Angiogenesis and Pancreatic Cancer: A role for tissue plasminogen activator (tPA)

A thesis presented by **RAM MOHAN.P.R**

in fulfillment of the requirements for

the degree of *Doctor of Philosophy* in Biomedicine at

the Department of Experimental and Health Sciences,

Faculty of Health and Life Sciences

Universitat Pompeu Fabra

This thesis was performed under the supervision of

Dr. Navarro Medrano Pilar in the Cancer Research Programme,

IMIM (Institut de Recerca Hospital del Mar)

Barcelona, May 2011

Ram Mohan. P.R
(PhD Candidate)

Navarro Medrano Pilar
(Thesis Supervisor)

Dedicated to my everloving Grandma Late Smt. C.P. Bharathy Amma

ACKNOWLEDGEMENTS

This thesis is a reflection of a great amount of help and support shown to me over these years by a large number of people.

I would firstly like to thank my supervisor Dr Navarro Medrano Pilar, for giving me the chance to be her PhD student, for all her support, guidance and kind advice throughout the years. I am grateful for the patience she showed and for her constructive criticism during the preparation of this manuscript. Her help was valued and much appreciated. I am very grateful for her patience, motivation, enthusiasm, and that, taken together, make her a great mentor.

I must thank the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) for my postgraduate scholarship which enabled me to undertake and complete my PhD . I would like to thank the Department of Cancer Biology, IMIM for providing the extra support I needed. The work was also supported from research grants SCII-FEDER(PI080421) from MICINN, Fundació La MaratóTV3 (051110) and Generalitat de Catalunya (2009SGR1409) to Pilar Navarro. Financial help for the publication of the thesis from Generalitat de Catalunya, Fundacio IMIM, and UPF is highly appreciated.

I would also like to thank Dr. Elena Ortiz for her helpful suggestions and for the protocols at the start of my molecular

biology work. She was very kind and found time in helping me inspite of her busy experimental schedules.

I thank Dr. David Pineda Tomas for his valuable moral support and helping with the experiments. He was very friendly and always had time to give me valuable tips in scientific and non-scientific side !

Special thanks to Neus Martinez Bosch for her moral support and for the good times we had.

Thank you to Professors, Inmaculada Hernández Muñoz,Anouchka Skoudy, Xavier, Antonio for their assistance.

Last but not least, very special thanks to Mohammad Massumi for his friendship, for all his support during my PhD years.. Thank you to Fabien Delaspre, Carles Romero for the fun we had during my early times.

You people have all been a great and generous family to me!

To my dear Parents and brother, thank you for all your love, support and encouragement over these years. Thanks for being there when I needed you most. I specially remember my late grandmother who always wished to see the best in me. Finally, I need to thank my fiancée Ms. Gayathri for her constant support and love during the last stages of my PhD.

