**

Fig. 23 Clonogenic anchorage agar assay overexpressing p19 or p19 (G12S) proteins separately in *HeLa* cells. In these assays, we found that the highest capacity of forming colonies was detected in pRK5-p19 (G12S) cell line. **A)** pRK5-C1 (control, one colony formed); **B)** pRK5-p19 (seven colonies formed); **C)** pRK5-p19 (G12S) protein (seventeen colonies formed).

Only one colony was found in pRK5-C1 cells, which was used as transfection control; besides the number of colonies formed in soft agar in pRK5-p19 cell line were seven, and in pRK5-p19 (G12S) were seventeen (table 40).

Cell line	Number of colonies formed
pRK5-C1	1
pRK5-p19	7
pRK5-p19 (G12S)	17

Table 40. Number of colonies formed in clonogenic anchorage assay in *HeLa* cell lines overexpressing pRK5-p19, or pRK5-p19 (G12S) proteins. Transfection control cell line was pRK5-C1, (empty vector)

Furthermore, colonies formed in the control were smaller than those colonies formed in pRK5-p19 (G12S) cell line (fig. 24). These results suggest that pRK5-p19 protein may inhibit cell growth in anchorage dependent as well as independent conditions in *in vitro* assay.

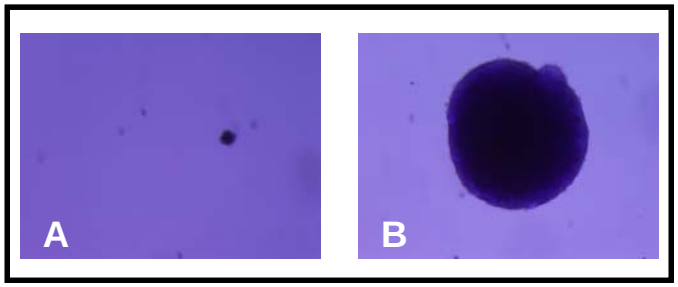


Fig. 24 Colonies formed in the clonogenic anchorage assay overexpressing pRK5-p19 (G12S) protein. **A)** colony formed in pRK5-C1 (control); **B)** colony formed in pRK5-p19 (G12S). Both images were taken with a magnifying glass 8(x).

5.2 P21 (G12S) protein showed the highest capacity of forming colonies in agar than p21 and p21 (Q61L).

In clonogenic anchorage agar assay overexpressing pRK5-p21, pRK5-p21 (Q61L) and pRK5-p21 (G12S) Costello mutant proteins separately, we can observed that pRK5-p21 has a higher capacity of forming colonies than pRK5-p19. Unexpectedly overexpression of p21 (Q61L) mutation did not contribute to increase the number of colonies formed and pRK5-p21 (G12S) Costello mutant was the cell line which formed more colonies than pRK5-p19 cell line (fig. 25).

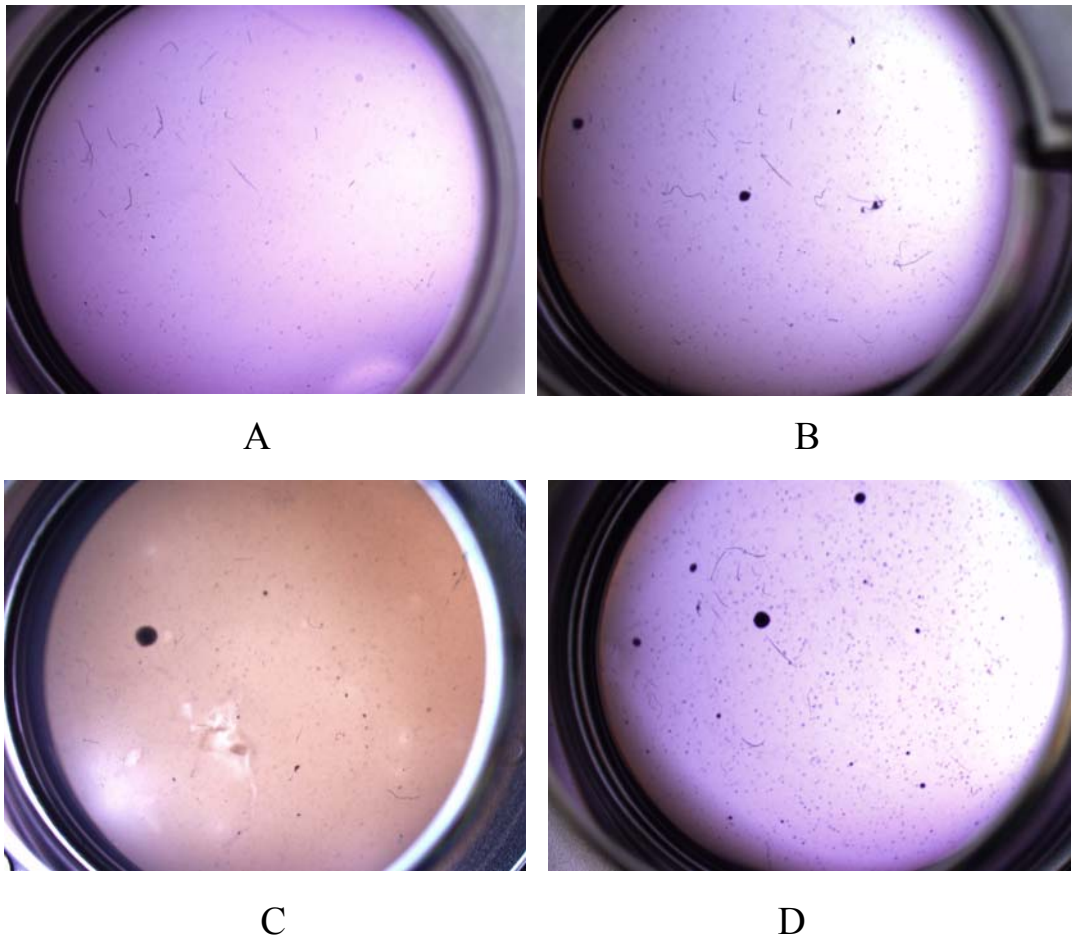


Fig. 25 Clonogenic anchorage agar assay overexpressing pRK5-p21, pRK5-p21 (Q61L) or pRK5-p21 (G12S) proteins in *HeLa* cells. A) pRK5-C1 (used as transfection control); **B**) pRK5-p21; **C)** pRK5-p21 (Q61L); and **D)** pRK5-p21 (G12S) Costello cell lines. The highest capacity of forming colonies was promoted by pRK5-p21 (G12S) Costello mutant protein overexpression.

In a general overview, all the cell lines which overexpressed pRK5-p21 proteins showed an increase in their capacity of forming colonies comparing with the control (pRK5-C1); so we counted 5-, 3- and 10-colonies formed in pRK5-p21, pRK5-p21 (Q61L), and pRK5-p21 (G12S) respectively, (table 41).

Cell line	Number of colonies formed
pRK5-C1	0
pRK5-p21	5
pRK5-p21 (Q61L)	3
pRK5-p21 (G12S)	10

Table 41. Number of colonies formed in clonogenic anchorage assays in *Hela* cell lines overexpressing pRK5-p21, pRK5-p21 (Q61L) or pRK5-p21 (G12S) proteins.

Besides, the colonies formed in pRK5-p21 (G12S) cell line were bigger than those colonies formed in the control cell line (pRK5-C1), (fig. 26).

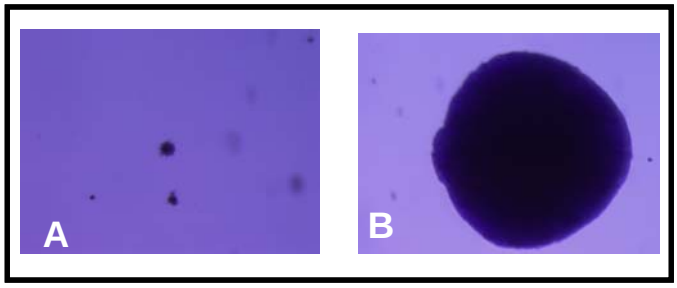


Fig. 26 Colonies formed in clonogenic anchorage assay overexpressing pRK5-p19 protein in *HeLa* cells. A) Colony formed in agar overexpressing pRK5-C1 (control), **B**) colony formed in agar overexpressing pRK5-p21 (G12S) Costello mutant in *HeLa* cells. Both images were taken with a magnifying glass 8 (x).

Further analyses of p19 c-H-Ras capacity of forming colonies were repeated in knock-out *H-Ras* (-/-); and double knock-out *H-Ras* (-/-), *N-Ras (-/-)* murine embryonic fibroblasts transfected with pEGFP-19 or pEGFP-p21. In these experiments knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with the empty vector was used as a transfection control and none colony was detected (fig. 27A); in other hand in *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-19 or pEGFP-p21 (formed one and two colonies respectively), (fig., 27B and 27C and table 42).

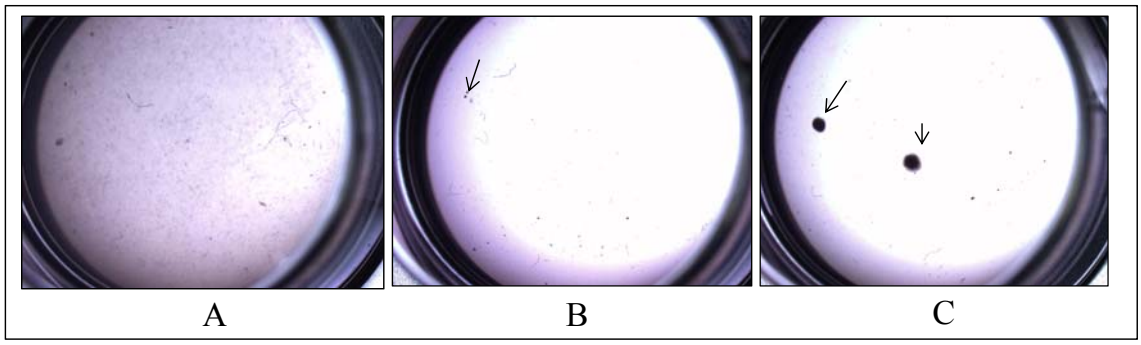


Fig. 27 Clonogenic anchorage agar assays of knock-out *H-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-19 or pEGFP-p21 proteins. A) knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-C1 (empty vector) used as a transfection control; **B)** knock-out *H-Ras* (-/-) murine embryonic fibroblasts pEGFP-p19, and **C)** knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p21. The arrows show the colonies formed in each cell line.

Cell line	Number of colonies formed
pEGFP-C1	0
pEGFP-p19	1
pEGFP-p21	2

Table 42. Number of colonies formed in clonogenic anchorage assays in knock-out *H-Ras* (-/-) murine embryonic fibroblasts, transfected with pEGFP-p19 or pEGFP-p21 separately.

In our double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-19 or pEGFP-p21, we confirmed that pEGFP-p19 has a lower capacity of forming colonies comparing with pEGFP-p21 (one and eight colonies formed respectively), (figures 28A and 28B). Moreover in pEGFP-p21 expression we found that the colonies formed were bigger (figures 28C and 28E).

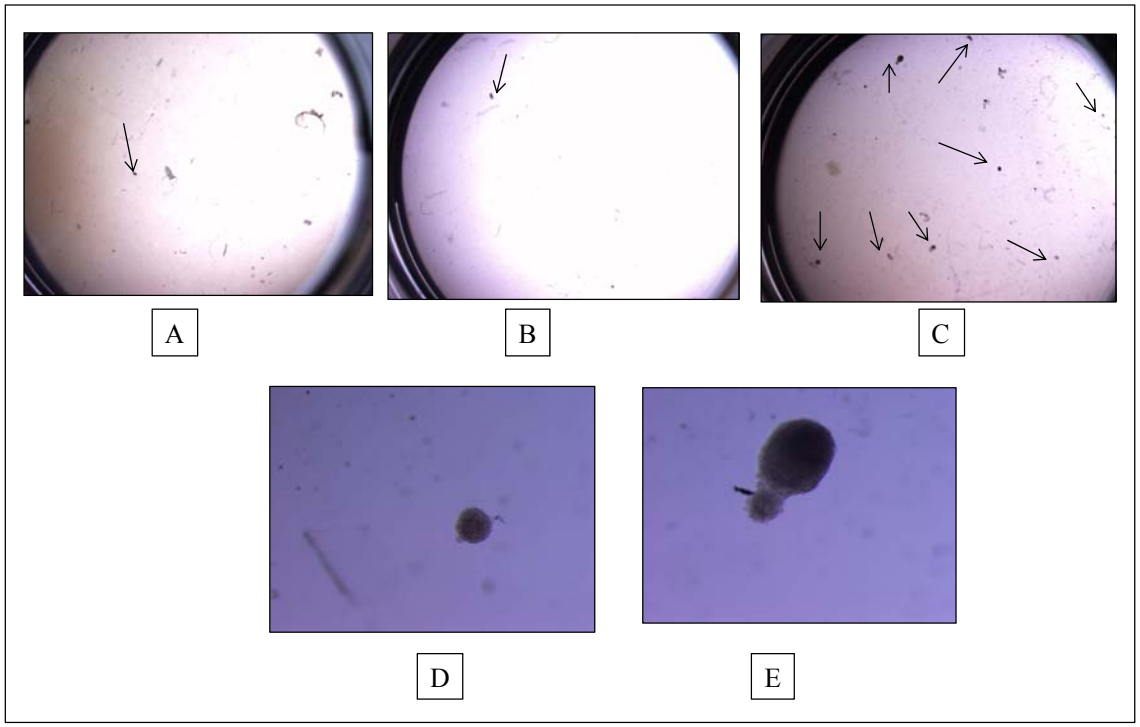


Fig. 28 Clonogenic anchorage agar assays of double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21 separately. A) pEGFP-C1 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts (MEFs); (cell line used as a transfection control). **B)** pEGFP-p19 double knock-out *H-Ras (-/-), N-Ras* (-/-) MEFs **C)** pEGFP-p21 double knock-out *H-Ras*, (-/-) *N-Ras* (-/-) MEFs **D)** colony formed in pEGFP-p19 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) MEFs **E)** colony formed in pEGFP-p21 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) MEFs.

The number of colonies formed in each cell line is shown in table 43.

Cell line	Number of colonies formed
pEGFP-C1	1
pEGFP-p19	1
pEGFP-p21	8

43. Number of colonies formed in clonogenic anchorage agar assays in double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts expressing pEGFP-p19 or pEGFP-p21 proteins separately.

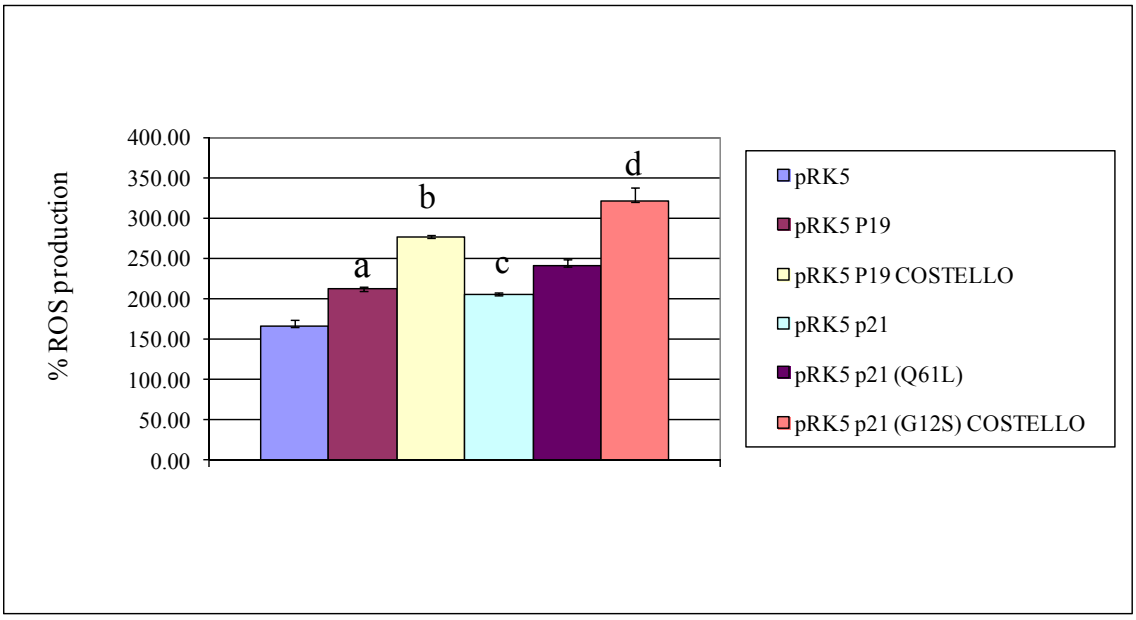
5. 6 Reactive oxygen species assay (ROS)

Excessive production of reactive oxygen species (ROS) is a feature of human malignancy that contributes to DNA damage, and activation of oncogenes. Moreover ROS production in the cell also can act as second messengers and can influence a variety of cellular process including growth factor responses and cell survival; besides their high concentration contributes to develop angiogenesis and tumorigenesis in ovarian and prostate cancer cells through misregulation of HIF-1 and VEGF (Xia et al., 2007). Due the importance of ROS production in carcinogenesis, angiogenesis and tumorigenesis, we determinate to evaluate the concentration of ROS production in our transient transfected *HeLa* cell lines (pRK5-p19, pRK5-p19 (G12S), pRK5-p21, pRK5-p21 (G12S), pRK5-p21 (Q61L)) and in our stable pEGFP-p19 or pEGFP-p21 knock-out *H-Ras* (-/-) murine embryonic fibroblasts.

For these assays we used an intracellular DCFH-DA (Dichlorodihydrofluorescin diacetate) staining method to measure the endogenous ROS levels in the transfected cells. Briefly, $1x10^5$ cells/well, of transfected *HeLa* cells were seeded, 24 h later were stained with DCFH-DA which penetrates into cell membrane and reacts with cellular esterases to DCFH (Dichlorodihydrofluorescin) that is rapidly oxidized to highly fluorescent 2' 7'-Dichlorodihydrofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS levels within the cell cytosol.

5.6.1 G12S mutation in p19 and p21 proteins overexpression increased ROS emission.

In ROS assays, we found that the ROS levels in pRK5-p19 (G12S) and pRK5-p21 (G12S) were higher than in other cell lines (276.69 and 321.40 % of ROS production respectively) and results for pRK5-p19 and pRK5-p21 ROS production were similar (211.42 and 205.13% respectively), (graphic 12, and table 44).



Graphic 12. Reactive oxygen species (ROS) production in *HeLa* cells overexpressing pRK5-p19, pRK5-p21 or their mutant variant proteins separately. All the assays were performance in the same plate, (n=12, a, b, c, d) means a statistical significance difference, Mann Whitney-U T test, $p \leq 0.0001$). The experiments were done at three times in an independent way and for triplicate each time. Control cell line refers to the cell line obtained with the transfection of pRK5-C1 (empty vector).

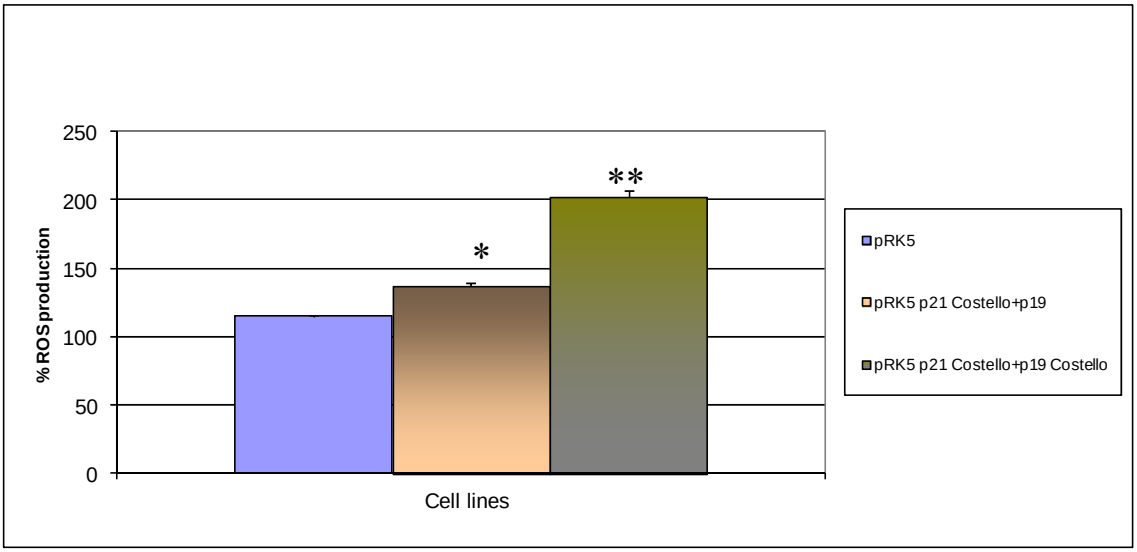
Cell line	% of ROS emission
pEGFP-C1	166.80
pEGFP-p19	211.42
pEGFP-p19 (G12S)	276.69
pRK5-p21	205.13
pRK5-p21 (Q61L)	240.83
pRK5-p21 (G12S)	321.40

Table 44. Percentage of ROS emission detected in *Hela* cells overexpressing pRK5-p19, pRK5-p21 or their mutant variant proteins. PRK5-C1 cell line was used as a transfection control; positive and negative controls were also included in these assays.

P19 and p21 produces a slight increment of the percentage of ROS emission maybe this was due of the transfection process (211.42 and 205.13% respectively), in other hand the presence of G12S and Q61L mutations contribute to a increment in the percentage of ROS, even though G12S mutation promotes a higher percentage of ROS emission than Q61L mutation, for example pRK5-p19 (G12S) and pRK5-p21 (G12S) produced a 276.69 and 321.40% of ROS respectively.

5.6.2 In cotransfected cell lines, p19 decreased ROS production

In further experiments we evaluated whether p19 was able to decrease ROS production, for these assays we cotransfected p19 or p19 (G12S) vectors with p21 (G12S) Costello mutant cell line, because in our previous ROS assays we detected that this cell line promoted a environment of high ROS production. In these cotransfection assays we found that the presence of p19 protein was enough to suppress ROS emission in an environment of high concentration of ROS; pRK5-p19/pRK5-p21 (G12S) cotransfection showed a 137 % of ROS emission and pRK5-p19 (G12S)/pRK5-p21 (G12S) cotransfection showed a 202% of ROS production but in this cell line a negative effect was not seen, which indicated that p19 (G12S) protein was not able to decrease ROS production (graphic 13).



Graphic 13. Reactive oxygen species (ROS) production decreased by overexpression of pRK5-p19 protein in *HeLa* cells. In these assays we cotransfected pRK5-p19/pRK5-p21 (G12S), or pRK5-p19 (G12S) / pRK5-p21 (G12S) in *HeL*a cells separately. The presence of p19 protein was able to decrease the high invasion capacity of pRK5-p19 (G12S) cell line. (*) and (*) means a statistical significance difference, Mann Whitney U T-test $p<0.001$. The experiments were done at two times in an independent way and for triplicate each time. Control cell line refers to the cell line obtained with the transfection of pRK5-C1 (empty vector)

Our results pointed out that p19 protein did not contribute to the increment of ROS production than p21 or their mutant variants proteins. Recent findings suggest that constitutive expression of Ras via GEFs promotes a strongly upregulated the production of both superoxide and hydrogen peroxide through the stimulation of NADPH oxidase (NOX) activity, that also promotes survival and the growth factor- independent proliferation, even

though Ras-induced ROS production specifically activating the p38 MAPK oxidative stress response, and expression of D cyclins (Hole et al., 2011). Due p19 did not bind GTP, is not possible that can promote the increment of percentage of ROS emission than p21 H-Ras proteins, and is important to notice that NADPH oxidase activity contributes to the increment of Warburg effect that in turn also contributes for surviving of cancer cells.

5.7 NM23H1 protein confers protection to the cell against ROS emission when p19 is overexpressed.

Run et al., 2008, reported that NM23H1 protein confers protection to the cell against ROS damage, in order to evaluate if p19 conferred protection to the cell against ROS emission, via upregulating NM23H1 protein expression; we performanced western blot analysis with whole extracts of pRK5-p19 or pRK5-p19 (G12S) *HeLa* cell lines collected 24 h post-transfection. Western blots were assayed for NM23H1 binding by inmmunoblot analysis with NM23H1 (c-20): sc-343 (1/1000) dilution; and GAPDH (GC5) sc:32233 dilution:1/500, both antibodies of Santa Cruz Biotechnology and antirabbit peroxidase conjugated secondary antibodies (1:10 000). Subsequently, the blots were washed and quimioluminiscence was developed with Super Signal ® West Femto Mazimun Sensivity Substrate (Thermo Scientific). We observed that NM23H1 protein was upregulated in *HeLa* cells transfected with pRK5-p19, but not with pRK5-p19 (G12S) Costello mutant cell line (fig. 29, and graphic 14); this result indicated that upregulation of NM23H1 was induced only by overexpression of p19 but not by p19 (G12S) protein overexpression.

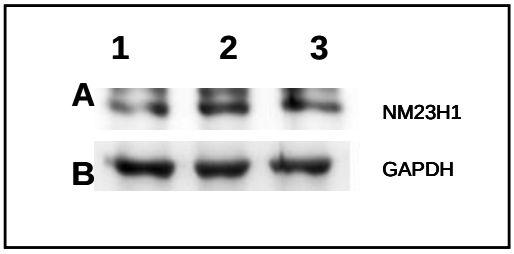
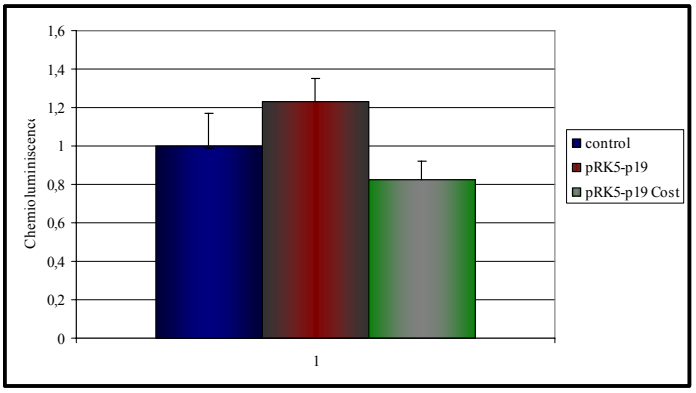


Fig 29 Western blot of NM23H1 protein in *HeLa* cells overexpressing pRK5-p19 or pRK5-p19 (G12S) proteins. A) Quimioluminiscence bands of NM23H1: A1) pRK5-C1 (transfection control); A2) pRK5-p19, A3) pRK5-p19 (G12S) Costello mutant. **B).** Quimioluminiscence bands of GAPDH: B1) pRK5-C1 (transfection control); B2) pRK5-p19, B3) pRK5-p19 (G12S) Costello mutant.



Graphic. 14 Quantification of quimioluminiscence of NM23H1 protein detected by western blot.
The chemiluminescence was quantified using Las-3000 and MultiGauge software V. 3.0; pRK5-C1= 1.0 (control of transfection); pRK5-p19= 1.23; pRK5-p19 (G12S) Costello mutant: 0.89 The experiments were done at three times in an independent way.

It had been reported recently by Tao et al., 2013 that RGS19 which is a regulator of G protein signalling can form complexes with NM23H1, Ras proteins or KSR (kinase suppressor of Ras), moreover RGS19 can suppress Ras mediated signalling via upregulation of NM23H1, hence is probably that p19 overexpression induces an upregulation of NM23H1 which in turn can form a complex with RSG19 and repress the downstream molecules of RAS/Raf/MEK/ERK signalling pathway.

VI. DISCUSSION

6.1 *H-Ras*: one gene, two different proteins

C-H-Ras gene is a proto-oncogene that renders two proteins by alternative splicing: p19 and p21; even though they have the same origin, they differ in their C-terminal amino acid sequences, and consequently in their functions and localizations. P21 c-H-Ras protein has a typical GTPase function of Ras proteins and it localizes in membrane at difference of p19 which it lacks of GTPase activity and exerts their functions forming protein complexes which localize in nuclear periphery.

In this PhD thesis we provide evidence that p19 c-H-Ras protein promotes an anticancerigen stage in the cell, increasing the expression of certain miRNAs downregulated in the development of several types of cancers and so on the expression of several signaling pathways. Besides we also included in our experiments the analyses of p21 and other H-Ras mutant variant proteins and we found evidences about their contribution in cancer process.

6.2 Protein complexes formed by p19 c-H-Ras

Even though p19 lacks of GTPase function, this protein exert their functions forming complexes with other proteins as such as RACK1, p73 α/β or neuron specific enolase (a truncate enolase isoform); these protein complexes activate intricate signaling pathways that regulate several process in the cell metabolism including miRNAs expression profile (fig. 30). RACK1 is a scaffold protein, similar as a cog that binds several proteins for activating different signaling pathways, for example PDE4D5, Src family kinases, IL-3, IL-5; β integrin cytoplasmatic domain; p85δ subunit of PI3K kinase, IGF-1 receptor and some isoforms of PKC are their parthers (McCahill et al., 2002). P19 also binds with PKCβII via RACK1, and they might be forming a trimeric complex that contributes to regulate the concentration of ROS and tumorigenesis (Inoguchi et al., 2003); an modulating cell cycle progression in the G1/M phase via Cdk (Giorgi et al., 2010).

P19/RACK1/PKCβII protein complex also phosphorylates directly ERK1 but not ERK2 activating the Raf/MEK/ERK signaling pathway and modulating downstream the routes Rheb/TSC1/TSC2/mTOR1 and p70S6K/S6/4E4BP1 (Camats et al., 2008a).

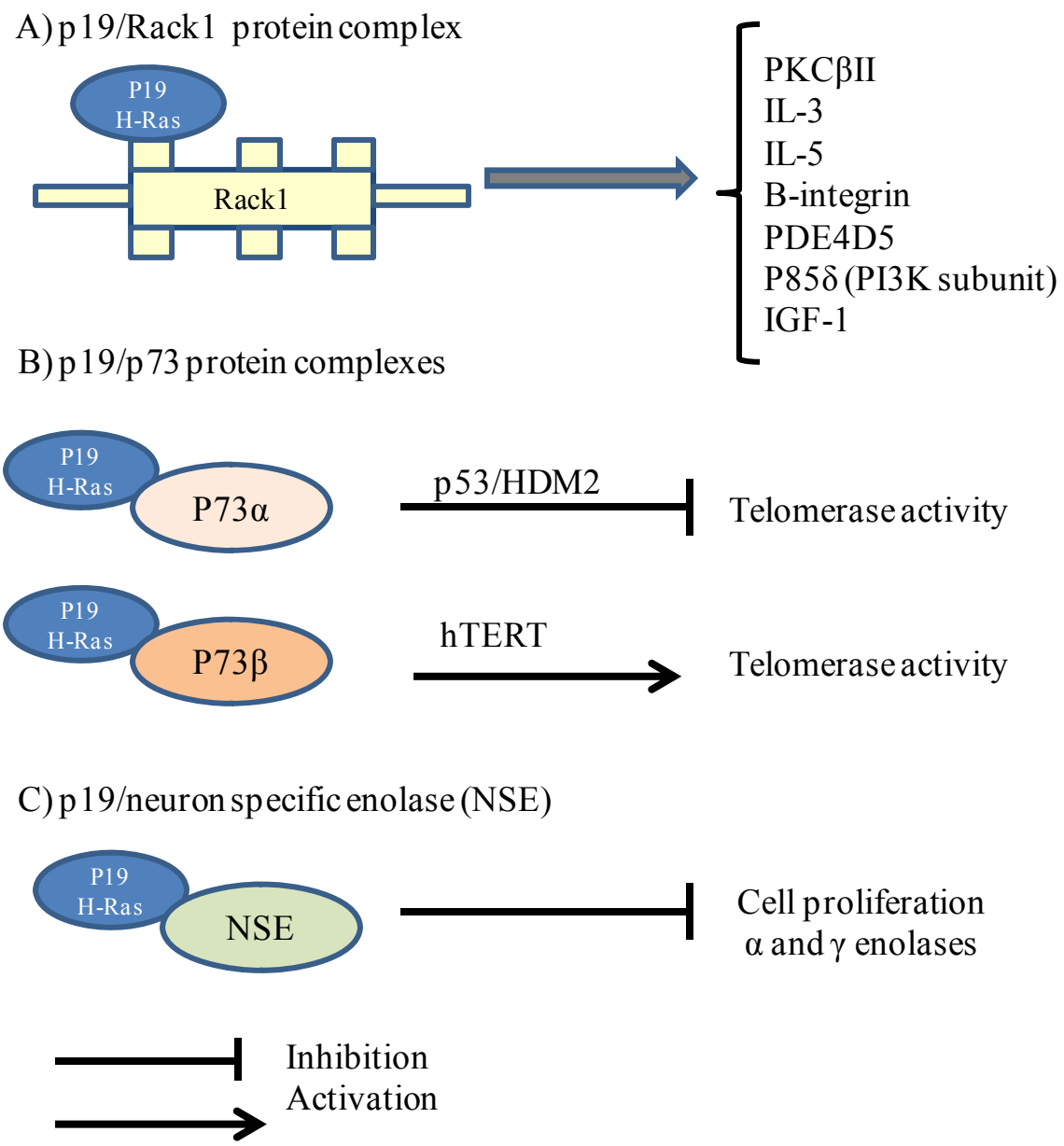


Fig. 30 Protein complexes formed by p19 c-H-Ras and their effects in metabolic pathways. A) p19/RACK1 complex can also interact with PKCβII which in turn inactivates Src and Raf/Mek/Erk signaling pathway, which suppress in turn TSC complex/mTor and P70S6K. This complex also modulates some interleukinas involved in inmunne response, integrins, PI3K/AKT/Mtor signaling pathway and PDE4D5 protein. **B)** p19 also can binds to p73 (both isoforms α and β) that can activates or repress telomerase activity. **C)** p19/NSE protein complex suppress cell proliferation in lung cancer, (MacCahill et al 2002; Camats et al., 2008a; Sang-Ming et al., 2009).

Moreover RACK1 also can interact with Src protein kinase and this binding could be enhanced by PKCβII, or platelet-derived growth factor, for this reason is possible that p19 can form a complex with RACK1/PKCβII and contributes to Src repression.

P19 also binds to p73 (α and β isoforms); the functions of these complexes depending on the type of p73 which is interacting with p19, for example p19/p73α inactivates strongly telomerase and p19/p73β, activates telomerase via hTERT (Camats-Malet, 2008, PhD thesis UAB). Activation of telomerase also modulated the DNA regulation damage mechanisms in absence of p53 protein, inducing the phosphorylation of c-Abl kinase (Zaika et al., 2011). The complex also contributes to reduce cell motility through Rac/Cdc42 proteins inducing a cell cycle arrest and apoptosis via E2F1, and increasing SLIT3 gene expression (Tophkhane et al., 2012; Rufini et al., 2011).

Neuron Specific Enolase (NSE) is a truncate isoform of enolase that in neurons serves as molecular markers of axon injury regeneration and target reinnervation (Kirino et al., 1983), nowadays NSE has been detected upregulated in patients with certain tumours, namely: neuroblastoma, small cell lung cancer, medullary thyroid cancer carcinoid tumours, pancreatic endocrine tumours and melanoma. Studies of NSE as a tumor marker have concentrated on patients with neuroblastoma and small cell lung cancer (SCLC). Measurements of NSE levels in patients with these two diseases can provide information about the extent of the disease and the patient's prognosis, and their response to treatments (Hartomo et al., 2013; Stovold et al., 2013). Furthermore p19 interacts with Neuron Specific Enolase (NSE) a truncate isoform of enolase, the interaction is enough to inhibit the enzymatic activity α and γ enolases, and repress lung cancer cell proliferation mostly increased by NSE in H1299 cells (Sang –Ming et al., 2009). Taken together, these results suggest that p19 is a novel regulator to suppress cell proliferation in lung cancer through the interaction with NSE.

6.3 Overexpression of p19, p21 and their mutant variant proteins induced an increment in miRNAs expression profile

In all miRNAs Taqman PCR Real Time evaluated in our *HeLa* cell lines overexpressing p19, p21 and their mutant protein variants we detected an upregulation in all miRNAs tested (all of them, except miR-138 and miR-206 that did not amplified in our transfected *HeLa* cells), but in a particular manner the increment was dramatic in pRK5-p19 (G12S) cell line.

In a general view, miR-342 was upregulated in all our cell lines overexpressing the proteins of our interest: pRK5-p19, pRK5-p21, pRK5-p21 (Q61L) and pRK5-p21 (G12S) (11.20, 11.0- , 6.11- and 6.94-fold change values respectively) with the exception of pRK5-p19 (G12S) that produces a dramatic increment in all the miRNAs evaluated and specifically in miR-335 (24.64-fold change). MiR-335 and miR-342 have been reported misregulated in several types of cancers, for example breast, gastric, colorectal, hepatic, prostate cancers as such as in leukaemias, adenomas and gliomas and some of them have been correlated with a high grade of proliferation and undifferentiated cancer cells that contribute to resistance against anticancer therapies.

It is probably, that the increment of miRNAs expression profile due overexpression of p19, p21 and their mutant variants proteins (including G12S and Q61L) are mediated by different mechanisms, in one way p19 can activate several signaling pathways by the complexes formed with other proteins whereas p21 and others mutant proteins might induce a constitutive activation of GTPase (G12S mutation) or in a decrease GTPase activity and constitutive signaling (Q61L mutation), that promotes a oncogenic activation downstream of several proteins and transcription factors that contributes to increase cell proliferation, metastasis, and apoptosis evasion (Su et al ., 2012; Holsenberg, 2006).

In all our *HeLa* cell lines overexpressing p19, p21 and their mutant variants we detected a upregulation in miR-126 expression. This miRNA had been downregulated in several cancer cell lines in which had been repressed by Src, that activates some proteins involved in cell migration and tumor cell invasion as such as Crk, ADAM9, MMP-7 and E-cadherin (Hamada et al., 2012; Meister and Schimidt, 2010; Png et al., 2010). Maybe RACK1/P19 protein complex inhibits Src and then Crk so p19 would be inhibiting invasiveness and tumorigenesis by this axis (Mamidiputy et al., 2004, Camats et al., 2009), upregulation of miR-126 also induces an arrest in G0/G1 cell cycle phase, repressing some proteins and transcription factors (IRS-1, SOX-2, CCND1, E1 and E2), (Hamada et al., 2012; Otsubo et al., 2011; Zhang et al., 2008).

Upregulation of miR-126 also contributes to modulate the following signalling pathways Ras/PI3K/AKT, MEK/ERK; and KF-KB signalling pathways (Felli et al., 2013); Ras/PI3K/AKT pathway has been reported misregulated in colon cancer cells (Guo et al., 2008), and NSCL (non-small cell lung cancer (Zhu et al., 2012), its restoration by miR-126 inhibits angiogenesis, repressing VEGF-A, diminished ROS production (Huang et al., 2012a; Chen et al., 2011c) and it contributes also to avoid resistance to anticancerigen agents inhibiting MMP1 (multidrug resistance associated with a ecreased effect of anticancer agent), (Quin et al., 2013) and cell surviving of mesenchymal stem cells, (Feng et al, 2012).

The increase on miR-330 expression contributes to represses several proteins involved in migration and mestastasis as such as CDC42, CDC44 and CDC43, three transmembrane glycoproteins misregulated in breast and colorectal cancer (Li et al., 2013; Jeyapalan et al., 2011); dCk (which confers gentamicine resistance in cancer cells), (Hodzic et al., 2011); and hypophosphorylates AKT and represses E2F1, which decreasing proliferation (Png et al., 2010; Lee et al., 2009).

Downregulation of miR-335 had been reported in gastric and adrenocortical carcinomas and it was taken as diagnostic marker for discriminating between these cancers (Schmitz et al., 2011). So restoration of miR-335 contributes to regulate several mechanisms that control proliferation, migration, metastasis. MiR-335 also induces a cell cycle arrest in phase G0/G1 repressing directly RB1 (Retinoblastoma 1 receptor) which also contributes to ameliorate apoptosis evasion and metastasis (Shi et al., 2012; Shu et al., 2011a). DNA damage elicits an increase of miR-335

expression in a p53-dependent manner, both molecules cooperate in a positive feedback loop to drive cell cycle arrest and together control cell proliferation balancing the activities of Rb and p53 tumor suppressor pathways (Scarola et al., 2010). MiR-335 also inhibits metastasis and migration through targeting the progenitor cell transcription factor SOX4 that regulates the transcription of SEMA3 and plexin, two proteins that facilitate cell proliferation and tumorigenesis *in vivo* in pancreatic cancer cells, in turn SOX4 can repress TGF-β, Wnt, Notch (Huang et al., 2012b); and extracellular matrix component tenascin C, and its misregulation in their expression is frequently detected in the majority of the primary tumours of breast cancer patients who relapse and have poor distal metastasis-free survival (Tavazoie et al., 2008). In gastric cancer cell lines downregulation of miR-335 was significantly associated with lymph-node metastasis, poor pT stage, poor pN stage and invasion of lymphatic vessels, so overexpression of miR-335 suppressed gastric cancer cell invasion and metastasis *in vitro* and *in vivo*, targeting Bcl-w and specificity protein 1 (SP1), that indirectly represses PI3K/AKT signaling pathway (Xu et al., 2011). In agreement to these data, we found also that bioinformatic tools predicted a set of genes that could be putative miR-335 targets and they are involved in actin cytoskeleton organization and biogenesis (DAAM1, ARPC5L, JAG1, MAP2 and RASA1), (Wang and Ruan, 2010a; Chen et al., 2011b; Tome et al., 2011; Ronchetti et al., 2008).

The upregulation detected in the set of miRNAs evaluated in our experiments implies that 1) restoration of several signalling pathways into a normal function, for example miR-342 upregulation decreases cell proliferation and activates apoptosis; which s suggests that it could function as a proapoptotic tumor suppressor (Grady et al., 2008), 2) and increase in the sensitivity to tamoxifen treatments in breast cancer with high grade of proliferation and undifferentiation (Van der Auwera et al., 2010; Zhao et al., 2012); and 3) an activation of mature B cells and promotes their transformation in chronic lymphocytic leukaemia, (Li et al., 2011b). Moreover miR-342 upregulation could foster inflammatory macrophage activation through direct target of Akt-1 which induces proinflammatory mediators, such as Nos2 and IL-6, in macrophages via the upregulation of miRNA-155 (Wei et al., 2013).

In this study we also found that p19 (G12S) protein overexpression contributes to upregulate miR-374 expression in a dramatic way (15.065-fold change value), which was not seen in p19

cell line (2.12- fold change value) and other variant Ras proteins evaluated. Upregulation of miR-374, stimulated by p19 (G12S) protein overexpression activates many genes involved in tumor invasion and metastasis as such as DNMT1 (DNA cytosine-5 methyltransferase which activates ADAM23 (also known as metalloproteinase 23); RASSF1A (Ras association RalGDS/AF-6 domain family member 1); AAMP (angio-associated, migratory cell protein), PDGFRA (platelet derived growth factor receptor, alpha polypeptide); all of these genes increase tumor invasion and metastasis (Van der Auwera, 2010), so this is another finding that confirms that p19 does not induce to a cancer stage in the cell at difference of p19 (G12S) protein.

Another interesting finding is the increase of let-7 expression in all our pRK5 vector cell lines; in these cell lines pRK5-p19 (G12S) showed the highest increment followed of pRK5-p21 (Q61L) and pRK5-p19 (13.76-, 5.16- and 3.05- fold change respectively), it seems that is more regulated by p19 (G12S) mutated protein than p19 or p21 proteins alone. Contribution of let-7 to cancer process is controversial because their up and downregulation have been involved in cancer processes (Boyerinas et al., 2010); for example downregulation of let-7 had been detected in ovarian, (Yang et al., 2012b); breast (Hu et al., 2013; Sun et al., 2012); colorectal (Ruzzo et al., 2012; Mosakhami et al., 2012) and prostate cancer (Kong et al., 2012; Liu et al., 2012a); neuroblastosma, medulloblastoma (Moleenar et al., 2012); and several types of leukaemias (Pelosi et al., 2012; Steineman et al., 2010), and it had been correlated also with poor outcome in patients which presented resistant to the treatments in pancreatic cancer (Buthia et al., 2013); and at the contrary, overexpression of let-7 members (a, c and 7g) had been associated with a dramatic downregulation of *Ras* gene which produces an aggressive stage, poor survival, diagnostic resection recurrence (Lu et al., 2012); and an increment on cell proliferation (Lee et al., 2010; He et al., 2010).

Nevertheless it had been reported that let-7 induces a direct downregulation of Ras gene expression (Johnson et al., 2005), our data suggest that it might be exist a double feedback between H-Ras proteins and let-7 expression because in our miRNAs Taqman PCR Real Time assyas, overexpression of H-Ras proteins increased let-7 expression. Another point that is important to discuss is that some literature references establish that overexpression of p19 induces a downregulation of p21 c-H-Ras protein and an increment in cell proliferation induced

by GAPs activation (Lee et al., 2010; He et al., 2010), which it means that p19 it would be acting as an oncogenic protein, even though our collegue Camats-Malet, 2008a, found that p19 overexpression activates total H-Ras and p21, although the endogenous p19/p21 ratio due to alternative splicing remains relatively unchanged (Camats et al., 2008c); and our findings in this PhD thesis corroborates that p19 is not an oncogenic protein (García-Cruz and Bach-Elias, 2013; and García-Cruz et al., submitted).

Downregulation of let-7 contributes to proliferation, survival, and apoptosis evasion, because let-7 targets several proteins, oncoproteins and transcription factors that regulate downstream all this routes.

Let-7 controls cell proliferation targeting the constitutive activation of Ras proteins (Lee et al., 2010; He et al., 2010), but also regulates several proteins and transcription factors involved in cell cycle progression, including hypophosphorylation of PI3K/AKT . Let-7 represses HMGA1 which is the principal protein that negatively regulates p16 ^{Ink} and p19 ^{Arf}, two proteins that inhibit Cdk4 and Cdk6 (Ikeda et al.,2012); NIRF an ubiquitin ligase also known as UHRF2, which is overexpressed in many carcinomas in which let-7a is known to be poorly expressed and its missregulation promotes that NIRF binds to methylated promoter regions of some TSG by interaction with HDAC1 (hystone deacetylase 1), (He et al., 2009; Zhang et al., 2012f; Wang et al., 2012f;), and multiple proteins including cylins (A2, B1, D1 and E), and p53 and pRB, suggesting that it works in thigh collaboration with the core cell cycle machinery, and it binds specifically to CDK2-cyclin E complex, inducing G1 arrest (Mori et al., 2011). Another point of restriction of cell proliferation driving by let-7 is the restriction of glucose metabolism which is necessary for initiating the Warburg effect in cancer cells in which cells produces a glucose dependent metabolism which utilize glycolysis instead of mitochondrial oxidative phosphorylation for glucose metabolism, this effect of glucose restriction can be reached by direct targeting of GSK-3 (a glycogen synthase kinase-3) that activates CCND1, cyclin E and c-Myc transcription factor that govern cell fate and differentiation, including c-Jun, β-canenin, GLI, Notch, Snail and sterol-regulatory-element-binding transcription factor 1 (SREBP1), (Zhu et al., 2011); and inactivating HIF-1α (hypoxia-inducible factor-1α), a transcription factor that

increases the expression of the glucose transporter GLUT1 and glycolytic enzymes ultimately leading to increase glucose uptake and glycolysis (fig.31), (Shaw and Cantley, 2006)

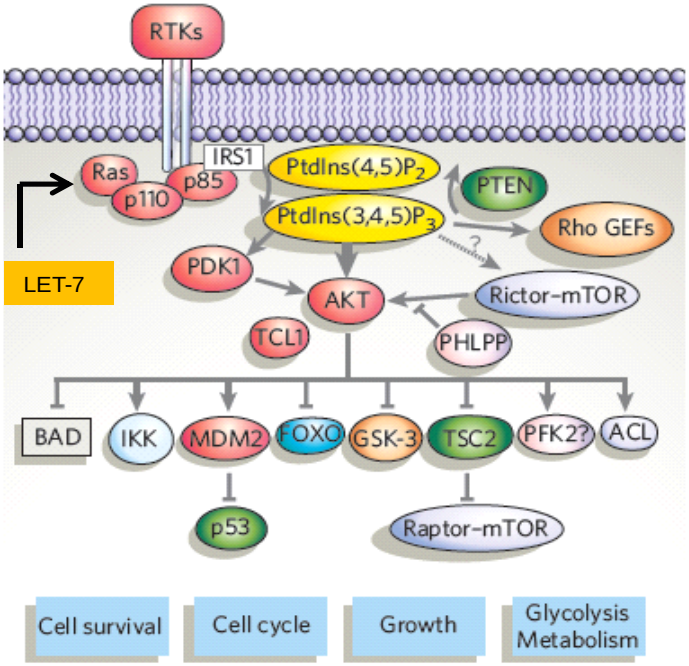


Fig. 31 Activation of PI3K/AKT/mTor signalling pathway via Let-7/Ras. Overexpression of p19 c-H-Ras increases let-7 expresssion which phosphorylates the D3 position of the inosytol ring of the plasma membrane lipid phosphatidylinositol 4,5-biphosphate to generate the second messenger phosphatidylinositol -3,4,5 triphosphate (PI3K). PI3K acts a membrane bound second messenger by localizing a subset of signalling proteins, where they become activated and initiate downstream signalling events. The most implicated in human cancer is AKT family of protein serine/threonine kinases, that activate different proteins involved in cell survival, cell cycle, growth and glycolisis metabolism. In other hand the protein complex formed by Rictor-Mtor (better known as mTor2), can activates AKT in a independent way of Ras/PI3K/AKT signalling (Shaw and Cantley, 2006).

P53 is also another direct target of let-7 and it might be a feedback loop between the expression of p53 and let-7-c and –a, and this effect may be relieve in response to DNA damage, in this function p68, and ATP-dependent RNA helicase is acting as coactivator of p53. P68 also promotes p21 c-H-Ras protein expression by excluding IDX exon (the exon responsible of forming p19 mRNA), in *H-Ras* splicing alternative (Camats-Malet et al., 2008c) and it had been related also of helping the unwinding stem loop containing pri-miRNAs, including let-7 as being part of the DROSHA complex. Let-7 also is a co-activator of p53 in response to DNA damage. So let-7 could be acting in a feedback loop promoting p21 expression not p19 expression and this miRNA subsequently increased let-7 expression in the cell.

Let-7 also controls apoptosis targeting directly caspase 3; Bcl-x, (Shimiku et al., 2010); BAD and IKB kinase, targeting IKB kinase (IKK)-NF-KB (nuclear factor-KB) pathway; and in a indirectly manner targeting Blimp-1 expression, a protein that orchestrates plasma cell differentiation by repressing genetic programs associated with activated B cells including those that control cell proliferation, or 2) by activating genetic programs associates with plasma cell functions, (Nie et al., 2010).

Increase of let-7 expression also contributes to suppress negatively oncostatin M (OSM), which is an autocrine/paracrine factor that promotes epithelial mesenchymal transition (EMT) in breast and lung cancers. In this via, OSM induces a constitutive activation of STAT3, a transcription factor which in turn also activates Lin28b transcription, STAT3 also stimulates the expression of HMGA2 which activates cell cycle via $p16^{Ink}$ and $p19^{Arf}$ (two important regulators of cell cycle progression); and transcription of Snail a key protein that participates in TGF-β-induced EMT in mammary epithelial cells (Guo et al., 2013).

Let-7 contributes also to repress several proteins involved in cytoskeleton pathway as such as PAK1, DIAPH2, RDX and ITGB8 that activates cell migration (Hu et al., 2013).

6.4 Contribution of Q61L mutation in the upregulation of miRNAs

Q61L mutation results in an amino acid substitution at position 61 in *H-Ras*, from a glutamine (Q) to a leucine (L), this mutation had been reported in the literature as a high oncogenic mutation because this mutation does not hydrolyze GTP, resulting in the constitutive activation of downstream effector proteins (Muraoka et al 2012); it had also reported as a frequent mutation in cutaneous squamous-cell carcinoma and lung adenocarcinoma which had been associated with the activation of mitogen-activated (MAPK) by direct interaction with Raf-1 and ERK mediated transcription (Su et al., 2012). Moreover oncogenic H-Ras (Q61L) induced signaling through inhibition of ERK correlates with a decrease in integrin activation, suggesting that one potential role for GTP-bound Ras mutants in human cancer is to decrease cell adhesion, thereby contributing to cell survival and proliferation in the absence of substratum and to metastasis. Upregulation of p21 (Q61L) increases miR-126 expression an endothelial cell-specific miRNA that plays an essential role in neoangiogenesis and in maintenance of vascular integrity. MiR-126 represses the expression of Spred1 (Sprouty-related EVH1 domain

containing 1) and PIK3R2, which negatively regulates VEGF (vascular endothelial growth factor) signaling via ERK and AKT pathways, respectively. VEGF, a highly specific mitogen for vascular endothelial cells, induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and plays a pivotal role in the regulation of vasculogenesis. And the expression of the VEGF gene is restrictedly, controlled by a hypoxia-inducible factor. Thus, in the absence of miR-126, Spred1/PIK3R2 expression is elevated, resulting in repression of angiogenic signaling. Conversely, miR-126 overexpression relieves the repressive influence of Spread-1/PI3K on the signaling pathways activated by VEFGF and FGF (Fibroblast Growth Factor) favoring angiogenesis (Wang et al., 2008; Meng et al., 2012)

6.5 p19:p21 splicing ratio alters miRNAs expression and cell growth

In order to further understand the contribution of p19 and p21 proteins have in miRNAs expression profile, we transfected in our knock-out *H-Ras* (-/-) murine embryonic fibroblasts with pEGFP-p19 or pEGFP-p21 vectors and it was possible to observe a differential expression in the miRNA expression. In these cell lines, miR-138 and miR-206 amplified and the fold change value was calculated in each cell line. PEGFP-p19 protein increase dramatically miR-206 expression (96.16- fold change value) at difference of pEGFP-p21 (18.80-fold change value); in other hand, miR-342 was differential expressed in pEGFP-p21 12.21-fold change value) at difference of pEGFP-p19 (7.11-fold change).

The dramatic increment of miR-206 expression induced by pEGFP-p19 contributes also to G1/S phase cell cycle delay, (Camats et al., 2009). MiR-206 is downregulated in ER α dependent BC, and their normal expression induce cell cycle arrest and inhibit estrogen-induced proliferation, (Dileva et al., 2010; O'Day et al., 2010; Yoshimoto et al., 2011); and limit the migration of metastatic cells, blocking anti-apoptotic activity of NOTCH 3 (Song et al., 2009); Re-establishing expression on miR-206 *in vitro,* suppresses filopodia formation regulating Cdc42 and MMP-2 and MMP-9 downstream (Li et al., 2010a) and contributes to develop highly aggressive and non responsive to hormonal therapy; and apoptosis evasion by misregulation of NOTCH3, KLF4 and PAX7 transcription factors (Gagan et al., 2012; Parasramka et al., 2012b; Lin et al., 2011a; Dey et al 2011; Song et al., 2009) and angiogenesis (Stahlhurt et al., 2012),

moreover miR-206 restoration also contributes to chromatin remodeling targeting directly CCND2 and Smarcd2 in gastric cancer cells (Zhang et al., 2013; Goljanek et al., 2012)

In rhabdomyosarcoma, a frequent neoplasia in Costello Syndrome patients miR-206 upregulation is a potential serum diagnostic marker (Miyachi et al., 2010); miR-206 normal expression in RB cell lines promotes myogenic differentiation and blocks tumour growth controlling directly c-Met expression, a receptor tyrosine kinase that is upregulated in RB and leads to cellular activation and it contributes to tumor growth, invasiveness and metastasis (Macquarrie et al., 2012; Gagan et al., 2012; Dongsheng et al., 2009; Taulli et al., 2009; Yan et al., 2009).

6.6 G12S and G12A mutations in Costello Syndrome and their contribution in miRNAs expression profile

In fibroblasts cell lines obtained from tumours of Costello Syndrom patients, which contain (G12A) or (G12S) mutations, a significant increment was detected in miR-330, miR-335 and miR-374; these results were similar to those obtained when p19 was overexpressed ectopically in *HeLa* cells. Moreover miR-206 and miR-126 were downregulated.

Recent studies have showed that miR-330 is a potential tumor suppressor; however their function and molecular mechanisms are still unraveled and further studies are needed. In some reports miR-330 upregulation or downregulation has been involved in cell proliferation, loss of adhesion, invasion, apoptosis evasion, tumor metastasis and resistance against cancer treatments (Qu et al., 2012; Hodzic et al., 2011; Lee et al., 2009). Is possible that miR-330 in Costello fibroblasts cell lines, miR-330 has a metastatic and oncogenic potential, mediated overexpression of c-Met proto-oncogene that promotes tumor cell migration and resistance to chemotherapy and radiotherapy (Navis et al., 2013; Yan et al., 2009) and inducing the Met/PI3K/Akt pathway which contributes to apoptosis evasion (Xiao-Hon et al., 2013).

Recently Cai et al., 2013 reported that upregulation of miR-374a activates Wnt/β-catenin signalling which drives epithelial mesenchymal transition and metastasis in both *in vivo* and *in vitro.* MiR-374a directly targeted and suppressed multiple negative regulators of Wnt/β-catenin signalling cascades, including WIF1, PTEN and WNT5A. Overexpression of miR-374a was

detected in primary tumour samples from patients with distant metastasis in breast cancer and was associated with poor metastasis-free survival. Our results suggest that overexpression of miR-374 could maintain constitutively activated Wnt/β-catenin signalling in Costello Syndrome but further studies are needed to confirm this hypothesis.

In accord with Taulli et al., 2009, we found a low expression of miR-206 in fibroblasts cell lines of Costello Syndrome patients, this lack of miR-206 promotes also a myogenic undifferentiation phenotype by modulation of more than 700 genes, including Met, RUNX1 and ZNF238, all of them are transcription factors downregulated in rhabdomyosarcomas (Macquarrie et al., 2012). Downregulation of miR-206 had been reported in primary paediatric rhabdomyosarcomas, and it was correlated with poor overall survival and with more aggressive tumours in metastatic embryonal and alveolar cases without PAX3/7-FOXO1 fusion genes. These clinical correlations are linked to the differentiation status of rhabdomyosarcoma and the propensity of undifferentiated cells to migrate and potentially metastasize (Missiaglia et al., 2010). Due p19 protein upregulates miR-206 expression and induces a downregulation of FOXO1 via PI3K/AKT a genetic therapy with p19 restoration can be a option for the treatment of rhabdomyosarcoma patients with Costello Syndrome, (Camats-Malet et al., 2009; Linardic, 2008).

6.7 Invasion capacity of H-Ras proteins and their mutant variants

Here, we report for the first time the regulatory effect of p19 protein in invasion cancer processes. We first demonstrated that overexpression of p19 (G12S) and p21 (G12S) proteins in HeLa cells increased dramatically invasion capacity in our experiments, followed by p21, p19 and p21 (Q61L) proteins, these results suggest that G12S mutation contributes to increased loss of adhesion and invasion more than a constitutive activation of Ras due p21 or the presence of Q61L mutation. G12S mutation in p21 protein disrupts guanine nucleotide binding causing a reduction in intrinsic and GAP induced GTPase activity resulting in constitutive activation of the RAS/MAPK signaling pathway which is one of the most important oncogenic pathways. In turn MAPK also activates transcriptional factors that influence in the expression of specific genes codifying for proteins implicated on cellular proliferation and/or differentiation control (Tydyman et al., 2009; Denayer et al., 2008). P21 c-H-Ras can induce the activation of Fyn, which is a critical mediator of Ras-stimulated invasive cell phenotype and is overexpressed in

multiple human cancers (prostate, melanoma, pancreatic, glioma, chronic myelogenous leukemia), activation of p21 c-H-Ras activates the PI3K/AKT pathway, and AKT phosphorylation but not MAPK or EGFR is necessary and sufficient for induction of Fyn by p21 c-H-Ras (Yadav et al., 2011). P19 (G12S) protein can induce a aberrant expression of RACK1 when they binding, this leads a constitutive signaling pathway that increases proliferation, chemoresistance and a constitutive phosphorylation of PI3K/Rac1 and eukaryotic initiation factor 4E (eIF4E) signaling pathways which led to preferential translation of the potent factors involved in growth, migration, survival and invasion capacity (Wu et al., 2013; Ruan et al., 2012).

Besides, overexpression of both H-Ras proteins, p19 and p21 containing (G12S) mutation acts as oncogenic signals and activates STAT3 pathway that is highly involved in tumor cell migration and invasion inducing an overexpression of IL-6 which in turns activates MMP2 and MMP-9, two matrix metalloproteases (MMPs) that lead to the degradation of collagen the major structural collagen of the basement membrane (Ataie-Kachoie et al., 2013) and also activates several cytokines and HIF-1α that contributes to Warburg effect in cancer cells (fig. 32).

The low capacity of invasion of p19 was not clearly detected when was expressed alone, because the transfection induces a stress stage in the cells, and invasion was increased slightly; for this reason, we decided to re-evaluated p19 invasion capacity in our *HeLa* cell lines cotransfected with pRK5-p21 (G12S) vector that confers a high environment of invasion, in these *in vitro* assays we demonstrated that p19 overexpression diminished the the high invasion environment promoted by overexpression of p21 (G12S); and due of these findings we can conclude that only (G12S) mutation of H-Ras isoforms mutation increased invasion capacity, whereas p19 overexpression diminished it.

It is possible that p19 conteracts the oncogenic function of p21 (G12S) by stimulating the transcriptional activity of the complex p19/p73β. Moreover, p73α and p53 also interact with p19 and abrogates the p73-MDM2 interaction (the major negative regulator of p53); (Harms and Chen, 2006).

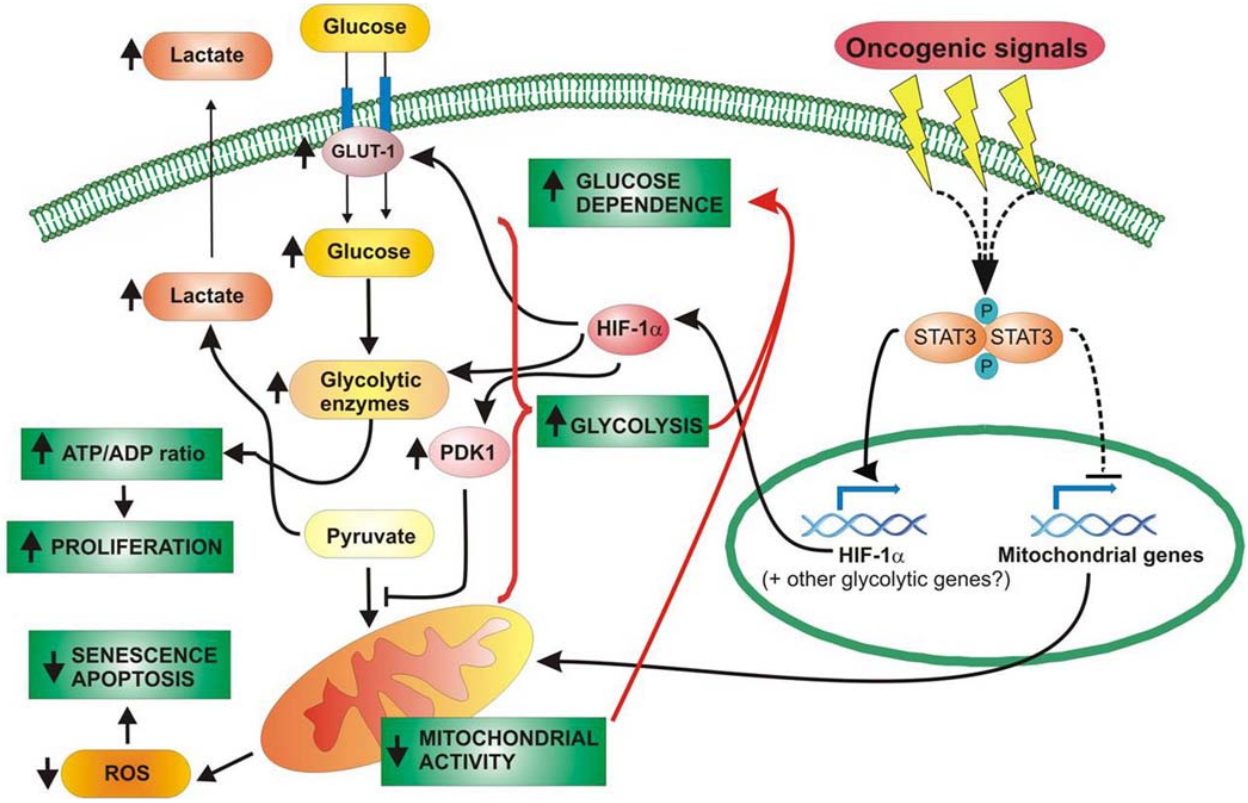


Fig. 32 Oncogenic activation of STAT3. STAT3 can be activated by oncogenic signals probably as such as (G12S) H-Ras proteins that promotes a constitutive GTPase activity in the case of p21 (G12S) overexpression or an aberrant binding between p19 (G12S) and RACK1 contribute to increase cell proliferation, chemoresistance and invasion.

In other hand, G12A mutation which constitutes a heterozygous nucleotide substitution, 35 G/C, which changes glycine to alanine at amino acid position 12; showed a lower capacity of invasion than G12S mutation in our fibroblasts cell lines obtained from Costello Syndrome patients, this finding is disagree with the correlation founded between genotype-phenotype malignancy risk, in which highest malignancy exists for those patients with a G12A mutation (which is very uncommon in Costello Syndrome patients) and they have more probabilities of developing ganglioneuroblastoma, rhabodmyosarcoma or bladder carcinoma, (Kerr et al 2006).

The G12A *H-Ras* substitution alters the phosphate-binding loop of the protein, and transforms cultured Rat1 cells *in vitro* and is predicted to decrease intrinsic Ras GTPase activity (Sovik et al., 2007).

6.8 Influence of H-Ras proteins upon cell proliferation and cell cycle

The proliferation was another important characteristic for identifying metastatic roles in p19 or p21 proteins, for these experiments pEGFP-p19 and pEGFP-p21 were expressed separately proteins in double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts (MEFs), is important to remark that this cell line does not show Ras genetic redundancy (Castellano et al., 2007) and the contribution of p21 and p19 to proliferation would be seen clearly.

In this assay pEGFP-p21 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts produced a high proliferation state in the cell, at difference of those double knock-out *H-Ras* (-/-); *N-Ras* (-/-) murine embryonic fibroblast transfected with pEGFP-p19 vector, in which expression of pEGFP-p19 protein promoted a quiescence state in G1/S cell cycle detected in flux citometry experiments. In order to confirm this quiescent stage in the cell cycle, we repeated the experiment in knock-out *H-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 or pEGFP-p21 vectors; once again we detected an increment in the percent of G1/S phase cell cycle only in knock-out *H-Ras* (-/-) murine embryonic fibroblasts that expressed pEGFP-p19.

Quiescent state in G0/G1 cell cycle induced by p19 was previously detected by our colleague Camats-Malet 2008, PhD thesis UAB and this effect was attributed by phosphorylation of ERK1, (that could be acting as negative modulator); and inhibition of telomerase activity via p19/p73β complex, mediated by MDM2 protein (Jeong et al., 2006); nevertheless, p19 can also induce the quiescence stage in cell cycle by binding NSE which repressed cell proliferation in lung cancer (Jang et al., 2010); or modulating the expression of some miRNAs, in particular miR-206 expression that in turn inactivates cell cycle progression.

6.9 Capacity of forming colonies of H-Ras proteins

The ability to initiate clonogenic growth -a determinant of tumorigenicity- is reflected by the potential to form colonies *in vitro* when seeded at very low density, minimizing cell-cell contact, when grown in an anchorage independent manner in low density soft agar. We therefore analyzed the clonogenic potential of our transfected *HeLa* cell lines overexpressing the proteins of our interest and in our knock-out *H-Ras* (-/-), and our double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts transfected with pEGFP-p19 and pEGFP-p21 separately.

Overexpression of p19 (G12S), significantly increased the number of colonies (17 colonies formed), compared to pRK5-C1 control, (fig. 23 A, B and C). PRK5-p19 showed a significant reduction colony formation (7 colonies formed). In pRK5-P19 (G12S) cell line colonies were bigger that in pRK5-p19. Furthermore pRK5-p21 (G12S) cell line showed also a high number of colonies formed (10 colonies) than pRK5-p21 (Q61L) mutation (3 colonies formed) and pRK5-p21 (5 colonies formed) comparing with the control pRK5-C1 cell line. Further anchorage-independent cell growth in soft agar assays were performance in our knock-out *H-Ras* (-/-) and double knock-out H-*Ras* (-/-), *N-Ras* murine embryonic fibroblasts transfected with pEGFP-p19 and pEGFP-p21 proteins for re-evaluating the ability of forming colonies of p19 and p21 proteins.

In pEGFP-p19 knock-out *H-Ras* (-/-) and pEGFP-p19 double knock-out *H-Ras* (-/-), *N-Ras* (-/-), murine embryonic fibroblasts, reduced both colony numbers and size in both cell lines than in pEGFP-p21 knock-out *H-Ras* (-/-) and pEGFP-p21 double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts.

Taken together, these results show a significantly reduced aggressiveness of p19 protein as shown by their reduced ability to give rise to cell colonies in all assays performed, this ability can be exert by direct regulation of TCTP, (translationally controlled tumor protein) which is an anti-apoptotic protein, that plays a role in cell growth, cell cycle and is expressed in several human cancer types and their overexpression had been correlate their increment with the grade of malignancy (Camats-Malet, 2008 PhD thesis UAB). It has been reported that TCTP controls negatively the stability of p53 tumor suppressor protein and interacts with the cellular

cytoskeleton. The regulation of the actin and cytokeratin cytoskeleton is responsible for the increase migratory activity of tumor cells and is linked with poor patient outcome. Recent studies indicate that cyclin A, a key regulatory of cell cycle, controls actin organization and negatively regulates cell motility via regulation of Rho A expression and there is an inverse relationship between the level of TCTP/RhoA and actin/p53/cyclin A, (Kloc e tal., 2012), so a misregulation of TCTP promotes high aggressiveness behavior in cancer and in this minicircuit p19 plays a crucial role regulating TCTP and avoiding tumorigenesis.