INTRODUCTION.. **18**

I.1 The Pancreas and Pancreatic Cancer.............................. **18**

I.1.1 *PDAC origin, characteristics, and molecular alterations.* **19**

I.1.1 *PDAC Treatments*... **24**

I.2 Angiogenesis and its molecular mechanisms……….. **26**

I.2.1 Soluble Factors in regulating Angiogenesis……………. **29**

I.2.1.1 *Vascular Endothelial growth factor*…………………….. **29**

I.2.1.2 *Angiopoietins and Tie receptors*……………………… **30**

I.2.1.3 *Fibroblast growth factor*………………………………. **31**

I.2.1.4 *Transforming Growth Factor-Beta*…………………….. **32**

I.2.1.5 *Interleukins*…………………………………………… **32**

I.2.1.6 *Matix Metalloproteinase's (MMPs)*……………………. **32**

I.3 Angiogenesis in Pancreatic Cancer………………… **34**

I.3.1 *Hypoxia in Pancreatic Cancer*……………………………. **40**

I.3.2 *Angiogenesis Inhibitors in PDAC*……………………… **42**

I.4 Plasminogen System………………………………… **45**

I.4.1 Components of Plasminogen system............................. **46**

I.4.1.1 *Plasminogen and Plasmin*……………………………. **46**

I.4.1.2 *The Urokinase-type Plasminogen activator*………….. **47**

I.4.1.3 *The Tissue- type Plasminogen activator*………………. **49**

I.4.1.4 *The Plasminogen activator inhibitors*…………………. **51**

I.4.1.4. 1 *Plasminogen activator inhibitor type-1*...................... **51**

I.4.1.4.2 *Plasminogen activator inhibitor type-2*...................... **52**

I.4.2 *The Plasminogen system in Cancer……………………* **53**

I.4.3 *The Plasminogen system and Angiogenesis…………….* **55**

I.4.4 *Tissue Plasminogen Activator in Cancer and PDAC….* **58**

I.5 Galectins………………………………………………… **62**

I.5.1 *Galectin Expression……………………………………..* **63**

I.5.2 *Galectin-1………………………………………………………* **64**

I.5.3 *Galectin-1 in Cancer…………………………………..............* **65**

I.5.4 *Galectin-1 in Pancreatic Cancer……………………………* **66**

I.5.5 *Galectin-1 in Angiogenesis………………………………….* **67**

OBJECTIVES... **70**

RESULTS... **72**

R.1 *Effect of tPA on Angiogenic factors*... **72**

R.2 *tPA induces endothelial cell Proliferation, Migration and Tubulogenesis* **75**

R.3 *tPA induces catalytic-independent activation of ERK1/2, AKT and JNK signalling pathways in Ecs*............................... **78**

R.4 *ERK1/2, AKT and JNK signalling pathways are responsible for tPA-mediated EC Proliferation, Migration and Tubulogenesis*.. **82**

R.5 *AnnexinA2, EGFR and Galectin-1 are involved in tPA-mediated signalling activation in endothelial cells*.................. **87**

R.6. *TGF-β and IL-1 induce tPA in HUVEC and PDAC cells..* **93**

R.7.*Hypoxia induces tPA in PDAC cells……………......................* **95**

DISCUSSION.. **98**

D.1 *tPA can have an indirect pro-angiogenic effect by inducing MMP-9 expression in tumoral pancreatic and endothelial cells* ……….…………………………………….…..... **98**
D.2 *tPA induces endothelial cell proliferation, migration and tubulogenesis by activation of ERK1/2, AKT and JNK signaling pathways and independently of tPA proteolytic activity* ………………... **101**
D.3 *Annexin A2, EGFR and Galectin-1 are involved in binding of tPA to initiate the activation of signaling pathways in endothelial cells* ………………………………………. **105**
D.4 *Cytokines (IL-1α and TGFβ) induce tPA expression in Pancreatic cancer and Endothelial cell lines* ………………………………….................................. **107**
D.5 *Hypoxia induces tPA expression in Pancreatic cancer cell lines* ………….. **109**
CONCLUSIONS…………………………………………… **112**
EXPERIMENTAL PROCEDURES................................. **113**
E.P.1 Cell Line Culture.. **113**
E.P.2 Cell treatments... **114**
E.P.3 siRNA oligonucleotde transfection.............................. **114**
E.P.4 shRNA oligonucleotde transfection............................ **116**
E.P.5 Total RNA isolation.. **117**
E.P.6 Semi Quantitative RT-PCR... **118**
E.P.7 Quantitative RT-PCR.. **122**
E.P.8 Protein Extraction... **124**
E.P.9 Western Blots.. **126**
E.P.10 Caesin Zymography.. **129**
E.P.11 Gelatin Zymography... **130**

E.P.12 Tubule formation Assay... 131
E.P.13 Proliferation Assay 132
E.P.14 Migration Assay... 133
REFERENCES.. 134