6.10 G12S mutant H-Ras proteins showed a higher rate of ROS

ROS production (Reactive oxygen species) promotes a microenvironment that induces apoptosis by p53 activation in normal cells. At the contrary, in cancer cells ROS microenviroment promotes proliferation and expansion of cancer cells by modulating various cell cycle proteins and tumour suppressor genes that together are able to modify cell adhesion, and migratory behavior. In our pRK5 transfected *HeLa* cell lines that contained G12S mutation in H-Ras proteins (pRK5-p19 (G12S) and pRK5-p21 (G12S) cell lines) we detected an increase in ROS emission; then in order to have more evidences of the contribution of p19 or p21 proteins in oxidative stress, we performanced further cotransfections assays overexpressing in the same cell line pRK5-p19/pRK5-p21 (G12S) or pRK5-p19 (G12S)/pRK5-p21 (G12S) cell lines, in these assays a decrease in ROS emission was detected when p19 was overexpressed, this effect was not seen in the cotransfection of pRK5-p19 (G12S)/pRK5-p21 cell lines.

Recent findings suggest that the constitutive expression of Ras via GEF's promotes a strongly upregulation in production of ROS species which promotes cell survival and growth factor independent proliferation (Hole et al., 2011); but p19 did not has GTPase function is not possible that can promotes an increment in the percentage of ROS emission than other proteins evaluated.

6.11 Overexpression of p19 induces an upregulation of NM23H1 protein and confers protection to the cell against ROS production

Run et al., 2008; reported that NM23H1 protein confers protection to the DNA damage against ROS production, for this reason we evaluated the expression of NM232H1 protein when p19 or p19 (G12S) proteins were upregulated (pRK5-P19 and pRK5-p19 (G12S) cell lines); our results showed that only overexpression of p19 was able to increase NM23H1 protein. This finding confers a key role of p19 in protecting DNA against ROS damage, the exactly mechanism is still unknown but it might be possible that p19 protein confers genomic stability involving the SEI1/SET/NM23H1 pathway. In this circuit SEI1 is an oncogene associated with chromosome instability; their overexpression contributes to develop a strong tumorigenic behavior in esophageal squamous cell carcinoma, which lead to genomic instability by increasing micronuclei formation and reducing the number of chromosomes. SEI1 upregulates SET and promotes a translocation of NM23H1 protein from the cytoplasm to the nucleus, then NM23H1 protein can induce DNA damage and activating apoptosis (Li et al., 2010b).

VII. CONCLUSIONS

Even though p19 protein lacks of GTPase activity, is able to bind other proteins that are key points of regulation and activation of multiple signaling pathways and mechanisms that confer protection against carcinogenesis, invasion and metastasis.

For all the evidences finding in the experimental development of this PhD thesis we conclude that:

- All H-Ras proteins including mutant variants that contained (G12S) or (Q61L) mutations increased the miR-342 and miR-335 expression profile, but p19 (G12S) increased dramatically all miRNAs expression profile.
- P19 expression protein showed a differential expression for miR-206; and p21 protein expression showed a differential expression for miR-342.
- In fibroblasts cell lines obtained from tumours of Costello Syndrome patients, the (G12S) mutation increased the expression of miR-374 and miR-330, whereas miR-335 and miR-330 were upregulated in (G12A) fibroblasts. In all these cell lines a downregulation of miR-206 was detected.
- The major invasion capacity was detected in the cell line that overexpressed p21 (G12S), followed by p21, p19 (G12S), p19 and p21 (Q61L) proteins. Besides p19 diminished the invasion of p21 (G12S) when they were cotransfected.
- In mutant fibroblasts of Costello Syndrome patients, (G12S) mutation was more invasive that (G12A) mutation.
- P19 protein showed their low proliferation capacity at difference of p21 protein.
- P19 protein induced a quiescent state in the cell at G0/G1 phase, this effect was also detected when p19 (G12S) was overexpressed in *HeLa* cells, even though the mutation was present, this cell cycle arrest was not detected when p21 was expressed in knock-out *H-Ras* (-/-) cell lines.
- The highest capacity of forming colony tumors was detected in p19 (G12S) mutant protein followed by p21 (G12S) , p19 , p21 and p21 (Q61L); in further experiments performanced in knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-), *N-Ras*

cell lines transfected with pEGFP-p19 or pEGFP-p21, p19 protein showed that was less tumorigenic than p21 protein.

- Reactive oxygen species (ROS) production was higher in those *HeLa* cell lines that overexpressed (G12S) mutant proteins (pRK5-p19 (G12S) and pRK5-p21 (G12S) cell lines). In further experiments using cotransfections we corroborated that p19 protein overexpression was able to diminish the high reactive oxygen species emission of p21 (G12S) *HeLa* cell line.
- Finally we established that p19 protein overexpression might confer protection to the cell against ROS production, via upregulating NM23H1 protein expression.

VIII. SUBMITTED ARTICLE

Article 1.

The Role of p19 and p21 c-H-Ras proteins and mutants in miRNA expression in cancer and Costello Syndrome cell model.

This article was submitted to pLoS One.

The Role of p19 and p21 c-H-Ras proteins and mutants in miRNA Expression in Cancer and a Costello Syndrome cell model.

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Running Title: miRNAs regulation by H-Ras proteins

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Key words: alternative splicing, IDX, H-ras, p19, p21, miRNAs, Costello syndrome, H-ras mutant

Abstract

P19 H-Ras a second product derived from the *H-Ras* gene, by alternative splicing induces to G1/s phase delay, thereby maintaining cells in a reversible quiescent state. This study describes how p19 affects the RNA world and shows that: i) miR-342, miR-206, miR-330, miR-138 and miR-99b are upregulated by p19 but not by p19W164A mutant, ii) anti-miR-206 can restore the 2 phase in the presence of p19; iii) p19 and p21Q61L regulate their own alternative splicing; iv) miR-206 and miR-138 are differentially regulated by p19 and p21 H-Ras and v) p19G12S Costello mutant show a clear upregulation of miR-374, miR-126, miR-342, miR-330, miR-335 and let-7. These results allow us to conclude that H-Ras G12S mutation plays an important role in miRNA expression and open up a new line of study to understand the consequences of this mutation on Costello Syndrome. Furthermore, our findings suggest that oncogenes may have a sufficiently important impact on miRNA expression to promote the development of numerous cancers.

Introduction

Ras, an important family of proto-oncogenes in humans, consists of three members (H-Ras, N-Ras and K-Ras) located on human chromosomes 1, 11 and 12 respectively (1,2). Indeed, Ras gene mutations have been implicated in up to 30% of all tumors tested. These mutations are different and depend on tissues involved, but most commoly result in pancreatic (90%), colon and thyroid tumours (50%), lung and myeloid leukemias (30%). A point mutation in Ras codons converts these genes into active oncogenes, decreased GTPase activity, thereby restricting the easy interchange of GDP to GTP and resulting in a constitutive activation of the downstream pathways, or loss of GAP function. Ras mutated proteins are indirectly involved in metastasic phenotype development, as they promote the acquisition of accumulative alterations in cellular pathways which result in cytoskeletal rearrangements, loss of cell adhesion (metalloproteases overexpression), tissue invasion, extravasation into lymphatic and blood vessels, and finally apoptosis evasion (3).

The H-Ras gene codifies for two different proteins, namely p19 H-Ras and p21 H-Ras, by alternative splicing (4-6). The mRNA structures of these alternative sequences are identical in their first coding exons (called 1, 2, 3 and 4A), and they have two non-codifying exons (0 and 4B located at the 5' UTR and 3' UTR regions respectively), separated by intronic

regions designated as A-E. The alternative exon known as IDX (82 nucleotides) is located between 3 and 4A exons. The pre-mRNA H-Ras is processed into two mRNAs, namely p21 mRNA (which excludes the IDX exon), and p19 mRNA (which includes it) , (4-6). p19 mRNA therefore codifies for a shorter protein than p21 mRNA. Furthermore, since IDX exon contains a premature stop codon, p19 does not contain the CAAX motif (5). P19 H-RAS induces a G1/S phase delay, thereby maintaining cells in a reversible quiescence state (6). P19 binds to RACK-1 and regulated telomerase activity upon interaction with p73α/β proteins, as well as inducing hypophosphorylation of Akt and p70SK6, and upregulating FOX1 (5-6). Although p19 overeexpression does not induce apoptosis (6) Kim et al., have shown that it stimulates p73 β induced apoptosis when both proteins are simultaneously overexpressed (7).

P19 RNAi increases cell growth, thereby having an opposite effect to the delay in G1/S phase described above. The H-Ras mutation, Q61L is frequently detected in different tumor cell lines, where it acts as a constitutive activator of the Ras-signalling pathway and is considered to be a strong activating mutant, by decreasing GTPase activity and increasing GDP/GTP exchange (1). G12S, another important H-Ras mutation is present in more than 90% of Costello Syndrome (CS) patients, a rare congenital disorder caused by germ-line activation of H-ras oncogene that affects p19 and p21 H-Ras (8).

Costello Syndrome (CS) is characterized by severe failure-to–thrive, cardiac abnormalities, including tachyarrhythmia and hypertrophic cardiomyopathy (HCM), a predisposition to papillomata and malignant tumours, and neurological abnormalities, including nystagmus, hypotonia developmental delay and mental retardation (9-11).

To better understand the role of p19 and p21 H-Ras proteins in the development of cancer, we transfected HeLa cells with p19 and p21 mutant sequences reported in the literature to be commonly detected in tumor cell lines and in Costello Syndrome (G12S). We also evaluated the expression of selected miRNAs involved in some aspects of metastasis and others related with aggressive small cell lung cancer.

Material and Methods

Cell lines, cell transfection and antibodies. HeLa cells were cultured and transfected as described elsewhere (5, 6). KO and DKO fibroblasts were obtained from Dr. E. Santos' Laboratory (12). KO and DKO cell lines stably expressing pEGFP (negative control),

pEGFP-p19 and pEGFP-21 were obtained by transfecting the fibroblasts with the specific vector and selecting with geneticin.

Plasmids. pEGFP-p19 and pEGF-p21, and pRK5-p19 W164A have been described previously (5,6). Therefore the other pRK5 plasmids were obtained in a similar manner (5-6). Other point mutations of p19 H-Ras and p21 H-Ras were performed by polymerase chain reaction site-directed mutagenesis using th QuickChange® Site-directed mutagenesis kit from Stratagene.

Isolation of small RNAs. miRNAs were extracted using the miRVANA ^{TM} miRNA isolation kit from Ambion Inc. (Austin Tx). Isolation was performed as manufacturer's protocol provided.

miRNA microarrays. miRNA microarray were perforemed as described previously (13).

microRNA Reverse Transcriptase Reaction. cDNA was reverse-transcribed from enriched miRNA samples (miRVANA kit) using specific miRNA primers from the TaqMan MicroRNA Assay and reagents from the TaqMan MicroRNA Reverse transcriptase kit (AB Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.33 μl of each resulting cDNA was amplified by PCR using TaqMan microRNA Assays primers with the TaqMan Universal Non amperase PCR Master Mix (in a total volume of 20 μl) and analyzed with a 7500 ABI PRISM Sequence Detector System according to the manufacturer's instructions. mRNA expression was calculated from the relevant signals by normalization with respect to the signal for U18.

RT-PCR amplification of miRNAs. *Quantitative reverse transcription PCR.* Stem-loop quantitative reverse transcription–PCR (RT-PCR) for mature miRNAs was performed as described previously using and Applied Biosystems ABI-7000 Real Time PCR system. All PCR reactions were run in triplicate and gene expression, relative to U18, calculated using the $2^{-\Delta\Delta}$Ct method (14).

PCR Real Time TAQMAN Assays. *Quantitative reverse transcription PCR detection of miRNA.* Total RNA was extracted from 10x10 ^{5} HeLa cells using TRIZOL reagent, (Life Technologies, Inc), as described previously. cDNA was reverse-transcribed from total RNA samples using SuperScript III ® from Invitrogen. The resulting cDNA was amplified by PCR using Taq Man Assay primers with the TaqMan Universal Non-amperase PCR Master Mix and analyzed with a 7500 ABI PRISM Sequence Detector System according to the

manufacturer's instructions. mRNA expression was calculated from the relevant signals by normalizacion with respect to the signal for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression.

The assay numbers of exons E3-IDX p19 H-Ras (Hs00978053_g1); E4A-E4B H-Ras total (Hs00978051_g1) and E3-E4A p21 H-Ras (Hs00610483_m1) and for GAPDH (Hs99999905_m1 housekeeping) were supplied by Applied Biosystems Gene Expression Assays (Applied Biosystems). Assays were run with Taqman Universal Master Mix.

Western blot analysis, cell proliferation and determination of cell cycle phase percentages. These assays were performed as described previously (5-6).

Results

P19 overexpression regulates specific miRNAs expression. We have shown previously that cell growth and metastatic genes are regulated by p19 (6). In this latter work we compared p19 expression with p19mut expression, where p19mut was a p19 mutant (W164A) that prevented p19-RACK1 and p19-p73α binding (6). This previous analysis is further complemented here by checking whether p19 can regulate other RNA genes, by studying miRNA expression in cells overexpressing p19 or p19 mut. Candidate miRNAs were found to be miR-342, miR-206, miR-330, miR-138 and miR-99b (Fig. 1A and supplemental 1 and 2), which vary with p19 but not with p19mut expression. The most relevant observation of this assay is that the expression of very few miRNAs varies upon overexpression of p19 versus p19mut. Interestingly however, 21 miRNAs were found to be upregulated upon overexpression of p19mut (supplemental 3).

miRNA upregulation by p19 H-Ras was re-validated by Real Time PCR with specific Taqman assays for mature miR-206, miR-342, miR-138 and miR-330 and was found to increase 2 fold, 1, 6, 16 and 2,5 fold respectively, upon overexpression of p19. miR-206 expression has recently been reported downregulated in metastatic cells and has also been shown to regulate cell migration and morphology (15). Our previous work showed that p19 H-Ras induces a G1/S phase delay, thereby maintaining cells in a reversible quiescence state (6). Taken together, these findings prompted us to study the contribution of miR-206 upregulation to the G1/S phase delay, both of which are induced by p19. Fig. 1B shows that anti-miR-206 partially antagonizes the effect of p19 and G1/S phases and restores the

G2 phase, thus indicating that miR-206 partially contributes to the G1/S delay observed upon p19 overexpression.

H-Ras mutants alter the H-Ras splicing rate. We have previously demonstrated that SR proteins (SFR's) activated inclusion of the alternative exon IDX and subsequently p19 expression (5, 16). p68 RNA helicase (DDX5) was also shown to inhibit p19 expression and reduce IDX inclusion, whereas SC-35 (SFRS2) and SRp40 (SFRS5) strongly increase IDX inclusion *in vivo* (5,16). Supplemental 1 and 2 show that miR-330 may target SC-35 (SFRS2) and many other SR proteins as well as an UsnRPN core protein, miR-342 may target SF4, and miR-206 may target p68 RNA helicase and many other SR proteins, therefore we decided to study whether the overexpression of p19 can also affect its own alternative splicing. Thus, two Taqman real-time assays were designed to map total/endogenous H-Ras mRNA (directed to E3-E4A exons) and the endogenous p19 level and the endogenous/total p21 H-Ras ratio determined in HeLa cells transiently overexpressing p19 (see Fig. 2A). Fig 2B shows that overexpression of p19 increases total endogenous H-Ras and endogenous p21 expression by 5.3 and 3.9-fold, respectively, and that this activation is reverted to endogenous basal levels by p19mut. The splicing rate for endogenous p21 formation was slightly reduced in cells overexpressing p19 (Fig 2B), but increased in the presence of p19mut (10% higher than negative control) thus indicating that p19mut can alter the p19:p21 ratio by acting on the H-Ras alternative splicing processes. In contrast, p19 was observed to directly regulate total mRNA H-Ras expression, but not to significantly alter the splicing rate of H-Ras pre-mRNA, thus indicating that the p19:p21 ratio is maintained.

P19:p21 splicing ratio alters miRNAs expression and cell growth. In other to understand how the alternative splicing of H-Ras alters miRNAs, we differentially expressed pEGFP-p19, or pEGFP-p21 in H-Ras $^{(-/-)}$ KO mice, and analyzed their effect on miR-206 and miR-138. Fig. 3A shows that p19 has a large effect on miR-206 expression whereas pEGFP-p21 incrasis it only slightly pEGFP-p19 also has a large effect on miR-138 in both KO mice and HeLa cells (Fig 3B and 3C, respectively), thus indicating that the alternative splicing of H-Ras affects the expression of several miRNAs. Fig. 4 shows that pEGFP-p21 increases cell growth in H, N-Ras $^{(-/-)}$/N-Ras $^{(-/-)}$ DKO mice fibroblasts whereas pEGFP-p19

does not. This finding is accordance with our previous results where we showed that p19 causes a G1/S delay in HeLa cells.

The p19 Costello mutant G12S alters miRNA expression. Aoki et al. (17) reported that Costello syndrome is a result of mutation in the H-Ras gene. The most common mutation is found on codon 12 with the amino acid change G12S (8). All human cells contain both p19 and p21 H-Ras proteins at different levels as a consequence of their alternative splicing, therefore when H-Ras gene is mutated in Costello patients, both proteins are present in their mutated forms. We obtained pRK5 plasmids containing p19 (G12S) and determined how this mutant alters a selected group of miRNAs in HeLa cells. These miRNAs included miR-342, and miR-330, which are already known to be upregulated by p19 overexpression (Fig. 1); miR-126 and miR-335 which significantly reduced the ability of CN34-LM1 and CN34-BoM1 cells to metastasize to the lung (15), miR-374 which is known to be associated with the aggressive small cell lung cancer (18) and let-7, which targets Ras genes (19). Fig. 5 shows that p19 (G12S) clearly upregulates all miRNAs (column 3), thus indicating that p19 Costello mutant contributes significantly to the syndrome by altering specific miRNA levels.

The p21 Costello mutant G12S also alters miRNA expression. We also analyzed how p21 mutants affect the same set of miRNAs by overexpresing pRK5-p21, pRK5-p21Q61L and pRK5-p21G12S in HeLa cells. Fig 5 shows that p21 and p21 mutants also alter the expression of these miRNAs although to a lesser extent than p19G12S. Thus p21 was found to upregulate miR-374, miR-126, miR-342, miR-335 and let-7 but not miR-330 and p21G12S was found to have the same effect as p21 on miR-374, miR-342 and miR-126. In contrast, p21G12S was found to have no effect on miR-335 and let-7 but upregulates miR-330 whereas p21 does not. This indicates that p21 and p21 mutants do not have such linear effect as p19 and p19G12S, but both p19 and p21 Costello mutants alter the expression of several miRNAs, which may contribute to the development of Costello Syndrome.

Discussion

P19 tumour mutants. Although H-Ras mutants have been extensively studied, this is not the case for specific p19 H-Ras mutants. However it is known that a mutation in the 5' splice-site of the IDX exon of H-Ras (position 2719, A to G change) induces a 10 –fold increase in p21 H-Ras level and corresponding increase in the transforming activity in a bladder carcinoma cell line (20). In addition we have previously reported that the mouse NIH 3T3 cell contains a mutation at position 165 which causes a D to G change (5).

Homeostatic regulation on H-Ras. P19 overexpression activates total H-Ras and p21, although the endogenous p19/p21 ratio due to alternative splicing remains relatively unchanged (Fig. 2). This H-Ras and p21 activation is dependent on the W164A amino acid (Fig. 2). Thus, whereas p19mut expression reverts endogenous total H-Ras to basal levels, p19mut alters alternative splicing in favor of p21 over p19 and total H-RAS.

In this study, we also found that the QG1L mutation in p21 produces a clear change in the p19:p21 ratio by doubling the amount of p19 mRNA compared to wild type.

We have also found that few proteins or miRNAs genes are up- or downregulated when p19 is overexpressed, showed in this work and (6). Indeed, the response mechanisms described in this work when p19, p19mut or the p21 mutant are overexpressed correlate well with observations in H-Ras knockout (KO) mice. These KO mice were found to be viable (21) and fibroblast obtained from them had few genes that modified their expression (12). These findings indicate that a cellular response mechanism is in place that overcomes the lack of H-Ras, thereby allowing the mice to survive, and that a strong homeostatic mechanism plays a role in controlling p21 and p19 levels, as p21 drives cell proliferation, whereas p19 maintains a reversible cellular quiescence state (6).

The role of p19 in cancer and metastasis processes. It may be significant that H-Ras is regulated by miRNAs, such as the let-7 miRNA family (19), that the alternative splicing of H-Ras, which favors p19 over p21, has consequences as regards the levels of certain miRNAs (Fig. 1A and supplemental 1-3). Two of the mRNAs upregulated by p19 (supplemental 1) have previously been reported to be of significant interest in cancer studies: miR-342 is one of the miRNA markers for acute promyeloytic leukemia (22) and miR-206 suppresses ER α in breast cancer cell lines and also plays a role in muscular dystrophy (23, 24). MiR-335, miR-206 and miR-126 have been shown to significantly

reduce the ability of certain cells to metastasize to the lung (15). These latter results have driven us to further study the regulation of miR-206 by H-Ras proteins as overexpression of p19 causes G1/S delay (6) and upregulates miR-206 (Fig. 1A). Herein we have shown that combining p19 overexpression with a miR-206 inhibitor, results in a partial decreased of the G1 phase with a clear recovery of the G2 phase, thus indicating that miR-206 is one of the factors, contributing to the delay of the G1/S phase. Additionally, we have shown that miR-206 is regulated by the alternative splicing of H-Ras (Fig. 3A), as the overexpression of p19 upregulates miR-206 more effectively than p21 H-Ras when pEGFP-p19 and pEGFP-p21 are stably expressed in KO H-Ras $^{(-/-)}$. miR-138, an miRNA that suppresses invasion and promotes apoptosis in some carcinoma cells (), was also studied Figs., 3B and 3C show that miR-138 is clearly upregulated in KO H-Ras $^{(-/-)}$ mice that stably express pEGF-p19, whereas it is unaffected in KO H-Ras$^{(-/-)}$ mice that stably express pEGP-p21 (Fig. 3B). This observation was corroborated by the transient expression of both proteins (Fig. 3C). Finally we also corroborate in our previous studies in which demonstrated that p19 H-Ras does not induce growth using DKO H-Ras$^{(-/-)}$/N-Ras$^{(-/-)}$ mice that stably express pEGFp-P19 (See Fig. 4).

P19 H-Ras and Costello Syndrome. H-Ras mutants have been described as a potential marker for Costello Syndrome (10, 25). Although the mutations in IDX sequences and the rasISS1 splicing silencer are described in (16) have not been observed in several patients with this syndrome (Katia Sol-Church, personnel communication). This finding strongly suggests that H-Ras related mutations in Costello Syndrome are likely to be found in the common amino acid sequences and thereby affect the complementary functions of p21 and p19. We can therefore conclude that both p21 and p19 must malfunction in order for a subject to develop Costello Syndrome. Around 90% of Costello patients have the G to A mutation that results in the G12S amino acid change. We therefore tested how this mutation affects a selected group of cancer-related miRNAs, and found a significant upregulation by p19 H-RAS G12S in all cases. This allowed us to conclude that the H-Ras G12S mutation plays an important role in miRNAs expression and therefore opens up a new line of study to understand the consequences of this mutation on Costello Syndrome. Furthermore, this finding has further consequences for many cancers, as our results indicate that oncogenes

may have a sufficiently important impact on miRNA expression to promote their development.

Supplemental Data

Supplemental data include additional tables. Microarray data accession number: E-TABM-494.

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References

1. Lowy DR, Willumsen BM. Function and regulation of ras. Annu Rev Biochem 1993;62:851-91.
2. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. J Cell Sci 2005;118:843-6.
3. HernandezAlcoceba R, delPeso L, Lacal JC. The Ras family of GTPases in cancer cell invasion. Cell Mol Life Sci 2000;57:65-76.
4. Cohen JB, Broz SD, Levinson AD. Expression of the H-ras proto-oncogene is controlled by alternative splicing. Cell 1989;58:461-72.
5. Guil S, de La Iglesia N, Fernandez-Larrea J, et al. Alternative splicing of the human proto-oncogene c-H-ras renders a new Ras family protein that trafficks to cytoplasm and nucleus. Cancer Res 2003;63:5178-87.

6. Camats M, Kokolo M, Heesom KJ, Ladomery M, Bach-Elias M. P19 H-ras induces G1/S phase delay maintaining cells in a reversible quiescence state. PLoS One 2009;4:e8513.

7. Kim JW, Kim WH, Jeong MH, et al. p19(ras) amplifies p73beta-induced apoptosis through mitochondrial pathway. Biochem Biophys Res Commun 2008;373:146-50.

8. Lin AE, Rauen KA, Gripp KW, Carey JC. Clarification of previously reported Costello syndrome patients. Am J Med Genet A 2008;146:940-3.

9. Gripp KW, Lin AE, Stabley DL, et al. HRAS mutation analysis in Costello syndrome: genotype and phenotype correlation. Am J Med Genet A 2006;140:1-7.

10. Gripp KW, Stabley DL, Nicholson L, Hoffman JD, Sol-Church K. Somatic mosaicism for an HRAS mutation causes Costello syndrome. Am J Med Genet A 2006;140:2163-9.

11. Sol-Church K, Stabley DL, Nicholson L, Gonzalez IL, Gripp KW. Paternal bias in parental origin of HRAS mutations in Costello syndrome. Hum Mutat 2006;27:736-41.

12. Castellano E, De Las Rivas J, Guerrero C, Santos E. Transcriptional networks of knockout cell lines identify functional specificities of H-Ras and N-Ras: significant involvement of N-Ras in biotic and defense responses. Oncogene 2007;26:917-33.

13. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci U S A 2004;101:9740-4.

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

15. Tavazoie SF, Alarcon C, Oskarsson T, et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008;451:147-52.

16. Guil S, Gattoni R, Carrascal M, Abián J, Stévenin J, Bach-Elias M. Roles of hnRNP A1, SR proteins, and p68 Helicase in c-H-ras Alternative Splicing Regulation. Mol Cel Biol 2003;23:2927-41.

17. Aoki Y, Niihori T, Kawame H, et al. Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nat Genet 2005;37:1038-40.

18. Miko E, Czimmerer Z, Csanky E, et al. Differentially expressed microRNAs in small cell lung cancer. Exp Lung Res 2009;35:646-64.

19. Bussing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. Trends Mol Med 2008;14:400-9.

20. Cohen JB, Levinson AD. A point mutation in the last intron responsible for increased expression and transforming activity of the c-Ha-ras oncogene. Nature 1988;334:119-24.

21. Esteban LM, Vicario-Abejon C, Fernandez-Salguero P, et al. Targeted genomic disruption of H-ras and N-ras, individually or in combination, reveals the dispensability of both loci for mouse growth and development. Mol Cell Biol 2001;21:1444-52.

22. Garzon R, Pichiorri F, Palumbo T, et al. MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene 2007.

23. Adams BD, Furneaux H, White BA. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha

messenger RNA and protein expression in breast cancer cell lines. Mol Endocrinol 2007;21:1132-47.

24. Gambardella S, Rinaldi F, Lepore SM, et al. Overexpression of microRNA-206 in the skeletal muscle from myotonic dystrophy type 1 patients. J Transl Med 2010;8:48.

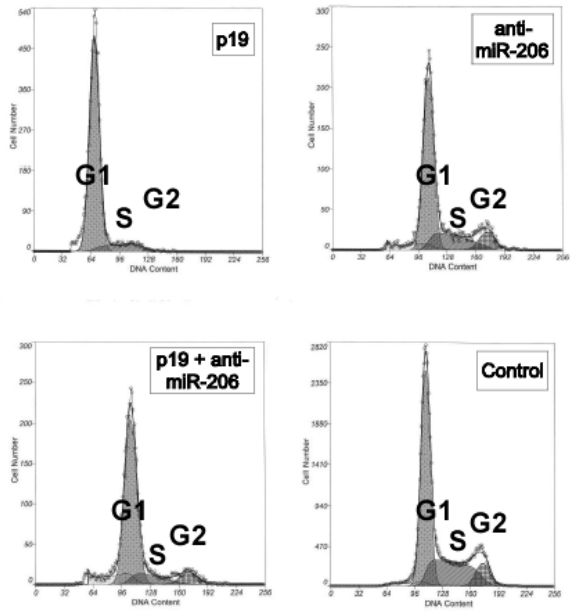
25. Dereure O. [Mutations in H-Ras proto-oncogen in Costello syndrome]. Ann Dermatol Venereol 2006;133:731.

FIGURE 1

A)

miRNA name	Fold Change
hsa-mir-342	4.98 (*2.40)
hsa-mir-206	3.37 (RT) (*1.00)
mmu-mir-330	2.07 (*1.00)
mmu-mir-138	2.61 (*1.60)
hsa-mir-99b	2.18 (*1.00)

B)



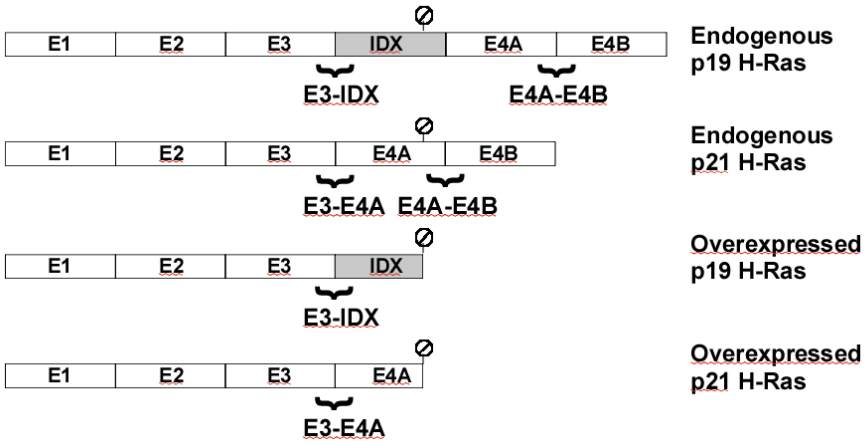
Legends to the Figures.

Figure 1. Anti-miR-206 partially antagonizes the effect of p19 on G1/S and G2 phases. A) Fold change in the value used to measure overexpression of pRK5-p19 as compared first to pRK5 empty vector and then to overexpression of pRK5-P19 mut for miRNA microarrays. (*) indicates the value obtained with pRK5-P19 mut. (**) additional gene information is

available in supplemental 1 and 2 ($P<0.01$). RT: miR-206 activation was confirmed by Taqman real time-PCR with a specific Taqman assay for mature miR-206 (Applied Biosystems). B) HeLa cells were first transfected with the pSuper-GFP vector containing the sequence of anti-miR-206 and incubated for 2 d under cell-culture conditions. Cells were then re-transfected with GFP-p19 vector and analyzed by FACS (fluorescent-activated cell sorting) the following day. A representative flow citometry histogram with GFP-p19, pSuper-GFP anti-miR-206, GFP-p19+pSuper-GFP anti miR-206 or GFP only (negative control) is shown in the upper portion, whereas G1, S, and G2 percentages are given below (*$P< 0.001$ as compared to GFP-p19 versus GFP-p19+anti-miR-206).

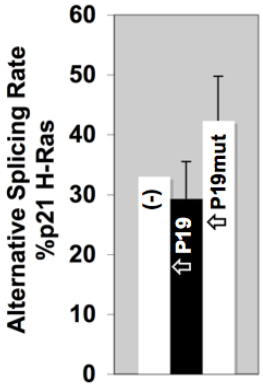
FIGURE 2

A)



B)

	EXPRESSION VECTOR		
	pRK5 (negative control)	pRK5-p19	pRK5-p19mut
REAL-TIME PCR PRIMERS	Fold Change	Fold Change	Fold Change
Total H-Ras (Taqman E4A-E4B)	1	5.3	0.8
P21 H-Ras (Taqman E3-E4A)	1	3.9	1



C)

	EXPRESSION VECTOR	
	pRK5 (negative control)	pRK5-p21Q61L
REAL-TIME PCR PRIMERS	Fold Change	Fold Change
Total H-Ras (Taqman E4A-E4B)	1	1.2
P19 H-Ras (Taqman E3-IDX)	1	2

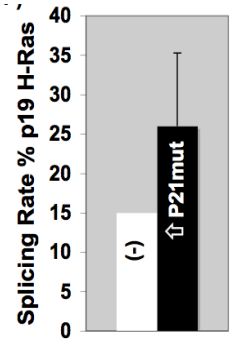


Figure 2. Gene expression and alternative splicing regulation by H-Ras proteins. A) Scheme of the Taqman assays used to determine endogenous p19, p21 and endogenous total H-Ras mRNA expression. E3-IDX, E3-E4A, and E4A-E4B recognize endogenous p19

H-Ras, P21 H-Ras, and both p19 and p21 H-Ras, respectively. Taqman assays were performed with E3-E4A and E4A-E4B for overexpressing p19 and with E3-IDX and E4A-E4B for overexpressing p21mut. B) Endogenous total H-Ras (Taqman E4A-E4B) and p21 (Taqman E3-E4A) mRNA levels change upon overexpression of p19 and p19mut. The results are presented as fold changes with respect to basal levels obtained for empty vector transfections, which were set to 1 (no change. Figure 2A: the percentage of alternative splicing p21/endogenous total H-Ras (Taqman E3-E4A/Taqman E4A-E4B) was obtained for each individual experiment and standard deviations of three separate experiments were calculated.

(-) p21 percent in transfections with empty vector set to 33%; (↑) p19 and (↑) p19 mut, % p21 in HeLa cells overexpressing p19 and p19mut, respectively. (↑) P=0.07; (↑) p19 mut, P=0.08 C) Endogenous total H-Ras (Taqman E4A-E4B) and p19 (Taqman E3-1DX) mRNA level changes upon overexpression of p21Q61L. The results are presented as fold changes with respect to the basal levels obtained for empty vector transfections, which were set to 1 (no change). Figure 2B, the percentage of alternative splicing p19/total H-Ras (Taqman E3-IDX/Taqman E4A-E4B) was obtained for each individual experiment and standard deviations of three separate experiments calculated. (-) % p19 in transfections with empty vector was set to 15%; (↑) p21 mut, % p19 in HeLa cells overexpressing p21Q61L, P=0.09.

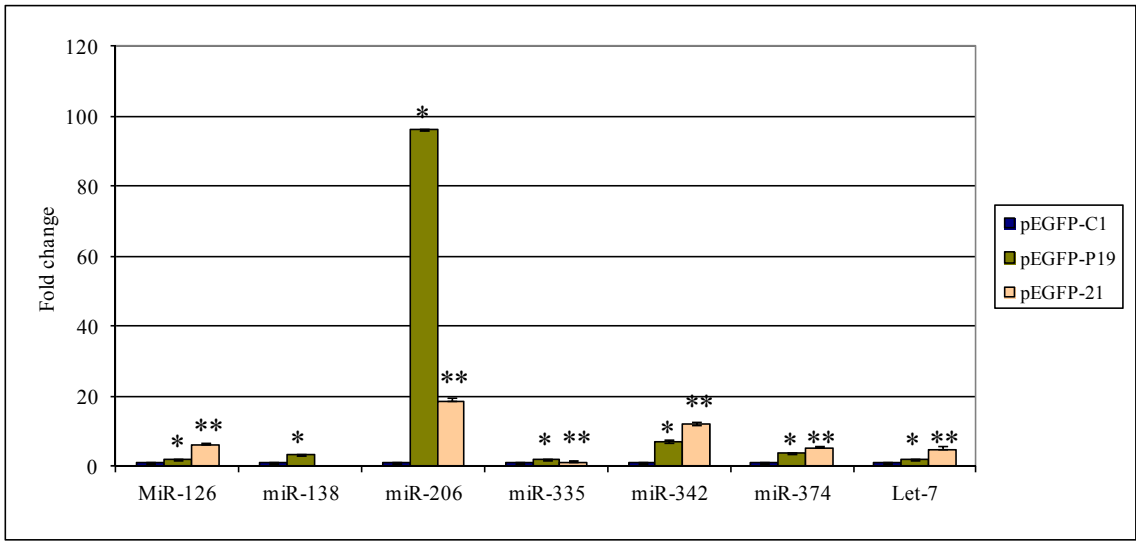


Figure 3. p19 and p21 H-Ras proteins differentially regulate miR-206 and miR-138. A) and B) show KO H-Ras $^{(-/-)}$ fibroblasts stably expressing pEGFP (negative control), pEGFP-p19 or pEGFP-p21. The regulation of miR-206 (A) and miR-308 (B) were analyzed in these cells with specific miRNA Taqman assays. C) Regulation of miR-138 in transient transfections in HeLa cells.

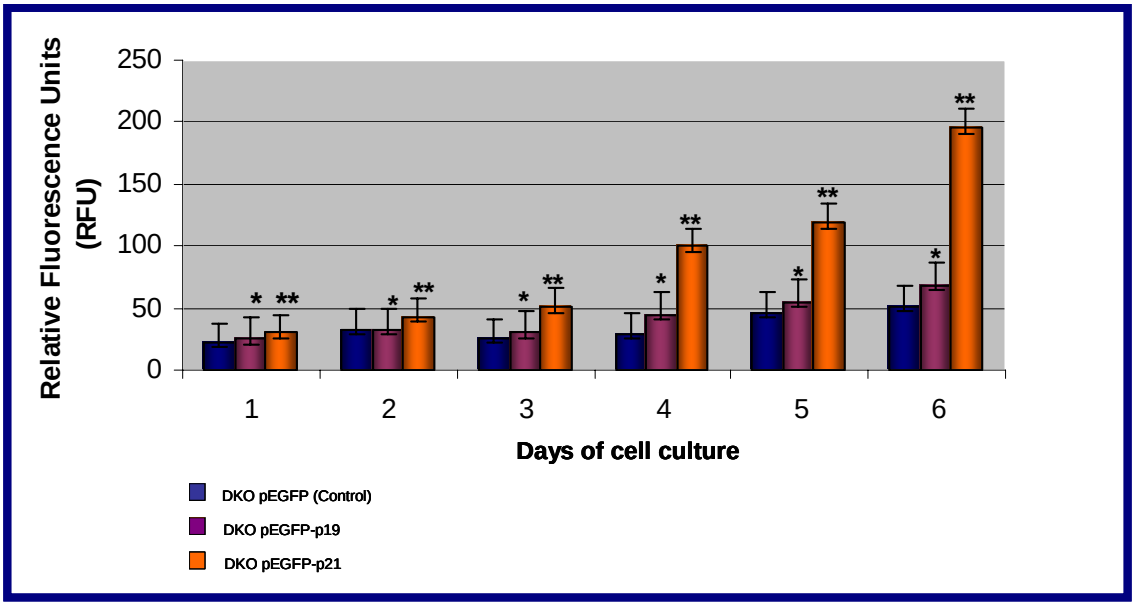
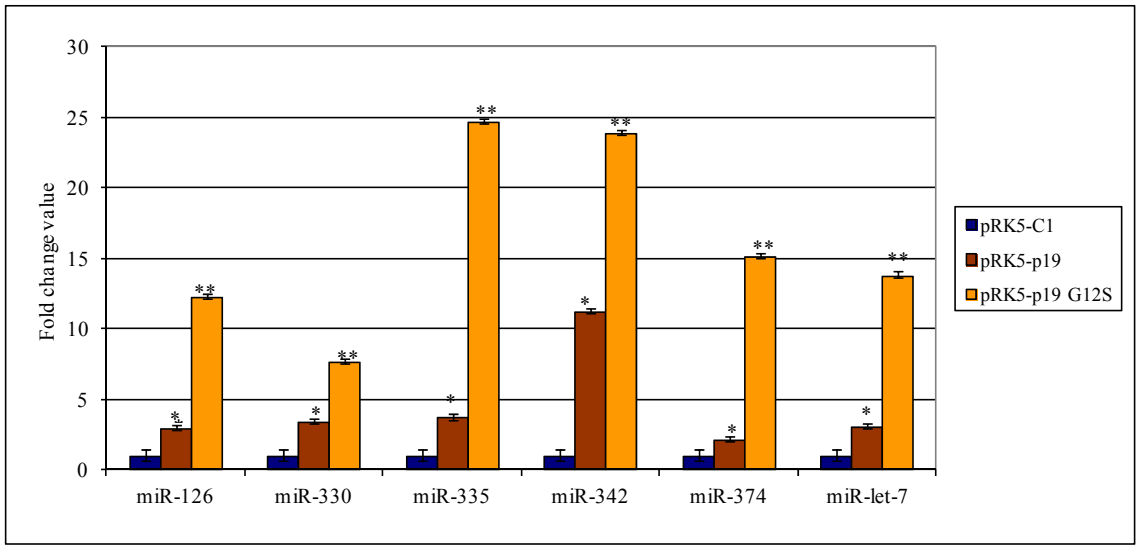


Figure 4. P19 H-Ras does not activate cell growth. DKO H-Ras $^{(-/-)}$/ N-Ras (-/-) fibroblast that stably express pEGFP (negative control). pEGFP-p19 and pEGFP-p21 were studied by direct cell proliferation assay. Cells were harvested and plated in 96 wells (10,000 cells/well) on six microplates in sextuplicate and incubate at 37°C, 5% CO_2 with DMEM/10% FCS. After the desired time, the microplates were washed and frozen. Cells were quantified with the green fluorescent dye, CyQuant (Invitrogen) according to the manufacturer's instructions. Fluorescence measurements were performed using a microplate reader with excitation at 485 nm and detection at 530 nm.

A)



B)

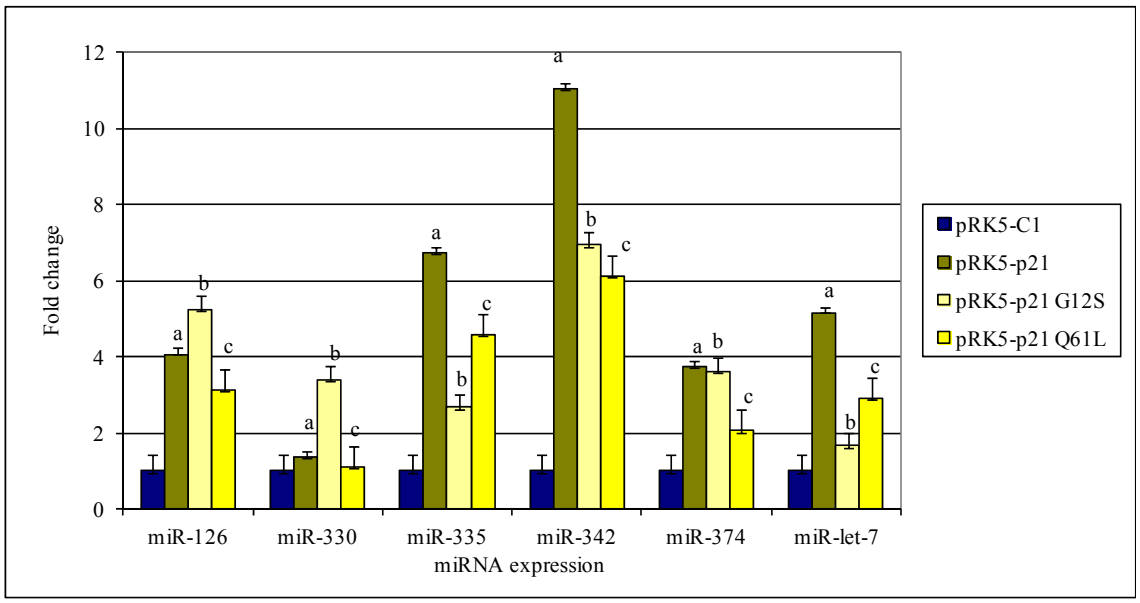


Figure 5. P19 H-Ras (G12S) Costello mutant clearly upregulates several miRNAs. *HeLa* cells transiently expressing: 5A 1) pRK5 (negative control); 2) pRK5-p19; 3) PRK5-p21G12S; 5B 1) pRK5-p21; 5) pRK5-p21Q61L; and 6) pRK5-p21G12S. MiRNAs levels were analyzed with specific miRNAs Taqman assays.

Supplemental 1

miRNAs regulated by p19 overexpression when compared to p19mut overexpression

miRNA name	Fold Change	Selected Target Genes**
hsa-mir-342	4.98 (*2.40)	***BCL2L1, BCL2L2, CSK, FOSB, IBRDC2, MAP3K7IP2, RAP2B, RASA1, RASSF1, SF4, SLA***
hsa-mir-206	3.37 **(RT)** (*1.00)	***ARHGAP21,CDK6, CDK9, DDX5, FOSB, JUND, MAP3K3, MET, MTSS1, RASA1, SFRS1, SFRS10, SFRS3, SFRS3. SFRS7, SFRS9,SFS7***
mmu-mir-330	2.07 (*1.00)	***CDC42, CDK11, DICER1, E2F1, GAS7, MAP2K1, PIK3R1, PRKCA/PKCα, PRKCB1/PKCβ1, PRKCE/PKCε, SFRS1, SFRS10, SFRS2, SFRS7, SFRS8, SFRS9, SLITRK4, SNRPD3***
mmu-mir-138	2.61 (*1.60)	***AIFM2, CCND3, CCNE1, DDX3X, MAP2K7, TCTP, MAPK4, MYBBP1A, TP53BP2, TP53INP1, TP53INP2, TP73L/p63***
hsa-mir-99b	2.18 (*1.00)	***MYCBP2, RalGPS1A***

Fold change is the value measuring overexpression of pRK5-p19 as compared first to pRK5 empty vector and then compared to overexpression of pRK5-p19mut for miRNAs microarrays. (*) indicates the value obtained with pRK5-p19mut. (**) additional gene information is in supplemental 1 of Table II. mRNA targets are described in http://microrna.sanger.ac.uk/sequences/ by using MIRANDA; TARGETSCAN, and PICTAR-VERT. (*P*<0.01). RT: mir-206 activation was confirmed by Taqman Real-PCR with an specific Taqman assay for mature miR-206 (Applied Byosystemes).

Supplemental 2

Supplemental of the Table II

hsa-miR-330

GENE TARGETS	ENSEMBL NUMBER	FUNCTION
CDC42	ENSG00000070831	Cell division control protein 42 homolog
CDK11	ENSG00000155111	Cyclin-dependent kinase (CDC2-like) 11
DICER1	ENSG00000100697	Endoribonuclease Dicer Helicase with RNase motif.
E2F1	ENSG00000101412	Transcription factor E2F1
GAS7	ENSG00000007237	Growth-arrest-specific protein 7
MAP2K1	ENSG00000169032	MAPK/ERK kinase 1
PIK3R1	ENSG00000145675	PI3-kinase p85-alpha subunit.
PRKCA/PKCa	ENSG00000154229	Protein kinase C, alpha type
PRKCB1/PKCb1	ENSG00000166501	Protein kinase C, beta type
PRKCE/PKCe	ENSG00000171132	Protein kinase C, epsilon type
SFRS1	ENSG00000136450	Splicing factor, arginine/serine-rich 1, SR protein, (pre-mRNA splicing factor SF2) (Alternative splicing factor ASF-1)
SFRS10	ENSG00000136527	splicing factor, arginine/serine-rich 10, SR protein, TRA2
SFRS2	ENSG00000161547	Splicing factor, arginine/serine-rich 2, SR protein SC35
SFRS7	ENSG00000115875	splicing factor, arginine/serine-rich 7, SR protein 9G8
SFRS8	ENSG00000061936	splicing factor, arginine/serine-rich 8, SR protein (suppressor-of-white apricot homolog, Drosophila, SWAP)
SFRS9	ENSG00000111786	Splicing factor, arginine/serine-rich 9, SR protein SRp30, (Pre-mRNA splicing factor SRp30C
SLITRK4	ENSG00000179542	slit and trk like 4 protein; slit and trk like gene 4

SNRPD3	ENSG00000100028	Small nuclear ribonucleoprotein Sm D3 (UsnRNP core protein D3) Splicing factor

hsa-miR-342

BCL2L1	ENSG00000171552	apoptosis regulator Bcl-X (Bcl-2-like 1 protein); apoptosis inhibitor.
BCL2L2	ENSG00000129473	apoptosis regulator Bcl-W (Bcl-2-like 2 protein); apoptosis inhibitor.
CSK	ENSG00000103653	c-src tyrosine kinase
FOSB	ENSG00000125740	Protein fosB,G0/G1 switch regulatory protein 3
IBRDC2	ENSG00000137393	p53-inducible RING finger protein.
MAP3K7IP2	ENSG00000055208	mitogen-activated protein kinase kinase kinase 7 interacting protein 2
RAP2B	ENSG00000181467	Ras-related protein Rap-2b
RASA1	ENSG00000145715	Ras GTPase-activating protein 1,Ras p21 protein activator.
RASSF1	ENSG00000068028	Ras association (RalGDS/AF-6) domain family 1. RASSF1A functions as a negative regulator of cell proliferation through inhibition of G1/S-phase progression.
SF4	ENSG00000105705	splicing factor 4
SLA	ENSG00000155926	Src-like-adapter protein 1

hsa-miR-206

ARHGAP21	ENSG00000107863	Rho GTAase activating protein p21;
CDK6	ENSG00000105810	cyclin depenent kinase 6
CDK9	ENSG00000136807	cyclin-dependent kinase 9
DDX5	ENSG00000108654	p68 RNA helicase
FOSB	ENSG00000125740	Protein fosB (G0/G1 switch regulatory protein 3)
JUND	ENSG00000130522	Jun D proto-oncogene,Transcription factor jun-D.
MAP3K3	ENSG00000198909	mitogen activated kinase 3
MET	ENSG00000105976	met proto-oncogene (hepatocyte growth factor receptor)
MTSS1	ENSG00000170873	metastasis suppressor 1.
RASA1	ENSG00000145715	Ras GTPase-activating protein 1,Ras p21 protein activator.
SFRS1	ENSG00000136450	Splicing factor, arginine/serine-rich 1, SR protein, (pre-mRNA splicing factor SF2) (Alternative splicing factor ASF-1)
SFRS10	ENSG00000136527	Arginine/serine-rich splicing factor 10, SR protein HTRA2- beta
SFRS3	ENSG00000112081	Splicing factor, arginine/serine-rich 3, SR protein SRP20
SFRS7	ENSG00000115875	splicing factor, arginine/serine-rich 7, SR protein 9G8
SFRS9	ENSG00000111786	splicing factor, arginine/serine-rich 9, SR protein SRp30

miR-138

AIFM2	ENSG00000042286	apoptosis-inducing p53-responsive gene
CCND3	ENSG00000112576	G1/S-specific cyclin D3.
CCNE1	ENSG00000105173	G1/S-specific cyclin E1.
DDX3X	ENSG00000124487	DEAD-box protein 3 (Helicase-like protein 2)
MAP2K7	ENSG00000076984	mitogen-activated protein kinase kinase 7,JNK kinase 2, c-Jun kinase 2) (JNK kinase 2)
MAPK4	ENSG00000141639	Mitogen-activated protein kinase 4, ERK-4, MAP kinase isoform p63
MYBBP1A	ENSG00000132382	MYB binding protein 1a; p53-activated protein-2
TP53BP2	ENSG00000143514	Apoptosis stimulating of p53 protein, p53-binding protein 2).
TP53INP1	ENSG00000164938	tumor protein p53 inducible nuclear protein 1
TP53INP2	ENSG00000078804	protein p53 inducible nuclear protein 2
TP73L/p63	ENSG00000073282	tumor protein p73-like; tumor protein63 kDa with strong homology to p53.

miR-99b

MYCBP2	ENSF00000004253	MYC binding protein 2
RalGPS1A	ENSG00000136828	Similar to Ral guanine nucleotide exchange factor

Supplemental 3

miRNAs regulated by p19mut overexpression when compared to p19 overexpression

miRNA	Fold Change
mmu-mir-145	4.80
mmu-mir-377	3.90
mmu-mir-294	3.60
hsa-miR-373*	3.50
mmu-mir-302	2.80
mmu-mir-196b	2.50
hsa-mir-185	2.40
hsa-mir-516	2.30
hsa-mir-213	2.30
hsa-mir147	2.00
hsa-mir-219	2.00
hsa-mir-361	2.00
mmu-mir-449	1.80
mmu-mir-32	1.80
mmu-mir-136	1.70
mmu-mir-138	1.70
mmu-mir-383	1.70
hsa-mir-30c	1.70
mmu-mir-331	1.60
hsa-mir-122a	1.60

Supplemental of the Table II
Fold change is the value measuring overexpression of pRK5-p19mut as compared first to pRK5 empty vector and then compared to overexpression of pRK5-p19 for miRNAs microarrays. miRNA targets are described in http://microrna.sanger.ac.uk/sequences/ by using MIRANDA; TARGETSCAN, and PICTAR-VERT

IX. MEMORIES PRESENTED IN DIFFERENT SYMPOSIUMS OF THIS PhD THESIS

1. Poster Title: **"MiRNAs new druggable players in human diseases"**, García-Cruz R., and Bach-Elias M. (2009) First IRB Barcelona PhD Student Symposium "The architecture of life", November 2-3, 2009.
2. Poster Title: **"El efecto antiproliferativo y quiescente de la proteína p19 c-H-Ras".** (2013). García-Cruz R. y Bach-Elias M. Pages: 222-224
3. Oral presentation: **"El papel de la proteína p19 c-H-Ras en la tumorigenesis y metástasis"**. (2013). García-Cruz R. y Bach-Elias M. Pages: 287-294

Memories 2 and 3 were presented in: Libro de memorias. Decimotercera Reunión de Ciencias Médicas. Departamento de Ciencias Médicas, División Ciencias de la Salud, Campus León. Universidad de Guanajuato, Guanajuato, México. April 24-26, 2013. **ISBN: 978-607-441-233-8**

X. APPENDICES

10.1 Restriction maps and multiple cloning sites of the vectors used for cell transfections for this PhD thesis

pRK5-C1 vector was employed for overexpressing p19, p21 or their mutant c-H-Ras variant proteins in *HeLa* cells.

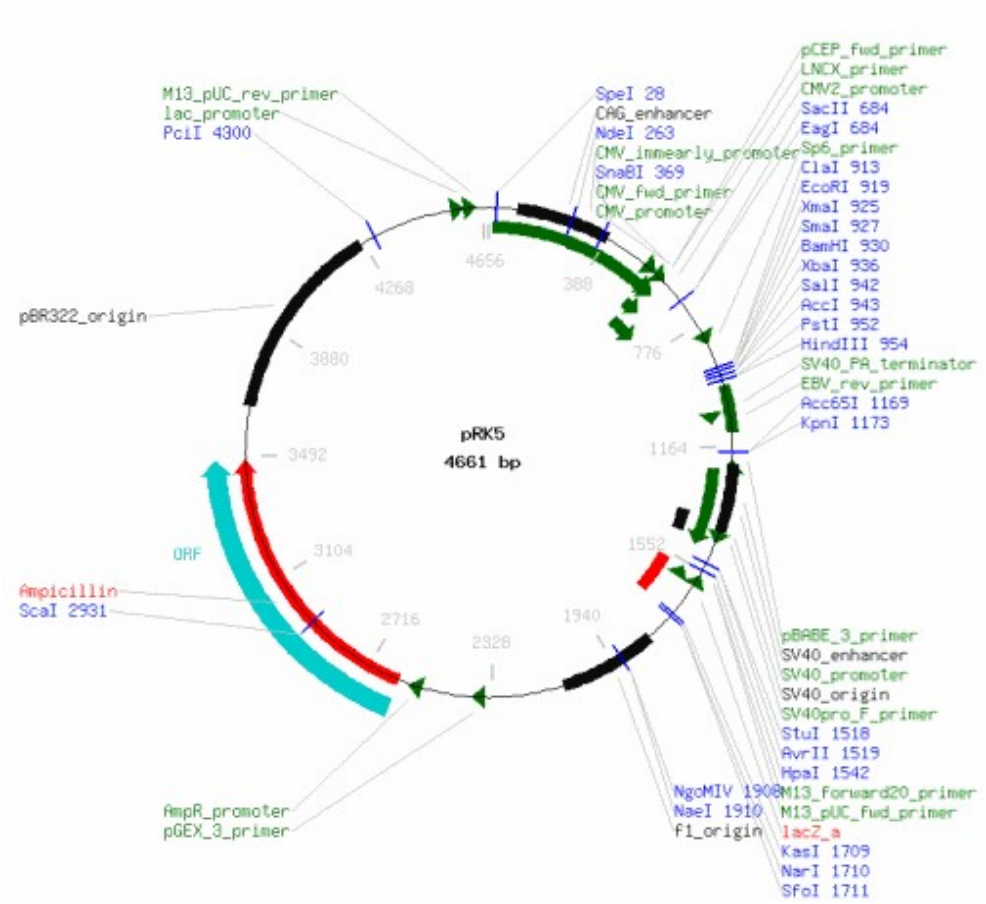
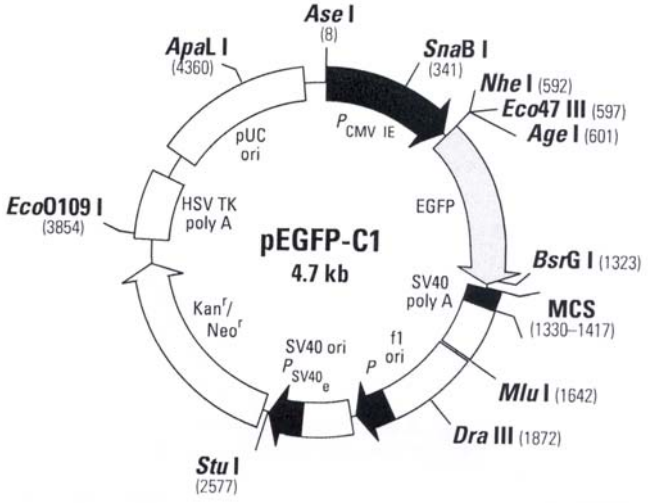


Fig. 33 pRK5 vector map. PRK5-C1 plasmid was used for oveexpressing p19, p21 and mutant variant proteins in *HeLa* cells**.**

Plasmid name	pRK5
Plasmid type	Mammalian
Promoter	CMV, SP6
Plasmid size	4754
Sequencing primer	SP6, pSG5-3' or SV40pA-R
Sequencing primer sequence	pSG5-3': GAA ATT TGT GAT GCT ATT GC
Bacterial resistance	Ampicillin

Table 45. Restriction map and multiple cloning site of pRK5-C1 vector. PRK5-C1 contains the CMV promotor and some introns that together can produces a high overexpression of mammalian proteins.

The second vector employeed for transfecting our knock-out *H-Ras* (-/-) and double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic cell lines was pEGFP-C1. This vector contains a tag which produces a chimeric green fluorescent protein of our interest, so in this manner is easier to detect those cells that were transfected properly.



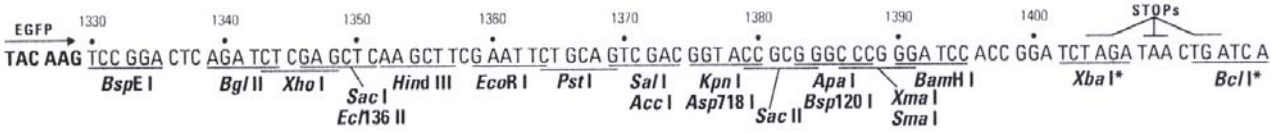


Fig. 34 PEGFP-C1 vector map. This vector was used for expressing pEGFP-p19 or pEGFP-p21 proteins in knock-out H-Ras (-/-) and double knock-out *H-Ras* (-/-), *N-Ras* (-/-) murine embryonic fibroblasts.

Plasmid Name	pEGFP`-C1
Plasmid Type	Mammalian
Promoter	CMV, SP6
Plasmid Size	4649
Sequencing Primer	SP6, pSG5-3' or SV40pA-R
Sequencing Primer	
Bacterial Resistance	Kanamycine/ Neomycine

Table 46. Restriction map and multiple cloning site (MCS) of pEGFP-C1vector

10.2 List of reagents

Reagent	Company	No. catalog
Cell culture reagents		
Lipofectamine TM Reagent	Invitrogen	183224-111 18324-012 18324-020
Plus reagent		
Fugene ® G Transfect Reagent	Roche	11815091001
Crystal Violet	Sigma	C3886
Methanol		
NaBH4		
Triton x 100		
Versene 1:5000 1X, liquid	Invitrogen	15040066
Select Agar	Invitrogen	30391-023
Glutaraldehyde	Fluca Sigma	49630
Cyquant ® cell proliferation assay kit for cells in culture	Invitrogen	C7026
Bovine Serum Albumin (BSA)	Sigma Aldrich	A7906-1006
SDS electrophoresis		
TEMED	Biorad	1610800
Acryl/Bis TM 29: 40% w/v solution	AMRESCO	0929b462
Ammonium peroxodisulfat $(NH4)_2S_2O_8$	Merk	1201
Bench Mark TM Prestained protein Ladder	Invitrogen	10748-010
DNA techniques reagents		
Agarose		
DNA polymerase 1000 Units 1U/µL	Biotools	10.003
PFU DNA polymerase 250 U 1U/µL	Biotools	10.502
Gene Ruler TM 100 bp plus DNA ladder plus 0.5 µg/µL 50 µg	Fermentas	SM0321
RNA tecniques reagents		
Trizol ® Reagent	Invitrogen	15596-018
DNA-free TM kit	Ambion	1906
Taqman ® MicroRNA Reverse Transcription kit	Applied Biosystems	43666596
mirVANA TM miRNA Isolation kit	Applied Biosystems	1560 1561
Acid Phenol: ChCl3 5:1 Solution pH 4.5 +1-0.2	Ambion	AM9720

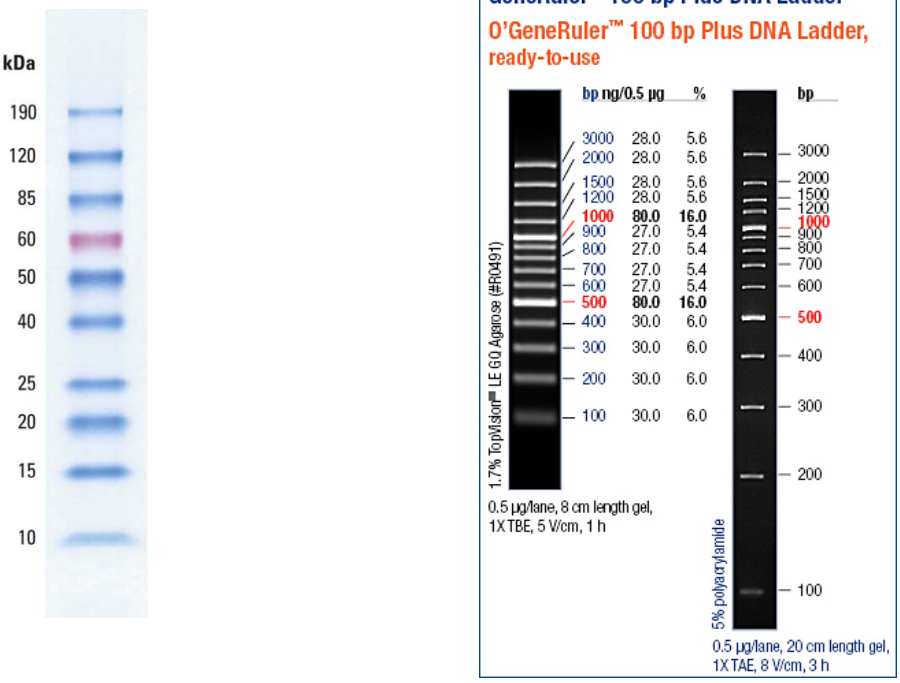
Table. 47 Table of reagents used for developing the experimental part of this PhD thesis.

10.3 List of buffers solutions and media

Cell culture	
Crystal violet 0.025%	10 ml of 0.1% crystal violet solution/20% methanol in PBS 1x, add 30 ml of methanol 20%/PBS 1x.
Crystal violet 1%/Methanol 20%	1 g crystal violet powder dilute in 10 ml of Methanol and added 80 ml of miliQ water.
Glutaraldehyde 0.5%	0.5 ml glutaraldehyde add 95 ml miliQ wáter
PBS 10 X p.H 7.4	Dissolve the following in 800 ml distilled miliQ water 80.0 g of NaCl 2.0 g of KCl 14.4 g of Na_2 HPO_4 2.4 of KH_2PO_4 Adjust pH to 7.4 Adjust volume to 1 L with additional distilled H2O Sterilize by autoclaving
NaBH4 (1 mg/mL)/PBS 1X	Prepare the solution until you will use it.
3% BSA/Tritón X 100 (0,2%)/PBS 1X	
Nonidet NP 40 10%/miliQ water Kathon 1:1000 as preservative	
Paraformaldehyde 4%/PBS 1X	2 g paraformaldehyde add 40 ml miliQ water and one drop of NaOH 10 M. Dilute until the precipitate dissapears, add 5 ml PBS 10 X, adjust pH until 7 and ad water until 50 ml.
NP-40 Lysis Buffer	1Sodium Chloride 150 mM 2NP-40 ---------- 1% 3Tris, pH 8.0 ---------- 50 mM NP-40 lysis buffer is one of the most commonly used lysis buffer. NP-40 is a nonionic detergent. Sometimes Triton X-100 can be used to replace NP-40.
DNA Tecniques	
TAE (50X)	242 G/L Tris Base 57.1 ml/l Acetic Glacial Acid 100 ml/0,5 M EDTA (p.H 8.0)
DNA sample buffer (5X) Undenaturalized gels	50% glycerol Orange 6

Table 48. Table of buffers and media used for developing this PhD thesis.

10.4 Molecular weight markers



A) **B)**

Fig. 35 Molecular weight markers for performing SDS-PAGE assays.
A) Protein weight Markers: Bench Mark TM Pre-Stained Protein Ladder of Invitrogen; catalog No. 10748-010. **B)** DNA weight Markers: Gene Ruler TM 100 bp plus DNA ladder Plus of Fermentas; TrackIt 100 bp DNA Ladder (Invitrogen) Cat. No. 10488-058 (Conc. 0.1 µg/µl).