ABREVIATIONS AND ACRONYMS INDEX

AKT: AKT8 virus oncogene cellular homolog
AnxA2: Annexin A2
bp: Base Pair
BSA: Bovine Serum Albumin
cDNA: Complementary DNA
CSC: Cancer Stem cells
DMEM: Dulbescco's Modified Eagle Medium
DMSO: Dimethyl Sulfoxide
DNA: Deoxyribonucleic acid
EC: Endothelial Cells
ECM: Extracellular Matrix
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
ERK: Extracellular-Regulated Kinase
FBS: Fetal Bovine Serum
FGF: Fibroblast Growth Factor
Gal-1: Galectin 1
HGH: Hepatocyte Growth Factor
HPRT: Hypoxanthine Guanine Phosphoribosyl Transferase
IF: Immunoflouresence
IGF: Insulin Growth Factor
IL: Interleukin
IP: Immunoprecipitation
JIP: JNK peptide inhibitor
JNK: Jun N-terminal kinase
kDa: Kilo Dalton
KO: Knock out
LPS: Lipopolysacchride
mAb: Monoclonal Antibody
MAPK: Mitogen-Activated Protein Kinase
MMP: Matrix metalloproteinase
mRNA: Messenger RNA
P-AKT: Phosphorylated AKT8 virus oncogene cellular homolog protein kinase
PBS: Phosphate Bufffered Saline
PDGF: Platelet Derived Growth Factor Receptor
P-ERK: Type I transmembrane ER-resident protein kinase
P-JNK: Phosphorylated Jun N-terminal kinase
qRT-PCR: Quantitative RT- PCR

SDS: Sodium Dodecyl Sulphate
shRNA: Short Hairpin RNA
siRNA: Short Interference RNA
TGFβ: Transforming Growth Factor Beta
TNFα: Tumor Necrosis Factor Alpha
tPA: Tissue type Plasminogen Activator
uPA: Urokinase Plasminogen Activator
uPAR: Urokinase Plasminogen Activator Receptor
UTR: Untranslated Region
VEGF: Vascualar Endothelial Growth Factor
VEGFR: Vascualar Endothelial Growth Factor Receptor
WB:WesternBlot
Wo: Wortmannin

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the fifth leading cause of cancer death in the developed countries and one of the most aggressive human tumors. Despite the key contribution of angiogenesis – the growth of new vessels from pre-existing ones- to the progression and spread of many cancers, its role in PDAC has been poorly characterized. Tissue plasminogen activator (tPA), a multifunctional protein regulating a broad range of cellular functions, has been reported to exert pro-angiogenic effects in *in vivo* models of PDAC, although the underlying molecular mechanism has not been analyzed. This work aims to elucidate the role of tPA in the angiogenesis of PDAC as well as to identify the factors responsible for tPA increase in pancreatic cancer. First, we demonstrated that tPA pro-angiogenic effects are both indirect and direct. On the one hand, tPA does not change the levels of the pro-angiogenic molecules VEGF, TGF-β, IL-1 or IL-8 produced by pancreatic tumoral cells or endothelial cells, but it is involved in MMP-9 –a potent stimulator of angiogenesis- upregulation and activation in pancreatic and endothelial cells, suggesting that this matrix metalloproteinase can indirectly mediate tPA angiogenic effects. On the other hand, we found that tPA, in a catalytic-independent way, directly promotes endothelial cell proliferation, migration and tubulogenesis. These direct effects of tPA are mediated by activation of ERK1/2, AKT and JNK signaling pathways in endothelial cells. In addition, using siRNA technology or chemical inhibitors, we found that AnnexinA2, Galectin-1 and

EGFR are required for tPA-mediated signaling activation in endothelium. Finally, we found that inflammatory cytokines and hypoxia, two hallmarks of PDAC and also angiogenic stimuli, lead to a sharp increase in tPA levels in pancreatic tumoral cells. These data support a feed-back loop between proangiogenic stimuli present in both tumoral and stromal cells and the increase of the proangiogenic molecule tPA.