10.5 Target genes of miRNAs selected

miR-138
oficial name: hsa miR-138
source: microRNA.org
mature sequence: 10-AGCUGGUGUUGUGAAUCAGGCCG-32 (hsa miR-138-5p)

Gene Abbreviation	Gene Name
AIFM2	Apoptosis inducing p53 responsive gene
CCND3	G1/S-specific cyclin D3
CCNE1	G1/S-specific cyclin E1
DDX3X	DEAD-box protein (Helicase-like protein 2)
MAP2K7	Mitogen-activated protein kinase kinase 7, JNK kinase 2, c-Jun kinase 2) (JNK kinase 2)
MAPK4	Mitogen-activated protein kinase 4, ERK-4, MAP kinase isoform p63
MYBBP1A	MYB binding protein 1a; p53 activated protein 2
TP53BP2	Apoptosis stimulating of p53 protein, p53-binding protein 2
TP53INP1	Tumor protein p53 inducible nuclear protein 1
TP53INP2	Protein p53 inducible nuclear protein 2
TP73L/p63	Tumor protein p73-like tumor protein 63 kDa with strong homology to p53

Table 49. Targets of miR-138

miR-126
oficial name: hsa-miR-126*
source: microRNA.org
mature sequence: 15-CAUUAUUACUUUUGGUACGCG-35 (hsa miR-126-5p)

Gene Abbreviation	Gene Name
PRTG	Protogenin homolog (Gallus gallus)
KL	Klotho
C5org47	Chromosome 5 open reading frame 47
ARSK	Arylsulfatase family, member K
SULT6B1	Sulfotransferase family, cytosolic, 6B, member 1
NDUFS1	NADH Deshidrogenase (ubquinone) Fe-S-protein 1 NADH-coenzyme Q reductase
HOXC8	Homeobox 8
PLCB1	Phospholipase C, beta 1 (Phosphoinositide-specific)
AKAP6	A kinase (PRKA) anchor protein 6
GRIK2	Glutamate receptor, ionotropic, kainite 2
DMRT2	Doublesex and mab-3 related transcription factor 2
GPR88	G protein –coupled receptor 88
SYT10	Synaptotagmin X
SLC4A10	Solute carrier family 4, sodium bicarbonate transporter, member 10

Table 50. Targets of miR-126

miRNA 206
oficial name: hsa-miR-206
source: http://www.microrna.org
mature sequence: 53- UGGAAUGUAAGGAAGUGUGUGG-74

Gene Abreviation	Gene Name
ARHGAP21	Rho GTAse activating protein 21
CDK6	Cyclin dependent kinase 6
CDK9	Cyclin dependent kinase 9
DDX5	P68 RNA helicase
FOSB	Protein Fos B (G0/G1, switch regularoty protein 3)
JUND	Jun D proto-oncogene (hepatocyte growth factor receptor)
MTS1	Metastasis supressor 1
RASA1	Ras GTPase-activating protein 1, Ras p21 protein activator
SFRS1	Splicing factor, arginine/serine-rich 1, SR protein (pre-mRNA splicing factor SF2), (Alternative splicing factor ASF-1)
SFRS10	Splicing factor, arginine/serine-rich 1, SR protein (pre-mRNA splicing factor SF2)
SFRS3	Arginine/serine-rich 1, rich splicing factor 3, SR protein SRP20
SFRS7	Splicing factor, arginine/serine-rich 3, SR protein SR protein 9G8
Sfrs9	Splicing factor, arginine/serine rich 9, SR protein SRp30

Table 51. Targets of miR-206

miR-330
oficial name: hsa miR-330
source: http://www.microrna.org
sequence: 22-UCUCUGGGCCUGUGUCUUAGGC-43 (mmu miR-330-5p)

Gene Abreviation	Gene Name
CDC42	Cell division control protein 42 homolog
CDK11	Cyclin-dependent kinase (CDC2-like) 11
DICER1	Endoribonuclease Dicer Helicase with RNAse motif
E2F1	Transcription factor E2F1
GAS7	Growth arrest specific protein 7
MAP2K1	MAPK/ERK kinase 1
PIK3R1	PI3 kinase p85 alpha subunit
PRKCA/PKC α	Protein kinase C, alpha type
PRKCB1/PKC ε	Protein kinase C, beta type
SFRS1	Splicing factor, arginine/serine-rich 1, SR protein (pre-mRNA splicing factor SF2) (Alternative splicing factor ASF-1)
SFRS10	Splicing factor, arginine/serine-rich 10, SR protein TRA2
SFRS2	Splicing factor, arginine/serine-rich 2, SR protein SC35
SFRS7	Splicing factor, arginine/serine rich 7, SR protein 9G8
SFRS8	Splicing factor, arginine/serine rich 8, SR protein (suppressor-ofwhite apricot homolog, Drosophila SWAP)
SFRS9	Splicing factor, arginine/serine-RICH 9, SR protein SRp30 (Pre mRNA splicing factor SRp30C)
SLITRK4	Slit and trk like 4 protein, slit and trk like gene 4
SNRPD3	Small nuclear ribonucleoprotein SmD3 (UsnRNP core protein D3) splicing factor

Table 52. Targets of miR-330

miR-335
official name: hsa miR-335
source: http://www.microrna.org
mature sequence: 16-UCAAGAGCAAUAACGAAAAAUGU-38 (hsa miR-335-5p)

Gene Abbreviation	Gene Name
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulador of chromatin, subfamily a, member 2
LMX1A	Homeobox transcription factor 1, alpha
PRDM2	Domain containing 2, with ZNF domain
FIGN	Fidgetin protein
DDX46	DEAD (Asp-Glut-Ala-Asp) box polypeptide 46
ZNF449	Zinc finger protein 449
SLC38A6	Solute carrier family 38, member 6
ARHGAP18	Rho GTPase activating protein 18
LASS5COG1	LAG1 homolog, ceramide synthase 5
PTPN22	Protein tyrosine phosphatase, non-recpetor tye 22 (lymphoid)
PIGY	Phosphatidylinositol glycan anchor biosynthesis, class Y
PITPNA	Phosphatidylinositol transfer protein, alpha
RAP1A	Member of Ras oncogene family
TTK	TTK protein kinase
GDE	Glycerophosphodiester phosphodiesterase 1
NUCB2	Nucleobindin 2
HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)

Table 53. Targets of miR-335

miR-342
official name: mmu-miR-342-3p
source: http://www.microrna.org
mature sequence: 19- AGGGGUGCUAUCUGUGAUUGAG-40 (mmu-miR-342-5p)

Gene Abbreviation	Gene Name
BCL2L1	Apoptosis regulador Bcl-X (Bcl-2-like 1 protein); apoptosis inhibitor
BCL2L2	Apoptosis regulador Bcl-X (Bcl-2-like 2 protein); apoptosis inhibitor
CSK	c-src tyrosine kinase
FOSB	Protein fos B G0/G1 switch regulador protein 3
IRBDC2	P53 inducible RING finger protein
MAP3K7IP2	Mitogen activated protein kinase kinase kinase 7 interacting protein 2
RAP2B	Ras- related protein Rap-2b
RASA1	Ras GTPase activating protein 1, Ras p21 protein activator
RASSF1	Ras association (rALgds/af-6) domain family 1, RASSF1A functions as a negative regulator of cell proliferation through inhibiton of G1/S-phase progression.
SF4	Splicing factor 4
SLA	Scr- like-adapter protein 1
Socs6	Suppressor of cytokine signalling 6
Nip7	Nuclear import 7 homolog

Table 54. Targets of miR-342

miR-374
official name: hsa-miR-374
source: http://www.microrna.org
mature sequence: 22- AUAUAAUACAACCUGCUAAGUG -43 (mmu-miR-374-5p)

Gene Abbreviation	Gene Name
Elf1	E74-like factor 1
Dimt1	Dimethyladenosine transferase 1-like
Cadm2	Cell adhesión molecule 2
Gimap8 GTPase	IMAP family member 8
Adamts9	Disintegrin-like and metallopeptidase (rerolysin type) with thrombospondin type 1 motif 9
Ptpn20	Protein tyrosine phosphatase, non -receptor type 20
Cpsf7	Cleaage and polyadenylation specific factor 7
Tmem47	Transmembrane protein 47
Zic3	Zinc finger protein of the cerebellum 3
Clcn3	Chloride channel 3
Nt5dc1	5' nucleotidase domain containing 1
Vezf1	Vascular endothelial zinc finger
Fgl2	Fibrinogen-like protein 2
Itgb8	Integrin beta 8
Pitx2	Paired-like homeodomain transcription factor 2

Table 55. Targets of miR-374

miR let-7
official name: hsa-let-7
source:http://www.microrna.org
mature sequences:
hsa-let-7a-5p: 6-UGAGGUAGUAGGUUGUAUAGUU-27
has-let-7a-3p: 57-CUAUACAAUCUACUGUCUUUC-77

Gene Abbreviation	Gene Name
C14orf28	Chromosome 14 open reading frame 28
HMGA2	High mobility group AT-hook 2
TRIM71	Tripartite motif-containing 71
DNA2	DNA replication helicase homolog
SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin subfamily, a containing DEAD/H box 1
ESR2	Estrogen receptor 2 beta
EDN1	Endothelin 1
FIGN	Fidgetin
FIGNL2	Fidgetin like-2
CTPS2	CTP synthase II
THRSP	Thyroid hormone responsive (SPOT14 homolog, rat)
ZNF644	Zinc finger protein
SNHG4	Small nucleolar RNA host gene 4 (non-protein coding)
PPP1R15B	Protein phosphatase 1, regulatory (inhibitor) subunit 15B
ERP29	Endoplasmic reticulum protein 29
RG9MTD2	RNA (guanine-9) methyltransferase domain containing 2
SFRS18	Splicing factor, argignine/serine-rich 18
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1

Table. 56. Targets of let-7

XI. BIBLIOGRAPHY

Abbot R. G., Forrest S., Pienta K.J. (2006). Simulating the hallmarks of cancer. Artificial Life 12: 617-634

Abdelrahim M., Safe S., Baker C., and Abudayyeh A (2006). RNAi and cancer: implications and applications. Journal of RNAi and Gene Silencing. 2 (1):136-145

Adams BD, Cowee DM, White BA. (2009). The role of miR-206 in the epidermal growth factor (EGF) induced repression of estrogen receptor-alpha (ERalpha) signaling and a luminal phenotype in MCF-7 breast cancer cells. Mol Endocrinol. 2009 Aug; 23(8):1215-30. Epub 2009 May 7. PMID: 19423651

Adams BD, Furneaux H, White BA. (2007). The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. Mol Endocrinol. 2007 May;21(5):1132-47. Epub 2007 Feb 20. PMID: 17312270

Adams JR., Schachter NF., Liu JC., Zacksenhaus E., and Egan SE. (2011). Elevated PI3K signaling drives multiple breast cancer. Oncotarget. 2 (6): 435-437

Adly A M. (2010). Oxidative stress and disease: an updated review. Research Journal of Immunology 3(2):129-145

Aggarwal B. Bharat and Prashasnika G. (2009). Inflammation and cancer: how friendly is the relationship for cancer patients?. Current opinion in pharmacology. (9): 351-369

Ahmedin J., Siegel R., Xu J., and Ward E. (2010). Cancer statistics, 2010. CA Cancer Journal for clinicians. (Vol. 60):277-300

Ali S, Ahmad A, Aboukameel A, Bao B, Padhye S, Philip PA, Sarkar FH. (2012). Increased Ras GTPase activity is regulated by miRNAs that can be attenuated by CDF treatment in pancreatic cancer cells. Cancer Lett. 2012 Jun 28;319(2):173-81. doi: 10.1016/j.canlet.2012.01.013. Epub 2012 Jan 17.

Ali S., Saleh H., Sethi S., Sarkar FH., Philip PA. (2012). MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. Br. J. Cancer. 107(8): 1354-60

Alves de Oliveira L., Artigiani-Neto R., Waisberg DR., Fernandez LC., Oliviera-Lima F., and Weisberg J. (2010). NM23 promote expression in colorectal carcinoma using TMA (tissue microarray) association with metastases and survival. Arq. Gastroentel. 47 (4): 361-367

Anderson C, Catoe H, Werner R. (2006). MIR-206 regulates connexin43 expression during skeletal muscle development. Nucleic Acids Res. 2006;34(20):5863-71. Epub 2006 Oct 24. PMID: 17062625

Aoki Y., Nihor T., Narumi Y., Shigeo K., and Matsubara Y. (2008). The RAS/MAPK syndromes novel roles of the RAS pathway in human genetic disorders. Human mutation. 29 (8):992-1006

Aoki Y., Niihori T., Kawame H., Kurosawa K., Ohashi H., Tanaka Y., Filocamo M., Kato K., Suzuki Y., Kure S., and Matsubara Y. (2005). Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nature Genetics (37): 1038-1040

Arora S, Ranade AR, Tran NL, Nasser S, Sridhar S, Korn RL, Ross JT, Dhruv H, Foss KM, Sibenaller Z, Ryken T, Gotway MB, Kim S, Weiss GJ. (2011). MicroRNA-328 is associated with (non-small) cell lung cancer (NSCLC) brain metastasis and mediates NSCLC migration. Int J Cancer. 2011 Dec 1;129(11):2621-31. doi: 10.1002/ijc.25939. Epub 2011 Mar 29. PMID: 21448905

Arozarena I., Calvo F., and Crespo P. (2011). Ras, an actor on many stages: postranslational modifications, localization, and site-specific events. Genes and Cancer 2(3).:182-194

Ataie-Kachoie P, Morris DL, Pourgholami MH. (2013). Minocycline suppresses interleukine-6, its receptor system and signaling pathways and impairs migration, invasion and adhesion capacity of ovarian cancer cells: in vitro and in vivo studies. PLoS One. 2013 Apr 8;8 (4):e60817. doi: 10.1371/journal.pone.0060817. Print 2013.

Ausubel, Frederick M.; Brent, Roger Kingstong, Robert E.; Moore, David D; Seidman, S.J.; Smith John A. and Sthuhl, Kevin. Short protocols in molecular biology. 4th ed. Wiley, USA, (1999). 1104 p. ISBN 0-471-32938-X.

Baljuls A., Beck M., Oenel A., Robubi A., Kroschwsku R., Hekman M., Rudel T., and Rapp U.R. (2012). The tumor suppressor DiRas3 forms a complex with H-Ras and C-RAf and regulates localization, dimerization and kinase activity to C-RAF. JBC. Papers in press. Published on may 17, 2012 as manuscript M112.343780

Bayani J., Kuzmanov U., Saraon P., Funf WA., Soosaipillai A., Squire JA., Diamandis EP. (2012). Copy-number and expression alterations of miRNAs in the ovarian cancer cell line OVCAR-3 impact on kalikrein G protein expression. Cin. Chem. (Epub ahead of print).

Bhutia YD, Hung SW, Krentz M, Patel D, Lovin D, Manoharan R, Thomson JM, Govindarajan R. (2013). Differential Processing of let-7a Precursors Influences RRM2 Expression and Chemosensitivity in Pancreatic Cancer: Role of LIN-28 and SET Oncoprotein. PloS One. 2013;8(1):e53436. doi: 10.1371/journal.pone.0053436. Epub 2013 Jan 15. PMID: 23335963

Black Douglas, L. (2003). Mechanisms of alternative pre-messenger RNA splicing. Ann.Rev. Biochem. 72:291-336

Blenkiron C., Leonard D Goldstein Natalie P Thorne, Inmaculada S. Suet-Feung Chin, Mark J Dunning, Nuno L Barbosa-Morais, Andrew E Teschendorff, Andrew R Green Ian O EllisSimon Tavaré, Carlos Caldas and Eric A Miska. (2007). MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype Genome Biology 2007, 8:R214 (doi:10.1186/gb-2007-8-10-r214).

Bode Ann M., and Dong Zigang. (2003). Mitogen-activated protein kinase activation in UV-induced signal transduction. Sci. STKE. 167, re2. (DOI: 10.1126/STKE.2003.167.RE2)

Boissan M., Dabernat S., Peuchant E., chlattner U., Lascu L., Lacombre ML. (2009). The mammalian NM23H1 NDPK family: from metastasis control to cillia movement. Mol. Cell Biochem. 329 (1-2):51-69 Doi: 10.1007/S11010-009-0120-7

Boominathan L. (2010). The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. PLoS One.5(5):e10615. PMID: 20485546

Bos Johannes L. (1989). Ras oncogenes in human cancer: a review. Cancer Res. 49: 4682-4689.

Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME.(2010). The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer. 2010 Jan 29;17 (1):F19-36. doi: 10.1677/ERC-09-0184. Print 2010 Mar. Review.

Breitbart R.E., Andreadis A., and Nadal-Ginard B. (1987). Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Ann. Rev. Biochem. 56:467-495.

Breving K., and Esquela-Kersher A. (2009). The complexities of microRNA regulation: mirandering around the rules. The International Journal of Biochemistry and Cell Biology. 42 (8): 1316-1329

Bruni L., Diaz M., Castellsagre X., Ferrer E., Bosh F.X., and de Sanjosé Silvia. (2010). Cervical Human Papillomavirus prevalence in 6 continents: meta-analysis of 1 millon women in normal cytological findings. The journal of infectious diseases. 202 (12):1789-1799

Bryant A., Palma AC., Jayaswal V., Yang YW., Lutherborrow M., Ma DF. (2012). MiR-10a is aberrantly overexpressed in neucleophosmin 1 mutated acute myeloid leukaemia and its suppression induces cell death. Mol. Cancer.

Büssing I, Slack FJ, Grosshans H. (2008). let-7 microRNAs in development, stem cells and cancer. Trends Mol Med. 2008 Sep;14 (9):400-9. doi: 10.1016/j.molmed.2008.07.001. Epub 2008 Jul 31.

Buxton ILO., and Yokdang N. (2011). Extracellular NM23 signalling in breast cancer: Incommodus verum. Cancers (Basel). 3(3): 2844-2857

Cai H., Wang JS., Zhang MX., Duan XY, Ma RA. (2011). Differential expression of hsa-miR-126 and has-miR-518b in esophageal squamous carcinoma. Nan Fang Yi Ke Da Xue Bao. 31 (1):23-7

Cai J, Guan H, Fang L, Yang Y, Zhu X, Yuan J, Wu J, Li M. (2013a). MicroRNA-374a activates Wnt/β-catenin signaling to promote breast cancer metastasis. J Clin Invest. 2013 Feb 1;123(2):566-79. doi: 10.1172/JCI65871. Epub 2013 Jan 16.

Cai WY, Wei TZ, Luo QC, Wu QW, Liu QF, Yang M, Ye GD, Wu JF, Chen YY, Sun GB, Liu YJ, Zhao WX, Zhang ZM, Li BA. (2013b). Wnt/β-catenin pathway represses **let-7** microRNAs expression via transactivation of Lin28 to augment breast cancer stem cell expansion. J Cell Sci. 2013 Apr 23. [Epub ahead of print]

Calin G.A., and Croce C.M. (2006). MicroRNA signatures in human cancers. Nature Rev. Cancer. 6:857-866.

Camats M, Kokolo M, Heesom KJ, Ladomery M, Bach-Elias M. (2009). P19 H-ras induces G1/S phase delay maintaining cells in a reversible quiescence state. PLoS One. Dec 30;4(12):e8513.

C**amats-Malet M.** (2008a). Mecanismes de Senyalitzacio intracellular regulats per la proteina p19 H Ras. Tesis de Doctorat. Departament de Bioquimica I Biologia Molecular. Unitat de Ciencies.Universitat Autonoma de Barcelona.

Camats-Malet., Calin G.A., Heesom, K.J., Liu cG., Volinia S., Croce M., Ladomery M., and Bach-Elias M. (2008b). P19 activates telomerase, regulates expression of proteins of the tuberous sclerosis (TSC) pathway and upregulate miRNA's expression. Submitted to Plos One.

Campbel S.L., Khosravi-Far R., Rossman K.L., Clark G.J. and Der C.J. (1998). Increasing complexity of Ras signaling. Oncogene. 17:1395-1413.
Cancer Lett. 2008 Oct 18;270(1):10-8. doi: 10.1016/j.canlet.2008.03.035. Epub 2008 May 23. Review.

Cancer Stats. (2011). Cancer Worldwide. World Health Organization. Cancer Research U.K.

Castaneda CA., Crotes-Funes H., Gomez HL., Ciruelos EM. (2010). The phosphatidyl inositol 3-kinase/AKT signalling pathway in breast cáncer. Cancer Metastasis Review: 29: 751-759

Castellano E, Guerrero C, Núñez A, De Las Rivas J, Santos E.(2009). Serum-dependent transcriptional networks identify distinct functional roles for H-Ras and N-Ras during

initial stages of the cell cycle.Genome Biol. 2009;10(11):R123. doi: 10.1186/gb-2009-10-11-r123. Epub 2009 Nov 6.

Castellano E, **Santos** E.(2011). Functional specificity of ras isoforms: so similar but so different.Genes Cancer. 2011 Mar;2(3):216-31. doi: 10.1177/1947601911408081.

Castro P., Soares P., Gusmao L., Seruca R., Sobrinho-Simoes. (2006). H-RAS 81 polymorphism is significantly associated with aneuploidy in follicular tumors of the thyroid. Oncogene. 25: 4620-4627

Cerne JZ., Stegel V., Gersa K., Navakovic SB (2012). K-Ras rs6176370 is associated with HER-2 overexpressed and poorly-differenciated breast cancer in hormone replacement therapy users: a case control study. BMC. Cancer. 12:105

Chan XH, Nama S, Gopal F, Rizk P, Ramasamy S, Sundaram G, Ow GS, Ivshina AV, Tanavde V, Haybaeck J, Kuznetsov V, Sampath P. (2012). Targeting glioma stem cells by functional inhibition of a prosurvival oncomiR-138 in malignant gliomas. Cell Rep. 2012 Sep 27;2(3):591-602. doi: 10.1016/j.celrep.2012.07.012. Epub 2012 Aug 24. PMID: 22921398

Chen AX, Yu KD, Fan L, Li JY, Yang C, Huang AJ, Shao ZM. (2011a). Germline genetic variants disturbing the Let-7/LIN28 double-negative feedback loop alter breast cancer susceptibility. PLoS Genet. 2011 Sep;7 (9):e1002259. Epub 2011 Sep 1.

Chen C, Wu CQ, Zhang ZQ, Yao DK, Zhu L. (2011b). Loss of expression of **miR-335** is implicated in hepatic stellate cell migration and activation. Exp Cell Res. 2011 Jul 15;317(12):1714-25. Epub 2011 May 7. PMID: 21586285

Chen JJ, Zhou SH. (2011c). Mesenchymal stem cells overexpressing MiR-126 enhance ischemic angiogenesis via the AKT/ERK-related pathway. Cardiol J.; 18(6):675-81.

Chen PS, Su JL, Cha ST, Tarn WY, Wang MY, Hsu HC, Lin MT, Chu CY, Hua KT, Chen CN, Kuo TC, Chang KJ, Hsiao M, Chang YW, Chen JS, Yang PC, Kuo ML. (2011d). miR-107 promotes tumor progression by targeting the let-7 microRNA in mice and humans. J Clin Invest. 2011 Sep;121 (9):3442-55. doi: 10.1172/JCI45390. Epub 2011

Chen X, Yan Q, Li S, Zhou L, Yang H, Yang Y, Liu X, Wan X. (2012a). Expression of the tumor suppressor miR-206 is associated with cellular proliferative inhibition and impairs invasion in ERα-positive endometrioid adenocarcinoma. Cancer Lett.314(1):41-53. PMID: 21983130

Chen YL, Yuan RH, Yang WC, Hsu HC, Jeng YM. (2012b). The stem cell E3-ligase Lin-41 promotes liver cancer progression through inhibition of microRNA-mediated gene silencing. J Pathol. 2012 Oct 24. doi: 10.1002/path.4130. [Epub ahead of print]

Chin LJ., Ratner E., Leng S., Zhai R., Nallur S., Babar I., Muller RU., Straka E., Su L., Burki EA, et al. (2008). A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. Cancer Res. 68:8535-8540

Cho William CS. (2007). OncomiRs: the discovery and progress of miRNAs in cancers. Molecular Cancer. 6:60

Christensen BC, Moyer BJ, Avissar M, Ouellet LG, Plaza SL, McClean MD, Marsit CJ, Kelsey KT. (2009). A let-7 microRNA-binding site polymorphism in the KRAS 3' UTR is associated with reduced survival in oral cancers. Carcinogenesis. 2009 Jun;30(6):1003-7. Epub 2009 Apr 20.

Cittelly DM, Das PM, Spoelstra NS, Edgerton SM, Richer JK, Thor AD, Jones FE.(2010). Downregulation of **miR-342** is associated with tamoxifen resistant breast tumors. Mol Cancer. 2010 Dec 20;9:317. PMID: 21172025

Cobb M.H., and Schaefer E.M. (1996). MAP Kinase signalling pathways, Promega Notes Magazine. 59: 37

Cohen J.B., Broz S.D., and Levinson A.D. (1989). Expression of the H-Ras proto-oncogene is controlled by alternative splicing. Cell. 58: 461-472

Colicelli J. (2004). Human Ras superfamily proteins and related GTPases. Sci.Signal 250: re13

Colotta F., Allavena P., Sia A., Cecilia Garlanda and Montovani A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30(7):1073-1081.

Conery AR., Sever S., Harlow E. (2010). Nucleoside diphosphate kinase NM23H1 regulates chromosomal stability by activating the GTPase dynamin during cytokinesis. PNAS. 107(35):15461-15466

Costello, J.M. (1977). A new syndrome: mental subnormality and nasal papillomata. Aust Paediat J. 13: 114-118.

Croce C.M. (2008). Oncogenes and cancer. Molecular origins of cancer. The new england journal of medicine. 358 (5):502-511.

Cullen P. J., and Lockyer P.J. (2006) Integration of calcium and Ras signalling. Nat. Rev. 3: 339-344.

Dacic S, Kelly L, Shuai Y, Nikiforova MN. (2010). miRNA expression profiling of lung adenocarcinomas: correlation with mutational status. Mod Pathol. 2010. (12):1577-82. Epub 2010 Sep 3.

Dang CB. (2011). There is no ROS for the weary. Cancer Discov. 2(4): 304-7

Daniluk J., Liu Y., Deng D., Chu J., Huang H., Gaiser S., Cruz-Monserrate Z., Wan H., Ji B., and Logsdon C.D. (2012). An NF-κB pathway –mediated positive feedback loop amplifies Ras activity to pathological levels in mice. The Journal of Clinical Investigation. 122 (4): 1519-1528

De Marchis ML, Ballarina M., Salvatori B., Puzzolo MC., Bozzoni L., Fatica AS. (2009). A new molecular network comprising PU 1 interferon regulatory factor proteines and miR-342 estimulates ATRA-mediated granulocytic differentiation of acute promelocytic leukemia cells. Leukemia 23 (5):856-62

De Vito C, Riggi N, Suvà ML, Janiszewska M, Horlbeck J, Baumer K, Provero P, Stamenkovic I.(2011).Let-7a is a direct EWS-FLI-1 target implicated in Ewing's sarcoma development.PLoS One. 2011;6(8):e23592. doi: 10.1371/journal.pone.0023592. Epub 2011 Aug 10.

DeFeo, D., M.A. Gonda, H.A. Young, E.H. Chang, D.r. Lowy, E. M. Scolnick, and Ellis R.W. (1981). Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci.USA 78:3328-332.

Denayer E., Parret A., Chmara M., Schubbert S., Vogels A., Devriendt K., Frijns JP., Rybin V., de Ravel T.J., Shannon K., Cools J., Scheffzek K., and Legius E. (2008) Mutation analysis in Costello syndrome: functional and structural characterization of the H-Ras p. Lys 117 Arg mutation. Human mutation 29 (2): 232-239.

Dessai B., Ma T., and Chellaiah M.A. (2008). Invadiopodia and matrix degradation, a new property of prostate cancer cells during migration and invasion. The Journal of Biological Chemistry. (283) 20: 13856-13866.

Dey BK, Gagan J, Dutta A. (2011). MiR-206 and -486 induce myoblast differentiation by downregulating Pax7. Mol Cell Biol. 2011. (1):203-14. Epub 2010 Nov 1. Erratum in: Mol Cell Biol. 2011 Mar;31(6):1329. PMID: 21041476

Di Leva G, Gasparini P, Piovan C, Ngankeu A, Garofalo M, Taccioli C, Iorio MV, Li M, Volinia S, Alder H, Nakamura T, Nuovo G, Liu Y, Nephew KP, Croce CM. (2010). (2010). MicroRNA cluster 221-222 and estrogen receptor alpha interactions in breast cancer. J Natl Cancer Inst. 2010 May 19;102(10):706-21. Epub 2010 Apr 13. PMID: 20388878

Díaz-Prado S.M., y Anton-Aparicio L.M. (2010) El papel de los microRNAs en el cáncer: desarrollo y potencialidad terapeútica. Capitulo I. Editorial Médica Panamericana. ISBN: 978-84-9835-315-0

DiFiore R., Fanale D., Drago-Ferrante R., Chiaradonna F., Giuliano M., De Blasio A., Amodeo V., Corsini LR., Bazan V., Tesoriere G., Vento R., Russo A. (2012). Genetic and molecular characterizacion of the human osteosarcoma 3AB-OS cancer stem cell line:

A possible moel for studying osteosarcoma orign and stemness. J. Cell Physiol. (Epub ahead of print) doi: 10.1002/jcp.24272

Dituri F., Mazzocca A., Giannelli G., and Antonaci S. (2011). PI3K functions in cancer progression, anticancer immunity and immune evasion of tumours. Clinical and Developmental Inmmunology. ID947858. Doi: 10.1155/2011/947858

Dohi O., Yasui K., Gen Y., Takada H., Endo M., Tsuji K., Konishi C., Yamada N., Mitsuyoshi H., Yaqi N., Naito Y., Tanaka S., Arii ., Yoshikawa T. (2012). Epigenetic silencing of miR-335 and its host gene MEST in hepatocellular carcinoma. Int. J. Oncol. (Epub ahead of print).

Domingo-Villanueva E. (2006). Alteracions via Ras-Far en Cáncer gastrointestinal amb defectes de la reparació genomica. Tesi doctoral. Departament de Genetica de la Facultat de Biología. Universitat de Barcelona.

Dong Q, Meng P, Wang T, Qin W, Qin W, Wang F, Yuan J, Chen Z, Yang A, Wang H.(2010). MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One. 2010 Apr 14;5(4):e10147.

Dong SW., Wang L., Sui J., Deng XY., Chen XD., Zhang ZW., Liu X., Liu ZM., Zhang JH., Yang QS., Jia YF., Song X. (2011). Expression patterns of ER, HER2 and NM23-H1 in breast cancer patients with differential menopausal status: correlations with metastasis. Mol. Diagn. Ther. 15 (4):211-9

Dongsheng Yang, Xiang Da (Eric) Dong, Xiaoyan Chen, Lihua Wang, Chunjing Lu, Wang Jiao, Jia Qu and Lili Tu. (2009). MicroRNA-1/206 targets C-Met and inhibits rhgabdomyosarcoma development. JBC. http://www. jbc.org/cgi/doi/10.1074/jbc.M109.020511.

Donnem T, Lonvik K, Eklo K, Berg T, Sorbye SW, Al-Shibli K, Al-Saad S, Andersen S, Stenvold H, Bremnes RM, Busund LT .(2011). Independent and tissue-specific prognostic impact of miR-126 in nonsmall cell lung cancer: coexpression with vascular endothelial growth factor-A predicts poor survival. Cancer. 117(14):3193-200. doi: 10.1002/cncr.25907.

Donnem T., Fenton CG., Lonvik K., Berg T., Eklo K., Andersen S., Slenvold H., Al-Xhibli K., Al-Saads, Bremnes RM., Busund LT. (2012). microRNA signatures in tumor tissue related to angiogenesis in non-small cell lung cancer. Plos One 7(1): e29671

Donnovan S., Shannon K. M., Bollag, G. (2002). GTPase activating proteins: critical regulators of intracellular signaling. Bioch. Biophy. Acta: 1602 (1):23-45

Downward, J. (2002). Targeting Ras signalling pathways in cancer therapy. Nat. Rev. Cancer 3: 11-22

Drosten M, Dhawahir A, Sum EY, Urosevic J, Lechuga CG, Esteban LM, Castellano E, Guerra C,Santos E, Barbacid M.(2010). Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival.EMBO J. 2010 Mar 17;29(6):1091-104. doi: 10.1038/emboj.2010.7. Epub 2010 Feb 11.

Ellis, R W., D. DeFeo, T, T.Y. Shih, M.A. Gonad, H. A. Young, N. Tsuchida, D.R. Lowy, and E. Scolnick. (1981). The p21 scr genes of Harvey and Kirsten sarcoa viruses originate from divergent members of a family of normal vertebrate genes. Nat. (London) 292:506-511.

Endo Y., Toyama T., Takahashi S., Yoshimoto N., Iwasa M., Asano T., Fujii y., Yamashita H. (2012). MiR-1290 and its potential targets are associated with characteristics of estrogen receptor α-positive breast cancer. Endocr. Relat. Cancer. (Epub ahead of print).

Erson AE., and Petty EM. (2008). MicroRNAs in development and disease. Clinical Genetics. 74: 296-306

Esquela-Kercher A., and Slack FJ. (2006). OncomiRs-microRNAs with a role in cancer. 2006. Nature reviews. 6: 259-269.

Fayyad-Kazan H, Bitar N, Najar M, Lewalle P, Fayyad-Kazan M, Badran R, Hamade E, Daher A, Hussein N, ElDirani R, Berri F, Vanhamme L, Burny A, Martiat P, Rouas R, Badran B. (2013). Circulating miR-150 and miR-342 in plasma are novel potential biomarkers for acute myeloid leukemia. J Transl Med. 2013 Feb 7;11:31. doi: 10.1186/1479-5876-11-31.

Felli N, Felicetti F, Lustri AM, Errico MC, Bottero L, Cannistraci A, De Feo A, Petrini M, Pedini F, Biffoni M, Alvino E, Negrini M, Ferracin M, Mattia G, Carè A.(2013). MiR-126 & miR-126* restored expressions play a tumor suppressor role by directly regulating ADAM9 and MMP7 in melanoma. PLoS One.8 (2):e56824. doi: 10.1371/journal.pone.0056824. Epub 2013 Feb 21.

Fenández-Medarde A., and Santos E. (2011). Ras in cancer and developmental diseases. Genes and Cancer. 2 (3):344-358

Feng R, Chen X, Yu Y, Su L, Yu B, Li J, Cai Q, Yan M, Liu B, Zhu Z. (2010). MiR-126 functions as a tumour suppressor in human gastric cancer. Cancer Lett. 2010 Dec 1;298(1):50-63. Epub 2010 Jul 8.

Feng X, Wang H, Ye S, Guan J, Tan W, Cheng S, Wei G, Wu W, Wu F, Zhou Y. (2012). Up-Regulation of microRNA-126 May Contribute to Pathogenesis of Ulcerative Colitis via Regulating NF-kappaB Inhibitor IκBα. PLoS One. 2012;7(12):e52782. doi: 10.1371/journal.pone.0052782. Epub 2012 Dec 28. PMID: 23285182

Finoux-AL., and Cjartrand P (2008). Micro-ARN: Oncogenes et suppresseurs de tumerurs. Medicine Scuence. 24 : 1049-1054

Frampton AE., Krell J., Jacbo J., Stebbing J., Castellano L., Jia LR. (2012) Loss of miR-126 is crucial to pancreatic cancer progression. Expert. Rev. Anticancer. Thr. 12 (7):881-4

Franken AP, Rodermond HM., Stap J., Haveman J. And Van Bree C. (2006).Clonogenic assay of cells in vitro. Nature Protocols vol (1) No. (5).:2315-2319.

Gagan J., Dey BK., Layer R., Yan Z., Dutta A. (2012). Notch 3 and Mef2c proteins are mutually antagonist via Mkp1 protein and miR-1/206 microRNAs in differentiating myoblast. J. Biol. Chem. 287; (48):40360-70

Gao L., Zhu W., Li L., Hou M., Ma L., Zhao, Zhou Q. (2010a). Comparative proteomics study on human high metastatic large cell lung cancer cell lines before and after transfecting with nm23-H1 gene. Chin J Lung Cancer. 13 (10):928-932

Gao W., Shen H., Liu L., Xu J., Xu J., Shu Y. (2010b). MiR-21 overexpression in human primary squamous cell lung carcinoma is associated with poor patient prognosis. J. Cancer Res. Clin. Oncol. 137 (4):557-66

García Cruz R., and Bach-Elias M. (2013). El efecto antiproliferativo y quiescente de la proteína p19 c-H-Ras- Trabajo libre oral presentado en la 13ª Reunión de Ciencias Médicas. Universidad de Guanajuato, México. Vol. 1: 22-224. ISBN: 978-607-441-233-8

García-Cruz R., Camats-Malet M., Calin GA., Chang-Gong L., Volinia S., Taccionli C., Croce CM and Bach-Elias M. (2012a) The role of p19 and p21 c-H-Ras proteins and mutants in miRNAs expression in cancer an a Costello Syndrome cell model. Article submitted to PLos One.

Giorgi C., Agnoletto C., Baldini C., Bononi A., Bonora M., Marchi S., Missiroli S., Patergnani S., Poletti F., Rimessi A, Zavan B., and Pinton P. (2010). Redox control of protein kinase C: cell-and disease-specific aspects. Antioxidants and Redox signaling. Vol. 13 (7):1051-1085

Girgis AH., Iakovlev VV., Beheshti B., Bayani J., Squirre JA., Bui A., Mankaruos M., Youssef Y., Khalil B., Khella HW., Pasic M., Yousef GM. (2012). A multilevel whole genome analysis reveals candidate biomarkers in clear cell renal cell carcinoma. Cancer Res. 72(20):5273-84

Goljanek-Whysall K., Pais H., Rathjen T., Sweeden D., Dalmary T., Munsterberg A. (2012). Regulation of multiple target genes by miR-1 and miR-206 is pivotal for C2C12 myoblasts differentiation. J. Cell. Sci. 125 (Pt15):3590-3600

Golubovskaya VM, Sumbler B, Ho B, Yemma M, Cance WG.(2013). MiR-138 and MiR-135 Directly Target Focal Adhesion Kinase, Inhibit Cell Invasion, and Increase Sensitivity to Chemotherapy in Cancer Cells.Anticancer Agents Med Chem. 2013 Feb 15. [Epub ahead of print]

Gong H, Song L, Lin C, Liu A, Lin X, Wu J, Li M, Li J. (2013). Downregulation of miR-138 Sustains NF-κB Activation and Promotes Lipid Rafts Formation in Esophageal Squamous Cell Carcinoma. Clin Cancer Res. 2013 Jan 14. [Epub ahead of print] PMID: 23319823 [PubMed - as supplied by publisher]

Gong Y., Reniquinta V., Himmerkus N., Zhang J., Reninguta A., Bleich M., Hou J. (2012). Claudin-14 regulates renal Ca++ transport in response to CaSR signaling via a novel microRNA pathway. EMBO. 31(8): 1999-2012

Grady WM., Parkin RK., Mitchell PS., Lee JM., Kim yh., Tsuchiya KD., Washington MK., Praskeva C., Willson Jk., Kaz AM., Kroh EM., Allen A., Fritz BR., Markowitz SD., Tewari M. (2008). Epigenetic silencing of the intronic microRNA hsa miR-342. Oncogene.27(27): 3880-8

Grechukhina O, Petracco R, Popkhadze S, Massasa E, Paranjape T, Chan E, Flores I, Weidhaas JB, Taylor HS. (2012). A polymorphism in a let-7 microRNA binding site of KRAS in women with endometriosis. EMBO Mol Med. 2012 Feb 3. doi: 10.1002/emmm.201100200. [Epub ahead of print]

Gremer L, De Luca A, Merbitz-Zahradnik T, Dallapiccola B, Morlot S, Tartaglia M, Kutsche K, Ahmadian MR, Rosenberger G.(2010). Duplication of Glu37 in the switch I region of HRAS impairs effector/GAP binding and underlies Costello syndrome by promoting enhanced growth factor-dependent MAPK and AKT activation. Hum Mol Genet. Vol. 19(5):790-802. doi: 10.1093/hmg/ddp548. Epub 2009 Dec 8.

Grewal T., Koese M., Tebar F., and Enrich C. (2011). Differential regulation of RasGAPs in cancer. Genes and Cancer 2(3): 288-297

Griffiths-Jones S., Grocock R., van Dongen S., Batex A., Enright A. (2006). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research. 34:140-144.

Gripp K.W., Innes A.M., Axelrad M.E., Gillan T.L., Parboosingh J.S., Davies C., Leonard N.J., Doyle D., Catalano S., Nicholson l., Stabley D., and Sol-Church K. (2008). Costello syndrome associated with novel germline H-Ras mutations: An attenuated phenotype? American Journal of Medical Genetics Part A. 146 A:683-690

Gripp K.W., Scott C.I., Nicholson L., McDonald-McGimm D.M., Ozeran J.D., Jones M.C., Lin A.E., and Zackai E.H. (2001). Five additional Costello syndrome patients with rhabdomyosarcoma: proposal for a tumour screening protocol. American Journal of Medical Genetics Part A. 108: 80-87

Gripp KW, Lin A.E, Stabley D., Nicholson L., Scott Jr. C.I., Doyle D., Aoki Y., Matsubara Y., Zachai E.H., Lapunzina P., Gonzalez-Meneses, A., Holbrook J., Agresta C.A., Gonzalesz I.L and Sol-Church K. (2006). HRAS mutation analysis in Costello Syndrome: Genotype and Phenotype correlation. Am J. Med. Genet.A 140 (1):1-7

Guang P., Yin Z., Li X., Wu W., Zhou B. (2012). Metanalysis of human lung cancer microRNA expression profiling studies comparing cancer tissues with normal tissues. J. Exp. Clin. Cancer. Res. 31(1):54

Guil S, de La Iglesia N, Fernández-Larrea J, Cifuentes D, Ferrer JC, Guinovart JJ, Bach-Elias M. (2003a). Alternative splicing of the human proto-oncogene c-H-ras renders a new Ras family protein that trafficks to cytoplasm and nucleus.Cancer Res. (2003 a) 1;63(17):5178-87.

Guil S, Gattoni R, Carrascal M, Abián J, Stévenin J, Bach-Elias M.(2003b). Roles of hnRNP A1, SR proteins, and p68 helicase in c-H-ras alternative splicing regulation. Mol Cell Biol. 23(8): 2927-41.

Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. (2008). The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer. 2008. 47(11):939-46.

Guo L, Chen C, Shi M, Wang F, Chen X, Diao D, Hu M, Yu M, Qian L, Guo N. (2013). Stat3-coordinated Lin-28-let-7-HMGA2 and miR-200-ZEB1 circuits initiate and maintain oncostatin M-driven epithelial-mesenchymal transition.

Guo R., Wu Q., Liu F., Wang Y. (2011). Description of the CD133+ subpopulation of the human ovarian cancer cell line OVCAR3. Oncol. Rep. 25(1):141-6

Haeng Ran P., Se-Heonk, Sei Young L., Ji Min S., Ae Ran P., Suki Kang BS, Baek Gil k., Yoon Pyo C., Yong-Baek K., and Nam Hoon Cho. (2011). Nuclear localization of NM23H1 in head and neck squamous cell carcinoma is associated with radiation resistance. Cancer: 1864-73.

Hafez MM., Hassan ZK., Zekri AR., Gaber AA., Al Rejaie SS., Sayed-Ahmed MM., Al Shabanah O. (2012). MicroRNAs and metastasis-related gene expression in Egyptian breast cancer patients. Asian Pac. J. Cancer Prev. 13 (2):591-8

Haigis MC., Deng CX., Finley LW, Kim HS, Gius D. (2012). SIRT3 is a mitochondrial tumor suppressor: a scientific tale tha connects aberrant ROS, the Warburg effect, and carcinogenesis. Cancer Res. 72 (10):2468-72

Hamada S, Satoh K, Fujibuchi W, Hirota M, Kanno A, Unno J, Masamune A, Kikuta K, Kume K, Shimosegawa T. (2012). MiR-126 acts as a tumor suppressor in pancreatic cancer cells via the regulation of ADAM9. Mol Cancer Res. 2012 Jan;10(1):3-10. doi: 10.1158/1541-7786.MCR-11-0272. Epub 2011 Nov 7. PMID: 22064652 [PubMed - indexed for MEDLINE] Free Article

Hamano R, Miyata H, Yamasaki M, Sugimura K, Tanaka K, Kurokawa Y, Nakajima K, Takiguchi S, Fujiwara Y, Mori M, Doki Y. (2012). High expression of Lin28 is

associated with tumour aggressiveness and poor prognosis of patients in oesophagus cancer. Br J Cancer. 2012 Apr 10;106(8):1415-23. doi: 10.1038/bjc.2012.90. Epub 2012.

Han ZB., Zhon L., Teng MJ., Fan JW., Tang HM., Wu JY., Chen HY., Wang ZW., Qui GQ., Peng ZH (2012). Identification of recurrence-related microRNAs in hepatocellular carcinoma following liver transplantation. Mol. Oncol. Epub ahead of print.

Hanagan D., and Weinberg RA (2011). Hallmarks of cancer: the next generation. Cell (144): 646-674

Hanagan D., and Weinberg RA (2000). The hallmarks of cancer. Cell (100): 57-70

Hancock JF. (2003). Ras proteins: Different signals from different locations. Nature Rev. Mol. Cell Biol. 4: 373-385.

Harms KL, Chen X.(2006). p19ras brings a new twist to the regulation of p73 by Mdm2.

Hartomo TB, Kozaki A, Hasegawa D, Van Huyen Pham T, Yamamoto N, Saitoh A, Ishida T, Kawasaki K, Kosaka Y, Ohashi H, Yamamoto T, Morikawa S, Hirase S, Kubokawa I, Mori T, Yanai T, Hayakawa A, Takeshima Y, Iijima K, Matsuo M, Nishio H, Nishimura N. (2013). Minimal residual disease monitoring in neuroblastoma patients based on the expression of a set of real-time RT-PCR markers in tumor-initiating cells. Oncol Rep.; 29(4):1629-36. doi: 10.3892/or.2013.2286. Epub 2013 Feb 12.

Hau A., Ceppi P., Peter ME. (2012) CD95 is part of a let-7/p53/miR-34 regulatory network. Plos One. 7 (11):e49636.

He YJ, Wu JZ, Ji MH, Ma T, Qiao EQ, Ma R, Tang JH.(2013). **miR-342** is associated with estrogen receptor-α expression and response to tamoxifen in breast cancer. Exp Ther Med. 2013 Mar;5(3):813-818. Epub 2013 Jan 22.

He X, Duan C, Chen J, Ou-Yang X, Zhang Z, Li C, Peng H. (2009). Let-7a elevates p21(WAF1) levels by targeting of NIRF and suppresses the growth of A549 lung cancer cells. FEBS Lett. **2009** Nov 3;583(21):3501-7. doi: 10.1016/j.febslet.**2009**.10.007. Epub **2009** Oct 8.

He XY, Chen JX, Zhang Z, Li CL, Peng QL, Peng HM. (2010). The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice.J Cancer Res Clin Oncol. 136 (7):1023-8. doi: 10.1007/s00432-009-0747-5. Epub 2009 Dec 22.

Helland A., Anglesio MS., George J., Cowin PA., Johnstone CN., House CM., Sheppard KE., Etemadmoghadam D., Melnyk N., Rustgi AK., Phillips WA., Johnsen H., Holm R., Kristensen GB., Birrer MJ., Australian OVrian Cancer Study Gropu, Pearson RB., Borresen Dale AL., Huntsman DG., DeFazio A., Creighton CJ., Smyth GK., Bowtell DD. (2011).Deregulation of MYCN, LIN28B and Let-7 in a molecular subtype of aggressive high-grade serous ovarian cancers. PLos One 6(4): e18064

Hernández-Alcoceba R., del Peso L., and Lacal J.C. (2000). The ras family of GTPases in cancer cell invasión. Cell. Mol. Life Sci. 55:65-76

Hernández-Aya LF., and González-Angulo. (2011). Targeting the phosphatidylinositol 3- kinase signaling pathway in breast cáncer. Oncologist 16 (4): 404-14

Hernández-Menéndez M., and Ríos-Hernández M. A (1999). Oncogenes y Cáncer. Revista Cubana de Oncología. 15(2):131-9.

Heyn H, Engelmann M, Schreek S, Ahrens P, Lehmann U, Kreipe H, Schlegelberger B, Beger C. (2011). MicroRNA **miR-335** is crucial for the BRCA1 regulatory cascade in breast cancer development. Int J Cancer. 2011 Dec 15;129(12):2797-806. doi: 10.1002/ijc.25962. Epub 2011 May 25. PMID: 21618216

Hodzic J., Giovannetti E., Diosdado B., Adema AD., Peters GJ. (2011). Regulation of deoxyytidine kinase expression and sensitivity to gemcitabine by microRNA-330 and promoter methylation in cancer cells. Nucleosides Nucleotides Nucleic Acids. 30 (12):1214-22

Hole PS, Pearn L, Tonks AJ, James PE, Burnett AK, Darley RL, Tonks A.(2011). **Ras-induced** reactive oxygen species promote growth factor-independent proliferation in human CD34+ hematopoietic progenitor cells.Blood. 2010 Feb 11;115(6):1238-46. doi: 10.1182/blood-2009-06-222869. Epub 2009 Dec 10.

Holsenber S.L. (2006). H-Ras and its oncogenic mutants Ras G12V and Ras Q6L. Ms.C Thesis. North Caroline State University.

Horak E. C., Heun Lee J., Marshall J.C, Shreeve S., and Steeg S. P. (2008). The role of metastasis suppressor genes in metastatic dormancy. Acta Pathologica, Microbiologica. et Immunologica Scandinavica (116):586-601

Hsieh HL., Wang HH., Wu WB., Chu PJ., Yang CM (2010). Transforming growth factor-β1 induces matrix metalloproteinase-9 and cell migration in astrocytes: roles of ROS-dependent ERK and JNK-FN-κB pathways. J. Neuroinflammation. 7:88

Hu H., Zhang Y., Cai XH., Huang JF., Cai L. (2012). Changes in microRNA expression in the MG-63 osteosarcoma cell line compared with osteoblasts. Oncol. Lett. 4(5): 1037-1042

Hu X, Guo J, Zheng L, Li C, Zheng TM, Tanyi JL, Liang S, Benedetto C, Mitidieri M, Katsaros D, Zhao X, Zhang Y, Huang Q, Zhang L.(2013). The heterochronic microRNA **let-7** inhibits cell motility by regulating the genes in the actin cytoskeleton pathway in breast cancer.Mol Cancer Res. 2013 Mar;11(3):240-50. doi: 10.1158/1541-7786.MCR-12-0432. Epub 2013 Jan 21.

Huang F, Zhu X, Hu XQ, Fang ZF, Tang L, Lu XL, Zhou SH.(2012a). Mesenchymal stem cells modified with miR-126 release angiogenic factors and activate Notch ligand

Delta-like-4, enhancing ischemic angiogenesis and cell survival. Int J Mol Med. doi: 10.3892/ijmm.2012.1200. [Epub ahead of print]. PMID: 23229021

Huang F., Hu LM., Liu JB., Zhang YX., Hu ZB. (2011). Relationship between genetic polymorphism of promoter region let-7 and genetic susceptibility to hepatocellullar carcinoma. Zhonghua Yu Fang Yi Xue Za Zhi 45(12):1093-8

Huang HY, Cheng YY, Liao WC, Tien YW, Yang CH, Hsu SM, Huang PH. (2012b). SOX4 Transcriptionally Regulates Multiple SEMA3/Plexin Family Members and Promotes Tumor Growth in Pancreatic Cancer. PLoS One. 2012;7(12):e48637. doi: 10.1371/journal.pone.0048637. Epub 2012 Dec 12. PMID: 23251334 [PubMed - in process] Free PMC Article

Iborra S., Soto M., Stark-Aroeira, Castellano E., Alarcón B., Alonso C., Santos E., and Fernandez-Malave E. (2012). H-ras and N-ras are dispensable for T-cell development and activation but critical for protective Th1 immunity. Blood. 117 (19):5102-5111.

Ikeda K., Ogawa K., Takeishi Y. (2012). The role of hmga2 in the proliferation and expansion of a hematopoietic cell in myeloproliferative neoplasms. Fukushima J. Med. Sci. 58 (2): 91-100

INEGI. Instituto Nacional de Estadística y Geografía (2011). "Estadísticas a propósito del día mundial contra el cáncer" Revisado el 4 de febrero del 2012. México. Documento en línea:
http://www.inegi.org.mx/inegi/contenidos/espanol/prensa/contenidos/estadisticas/2011/cancer11.asp?s=inegi&c=2780&ep=50

Inoguchi T, Sonta T, Tsubouchi H, Etoh T, Kakimoto M, Sonoda N, Sato N, Sekiguchi N, Kobayashi K, Sumimoto H, Utsumi H, Nawata H.(2003). Protein kinase C-dependent increase in reactive oxygen species (ROS) production invascular tissues of diabetes: role of vascular NAD(P)H oxidase. J Am Soc Nephrol. 2003 Aug;14(8 Suppl 3):S227-32. Review.

Iorio M., and Croce CM. (2012). MicroRNAs involvement in human cancers. Carcinogenesis. 33 (6): 1126-33

Ishihara K., Sasaki D., Tsuruda K., Inokuchi N., Nagai K., Hasegawa H., Yanagihara K., Kamihira S. (2012). Impact of miR-155 and miR-126 as novel biomarkers on the assessment of disease progression and prognosis in adult T-cell leukemia. Cancer Epidemiol. (Epub ahead of print)

Jakymiw A, Patel RS, Deming N, Bhattacharyya I, Shah P, Lamont RJ, Stewart CM, Cohen DM, Chan EK. (2010). Overexpression of dicer as a result of reduced let-7 MicroRNA levels contributes to increased cell proliferation of oral cancer cells.Genes Chromosomes Cancer. 49(6):549-59. doi: 10.1002/gcc.20765.

Jalali S., Ramanathan GK., Parthasarathy PT., Aljubran S., Galam L., Yunus A., Garcia S., Cox RR, Jr., Lockey RF., Kalliputi N. (2012). Mir-206 regulates pulmonary artery smooth muscle cell proliferation and differentiation. Plos One. 7(10): E46808.

Janardhan SV, Praveen K., Marks R., Gajewski TF (2011). Evidence implicating the Ras pathway in multiple CD28 costimulatory functions in CD4+ T cells. Plos One 6(9): e24931. Doi: 10.1371/journal.pone.0024931

Jang SM, Kim JW, Kim CH, Kim D, Rhee S, Choi KH. (2010) p19(ras) Represses proliferation of non-small cell lung cancer possibly through interaction with Neuron-Specific Enolase (NSE). 289(1):91-8. doi: 10.1016/j.canlet.2009.08.005. Epub 2009 Aug 26.

Jarret S.G., Novak M., Dabernat S., Daniel J.Y., Mellon I., Zhang Q., Harris N., Ciesielski M.J.., Fenstermaker R.A., Kovacic D., Slominski A., Kaetzel D.M. (2012a). Metastasis suppressor NM23H1 promotes repair of UV-induced DNA damage and suppresses UV-induced melanomagenesis. Cancer Res. 72 (1)133-143.

Jarret S.G., Novak M., Harris N., Merlino G., Slominski A., KaetzeL D.M. (2012b). NM23 deficiency promotes metastasis in a UV radiation-induced mouse model of human melanoma. Clin. Exp. Metastasis. Epub ahead of print.

Jemal A., Siegel R., Xu J., Wand E. (2010). Cancer statistics. CA Cancer Journal for Clinicians. (Vol. 60):277-300.

Jeong MH., Bae J., Kim WH., Yoo SM., Kim JW., Son PI, Choi KH. (2006). P19 ras interacts with and activates p73 by involving the MDM2 protein. The Journal of Biological Chemistry. 281(13):8707-8715

Jeyapalan Z, Deng Z, Shatseva T, Fang L, He C, Yang BB. (2011). Expression of CD44 3'-untranslated region regulates endogenous microRNA functions in tumorigenesis and angiogenesis. Nucleic Acids Res. 2011 Apr;39(8):3026-41. Epub 2010 Dec 10. PMID: 21149267

Ji J., and Wang XW. (2010). A Ying-Yang balancing act of the lin 28/ let-7 link in tumorigenesis. J. Hepatol. 53(5): 974-5

Jiang J., Sun X., Wang W., Jin X., Bo X., Li Z., Bian A., Jiu J., Wang X., Liu D., Hui X., Wang Y., Wang A., Ding L. (2012a). Tumor microRNA-335 expression is associated with poor prognosis in human glioma. Med. Oncol. 29 (5):3472-77

Jiang L, Dai Y, Liu X, Wang C, Wang A, Chen Z, Heidbreder CE, Kolokythas A, Zhou X. (2011). Identification and experimental validation of G protein alpha inhibiting activity polypeptide 2 (GNAI2) as a microRNA-138 target in tongue squamous cell carcinoma. Hum Genet. 129(2):189-97. Epub 2010 Nov 16. PMID: 21079996

Jiang L, Liu X, Kolokythas A, Yu J, Wang A, Heidbreder CE, Shi F, Zhou X. (2010). Downregulation of the Rho GTPase signaling pathway is involved in the microRNA-138-mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. Int J Cancer. 1; 127(3):505-12. PMID: 20232393

Jiang Y., Qin Z., Hu Z., Guan X., Wang Y., He Y., Xue J., Liu X., Chen J., Dai J., Jin G., Ma H., Wang S., Shen H. (2012b). Genetic variation in a hsa-let-7 binding site in RAD52 is associated with breast cancer susceptibility. Carcinogenesis. (Epub ahead of print).

Jiao L.R., Frampton A.E., Jacob J., Pellegrino L., Krell J., Giamas G., Tsim N., Vlavianos P., Cohen P., Ahmad R., Keller A., Habib NA., Stebbing J., Castellano L. (2012). MicroRNAS targeting oncogenes are down-regulated in pancreatic malignant transformation from benign tumors. Plos One. 7(2):e32068.

Jin Y., Wang C., Liu X., Mu W., Chen Z., Yu D., Wang A., Dai Y, Zhou X. (2011a). Molecular characterization of the microRNA-138 –Fos-like antigen 1 (FOS L1) regulatory module in squamous cell carcinoma. J. Biol.Chem. 286 (46): 40104-9

Jin H, Lv S, Yang J, Wang X, Hu H, Su C, Zhou C, Li J, Huang Y, Li L, Liu X, Wu M, Qian Q. (2011b). Use of microRNA Let-7 to control the replication specificity of oncolytic adenovirus in hepatocellular carcinoma cells. PLoS One. 2011;6(7):e21307.

Jin L., Liu G., Zhan C., Lu C., Xiong S., Fen-Ge, He Q., Kobayashi N., and Wang Y. (2009). NM23-H1 regulates the proliferation and differentiation of the myeloid leukaemia K562 cell line: a functional proteomics study. Life Sciences 84: 458-467.

John-Aryankalayil M, Palayoor ST, Makinde AY, Cerna D, Simone CB 2nd, Falduto MT, Magnuson SR, Coleman CN. (2012). Fractionated radiation alters oncomir and tumor suppressor miRNAs in human prostate cancer cells.Radiat Res. 2012 Sep;178(3):105-17.

Johnson G. L., and Razvan Lapadat. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 298: 1911-1912

Johnson RF, Perkins ND (2012). Nuclear factor κb-P53 and mitochondria: regulation of cellular metabolism and the Warburg effect: Trends. Biochem. Sci (Epub ahead to print).

Johnson SM., Helge Grosshans, Shingara J., Byrom M., Jarvis R., Cheng A., Labourier E., Reinert K.L., Brown D. y Slack F.J. (2005). Ras is regulated by the let-7 microRNA Family. Cell. 120: 635-647.

Jusufovic E., Rijavec M., Keser D., Korosec P., Sodja E., Iljazovic E., Radojevic Z., Kosnik M. (2012). Let-7b and miR-126 are down-regulated in tumor tissue and correlate with microvessel density and survival outcomes in non-small-cell-lung cancer. Plos One. 7(9): e45577.

Kai Z.S., Finnegan EF., Huang S., Pasquinelli AE. (2013). Multiplw cis-element and tras-acting factors regulate dynamia spatio-temporal transcription of let-7 in Caernohabditis elegan. Dev. Biol. 374 (1): 223-33

Kang XH, Xu ZY, Gong YB, Wang LF, Wang ZQ, Xu L, Cao F, Liao MJ. (2013). Bufalin Reverses HGF-Induced Resistance to EGFR-TKIs in EGFR Mutant Lung Cancer Cells via Blockage of Met/PI3k/Akt Pathway and Induction of Apoptosis. Evid Based Complement Alternat Med. 2013;2013:243859. doi: 10.1155/2013/243859. Epub 2013 Feb 28.

Karar J., and Maity A. (2011). PI3K/AKT/MTOR pathway in angiogenesis. Frontiers in Molecular Neuroscience. Vol. 4. Article 51; 1-8

Kassi E., and Moutsatsou P. (2010). Estrogen receptor signaling and its relationship to cytokines in Systemic Lupus Erithematosus. Journal of Biomedicine Biotechnology. ID: 317452.

Kent O.Z., and Mendell J.T .(2006). A small piece in the cancer puzzle: microRNAs as tumor supressors and oncogenes. Oncogene 25:6188-6196

Kerr B. Allanson J., Delrue M.A., Gripp K.W. Lacombe D., Lin A.E., and Raven K.A. (2008). The diagnosis of Costello syndrome nomenclature in RAS/MAPK pathway disorders. American Journal of Medical Genetics. Part A. 146A: 1218-1220.

Kerr B., Delrue M.A., Sigaudy S., Perueen R., Marche M., Burgelin I., Stef M., Tang B., Eden O.B., Sullivan J.O., de Sandre-Giovannoli A., Reardon W., Brewer C., Bennet O.Q., Cann W.M., Donnai D., Stewart F., Hennekam R., Cave H., Verloes A., Philip N., Lacombe D., Levy N., Arveiler B., Black G. (2006). Genotype-phenotype correlation in Costello syndrome: H-Ras mutation analyses in 43 cases. American Journal of Medical Genetics. 43: 401-405.

Kim JW, Kim WH, Jeong MH, Jang SM, Song KH, Park SI, Song PI, Kang KH, Choi KH.(2008). p19(ras) amplifies p73beta-induced apoptosis through mitochondrial pathway.Biochem Biophys Res Commun. 2008 Aug 15;373(1):146-50. doi: 10.1016/j.bbrc.2008.06.010. Epub 2008 Jun 12.

Kim SJ., Shin JY., Lee KD., Bae YK., Sung KW., Nan SJ., Chun KH. (2012). MicroRNA let-7a suppresses breast cancer cell migration and invasion through downregulation of C-C chemokine receptor type 7. Breast Cancer Res. 14 (1): R14.

Kinoshita T., Hanazawa T., Nohata N., Kikkawa N., Enokida H., Yoshino H., Yamasaki T., Hidaka H., Nakagawa M., Okamoto Y., Seki N. (2012). Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma. Oncotarget. (Epub ahead of print).

Kirino T, Brightman MW, Oertel WH, Schmechel DE, Marangos PJ. (1983). Neuron-specific enolase as an index of neuronal regeneration and reinnervation. J Neurosci. **1983** May;3(5):915-23.

Kjersem JB., Ikdahl T., Guren T., Skovlond E., Sorbye H., Hamfjord J., Pfeiffer P., Glimelius B., Kersen C., Solvang H., Treit KM., Kure EH (2012). Let-7 miRNA-binding site polymorphism in the KRAS 3′UTR; colorectal cancer screening population prevalence and influence on clinical outcome in patients with metastatic colorectal cancer treated with 5-fluorouracil and oxaliplatin +/- cetuximab. BMC Cancer. 12(1):534.

Kloc M, Tejpal N, Sidhu J, Ganachari M, Flores-Villanueva P, Jennings NB, Sood AK, Kubiak JZ, Ghobrial RM.(2012). Inverse relationship between TCTP/RhoA and p53 /cyclin A/actin expression in ovarian cancer cells. Folia Histochem Cytobiol. 2012 Oct 8;50(3):358-67. doi: 10.5603/19745.

Kondo N, Toyama T, Sugiura H, Fujii Y, Yamashita H. (2008). **miR-206** Expression is down-regulated in estrogen receptor alpha-positive human breast cancer. Cancer Res. 2008 Jul 1;68(13):5004-8. PMID: 18593897

Kong D, Heath E, Chen W, Cher ML, Powell I, Heilbrun L, Li Y, Ali S, Sethi S, Hassan O, Hwang C, Gupta N, Chitale D, Sakr WA, Menon M, Sarkar FH. (2012). Loss of let-7 up-regulates EZH2 in prostate cancer consistent with the acquisition of cancer stem cell signatures that are attenuated by BR-DIM.PLoS One. 2012;7(3):e33729. doi: 10.1371/journal.pone.0033729.

Kopnin B.P. (2000). Targets of oncogenes and tumor suppressors: key for understanding basic mechanisms of carcinogenesis. Biochemistry. 65 (1):2-27

Kuhnert F, Mancuso MR, Hampton J, Stankunas K, Asano T, Chen CZ, Kuo CJ. (2008). Attribution of vascular phenotypes of the murine Egfl7 locus to the microRNA miR-126. Development. 2008 Dec;135(24):3989-93. doi: 10.1242/dev.029736. Epub 2008 Nov 5.

Lee RC., Fienbaum RL., Ambros V. (1993). The C. elegans heterochronic gen lin-4 encodes smal RNAs with antisense complementary tool in-14. Cell. 75(5):843-54

Lee JH, Voortman J, Dingemans AM, Voeller DM, Pham T, Wang Y, Giaccone G. (2011). MicroRNA expression and clinical outcome of small cell lung cancer. PLoS One. 2011;6(6):e21300. Epub 2011 Jun 22. PMID: 21731696

Lee JY, Kim HJ, Yoon NA, Lee WH, Min YJ, Ko BK, Lee BJ, Lee A, Cha HJ, Cho WJ, Park JW.(2013). Tumor suppressor p53 plays a key role in induction of both tristetraprolin and **let-7** in human cancer cells.Nucleic Acids Res. 2013 Apr 16. [Epub ahead of print]

Lee KH, Chen YL, Yeh SD, Hsiao M, Lin JT, Goan YG, Lu PJ.(2009). MicroRNA-330 acts as tumor suppressor and induces apoptosis of prostate cancer cells through E2F1-

mediated suppression of Akt phosphorylation. Oncogene. 2009 Sep 24;28(38):3360-70. Epub 2009 Jul 13. PMID: 19597470

Lee ST., Chu K., Oh HJ., Im Ws., Lim JY., Kim SK., Park CK., Jung KH., lee SK., Kim M and Roh JK. (2010). Let-7 microRNA inhibits the proliferation of human glioblastoma cells. J. Neurooncol. 102:19-24

Lee YC, Tzeng WF, Chiou TJ, Chu ST. (2012). MicroRNA-138 Suppresses Neutrophil Gelatinase-Associated Lipocalin Expression and Inhibits Tumorigenicity. PLoS One. 7(12):e52979. doi: 10.1371/journal.pone.0052979. Epub 2012 Dec 31. PMID: 23300839 [PubMed - in process]

Legendre M., Ritchie W., Lopez F., and Gautheret D. (2006). Differential repression of alternative transcripts: a screen for miRNA targets. Plos One. 2(5):e43

Leite KR, Tomiyama A, Reis ST, Sousa-Canavez JM, Sañudo A, Camara-Lopes LH, Srougi M. (2011). MicroRNA expression profiles in the progression of prostate cancer-from high-grade prostate intraepithelial neoplasia to metastasis. Urol Oncol. 2011 Aug 29. [Epub ahead of print] PMID: 21880514

Leivonen SK, Mäkelä R, Ostling P, Kohonen P, Haapa-Paananen S, Kleivi K, Enerly E, Aakula A, Hellström K, Sahlberg N, Kristensen VN, Børresen-Dale AL, Saviranta P, Perälä M, Kallioniemi O. (2009). Protein lysate microarray analysis to identify microRNAs regulating estrogen receptor signaling in breast cancer cell lines. Oncogene. 2009 Nov 5;28(44):3926-36. Epub 2009 Aug 17. PMID: 19684618

Li HL., Xiang Z., Zhao T. (2010a). Expression of surviving, MMP2, TIMP1, CD444 and NM23 of two tumours originating from different inmmunosurveillance in the same patients. Nan Fang Yi Ke Da Xue Xue Bao 30 (6):1291-1294

Li Q., Lee J.A., and Black D.L. (2007). Neuronal regulation of alternative pre-mRNA splicing. Nat. Rev. Neuroscience. 8: 819-831

Li S., Moffet HF., Lu J., Werner K., Zhang H., Ritz j., Neuberg D., Wucherpfenning KW., Brown JR., Novina CD (2011b). MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells. Plos Ones. 6(3): e16956.

Li T., Chen JX., Fu XP., Yang S., Zhang Z., Chen kh, Li Y. (2011c). MicroRNA expression profiling of nasopharyngeal carcinoma. Oncol. Rep. 25 (5):1353-63

Li X., Hu R., Qu J., He Q., Chen Y., Li J., Ye X., Xiang Y., Hong Y. (2012a). Identification of nm23-H1 as a metastatic suppressor and prognostic factor in nasopharyngeal carcinoma by proteomic analysis. J. Cent. South Unive. (Med. Sci). 37 (1):17-26

Li X., Zhang g., Luo F., Ruan J., Huang D., Feng D., Xian D., Zeng Z., Chen X., Wu X. (2012b). Identification of aberrantly expressed microRNAs in colorectal cancer. Oncolo. Rep. 28 (1):77-84

Li Y, Zhu X, Xu W, Wang D, Yan J. (2013). miR-330 regulates the proliferation of colorectal cancer cells by targeting Cdc42.Biochem Biophys Res Commun. 2013 Feb 15;431(3):560-5. doi: 10.1016/j.bbrc.2013.01.016. Epub 2013 Jan 18.

Li Y., Nie CJ., Hu L., Qin Y., Liu HB., Zeng TT., Chen L., Fu L., Deng W., Chen SP., Jia WH., Zhang C., Xie D., Guan XY. (2010b). Characterization of a novel mechanism of genomic instability involving the SEI 1/SET/NM23H1 pathway in esophageal cancers. Cancer Res. 70 (14): 5695-705

Li Z., Gu X., Fang Y., Xiang J., Chen Z. (2012c). MicroRNA expression profiles in human colorectal cancers with brain metastasis. Oncol. Lett. 3(2):346-350 CAMBIAR DE 2012 A A C

Lilly AJ., Khanim FL., Hayden RE., Luong QT., Drayson MT., Brunce CM. (2011). NM23-H1 indirectly promotes the survival acute myeloid leukemia blast cells by binding to more mature components of the leukemia clone. Cancer Res. 71(3):1177-86

Limana F, Esposito G, D'Arcangelo D, Di Carlo A, Romani S, Melillo G, Mangoni A, Bertolami C, Pompilio G, Germani A, Capogrossi MC. (2011). HMGB1 attenuates cardiac remodeling in the failing hearth via enhanced cardiac regeneration and miR-206 mediated inhibition of TIMP-3. PLoS One. 2011;6(6):e19845. PMID: 21731608

Lin AE, Rauen KA, Gripp KW, Carey JC.(2008). Clarification of previously reported **Costello syndrome** patients.Am J Med Genet A. 2008 Apr 1;146(7):940-3. doi: 10.1002/ajmg.a.32164. No abstract available.

Lin CC, Liu LZ, Addison JB, Wonderlin WF, Ivanov AV, Ruppert JM. (2011a) RelatedA KLF4-miRNA-206 autoregulatory feedback loop can promote or inhibit protein translation depending upon cell context. Mol Cell Biol. 2011 Jun;31(12):2513-27. Epub 2011 Apr 25. PMID: 21518959

Linardic CM. (2008). **PAX3-FOXO1 fusion gene** in rhabdomyosarcoma. Cancer Lett. 2008 Oct 18;270(1):10-8. doi: 10.1016/j.canlet.2008.03.035. Epub 2008 May 23. Review.

Lionello M., Blandamura S., Agostini M., Staffieri C., Lovato a., Tealdo G., Favoretto N., Giacomelli L., Loreggian L., taffieri A., Marioni G. (2012). A prognostic role for NM23-H1 in laryngeal carcinoma treated with postoperative radiotherapy: an introductory investigacion. Eu. Arch. Otorhinolaryngol. Epub ahead of print.

Liu X., Wang C., Chen Z., Jin Y., Wang Y., Kolokythas A., Dai Y., Zhou X. (2011a). MicroRNA-138 suppresses epithelial-mesenchymal transition in squamous cell carcinoma cell lines. Biochem. J. 440 (1):23-31

Liu B., Xiao-Chun P., Xiao-Li Z., Wang J., Young WQ. (2009a). MiR-126 restoration downregulated VEGF and inhibit the growth of lung cancer cell lines in vitro and in vivo. Lung cancer. 66 (2): 169-175.

Liu C., Kelnar K., Vlassov AV., Brown D., Wang J., Tang DF. (2012a). Distinct microRNA expression profiles in prostate cancer stem/progenitor stem cells and tumor suppressive functions of let-7. Cancer Res. 72(13): 3393-404.

Liu H, Cao YD, Ye WX, Sun YY. (2010a). Effect of microRNA-206 on cytoskeleton remodelling by downregulating Cdc42 in MDA-MB-231 cells. (2010). Tumori. 96 (5):751-5. PMID: 21302623

Liu S, Gao S, Wang XY, Wang DB. (2011b). Expression of miR-126 and Crk in endometriosis: miR-126 may affect the progression of endometriosis by regulating Crk expression. Arch Gynecol Obstet. [Epub ahead of print] PMID: 22012249

Liu X, Chen Z, Yu J, Xia J, Zhou X. (2009b). MicroRNA profiling and head and neck cancer. Comp Funct Genomics. 837514. Epub 2009 Jun 1. PMID: 19753298

Liu X, Jiang L, Wang A, Yu J, Shi F, Zhou X. (2009c). MicroRNA-138 suppresses invasion and promotes apoptosis in head and neck squamous cell carcinoma cell lines. Cancer Lett. 286(2):217-22. Epub 2009 Jun 21. PMID: 19540661

Liu X., Lu XB., Wang XP., Sang Y., Xu S., Hu K., Wu M., Liang Y., Liu P., Tang J., Lu W., Feng QS., Chen LZ., Qian CN., Bei JT., Kang T., Zeng YM (2012b). Micro-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene. Cell Cycle. 11(13):2495-506

Livak KJ, and Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T Method. Methods. 25: 402–8

Long G, Wang F, Duan Q, Chen F, Yang S, Gong W, Wang Y, Chen C, Wang DW. (2012).Human circulating microRNA-1 and microRNA-126 as potential novel indicators for acute myocardial infarction.Int J Biol Sci. 2012;8(6):811-8. doi: 10.7150/ijbs.4439. Epub 2012 Jun 7.PMID: 22719221 [PubMed - indexed for

Lopez Bergami P., Habelhan H., Bhoumik A., Zhang W., Wang L.H. and Ronai Z. (2005). RACK 1 mediates activation of JNK by protein kinase C. Moll. Cell. 19 (3): 309-320.

Lopez-Camarillo C., Marchat LA., Arechaga-Ocampo E., Perez-Plascencia C., Moral-Hernandez O., Castañeda-Ortiz EJ., and Rodriguez –Cuevas S. (2012). MetasmiRs: non-coding microRNAs driving cáncer invasión and metástasis. Int. J. Mol. Sci. 13:1347-1379

Lopéz-Otín C and Matrisian M. Lynn. (2007). Emerging roles of proteases in tumour suppression. Nature reviews. Cancer. (7): 800-809.