INTRODUCTION

I.1 The Pancreas and Pancreatic Cancer

Pancreas is an elongated dual functioning gland which lies in the abdominal cavity consisting of both endocrine and exocrine units which help in the glucose metabolism and food digestion. It is located deep in the abdomen sandwiched between the stomach and the intestine **Fig.I.1**. Pancreas is described having a head,body and tail. The exocrine unit of the pancreas is composed of acinar cells which produce and secrete zymogens which pass through the duct and into the gastro intestinal tract which then add mucous and bicarbonate to the enzyme mixture. The centroacinar cells are found at the junction of the ducts and the acini. The endocrine units,or islets of Langerhans,are most numerous in the tail region of pancreas. The hormones they produce are secreted into the blood. The major endocrine cell types present are α, β, δ and PP cells which secrete glucagon, insulin, somatostatin, and pancreatic polypeptide respectively.

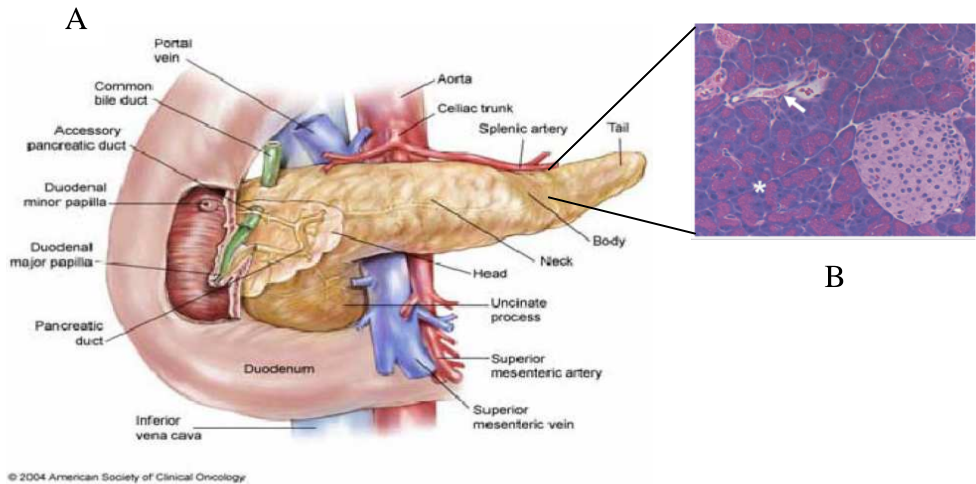


Fig.I.1.- A. Morphological structure and location a of Pancreas in the body. B Hematoxylin and eosin staining of the pancreas with its major components: an islet of Langerhans in the lower right side, acini (asterisk), and a duct (arrow).

Pancreatic cancer has one of the poorest prognoses of all cancers. Currently, it is the fourth leading cause of cancer-related deaths in Western industrialized countries, and the incidence has been increasing throughout the past decades [1]. Pancreatic ductal adenocarcinoma (PDAC) is by far the most frequent type of pancreatic cancer accounting for approximately 85% of all pancreatic tumors [2].

I.1.1 PDAC origin, characteristics, and molecular alterations.

The ductal morphology of PDAC led to postulate that ductal cells were at the origin of transformation. Supporting this hypothesis, PDAC occurs with high frequency in association with dysplastic and hyperplastic ductal lesions [4 ,5]. However, other data using experimental animal models have suggested that PDAC may derive

from acinar, endocrine or centroacinar cells [223]. In addition, independently of their origin, putative cancer stem cells, defined by their ability to self-renew, to differentiate into the bulk tumor population and their potential for tumor formation have been recently identified from human PDAC using cell surface markers [23,24].

The risk factor known for PDAC includes advance age, smoking, constantly recurring chornic pancreatitis, diabetes and obesity [6-7]. A recent analysis of 24 pancreatic cancers suggested that the mature pancreatic cancer cell carries on an average 63 genetic alterations per cancer [8]. Non invasive precurson lesions have also been identified, being able to link multi step progression of pancreatic cancer with the subsequent genetic alterations.