Lorenz S., Petersen C., Kordab U., Seidel H., Zenker M., Kutsche K. (2012). Two cases with severe course of Costello syndrome associated with H-Ras p-612C and p.G12D. Eur. J. Med. Genet. 55(11):615-9

Lowy D.R., and Willumsen B.M. (1993). Function and regulation of Ras. Ann. Rev. Biochem. 62:851-891.

Lu Y., Govindan R., Wang L., Liu PY., Goodgame B., Wen W., Sezhiyan A., Pfeifer J., Li YM, Hua X, Wang Y, Yang P., You M. (2012). MicroRNA profiling and prediction of recurrence/relapse-free survival in stage I lung cancer. Carcinogenesis 33(5):1046-54

Luo XQ., Xu L., Ke ZY., Huang LB., Zhang XL (2011). MicroRNA expression in childhood acute granulocytic leukemia and its subtypes. Zhonghua Zhong Liu Za Zhi. 33 (11):831-5

Lv K., Liu L., Wang L., Yu J., Liu X., Cheng Y., Dong M., Teng R., Wu L., Fu P., Deng W., Hu W., Teng L. (2012). Lin 28 mediates praclitaxel resistance by modulating p21, Rb, and let-7a, miRNA in breast cancer cells. Plos One 7 (7): e40008

Lynch J., Fay J., Meehan M., Bryan K., Watters K., Murphy DM., Stallings RL. (2012). miRNA-335 suppresses neuroblastoma cell invasiveness by targeting of multiple genes from the non-canonical TGF-β signaling pathway. Carcinogenesis. 33 (5):976-85

Lynn H.K., and Chen X. (2006). P19 ras brings a new twists to the regulation of p73 by Mdm2. SCi STKE 3(37) pe24. DOI: 10.1126.Stke. 3372006pe24

Macaluso M., Russo G., Cinti C., Bazan V., Gebbia N., and Russo A. (2002). Ras family genes: an interesting link between cell cycle and cancer. J. Cell Physiol. 192: 125-130.

MacQuarrie KL., Yao Z., Young JM., Cao Y., Tapscott SJ (2012). MiR-206 integrates multiple components of differentiation pathways to control the transition from growth to differentiation in rhabdomyosarcoma cells. Skeletal muscle 2(1):7.

Macville M., Schröck E., Padilla-Nash H., Keck C., Ghadimi Michael b., Zimonjic D., Popescu N., and Ried T. (1999). Comprehensive and definitive molecular cytogenetic characterization of HeLa cells by spectral karyotyping. Cancer Res. 59: 141-150.

Madhukara J.A., and Kumaran M, S. (2007). Costello syndrome. Indian Journal of Dermatology, Venereology and Leoprology. 406-8.

Mahajan A, Liu Z, Gellert L, Zou X, Yang G, Lee P, Yang X, Wei JJ. (2010). HMGA2: a biomarker significantly overexpressed in high-grade ovarian serous carcinoma. Mod Pathol. 2010 May; 23(5):673-81. Epub 2010 Mar 12. PMID: 20228781

Majid S., Dar AA., Saini S., Arora S., Shahryari V., Zaman MS., Chang I., Yamamura S., Tanaka Y., Deng G., Dahiya R. (2012). MicroRNA-23b represses proto-oncogene Src kinase functions as a methylation-silenced tumor suppressor with diagnostic and prognostic significance in prostate cancer. Cancer Res. (Epub ahead of print).

Malaney S., and Daly R.J. (2001). The Ras signalling pathway in mammary tumorigenesis and metastasis. J. Mammary Gland Biol Neoplasia. 6(1):101-113

Malumbres M., and Barbacid M. (2003). Ras oncogenes: the first 30 years. Nature Reviews 3,7-13.

Malvezzi M., Bosseti C., Chatenoud L., Rodriguez R., Levi F., Negri E., and la Vecchia. (2004). Trends in cancer mortality in Mexico, 1970-1999. Annals of Oncology. (15):1712-1718.

Mamidipudi V, Zhang J, Lee KC, Cartwright CA. (2004). RACK1 regulates G1/S progression by suppressing Src kinase activity. Mol Cell Biol. **2004** Aug;24(15):6788-98.

Manikandan J., Aarthi JJ., Kamar SD., and Pushparaj PN. (2008). OncomiRs: the potential role of non-coding microRNAs in understanding cancer. Bioinformation 2(8): 330-334.

Marioni G., Ottaviano G., Lionello M., Lora L., Lovato A., Staffieri C., Favoretto N., Giacomelli L., Stellini E., Staffieri A., Blandamura S. (2012). Nm23-H1 nuclear expression is associated with a more favourable prognosis in laryngeal carcinoma: univariate and multivariate analysis. Histopathology. Epub ahead of print.

Martin SE., Susen M., Joseph A., Kingham BF., Martin ES. (2012). Identification of a HMGA2-EFCAB6 gene rearrangement following next generation sequencing in a patient with a t(12:22) (q143; q13.2) and JAK2VG17F-positive myeloproliferative neoplasm. Cancer Genet. 205(6):295-303

Martinez J.D., Parker M.T., Fultz K.E., and Gerber E.W. (2003). Chapter One. Molecular biology of cancer. Burger's Medicinal Chemistry and Drug Discovery. Sixth Edition, Volume 5: Chemoterapeutic Agents. Edited by Donald J. Abraham John Wiley & sons, Inc. ISBN 0-471-37031-2

Martinez-Outschoorn UE., Balliet RM., Rivadeneira D., Chiavanna B., Pavlides S., Wang C., Whitaker-Menezes D., Daumer KM., Lin Z., Whikiewicz AK., Flomemberg N., Howell A., Pestell RG., Knudsen ES, Sotgia F., Lisanti MP. (2010). Oxidative stress in cancer associated fibroblast drives tumor-stroma co-evolution. Cell cycle. 9(16): 3256-3276

Marzi MJ, Puggioni EM, Dall'Olio V, Bucci G, Bernard L, Bianchi F, Crescenzi M, Di Fiore PP, Nicassio F. (2012). Differentiation-associated microRNAs antagonize the Rb-

E2F pathway to restrict proliferation.J Cell Biol. 2012 Oct 1;199(1):77-95. doi: 10.1083/jcb.201206033.

Mattes J, Collison A, Plank M, Phipps S, Foster PS. (2012). Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. Proc Natl Acad Sci U S A. 2009 Nov 3;106(44):18704-9. doi: 10.1073/pnas.0905063106. Epub 2009 Oct 20. PMID: 19843690 [PubMed - indexed for MEDLINE].

McCahill A., Warwicker J., Graeme B. B., Miles D.H., and Yarwood S.J. (2002). The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. Molecular Pharmacology. Vol.62 (6):1261-1273

Meister J, and Schmidt MHH (2010). miR-126 and miR-126*: new players in cancer. The Scientific World Journal 10_2090-2100 doi: 10.11.00&tsw.2010.198

Mendez-Samperio P., Perez A., Alba L. (2010). Reactive oxygen species-activated p38/ERK ½ MAPK signaling pathway in the Mycobacteriun bovis bacillus Calmette Guerin (BCG)-induced CCL2 secretion in human monocytic cell line THP-1. Arch. Med.Res. 41(8):579-85

Meng S, Cao JT, Zhang B, Zhou Q, Shen CX, Wang CQ.(2012). Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1. J Mol Cell Cardiol. 2012 Jul;53(1):64-72. doi: 10.1016/j.yjmcc.2012.04.003. Epub 2012 Apr 16.

Miao Y., Li J., Qiu X., Li Y., Wang Z., Luan Y. (2013). MiR-27a regulates the selfrenewal of the H46 small cell lung cancer cell line in vitro. Oncol. Rep. 29(1):161-8

Mi-Jin L., Dong-Yuang Z., Hua Li, Goung-Ran y., Sun-He L., In-Sun C., In-Hee K., and Dae-Ghon K. (2012). Pro-oncogenic potential of NM23-H2 in hepatocellular carcinoma. Experimental and Molecular Medicine. 44 (3):214-224

Miko E., Margitai Z., Czimmerer Z., Varkonyi I., Dezso B., Lanyi A., Bacso Z., Scholtz B. (2011). MiR-126 inhibits proliferation of small lung cancer cells by targeting SLC7A5. FEBS Letter. 585 (8): 1191-6

Miko E, Czimmerer Z, Csánky E, Boros G, Buslig J, Dezso B, Scholtz B.(2009). Differentially expressed microRNAs in small cell lung cancer. Exp Lung Res. 2009 Oct;35(8):646-64.

Milenkovic D, Deval C, Gouranton E, Landrier JF, Scalbert A, Morand C, Mazur A. (2012). Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: a new mechanism of the action of polyphenols. PLoS One. 2012;7(1):e29837. Epub 2012 Jan 10. PMID: 22253797

Miller TW., Hennessy BT., González-Angulo AM., Fox EM., Mills GB., Chen H., Higham C., García-Echeverria C., Shyr Y., and Artega CL. (2010). Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. The Journal of Clinical Investigation. 120 (7):2406-2413

Min DJ., Ezponda T., Kim MK., Will CM., Martinez Garcìa E., Popovic R., Bashur V., Elenitoba-Johnson KS., Licht ND. (2012). MMSET stimulates myeloma cell growth through microRNA-mediated modulation of c-Myc. Leukemia (Epub ahead of print).

Miñarro R., Black RJ., Martínez C., Navarro C., Garau I., Izarzugaza I., Ardanar E., Vergara A., Galcerán J., Alonso R., Mateos A., y Rodríguez M. (2000). Incidencia y mortalidad por cáncer en España. Patrones y Tendencia. Revista Española de Salud Publica. (Vol. 74) 581-582.

Missiaglia E, Shepherd CJ, Patel S, Thway K, Pierron G, Pritchard-Jones K, Renard M, Sciot R, Rao P, Oberlin O, Delattre O, Shipley J.(2010). MicroRNA-206 expression levels correlate with clinical behaviour of rhabdomyosarcomas. Br J Cancer. 2010 Jun 8;102(12):1769-77. Epub 2010 May 25. PMID: 20502458

Mitin N., Rossman L. K., and Der C. J. (2005). Signaling interplay in Ras superfamily function. Current Biology15 (14): R563 - R574.

Mitomo S, Maesawa C, Ogasawara S, Iwaya T, Shibazaki M, Yashima-Abo A, Kotani K, Oikawa H, Sakurai E, Izutsu N, Kato K, Komatsu H, Ikeda K, Wakabayashi G, Masuda T. (2008). Downregulation of **miR-138** is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. Cancer Sci. 99(2):280-6. Epub 2008 Jan 14. PMID: 18201269

Miyachi M, Tsuchiya K, Yoshida H, Yagyu S, Kikuchi K, Misawa A, Iehara T, Hosoi H. (2010). Circulating muscle-specific microRNA, **miR-206**, as a potential diagnostic marker for rhabdomyosarcoma. Biochem Biophys Res Commun. 2010 Sep 10;400(1):89-93. Epub 2010 Aug 7. PMID: 20696132

Mocharla P., Briand S., Giannotti G., Dorries C., Jacob p., Paneni F., Lusher T., Landmesser Y. (2012). AngiomiR-126 expression and secretion from circulating CD34+ and CD14+ PBMC`s: role of pro-angiogenic effects ad alterations in type-2 diabetics. Blood (Epub ahead of print).

Mohammad G., Kowluru RA. (2011). The role of Raf-1 kinase in diabetic retinopathy. Expert. Opin. Thr. Targets. 15 (4): 357-64

Molenaar JJ., Domingo-Fernandez R., Ebus ME., Lindner S., Koster J., Drabek K., Mestdagh P., van Sluis P., Valentijn LJ., Van Nes J., Broekmans M., Haneveld F., Volckmann R., Bray I., Heukamp L., Sprussel A., Thror T., Lieckbusj k., Klein-Hitpass L., Fisher M., Van de sompele J., Schramm A., van Noesel MM., Varesio L., Speleman F., Eggert A., Starling RL., Caron HN., Versteeg R., Schulte JH. (2012).

LIN 28B induces neuroblastoma and enhances MYCN levels vial et-7 suppression. Nat. Genet. (Epub ahead of print).

Molina J.R., and Adjei A. A. (2006). The Ras/Raf/MAPK pathway. J. Thorac. Oncol. 1:7-9

Mori K., Hata M., Neya S., Hoshino T. (2002) A study on the role of Mg+" in a Ras protein by MD simulation. CBIJ. 2 (4): 147-155

Mori T, Ikeda DD, Fukushima T, Takenoshita S, Kochi H.(2011). NIRF constitutes a nodal point in the cell cycle network and is a candidate tumor suppressor. Cell Cycle. 2011 Oct 1;10 (19): 3284-99. doi: 10.4161/cc.10.19.17176. Epub 2011 Oct 1.

Mosakhani N, Lahti L, Borze I, Karjalainen-Lindsberg ML, Sundström J, Ristamäki R, Osterlund P, Knuutila S, Sarhadi VK. (2012). MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF .Cancer Genet. 2012 Nov;205(11):545-51. doi: 10.1016/j.cancergen.2012.08.003.

Mott J.L. (2009). MicroRNAs involved in tumor suppressor and oncogene pathways: implications for hepatobiliary neoplasia. Hepatology 50:630-637

Muraoka S, Shima F, Araki M, Inoue T, Yoshimoto A, Ijiri Y, Seki N, Tamura A, Kumasaka T, Yamamoto M, Kataoka T. (2012). Crystal structures of the state 1 conformations of the GTP-bound H-Ras protein and its oncogenic G12V and Q61Lmutants. FEBS Lett. 2012 Jun 12;586(12):1715-8. doi: 10.1016/j.febslet.2012.04.058. Epub 2012 May 11.

Musiyenko A., Bitko V., Barik SJ. (2008). Ectopic expression of miR-126*, an intronic product of the vascular endothelial EGF-like 7 gene, regulates protein translation and invasiveness of prostate cancer LNCaP cells. J. Mol. Med. 86(3):313-22

Nadiminty N, Tummala R, Lou W, Zhu Y, Zhang J, Chen X, eVere White RW, Kung HJ, Evans CP, Gao AC.(2012). MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells. J Biol Chem. 2012 Jan 6;287(2):1527-37. Epub 2011 Nov 28.

Nair VS., Maeda LS and Ionnidis JP (2012). Clinical outcoe prediction by miRNAs in human cancer : a systematic review. J. Natl. Cancer Inst. 104 (7): 528-40

Namlos HM., Meza-Zepeda LA., Baroy T., Ostenten IH., Kresse Sh., Kuijjer ML., Serra M., Burger H., Clenton-Jansen AM., Myklebost O. (2012). Modulation of osteosarcoma expression phenotype by microRNAs. Plos One. 7(10): e48086.

Navarro A, Quirós L, Casado M, Faria M, Carrasco L, Benejam L, Benito J, Díez S, Raldúa D, Barata C, Bayona JM, Piña B. (2009). Physiological responses to mercury in

feral carp populations inhabiting the low Ebro River (NE Spain), a historically contaminated site. Aquat Toxicol. 93(2-3):150-7. Epub 2009 May 3.

Navis AC, Bourgonje A, Wesseling P, Wright A, Hendriks W, Verrijp K, van der Laak JA, Heerschap A, Leenders WP. (2013). Effects of dual targeting of tumor cells and stroma in humanglioblastoma xenografts with a tyrosine kinase inhibitor against c-MET and VEGFR2. PLoS One. 2013;8(3):e58262. doi: 10.1371/journal.pone.0058262. Epub 2013 Mar 4.

Negrini M., and Calin G.A. (2008). Breast cancer metastasis: a micro RNA story. Breast cancer research. 10: 303-311doi: 10118661/bcr1867

Nie K, Zhang T, Allawi H, Gomez M, Liu Y, Chadburn A, Wang YL, Knowles DM, Tam W. (2010). Epigenetic down-regulation of the tumor suppressor gene PRDM1/Blimp-1 in diffuse large B cell lymphomas: a potential role of the microRNA let-7. Am J Pathol. 177(3):1470-9. Epub 2010 Jul 22.

Niitsu N., Nakamine H., and Okamoto M. (2011). Expression of nm23-H1 is associated with poor prognosis in peripheral T-cell lymphoma, not otherwise specified. Clin. Cancer Res. 17:2993-2899

No authors listed. (2012). LIN28B Is a Neuroblastoma Oncogene.Cancer Discov. 2012 Dec;2(12):OF15. doi: 10.1158/2159-8290.CD-RW2012-180. Epub 2012 Oct 18.PMID:23230196[PubMed - in process] Related citations

Nohata N, Hanazawa T, Enokida H, Seki N. (2012). microRNA-1/133a and microRNA-206/133b clusters: Dysregulation and functional roles in human cancers. Oncotarget. 2012 Feb;3(1):9-21. PMID: 22308266

Nordentoft I., Birkenkamp-Demtroder K., Agerbaek M., Theodorescu D., Ostenfeld ms., Hartmann A., Borre M., Orntoft tf., Dyrsjot L. (2012). MicroRNAs associated with chemosensitivity in cell lines and in advanced bladder cancer. BMC Med. Genomics. 6 (5):40

Nurtdinov R., Neverov A.D., Favorov A.V., Mironov A. A., and Gelfand M. (2007).Conserved and species-specific alternative splicing in mammalian genomes. BMC Evolutionary Biology. 7:249-260.

O'Day E, Lal A. (2010). MicroRNAs and their target gene networks in breast cancer. Breast Cancer Res. 2010;12(2):201. Epub 2010 Mar 19. Review. PMID: 20346098

Okabe-Kado J., Kasukabe T., Kaneko J. (2012). Extracellular NM23 protein as a therapeutic target for hematologic malignancies. Adv. Hematol. 879368. Epub ahead of print.

Oliviera-Carvalho V., Silva MM., Guimaraes GV., Bacal F., Bocchi EA. (2012). MicroRNAs: new players in heart failure. Mol. Biol. Rep. (Epub ahead of print).

Oncogene. 2013 Jan 14. doi: 10.1038/onc.2012.573. [Epub ahead of print]

Oshima K., Inoue K., Fukiwara A., Hatekeyama K., Kanto K., Watanabe Y., Muramatsu K., Fukuda Y., Ogura S., Yamaguchi K., Mozhizuki T. (2010). Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gatric cancer cell line. Plos One 5(10): e13247.

Otsubo T, Akiyama Y, Hashimoto Y, Shimada S, Goto K, Yuasa Y. (2011). MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. PLoS One. 6(1):e16617.

Paez Guillermo J., and Sellers W.R. (2002). PI3K/PTEN/Akt pathway. A critical mediator of oncogenic signalling. Book title, 1-6. Web site: http://reseach.dfci.harvard.edu/ sellers/ab/publications/Paez_bkchapter_2002.pdf

Parasramka MA., Dashwood WM., Wang R., Abdelli A., Bailey GS., Williams DE., Ho E., Sashwood RH. (2012a). MicroRNA profiling of carcinogen-induced rat colon tumors and the influence of dietary spinach. Mol. Nutr. Food Res. (Epub ahead of print).

Parasramka MA., Dashwood WM., Wang R., Saeed HH., Williams DE, Ho E., Dashwood RH. (2012b). A role for low abundance miRNAs in colon cancer: the miR-206 Kruppel-like factor 4 (KLF4) axis. Clin. Epigenetics 4(1): 16

Park HR., Se-Heon K., Sei-Young L., Ji-Min S., Ae Ran P., Suki K., Baek G., Yoon PC, Yong-Bae K., and Nam Hoon C. (2011). Nuclear localization of NM23-H1 in head and neck squamous cell carcinoma is associated with radiation resistance. Cancer. 117: 1864-73.

Paun BC., Chen Y., Leggett BA., Young J., Meltzer SJ., Mori Y. (2009). Screening for microsatellite instability identifies frequent 3'-untranslated region mutation of the RB1 inducible coiled-coil 1 gene in colon tumours. Plos One. 4(11):e7715.

Pelosi A., Careccia s., Lulli V., Romania P., Marziali G., Testa U., Lavargna S., Lo-Coco F., petti MC., Calabretta B., Levrero M., Piaggio G., Rizzo MG (2012). Micro let-7-c promotes granulocytic differentiation in acute myeloid leukemia. Oncogene. (Epub ahead of print).

Perou, C.M., Sorlie T., Einen M.B., van de Rijn, M., Jeffrey S.S., Rees, C.A., Pollack J.R., Ross D.T., Hohnsen H., Akslen L., A, Fluge O., Pergamenschikov A., Williams C., Zhu S.X., Lonning P.E., Borresen-Dale., A.L., Brown P. O, Botstein D. (2000). Molecular portraits of human breast tumours. Nature. 406: 747-752.

Pignot G., Cizeron-Clairac G., Vacher S., Susini A., Tozlu S., Viellenfond A., Zerbib M., Lidereau R., Debre B., Amsellem-Oyazana D., Bieche L. (2012). MicroRNA expression profile in a large series of bladder tumours: identification of a 3 mirna signature associated with aggressiveness of muscle –invasive bladder cancer- Int. J. Cancer. (Epub ahead of print). PMID: 22113756 [PubMed - indexed for MEDLINE]

Piskounova E, Polytarchou C, Thornton JE, LaPierre RJ, Pothoulakis C, Hagan JP, Iliopoulos D, Gregory RI.(2011). Lin28A and Lin28B inhibit let-7 microRNA biogenesis by distinct mechanisms. Cell. 2011 Nov 23;147(5):1066-79.

Png KJ., Halberg N., Yoshida M., and Tavazoie SF. (2011a). A microRNA regulon that mediates endothelial recruitment and mestastasis by cancer cells. Nature. 481: 190-194.

Png KJ., Yoshida M., Zhang XH, Shu W, Lee H., Rimner A., Chang TA., Comen E., Andrade VP., Kim SW., King TA., Hudis CA., Norton L., Hicks J., Massague J., and Tavazoie SF (2011b). MicroRNA-335 inhibits tumor reinitiacion and is silenced through genetic and epigenetic mechanisms in human breast cancer. Genes Dev. 25 (3): 226-31

Png Y., Young Charles YF-. Yuan H. (2010). MicroRNAs and prostate cancer. Acta Biochim. Biophys. Sin. 42 (6):363-369

Poliseno L., Haimovic A., Segura MF., Hanniford D., Christos PJ., Darvishian F., Wang J., Shapito RL., Pavlick AC., Berman RS., Hernando E., Avadil J., Osman I. (2012). Histology-specific microRNA alteration in melanoma. J. Invest. Dermatol. Doi: 10.1038/jid.2011.451

Polytarchou C., Lliopoulos D., Struhl K. (2012). An integrated transcriptional regulatory circuit that reinforces the breast cancer stem cell state. PNAS. 109 (36): 14470-5

Poulsen HE., Prieme H and Loft S. (1998). Role of oxidative DNA damage in cancer, initiation and promotion. European Journal of Cancer Prevention. 7:9-16.

Presneu N., Eskandapour M., Shemais T., Henderson S., Halai D., Tirabosco R., Flanagan AM. (2012). MicroRNA profiling of periphereal nerve sheath tumour identifies miR-29c as a tumour suppressor gene involved in tumor progression. Br. J. Cancer. (Epub ahead of print).

Prior I.A., and Hancock J.F. (2001). Compartmentalization fo Ras proteins. J.Cell Sci. 114(9)1603-1608.

Ptfaffl M.W. (2001). A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Research (29): 2003-2007.

Qin A, Wen Z, Zhou Y, Li Y, Li Y, Luo J, Ren T, Xu L. (2013). MicroRNA-126 regulates the induction and function of CD4(+) Foxp3(+) regulatory T cells through PI3K/AKT pathway. J Cell Mol Med. 2013 doi: 10.1111/jcmm.12003. [Epub ahead of print].

Qin X., Liu B., Li Y., You J., Zhou Q. (2011a). Screening and identification of microRNAs related to acquired gefitinib-resistance in lung adenocarcinoma cell lines. Zhong guo Fei Ai Za Zhi. 14 (6):478-83

Qin Z., Dai L., Toole B., Robertson E., Parsons C. (2011b). Regulation of NM23H1 and cell invasivenes by Kaposis¿ sarcoma-associated herpesvirus. J. Virol. 85 (7):3596-3606

Qu S., Yao Y., Shang C., Xue Y., Ma J., Li Z., Liu y. (2012). MicroRNA-330 is an oncogenic factor in glioblastoma cells by regulating SH36L2 gene. Plos One. 7(9): e46010.

Rahbari R, Holloway AK, He M, Khanafshar E, Clark OH, Kebebew E. (2010). Identification of differentially expressed microRNA in parathyroid tumors. Ann Surg Oncol. 18(4):1158-65.

Rauen K., A., Hefner E., Carrillo K., Taylor J., Messier L., Aoki Y., Gripe K.E., Matsubara Y., Proud V.K., Hammond P., Allanson J.E., Delrue M.A., Axelrad M.E., Lin A.E., Doyle D.A., Kerr B., Caray J.C., Mc Cormick F., Silva A.J., Kieran M.W., Hinek A., Nguyen T.T., Schoyer L. (2008). Molecular aspects, clinical aspects and possible treatment modalities for Costello Syndrome. Proccedings from the 1st International Costello Symposium 2007. American Journal of Medical Genetics Part A. 146 A:1205-1217

Rehman H., and Bos J. (2004) Thumbs up for inactivation. Nature. 249: 138-139
Ricarte-Filho JC, Fuziwara CS, Yamashita AS, Rezende E, da-Silva MJ, Kimura ET.(2009). Effects of let-7 microRNA on Cell Growth and Differentiation of Papillary Thyroid Cancer. Transl Oncol. 200. (4):236-41.

Ricarthe-Filho JC., Fuziwara CS., Yamashita AS., Rezende E., Da Silva M., Kimura ET. (2009). Effects of let-7 microRNA on cell growth and differentiation of papillary thyroid cancer. Transl. Oncol. 2(4): 236-41

Rodriguez-Viciana P., Sabatier C., and McCormick F. (2004) Signalling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulated. Mol. Cell. Biol. 24(11):4943-4954.

Romero-Perez L., Castilla MA., Lopez-García MA., Díaz-Martin J., Biscuola M., Ramiro-Fuentes S., Oliva E., Matias-Guiu X., Prat J., Cano A., Moreno-Bueno G., Palacios J. (2012). Molecular events in endometrial carcinosarcomas and the role of high mobility group AT-hook 2 in endometrial carcinogénesis. Human Pathol. (Epub ahead of print) doi:pii:S0046-8177(12)00188-8

Rommel C., Camps M and Ji H. (2007). PI3Kδ and PI3Kγ: partners in crime in inflammation in rheumatoid arthritis and beyond? Nature reviews. Inmmunology. 7: 191-201

Ronchetti D., Lionetti M., Mosca L., Agnelli L., Andronache A., Fabris S., Deliliers GL., Neri A. (2008). An integrative genomic approach reveals coordinated expression of intronic miR-335, miR-342 and miR-561 with deregulated host genes in multiple myeloma. BMC Med Genomics 13; 1-37.

Rosanna DC and Salvatore C. (2012). Reactive oxygen species, inflammation, and lung disease. Curr. Pharm. Des. 18(26):3889-900.

Ruan Y, Sun L, Hao Y, Wang L, Xu J, Zhang W, Xie J, Guo L, Zhou L, Yun X, Zhu H, Shen A, Gu J. (2012). Ribosomal RACK1 promotes chemoresistance and growth in human hepatocellular carcinoma. J Clin Invest. 2012 Jul 2;122(7):2554-66. doi: 10.1172/JCI58488. Epub 2012 Jun 1.

Rufini A, Agostini M, Grespi F, Tomasini R, Sayan BS, Niklison-Chirou MV, Conforti F, Velletri T, Mastino A, Mak TW, Melino G, Knight RA. (2011). p73 in Cancer. Genes Cancer. 2011 Apr;2(4):491-502. doi: 10.1177/1947601911408890.

Run A., Chu Y., Tian C., Dai XA., Chen J., Shi Q., Han J., Dong X. (2008). Overexpression of NM23H1 in *HeLa* cells provides cells with higher resistance to oxidative stress possibly due to raising intracellular p53 and GPX1. Acta Pharmacology Sin. 29 (12):1451-1458

Rustamadji P. (2012). NM23H1 as marker of metastasis in invasive ductal breast cancer. Acta. Med. Indones. 44 (1):40-6

Rutkowski R, Dickinson R, Stewart G, Craig A, Schimpl M, Keyse SM, Gartner A. (2011). Regulation of Caenorhabditis elegans p53/CEP-1-dependent germ cell apoptosis by Ras/MAPK signaling. PLoS Genet. (8):e1002238. Epub 2011 Aug 25.

Ruzzo A., Graziano F., Vicenzi B., Canestrari E., Perrone G., Gallucuo N., Catalano V., Loupakis F., Rabitti C., Santini D., Tonini G., Fiorentini G., Rossi D., Falcone A., Magnani M. (2012). High let-7a microRNA levels in KRAS mutated colorectal carcinomas may rescue anti-EGFR therapy in patients with chemotherapy-refractory metastatic disease. M. Oncologist. 17(6): 823-9

Sahai E. (2007). Illuminating the metastatic process. Nature Reviews. Cancer. 7:737-749

Saito Y., Susuki H., Matsuyra M., Sato A., Kasai Y., Yamada K., Saito H., Hibi T (2011). MicroRNAs in hepatobiliary and pancreatic cancers. Front. Genet. 2:66

Saito Y., Suzuki H., Imaheda H., Matsuzaki J., Hirata K., Tsugawa H., Hibino S., Kanai Y., Saito H., Hibi T. (2012). The tumor suppressor microRMAs-29c is downregulated and restored by celecoxib in human gastric cancer cells. Int. J. Cancer (Epub ahead of print).

Sakurai M., Miki Y., Masuda M., Hata s., Shibahara Y., Hirakawa H., Suzuki T., Sasano H. (2012). LIN 28 a regulator of tumor suppressing activity of let-7 microRNA in human breast cancer. J. Steroid. Biochem. Mol. Biol. 131 (3-5): 101-6.

Saleh AD, Savage JE, Cao L, Soule BP, Ly D, DeGraff W, Harris CC, Mitchell JB, Simone NL. (2011). Cellular stress induced alterations in microRNA let-7a and let-7b

expression are dependent on p53. PLoS One. 2011;6(10):e24429. Epub 2011 Oct 11. PMID: 22022355

Sanchez M.J., Payer T., De Angelis R., Larrañaga N., Capocaccia R., and Martinez C. (2010). Cancer incidence and mortality in Spain : estimates and projections for the period 1981-2012. Annals of Oncology. 21 (Supplement 3): iii30-iii36

Sang-Min J., Jung-Woong K., Chul-Hong K., Daehwan K., Sangmyung R., Kyung-Hee C. (2009). P19 RAS represses proliferation of non-small cell lung cancer possibly throught interaction with neuron-specific enolase (NSE). Cancer Letters. 289:91-98

Santarelli L., Strafela E., Staffolani S., Amati M., Emanuelli M., Sartini D., Pozzi V., Carbonari D., Bracci M., Pignotti E., Mazzanti P., Sabbatini A., Ranaldi R., Gasparini S., Neuzil J., Tomassetti M. (2011). Association of miR-126 with soluble mesothelin-related peptides, a marker for malignant mesothelioma. Plos One. 6(4): e18232.

Santoriello C., Deflonan G., Pezzimenti F, Kawakami K., Lanfrancone L., DI Fagagna DF and Mione M. (2009). Expression of H-Ras V12 in a zebrafish model of Costello syndrome causes cellular senescence in adult proliferating cells. Disease models and mechanisms. 2:56-67

Sasahira T., Kurihara M., Bhawal UK., Ueda N., Shimomoto T., Yamamoto K., Kiritia T., and Kuniyasu H. (2012). Downregulation of miR-126 induces angiogenesis and lymphagiogenesis by activation of VEGF-A in oral cancer. British Journal of Cancer. Epub ahead of print. Doi: 10.1038/bjc. 2012.330

Savad S., Mehdipour P., Miryounesi M., Shrkoohi R., Fereidooni F., Mansouri F., Moderressi MH. (2012). Expression analysis of miR-21, miR-205 and miR-342 in breast cancer in Iran. Asian Pac. J. Cancer Prev. 13 (3): 873-7

Scarola M., Schoeftner S., Schneider C., Benetti R. (2010). MiR-335 directly targets RB1 (pRB/p105) in a proximal connection to p53-dependent stress response. Cancer Res. 70 (17):6925-33

Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J. 2007 Apr 4;26(7):1749-60. Epub 2007 Mar 8.

Schmitz KJ, Helwig J, Bertram S, Sheu SY, Suttorp AC, Seggewiss J, Willscher E, Walz MK, Worm K, Schmid KW. (2011). Differential expression of microRNA-675, microRNA-139-3p and microRNA-335 in benign and malignant adrenocortical tumours. J Clin Pathol. 2011 Jun;64(6):529-35. Epub 2011 Apr 6. PMID: 21471143

Schuhmacher AJ., Guerra C., Sauzeau V., Cañamero M., Busteo XR and Barbacid M (2008). A mouse model for Costello syndrome reveals and Ang II-mediated hypertensive condition. The Journal of Clinical Investigation. 118 (6): 2169-2179

Schultz A.L., Albrecht B., Arici C., Ivander B., Buske A., Gillessen-Kaesbach G., Heller R., Horn D., Hubner CA., Korence GC., Going R., Kress W., Kruger G., Meinecke P., Mücke K., Plecki B., Rossier E., Schinzel A., Schulze A., Seemanova E., Seidel H., Spranger S., Tuysuz B., Uhrig S., Wieczorek D., Kutsche K., and Zenker M. (2008a). Mutation and phenotypic spectrum in patients with cardio-facio-cutaneous and Costello syndrome. Clin Genet (73):62-70

Schultz J, Lorenz P, Gross G, Ibrahim S, Kunz M.(2008b). MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res. 2008.(5):549-57. PMID: 18379589
Sci STKE. 2006 May 30;2006(337):pe24. Review.

Sebio A, Paré L, Páez D, Salazar J, González A, Sala N, Del Río E, Martín-Richard M, Tobeña M, Barnadas A, Baiget M. Pharmacogenet Genomics. (2013) The LCS6 polymorphism in the binding site of let-7 microRNA to the KRAS 3'-untranslated region: its role in the efficacy of anti-EGFR-based therapy in metastatic colorectal cancer patients. [Epub ahead of print] PMID:23324806

Shang –Ying W., Sheng-Hui L, Da-En C., Wei-Kai C., Cheng-Huang S., Ying-Ray L., Zuchin R., and Hsia-Shen-Liu. (2011). Ras-related tumorigenesis is suppressed by BNIP3 mediated atuophagy through inhibition of cell proliferation. Neoplasia. 13 (12):1171-1182.

Shaw R J, and Cantley LC. (2006).
Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature. 25; 441(7092):424-30.

She S., Xu B., He M., Lan X., Wang Q. (2010). NM23H1 suppresses hepatocarcinoma cell adhesion and migration on fibronectin by modulating glycosylation of integrin beta1. J. Exp. Clin. Cancer Res. 11: 29-93.

Sherr CJ. (2004) Principles of tumor suppression. Cell. 116:235-246

Shi L., Jiang D., Sun G., Wan Y., Zhang S., Zeng Y., Pan T., Wang Z. (2012). MiR-335 promotes cell proliferation by directly targeting RB1 in meningiomas. J. Neuroncol. 110(2):155-62.

Shimiku, K., M. Goldfarb, M. Perucho, and M. Wigler. (1983). Isolation of preliminary characterization of the transforming gene of a human neuroblastoma cell line. Proc. Natl. Acad. Sci. USA 80:383.387.

Shomron N., and Levy C. (2009). MicroRNA-biogenesis and pre-mRNA splicing crosstalk. Journal of Medicine and Biotechnology. Articule ID 594678, 6 pages, doi:10.1155/2009/594678

Shu M., Zheng X., Wu S., Lu H., Leng T., Zhu W., Zhou Y., Ou Y., Lin X., Lin Y., Xu D., Yang G. (2011a). Targeting oncogenic miR-335 inhibits growth and invasion of malignant astrocytoma cells. Mol. Cancer 10: 59

Shu M., Zhou Y., Zhu W., Zhang H., Wu S., Chen J., Yan G. (2012). MicroRNA-335 is required for differentiation of malignant glioma cells induced by activation of cAMP/protein kinase A pathway. Mol. Pharmacol. 81(3):292-8

Shu M., Zhou Y., Zhu W., Zhang H., Wu S., Chen J., Yang G. (2011b). MiR-335 is required for differentiation of malignant glioma cells induced by activatin of cAMP/ PKA pathway. Mol. Phaarmacol. PMID: 22172575

Shubbert S., Shannon K., and Bollag G. (2007). Hyperactive Ras in developmental disorders and cancer. Nature Review of Cancer. 7:295-308

Slaby O., Redova M., Proprach A., Nekvindoka J., Illiev R., Radova L., Lakomy R., Svodoba M., Vyzuña R. (2012). Identification of miRNAs associated with early relapse after nephrectomy in renal cell carcinoma patients. Genes Chromosomes Cancer- 51 (7):707-16

Snowdown J., Boag S., Feilotter H., Izard J., Siemmens DR. (2012). A pilot study of urinary microRNA as a biomarker for utothelial cancer. Can. Urol. Assoc. J. 15:1-5 doi: 10.5489/cuaj.11115

Sociedad Española de Oncología Médica, http://www.seom.org

Sohn EJ., Park J., Kang SI., Wu YP. (2012). Accumulation of pre-let-7g and downregulation of mature let-7g with depletion of EWS. Biochem. Biphys. Res. Commun. 426 (1):89-93

Sol-Church K., and Gripp K.W. (2009). The molecular aspects of Costello Syndrome. Monogr Hum Genet Basel, Karger 17:94-103

Song G, Zhang Y, Wang L. (2009). MicroRNA-206 targets notch3, activates apoptosis, and inhibits tumor cell migration and focus formation. J Biol Chem. 2009 Nov 13;284(46):31921-7. Epub 2009 Sep 1. PMID: 19723635

Song T, Zhang X, Wang C, Wu Y, Cai W, Gao J, Hong B. (2011). **MiR-138** Suppresses Expression of Hypoxia-inducible factor 1? (HIF-1?) in Clear Cell Renal Cell Carcinoma 786-O Cells. Asian Pac J Cancer Prev.12(5):1307-11. PMID:

Søvik O, Schubbert S, Houge G, Steine SJ, Norgård G, Engelsen B, Njølstad PR, Shannon K, Molven A.(2009). De novo HRAS and KRAS mutations in two siblings with short stature and neuro-cardio-facio-cutaneous features.BMJ Case Rep. 2009;2009. doi:pii: bcr07.2008.0550. 10.1136/bcr.07.2008.0550. Epub 2009 Jan 23.

Stalhut C., Suarez Y., Lu J., Mishima Y., Giraldez AJ (2012). MiR-1 and miR-206 regulate angiogenesis by modulating VegfA expression in zebrafish. Development. 139 (23): 4356-65.

Steeg P. (2003). Metastasis suppressors alters the signal transduction of cancer cells. Nature Reviews Cancer. 3:55-63.

Steineman D., Tausher M., Praudich L., Niemeyer CM., Flotho C., Schlegelberger B. (2010). Mutations in the let-7 binding site a mechanism of RAS activation in juvenile myelomonocytic leukemia. Haematologica 95(9): 1616

Stornetta RL., and Zhu JJ. (2011). Ras and Rap signaling in synaptic plasticity and metal disorders. Neuroscientsis. 17 (1); 54-78

Stovold R, Meredith SL, Bryant JL, Babur M, Williams KJ, Dean EJ, Dive C, Blackhall FH, White A. (2013). Neuroendocrine and epithelial phenotypes in small-cell lung cancer: implications for metastasis and survival in patients. Br J Cancer. **2013** Apr 30;108(8):1704-11. doi: 10.1038/bjc.**2013**.112. Epub **2013** Mar 21

Su F, Viros A, Milagre C, Trunzer K, Bollag G, Spleiss O, Reis-Filho JS, Kong X, Koya RC, Flaherty KT, Chapman PB, Kim MJ, Hayward R, Martin M, Yang H, Wang Q, Hilton H, Hang JS, Noe J, Lambros M, Geyer F, Dhomen N, Niculescu-Duvaz I, Zambon A, Niculescu-Duvaz D, Preece N, Robert L, Otte NJ, Mok S, Kee D, Ma Y, Zhang C, Habets G, Burton EA, Wong B, Nguyen H, Kockx M, Andries L, Lestini B, Nolop KB, Lee RJ, Joe AK, Troy JL, Gonzalez R, Hutson TE, Puzanov I, Chmielowski B, Springer CJ, McArthur GA, Sosman JA, Lo RS, Ribas A, Marais R. (2012).RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAFinhibitors. N Engl J Med. 2012 Jan 19;366(3):207-15. doi: 10.1056/NEJMoa1105358.

Sugimura K, Miyata H, Tanaka K, Hamano R, Takahashi T, Kurokawa Y, Yamasaki M, Nakajima K, Takiguchi S, Mori M, Doki Y. (2012). Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma. Clin Cancer Res. 2012 Sep 15;18(18):5144-53. doi: 10.1158/1078-0432.CCR-12-0701. Epub 2012 Jul 30.

Sun X., Fan C., Hu LJ., Du N., Xu CW., Ren H. (2012). Role of maintaining characteristics of breast cancer stem cells. Xia Bao YU Fen Zi Mian Yi Xue Za Zhi. 28(8) 789-92.

Sun Y., Bai Y., Zhang F., Wang Y., Guo Y., Guo L.(2010). MiR-126 inhibits non-small cell lung cancer cells proliferation by targeting EGFL7. Biochem. Biophys. Res Commun. 391(3):1483-9.

Svoboda M., Sana J., Fabian p., Kocakova I., Gambosova J., Nekvindova j., Radova K., Vyzula R., Slaby O. (2012). MicroRNA expression profile associated with response to neoadjuvant chemoradiotherapy in locally advanced rectal cáncer patients. Radiation Oncology. 7: 195.

Taipale J., and Beach P.A. (2001). The hedgehog and Wnt signalling pathways in cancer. Nature (411) 394-354.

Tarpwsli M., Schneider G., Amann G., Clark G., Houghton P., Barr FG., Kenner l., Ratajczak MZ., Kucia M. (2012). Ras GRF1 regulates proliferation and metastatic behaviour of human alveolar rhabdomyosarcomas. Int. J. Oncol. 41(3): 995-1004.

Taulli R, Bersani F, Foglizzo V, Linari A, Vigna E, Ladanyi M, Tuschl T, Ponzetto C.(2009). The muscle-specific microRNA **miR-206** blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J Clin Invest. 2009 Aug;119(8):2366-78. doi: 10.1172/JCI38075. Epub 2009 Jul 20. PMID: 19620785

Tavazoie S.F., Alarcón C., Oskarsson T., Padua D., Qiongqing Wang., Paula D. Bos, William L. Gerald and Joan Massague. (2008). Endogenous human microRNAS that suppress breast cancer metastasis. Nature. 451: 147-154

Tidyman W.E., and Rauen K.A. (2009). The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation. Current Opiniong Genetic Development 19 (3): 230-236

Tomassetti M., Staffolani S., Nocchi L., Neuzil J., Stratatella E., Manzella N., Mariotti L., Bracci M., Valentino M., Amati M., Santarelli L. (2012). Clinical significance of circulaing miR-126 quantification in malignant metothlioma patients. Clin. Biochem. 45 (7-8= :575-81.

Tome M., Lopéz-Romero P., Albo C., Sepúlveda JC, Fernández-Gutiérrez B., Dopazo A., Bernad A., González MA. (2011). MiR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. Cell Death Diff. 18 (6); 985-95

Tophkhane C, Yang SH, Jiang Y, Ma Z, Subramaniam D, Anant S, Yogosawa S, Sakai T, Liu WG, Edgerton S, Thor A, Yang X. (2012). p53 inactivation upregulates p73 expression through E2F-1 mediated transcription. PLoS One. 2012;7(8):e43564. doi: 10.1371/journal.pone.0043564. Epub 2012 Aug 30.

Towbin H., Wenter P., Guennewing B., Imig J., Zagalak JA., Gerer AP, Hall (2012). Systematic screens for proteins binding to synthetic microRNA precursosrs. J. Nucleic Acids Res. (Epub ahead of print).

Tso PH, Wang Y, Yung LY, Tong Y, Lee MM, Wong YH.(2013). **RGS19** inhibits Ras signaling through Nm23H1/2-mediated phosphorylation of the kinase suppressor of Ras.Cell Signal. 2013 May;25(5):1064-74. doi: 10.1016/j.cellsig.2013.02.010. Epub 2013 Feb 14.

Urano T., Emkey , R., and Feig L.A. (1996).Ral GTPases mediate a distinct downstream signalling pathway from Ras that facilitates transformation. EMBO J. 15: 810-816

Usacheva A., Tian X., Sandoval- Raudel, Salvi D., Levy D., and Colamonici O. (2003). The WD Motif-Containing Protein RACK-1 Functions as a Scaffold Protein Within the Type I IFN Receptor-Signaling Complex1. The Journal of Immunology 171: 2989–2994

Vairaktaris E., Goutzanis L., Yapijakis C., Vassilliou S., Spyridonidou S., Vylliotis A., Nkenke E., Lazaris AC., Strantzias P., Patsouris E. (2009). Diabets enhances the expression of H-Ras and suppresses the expression of EGFR leading to increased cell proliferation. Histol. Hystopathol. 24 (5): 531-9

Valentijn LJ., Koster J., Versteeg R. (2006). Read through transcript from NM23H1 into the neighborin NM23H2 gene encodes a novel protein N23-LV. Genomics. 87: 483-489.

Van der Auwera I, Limame R, van Dam P, Vermeulen PB, Dirix LY, Van Laere SJ. (2010). Integrated miRNA and mRNA expression profiling of the inflammatory breast cancer subtype. Br J Cancer. 2010 Aug 10;103(4):532-41.PMID: 20664596

Van Wynsberghe PM, Kai ZS, Massirer KB, Burton VH, Yeo GW, Pasquinelli AE. (2011). LIN-28 co-transcriptionally binds primary let-7 to regulate miRNA maturation in Caenorhabditis elegans. Nat Struct Mol Biol. 2011 Mar;18(3):302-8. Epub 2011 Feb 6. PMID: 21297634

Ventura A., and Jacks T. (2009). MicroRNAs and cancer: short-RNAs go along way. Cell. 136:586-591

Vickers MM, Bar J, Gorn-Hondermann I, Yarom N, Daneshmand M, Hanson JE, Addison CL, Asmis TR, Jonker DJ, Maroun J, Lorimer IA, Goss GD, Dimitroulakos J.(2012). Stage-dependent differential expression of microRNAs in colorectal cancer: potential role as markers of metastatic disease.Clin Exp Metastasis. 29(2):123-32. doi: 10.1007/s10585-011-9435-3.

Võsa U, Vooder T, Kolde R, Vilo J, Metspalu A, Annilo T. (2012). Meta-analysis of microRNA expression in lung cancer. Int J Cancer. 2012 Dec 6. doi: 10.1002/ijc.27981. [Epub ahead of print]. PMID: 23225545

Vriens MR., Weng J., Suh I., Huynh N., Guerrero MA., Shen WT., Duh QU., Clarck OH., Kebebew E. (2012). MicroRNA expression profiling is a potential diagnostic tool for thyroid cancer. Cancer. 118 (3): 3426-32

Wang DJ., Legesse-Miller A., Johnson EL., Coller HA. (2012a). Regulation of the let-7a-3 promoter by NF-KB. Plos One 7(2):e31240

Wang H., Wu J., Meng x., Ying X., Zao Y., Liu R, Pan Z, Kang T, Huang w. (2011a). MicroRNA-342 inhibits colorectal cancer cell proliferation and invasion by directly targeting DNA methyltransferase 1. Carcinogenesis. 32 (7):1033-42

Wang J., Ruan K. (2010b). MiR-335 is involved in the rat epididymal development by targeting the mRNA of RASA1. Biochem. Biophys. Res Comm. 402 (2): 222-7

Wang P., Fu T., Wang X., Zhu W. (2010c). Primary study of miRNA expression patterns in laryngeal carcinoma by microarray. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi. 24(12): 535-8

Wang Q, Li P, Li A, Jiang W, Wang H, Wang J, Xie K.(2012b).Plasma specific miRNAs as predictive biomarkers for diagnosis and prognosis of glioma. J Exp Clin Cancer Res. 2012 Nov 22;31(1):97. [Epub ahead of print] PMID: 23174013 [PubMed - as supplied by publisher

Wang Q, Tang H, Yin S, Dong C.(2013). Downregulation of microRNA-138 enhances the proliferation, migration and invasion of cholangiocarcinoma cells through the upregulation of RhoC/p-ERK/MMP-2/MMP-9.Oncol Rep. 2013 May;29(5):2046-52. doi: 10.3892/or.2013.2304. Epub 2013 Feb 26.

Wang Q., Zhong M., Liu W., Li Z., Huang J., Zheng L. (2011b). Alterations of microRNAs in cisplatin resistant human non-small cell lung cancer cells (A549-DD9). Exp. Lung Res. 37 (7):427-34

Wang R., Hu Y., Song G., Hao CJ, Cui Y., Xia HF., Max. (2012c). miR-206 regulates neural cell sproliferation and apoptosis via Otx2 . Cell. Physiol. Biochem 29 (3-4):381-90

Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. (2008a). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell. 2008 Aug;15(2):261-71.

Wang W, Zhao LJ, Tan YX, Ren H, Qi ZT. (2012d) Identification of deregulated miRNAs and their targets in hepatitis B virus-associated hepatocellular carcinoma. World J Gastroenterol. 2012 Oct 14;18(38):5442-53. doi: 10.3748/wjg.v18.i38.5442.

Wang W., Zhao LJ., Tan YX., Ren H., and Qi ZT. (2012e). MiR-138 induces cell cycle arrest by targeting cyclin D3 in hepatocellular carcinoma. Carcinogenesis. 33(5):1113-1120.

Wang X, Ling C, Bai Y, Zhao J. (2011c). MicroRNA-206 is associated with invasion and metastasis of lung cancer. Anat Rec (Hoboken). 294(1):88-92. doi: 10.1002/ar.21287. PMID: 21157919

Wang X., Yao L., Wang Y., Wang X., Liu N., You Y (2012f). Regulation of let-7 and its target of let-7 and its target oncogenes. (Review). Oncology letters 3: 955-960

Wang XY, Wu MH, Liu F, Li Y, Li N, Li GY, Shen SR. (2010c). Differential miRNA expression and their target genes between NGX6-positive and negative colon cancer cells. Mol Cell Biochem. (1-2):283-90. Epub 2010 Sep 22.

Wang Y, Huang JW, Li M, Cavenee WK, Mitchell PS, Zhou X, Tewari M, Furnari FB, Taniguchi T. (2011d). MicroRNA-138 modulates DNA damage response by

repressing histone H2AX expression. Mol Cancer Res. 9(8):1100-11. Epub 2011 Jun 21. PMID: 21693595

Wang Y., Hu X., Greshock J., Shen I., Yang X., Shao z., Liang S., Tanyi JL., Sood AK., Zhang L. (2012g). Genomic DNA copy number alteration of the let-7 family in human cancers. Plos one. 7(9):e44399..

Wang YX, Zhang XY, Zhang BF, Yang CQ, Chen XM, Gao HJ. (2010d). Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis. J Dig Dis.(2010) Feb;11(1):50-4. doi: 10.1111/j.1751-2980.2009.00413.x. PMID: 20132431 [PubMed - indexed for MEDLINE]

Waris G., and Absan H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. Journal of carcinogenesis. 5:14 Doi:10.11861 1477-3163-5-14

Watahari A., Wang Y., Morris J., Dennis K., O` Dwyer HM., Gleave M., Gout PW., Wang Y. (2011). MicroRNAs associated with metastatic prostate cancer. Plos One. 6(9):e24950.

Watanabe S, Ueda Y, Akaboshi S, Hino Y, Sekita Y, Nakao M. (2009). HMGA2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells. Am J Pathol. 2009 Mar;174(3):854-68. Epub 2009 Jan 29.

Wei Y, Nazari-Jahantigh M, Chan L, Zhu M, Heyll K, Corbalán-Campos J, Hartmann P, Thiemann A, Weber C, Schober A. (2013). The microRNA-342-5p Fosters Inflammatory Macrophage Activation Through an Akt1- and microRNA-155-Dependent Pathway During Atherosclerosis. Circulation. 2013 Apr 16;127(15):1609-19. doi: 10.1161/CIRCULATIONAHA.112.000736. Epub 2013 Mar 19

Weigelt B., Geyer F.C., Reis-Filho J. S. (2010). Histological types of breast cancer: how special are they?. Molecular Oncology. 4: 192-208

Wennerber K., Rossman K.L., and Der C.L. (2005).The Ras superfamily at glance. J. Cell Sci.118:843-846.

White NM., Bao TT., Grigull J., Youssef YM., Girgis A., Diamandis M., Fatoohi E., Metias M., Honey RJ., Stewart R., Pace KT., Bjarnason GA., Yousef GM. (2011). MiRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. J. Urol. 186 (3): 1077-83

Winbanks CE, Wang B, Beyer C, Koh P, White L, Kantharidis P, Gregorevic P. (2011). TGF-beta regulates **miR-206** and miR-29 to control myogenic differentiation through regulation of HDAC4. J Biol Chem. 2011 Apr 22;286(16):13805-14. Epub 2011 Feb 15. PMID: 21324893

Winter J., Jung S., Séller S., Gregory R.i., and Diederich S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature Cell Biology. Vol 11 (3): 228-234.

Wong TS, Man OY, Tsang CM, Tsao SW, Tsang RK, Chan JY, Ho WK, Wei WI, To VS. (2011). MicroRNA let-7 suppresses nasopharyngeal carcinoma cells proliferation through downregulating c-Myc expression. J Cancer Res Clin Oncol. 2011 Mar;137(3):415-22. Epub 2010 May 4.

Wong TS., Liu XB., Wong BY., NG RW., Yuen AP., Wei WI. (2008). Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin. Cancer Res. 4(9): 2588-92

World Health Organization. (2011). International Agency for Researcher on Cancer. Cancer Stats. Cancer Worldwide. Cancer Research U.K

Wu H., Ye C., Ramirez D., Manjunath N. (2009). Alternative processing of primary microRNA transcripts by Drosha generates 5' end variation of mature microRNA. PLos One 4(10): e7566. doi:10.1371/journal.pone.0007566

Wu J, Meng J, Du Y, Huang Y, Jin Y, Zhang J, Wang B, Zhang Y, Sun M, Tang J. (2013). RACK1 promotes the proliferation, migration and invasion capacity of mouse hepatocellular carcinoma cell line in vitro probably by PI3K/Rac1 signaling pathway. Biomed Pharmacother. 2013 Feb 18. pii: S0753-3322(13)00014-0. doi: 10.1016/j.biopha.2013.01.011. [Epub ahead of print]

Wu Y., Li XF., Yang JH., Liao XY., Chen YZ. (2012a). MicroRNA expression profile in acute promyelocytic leukaemia cell differentiation induced by all-trans retinoic acid and arsenic trioxide. Zhonghua Xue Ye Xue Za Zhi. 33 (7):546-51

Wu Z., Sun L., Wang H., Yao J., Jiang c., Xu W., Yang Z. (2012b). MiR-328 expression is decreased by high grade gliomas and is associated with worse survival in primary glioblastoma. Plos One. 7(10): e47270

Xia C., Meng Q., Liu L., Rojanasakul Y., Wang X., and Jiang B. (2007). Reactive oxygen species regulated angiogenesis and tumor growth through vascular endothelial growth factor. Cancer Research. 67:10823-10830.

Xu C, Fu H, Gao L, Wang L, Wang W, Li J, Li Y, Dou L, Gao X, Luo X, Jing Y, Chim CS, Zheng X, Yu L. (2012a). BCR-ABL/GATA1/miR-138 mini circuitry contributes to the leukemogenesis of chronic myeloid leukemia..Oncogene. doi: 0.1038/onc.2012.557. [Epub ahead of print]. PMID: 23208504 [PubMed - as supplied by publisher].

Xu Y., Zhao F., Wang Z., Song Y., Luo Y., Zhang X., Jiang L., Son Z., Miao Z. Xu H. (2012b). MicroRNA-335 acts as a metastasic suppressor in gastric cancer by targeting Bcl-w and specific protein 1. Oncogen 15 (11):1398-1407

Xu Y., Zhao F., Wang Z., Song Y., Luo Y., Zhang X., Jiang L., Sun Z., Miao Z., Xu H. (2011). MicroRNA-335 acts as a metastasis suppressor in gastric cancer by targeting Bcl-w and specificity protein. Oncogene. 31 (11):1398-1407.

Yadav V, Denning MF. (2011) Fyn is induced by Ras/PI3K/Akt signaling and is required for enhanced invasion/migration). Mol Carcinog. 2011 May;50(5):346-52. doi: 10.1002/mc.20716. Epub 2010 Dec 10.

Yamada Y., Hidaka H., Seki N., Yoshiro H., Yamasaki T., Itesako T., Nakagawa M., Enokida H. (2012) Tumor suppressive microRNA-135 a inhibits cancer cell proliferation by targeting the c-Myc oncogene in renal cell carcinoma. Cancer Sci. (Epub ahead of print).

Yan D, Dong Xda E, Chen X, Wang L, Lu C, Wang J, Qu J, Tu L.(2009). MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. J Biol Chem. 2009 Oct 23;

Yan Z, Xiong Y, Xu W, Gao J, Cheng Y, Wang Z, Chen F, Zheng G.(2012a). Identification of hsa-miR-335 as a prognostic signature in gastric cancer. PLoS One. 2012;7(7):e40037. doi: 10.1371/journal.pone.0040037. Epub 2012 Jul 3. PMID: 22802949 [PubMed - indexed for MEDLINE] Free PMC Article

Yang J., Zhang Q., Dong JQ., Chang XH., He XJ. (2012b). Overexpression of high mobility group AZ and its correlation with microRNA let-7 family in serous ovarian cancers. Beijing Da Xue Xue Bao. 44 (5):749-54

Yang R, Dick M, Marme F, Schneeweiss A, Langheinz A, Hemminki K, Sutter C, Bugert P, Wappenschmidt B, Varon R, Schott S, Weber BH, Niederacher D, Arnold N, Meindl A, Bartram CR, Schmutzler RK, Müller H, Arndt V, Brenner H, Sohn C, Burwinkel B. (2011). Genetic variants within **miR-126** and miR-335 are not associated with breast cancer risk. Breast Cancer Res Treat. 127(2):549-54.

Yang X, Lin X, Zhong X, Kaur S, Li N, Liang S, Lassus H, Wang L, Katsaros D, Montone K, Zhao X, Zhang Y, Bützow R, Coukos G, Zhang L.(2010). Double-negative feedback loop between reprogramming factor LIN28 and microRNA let-7 regulates aldehyde dehydrogenase 1-positive cancer stem cells. Cancer Res 70(22):9463-72. Epub 2010 Nov 2. PMID: 21045151

Ye D, Wang G, Liu Y, Huang W, Wu M, Zhu S, Jia W, Deng AM, Liu H, Kang J.(2012). MiR-138 promotes induced pluripotent stem cell generation through the regulation of the p53 signaling. Stem Cells. 2012 Aug;30(8):1645-54. doi: 10.1002/stem.1149. PMID: 22696098 [PubMed - in process]

Yeh YM, Chuang CM, Chao KC, Wang LH.(2013)MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1α Int J Cancer. 2013 Feb 6. doi: 10.1002/ijc.28086. [Epub ahead of print] PMID:23389731

Yeo G., Holste D., Kreiman G., and Burge C.B. (2004).Variation in alternative splicing across human tissues. Genome Biol. 5:R74

Yip L, Kelly L, Shuai Y, Armstrong MJ, Nikiforov YE, Carty SE, Nikiforova MN. (2011). MicroRNA signature distinguishes the degree of aggressiveness of papillary thyroid carcinoma. Ann Surg Oncol. Jul;18(7):2035-41. Epub 2011 May 3. PMID: 21537871

Yoshikawa T., Rae V., Brunis-Slot W., Van den Berg J., Taylor J.R., and Streilein J.W. (1990). Susceptibility to effects of UVB radiation on induction of contact hypersensibility as a risk factor for skin cancer in humans. The Journal of investigate dermatology. 95 (5): 530-536

Yoshimoto N, Toyama T, Takahashi S, Sugiura H, Endo Y, Iwasa M, Fujii Y, Yamashita H. (2011). Distinct expressions of microRNAs that directly target estrogen receptor α in human breast cancer. Breast Cancer Res Treat. (1):331-9. PMID: 41755340

Young-Lee M., Jeong W., Oh J., Choi K. (2009). NM23H2 inhibits EGF- and Ras-induced proliferation of NIH3T3 cells by blocking the ERK pathway. Cancer Letters. 275:221-226.

Yu J., Cai X., He J., Zhao W., Wang Q., Liu B. (2012). Microarray-based analysis of gene regulation by transcription factors and microRNA in glioma. Neurol. Sci. (Epub ahead of print).

Yuan Y., Chen HZ., Li BL., Gong DJ., Zhuang CW., Wang SG., Zeng ZY., Huang SD. (2012). MicroRNA-98 and microRNA-214 post-transcriptionally regulate enhancer of zeste homolog 2 and inhibit migration and invasion in human esophageal squamous cell carcinoma. Mol. Cancer. 11(1):51.

Zaika E, Wei J, Yin D, Andl C, Moll U, El-Rifai W, Zaika AI. (2011). p73 protein regulates DNA damage repair.FASEB J. **2011** Dec;25(12):4406-14. doi: 10.1096/fj.11-192815. Epub **2011** Sep 2.

Zaman MS., Maher DM., Khan S., Jaggi M., Chauhan SC. (2012). Current status and implications of microRNAs in ovarian cancer diagnosis and therapy. J. Ovarian Res.5(1):44.

Zampetaki A., Kiechl S., Drozdor I., Willeit P., Mayr U., Prokopi M., Myur A., Weger S., Oberhollenzer F., Bonova E., Shan A., Willeit j., Mayr M. (2010). Plasma microRNA profiling reveals lost of endothelial miR-126 and other microRNAs in type 2 diabetes. Circulation Research. 107 (6): 810-7

Zaravinos A., Radojicic J., Lambrou GL., Volaris D., Delakas D., Stathopoulus EN., Spandidos D. (2012). Expression of microRNAs involved in angiogenesis tumor proliferation, tumor suppressor inhibition, epitelial-mesenchymal transition and activation of metastasis in bladder cáncer. J. Urol. 188 (2):615-23

Zeng Y. (2006). Principles of micro-RNA production and maturation. Oncogene. 25: 6156-6162

Zhang R., He Y., Zhang X., Xing B., Sheng Y., Lu H., Hei Z. (2012b). Estrogen receptor-regulated contribute to the BCL-2/BAX in endometrial adenocarcinoma and precancerous lesions. Cancer Lett. 4(2): 155-65

Zhang S., Kim K., Jin UH., Pfent C., Cao H., Amendt B., Liu X., Wilson-Robles H., Safe S. (2012c). Aryl Hydrocarbon receptor agonists induce microRNA-335 expression and inhibit lung metastasis of estrogen receoptor negative breast cancer cells. Mol. Cancer Ther. (1):108-18

Zhang T., Liu M., Wang C., Lin C., Sun Y., and Jin D. (2012d). Downregulation of miR-206 promotes proliferation and invasion of laryngeal cancer by regulating VEGF-expression. Anticancer Res. 31(11)3859-63

Zhang B., Pan X., Cobb G., and Anderson TA. (2007). MicroRNAs as oncogenes and tumor suppressors. Developmental Biology. 302:1-12

Zhang H, Zhang H, Zhao M, Lv Z, Zhang X, Qin X, Wang H, Wang S, Su J, Lv X, Liu H, Du W, Zhou W, Chen X, Fei K. (2013a). **MiR-138** inhibits tumor growth through repression of EZH2 in non-small cell lung cancer. Cell Physiol Biochem. 2013;31(1):56-65. doi: 10.1159/000343349. Epub 2013 Jan 15. PMID: 23343715

Zhang J, Du YY, Lin YF, Chen YT, Yang L, Wang HJ, Ma D. (2008). The cell growth suppressor, mir-126, targets IRS-1. Biochem Biophys Res Commun. 377(1):136-40. Epub 2008 Oct 1. PMID: 18834857

Zhang L, Liu X, Jin H, Guo X, Xia L, Chen Z, Bai M, Liu J, Shang X, Wu K, Pan Y, Fan D. (2013b). **miR-206** inhibits gastric cancer proliferation in part by repressing cyclinD2.Cancer Lett. 2013 May 10;332(1):94-101. doi: 10.1016/j.canlet.2013.01.023. Epub 2013 Jan 21.

Zhang W, Winder T, Ning Y, Pohl A, Yang D, Kahn M, Lurje G, Labonte MJ, Wilson PM, Gordon MA, Hu-Lieskovan S, Mauro DJ, Langer C, Rowinsky EK, Lenz HJ. (2011b). A let-7 microRNA-binding site polymorphism in 3'-untranslated region of KRAS gene predicts response in wild-type KRAS patients with metastatic colorectal cancer treated with cetuximab monotherapy. Ann Oncol. 2011 Jan;22(1):104-9. Epub 2010 Jul 5. PMID: 20603437

Zhang W., Qian JX., Yi HL., Yang ZD., Wang CF., Chen JY., Wei XZ., Fu Q., Ma H. (2012e). The microRNA-29 plays a central role in osteosarcoma pathogenesis and progression. Mol. Biol. 46(4): 622-7

Zhang X., Zhang Z., Cheng J., Li M., Wang W., Xu W., Wang H., Zhang R. (2012f). Transcription factor NFAT1 activates the medm2 oncogene independent of p53. J. Biol. Chem. (Epub ahead of print)

Zhang XY., Ding JX., Tao Z., Hua KQ. (2012g). FSH stimulates expression of the embryonic gene HMGA2 by regulating let-7 in normal fimbrial epithelial cells in ovarian high-grade serous carcinomas. Exp. Ther. Med. 5(1):350-354

Zhao L., Sun Y., Hou Y., Peng Q., Wang L., Luo H., Tang X., Zeng Z., Liu M. (2012). MicroRNA expression analysis of cancer associated fibroblasts and normal fibroblast in breast cancer. Int. J. Biochem. Cell. Biol. 44 (11):2051-9.

Zhao X, Yang L, Hu J, Ruan J. (2010). MiR-138 might reverse multidrug resistance of leukemia cells.Leuk Res. 2010 Aug;34(8):1078-82. doi: 10.1016/j.leukres.2009.10.002. Epub 2009 Nov 6.

Zhao Y, Deng C, Lu W, Xiao J, Ma D, Guo M, Recker RR, Gatalica Z, Wang Z, Xiao GG. (2011). Let-7 microRNAs Induce Tamoxifen Sensitivity by Down-Regulation of Estrogen Receptor Alpha Signaling in Breast Cancer. Mol Med. doi: 10.2119/molmed.2010.00225. [Epub ahead of print] PMID: 21826373

Zhou CH., Yang SF., Li PQ. (2012). Human lung cancer cell line SPC-A1 contains cells with characteristics of cancer stem cells. Neoplasm. 59 (6):685-92

Zhou J, Tian Y, Li J, Lu B, Sun M, Zou Y, Kong R, Luo Y, Shi Y, Wang K, Ji G.(2013). **miR-206** is down-regulated in breast cancer and inhibits cell proliferation through the up-regulation of cyclinD2.Biochem Biophys Res Commun. 2013 Apr 5;433(2):207-12. doi: 10.1016/j.bbrc.2013.02.084. Epub 2013 Mar 1.

Zhou L, Chen J, Li Z, Li X, Hu X, Huang Y, Zhao X, Liang C, Wang Y, Sun L, Shi M, Xu X, Shen F, Chen M, Han Z, Peng Z, Zhai Q, Chen J, Zhang Z, Yang R, Ye J, Guan Z, Yang H, Gui Y, Wang J, Cai Z, Zhang X. (2010). Integrated profiling of microRNAs and mRNAs: microRNAs located on Xq27.3 associate with clear cell renal cell carcinoma. PLoS One. 2010. ;5(12):e15224.

Zhu DX., Zhu W., Fang C., Fan L., Zou ZJ., Wang YH., Liu P., Hong M., Miao kr., Liu P., Xu w., Li JY. (2012a). miR-181 a/b significantly enhances drug sensitivity in chronic lymphocytic leukaemia cells via targeting multiple anti-apoptosis genes. Carcinogenesis. 33(7):1294-301

Zhu H., Fan GC. (2011a). Role of microRNAs in the reperfused myocardium towards post-infarct remodeling. Cardiovascular Res. 95(3):165-167.

Zhu N, Zhang D, Xie H, Zhou Z, Chen H, Hu T, Bai Y, Shen Y, Yuan W, Jing Q, Qin Y. (2011b). Endothelial-specific intron-derived miR-126 is down-regulated in human breast cancer and targets both VEGFA and PIK3R2. Mol Cell Biochem. 351(1-2):157-64. Epub 2011 Jan 20. PMID: 21249429

Zhu X., Li H., Long L., Hui L., Chen H., Wang X., Shen H., Xu w. (2012b). miR-126 enhances the sensivity of non-small cell lung cancer cells to anticancer agents by targeting vascular endothelial growth factor A. Acta Biochimi Biophys. Sin (Shangai) 44(6):519-26

Zhu XM., Wu LJ., Xu J., Yang R., Wu FS. (2011c). Let-7 microRNA expression and clinical significance in hepatocellular carcinoma. J. Int. Med. Res. 39(6): 2323-9

Zhu J, Cui G, Chen M, Xu Q, Wang X, Zhou D, Lv S, Fu L, Wang Z, Zuo J. (2013). Expression of R132H Mutational IDH1 in Human U87 Glioblastoma Cells Affects the SREBP1a Pathway and Induces Cellular Proliferation.

Zou J, Li WQ, Li Q, Li XQ, Zhang JT, Liu GQ, Chen J, Qiu XX, Tian FJ, Wang ZZ, Zhu N, Qin YW, Shen B, Liu TX, Jing Q. (2011). Two functional microRNA-126s repress a novel target gene p21-activated kinase 1 to regulate vascular integrity in zebrafish.Circ Res. 2011 Mar 4;108(5):e10. PMID: 21148433

Zu Y., Ban J., Xia Z., Wang J., Cai Y., Ping W., Sun W. (2012). Genetic variation in a miR-335 binding site in BIRC5 alters susceptibility to lung cancer in Chinese Han populations. Biochem., Biophys., Res. Commun. (Epub ahead of print).