Three different ductal preneoplastic lesions have been described: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMNs). Common and distinct molecular events have been found among these lesions, suggesting that each precursor lesion may reflect variations leading to malignant transformation [10] (**Fig.I.2**). Activating mutations of K-RAS can be found in 30% of early neoplasms but are present in almost 100% of advanced PDAC's indicating a central role for K-RAS mutations in tumorigenesis [11]. Mutations in K-RAS, including the frequent glycine to aspartate mutation at codon 12 are most frequently located near or at the catalytic site of the molecule, thereby reducing

the rate of GTP hydrolysis. K-RAS activation seems to be essential for PDAC maintenance as has been demonstrated by dominant negative mutants as well as RNAi knockdown studies [12, 13]. In addition to K-RAS activating mutations, loss of expression of tumor suppressor genes has also been reported in PDAC, as CDKN2A (80-95% of PDAC, [11] or SMAD/DPC4 (30 % of pancreatic cancers [14].

Desmoplasia is a hallmark in pancreatic cancer [3], in which extracellular matrix, stromal fibroblasts, inflammatory cells and proliferating endothelial cells are accumulated surrounding the tumor epithelial cells [4]. The pancreatic stellate cells (myofibroblast) play a critical role in the formation and turn over of the stroma. On activation of growth factors such as transforming growth factor-1 (TGF-β1), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF), these cells secrete collagen and other components of extracellular matrix. In addition, stellate cells also appear to be responsible for the poor vascularisation that is characteristic of pancreatic cancer. Other factors produced by fibroblasts, immune and inflammatory cells, adipocytes, etc. are involved in tumor- stroma interaction and may contribute to the development of pancreatic cancer, as shown in **FigI.3**

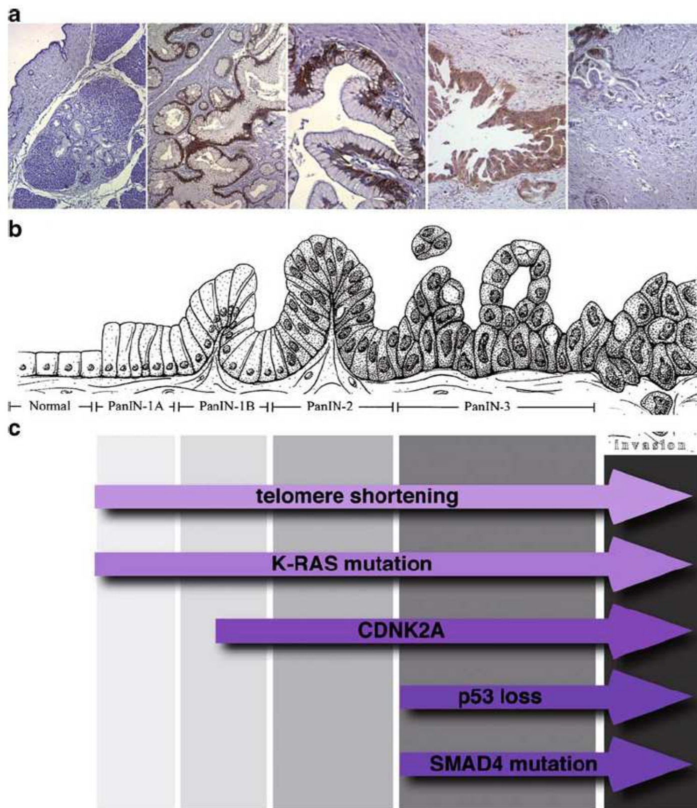


Fig.I.2.-PanIN-to-PDAC progression. As pancreatic ductal cells acquire successive molecular alterations, they develop from low-grade PanINs to high-grade PanINs. a) Histopathological images of normal pancreatic parenchyma, consecutive PanIN lesions with progressive histological changes from PanIN1, 2, 3 and to PDAC. b) Schematic drawing of the histopathological features. c) arrows indicating some alterations occur early and some late. Adapted from Maitra *et a*l, Mod Pathol, 2003. 16(9):p. 902-12

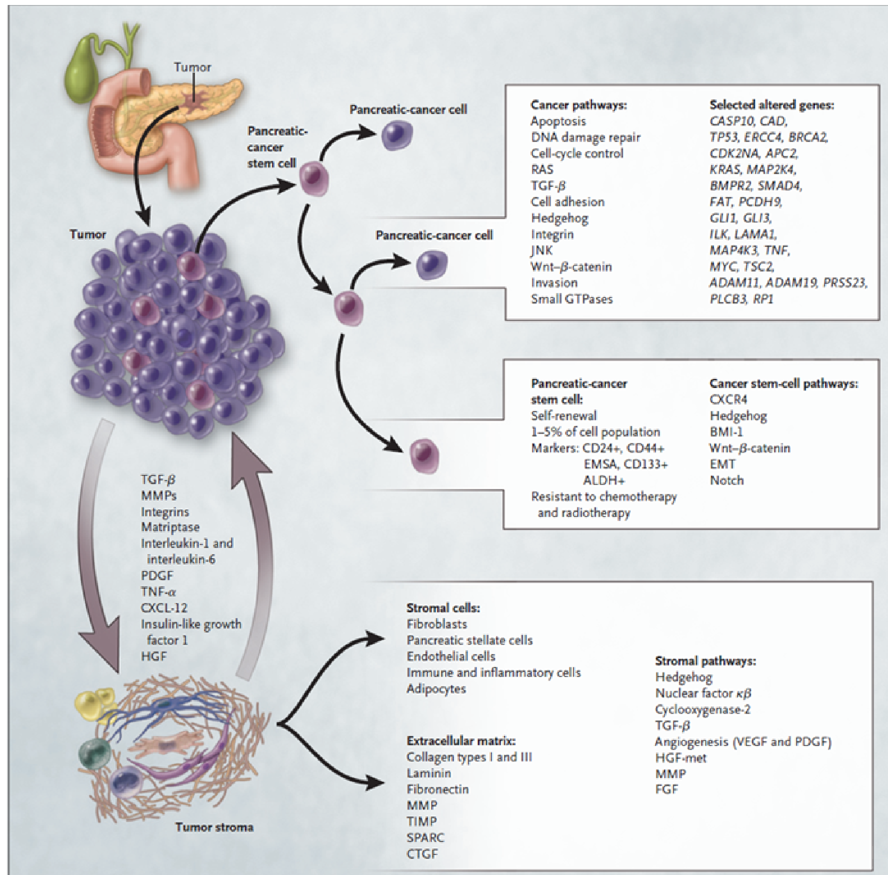


Fig.I.3.- Interaction between tumor and stroma in Pancreatic cancer. Adapted from Hidalgo, N Engl J Med 2010; 362:1605-17

Recently, Jones et al.using a genome-wide genetic analysis have found that genetic alterations in PDA define a core set of signalling pathways and processes that were each altered in the great majority of pancreatic cancers. In this analysis, 1,562 somatic mutations, 198 homozygous deletions, and 144 amplifications were identified, indicating the genetic complexity and diversity of PDAC. However, 69 gene sets were identified that were genetically altered in the

majority of the 24 tumors, and 31 of these sets could be grouped into 12 core signaling pathways [8] as shown in **Fig.I.4**.

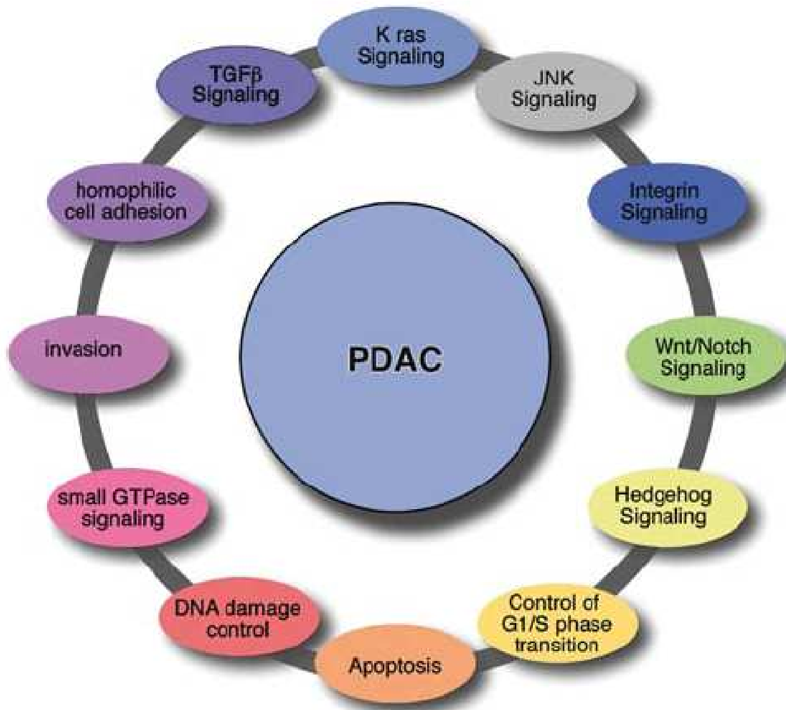


Fig.I.4. - Core signaling pathways altered in Pancreatic Cancer. The signaling pathways listed are altered in 69-100% of PDACs. Adapted from Jones et al, Science 2008.

I.1.2. PDAC Treatments

Most patients with pancreatic cancer present with locally advanced or metastatic disease. Conventional treatments include surgery, gemcitabine (a nucleoside analogue) and erlotinib (an EGFR tyrosine kinase inhibitor). Overexpression of EGFR occurs in many pancreatic tumors and has been found to correlate wih poor

prognosis and disease progression [16]. In xenograft animal model studies it was shown that antagonizing EGFR signaling could inhibit growth and metastatis of pancreatic tumors [17] . Treatment with Erlotinib along with gemcitabine used as chemotherapy had an improvement in median overall survival compared with those who recived gemcitabine alone but with side effects like rashes and diarrhea [18]. In the absence of effective screening methods, considerable efforts have been made during the past decade to identify better systemic treatments. Unfortunately most trials have not shown a survival advantage for most therapies. The studies that K-Ras mutations which occur in 90% of pancreatic cancer [15] paved the way to check whether inhibition of K-ras will have any effect in the progression of the disease. However this strategy of using inhibitors failed to make any progress. The reason for these result might be that K-Ras mutations occur early in the development of pancreatic cancer and cancer cells become less dependent on this pathway as disease progression occurs. Pancreatic cancer development seems to be related to alterations in group of genes involved in specific pathways and process [8] but for each idividual the genes and pathways affected were varied which indicated that individualised approach is likely to be required and hence targeted agents fail to provide a benefit for patients with pancreatic cancer in clinical trials. The identification of pancreatic cancer stem cells defined the new treatment strategy based on agents that could target developmental pathways such as Notch and Wnt signaling which are quiesent in normal pancreas but found to be overexpressed in specimens of pancreatic cancer where it is beleived they could

sustain tumor growth and responsible for resistance to chemotherapy [19].

I.2 Angiogenesis and its molecular mechanisms

Angiogenesis is the formation of new capillaries and blood vessels from pre-existing blood vessels. It is an important biological process as well as in a variety of diseases including cancer, diabetic retinopathy and rheumatoid arthritis [20]. Angiogenesis is a tightly regulated process by which variety of circulating and sequestered inhibitors suppress proliferation of vascular endothelium [21] thereby maintaining a balance. Like normal tissues, tumors require an adequate supply of oxygen, metabolites and an effective way to remove waste products [22] but these requirements do change over the course of tumor progression and varies among tumor types [23]. Induction of tumor vasculature called 'angiogenic switch' **Fig.I.5** is activated when induced by physiologic stimuli such as hypoxia, angiogenic inducing factors such as VEGF family, angiopoietins, TGF–β, PDGF, tumor necrosis factor –α (TNF-α), interleukins and the members of the fibroblast growth factor (FGF) family, membrane bound proteins, cell-matrix and cell-cell interactions etc [22, 24].

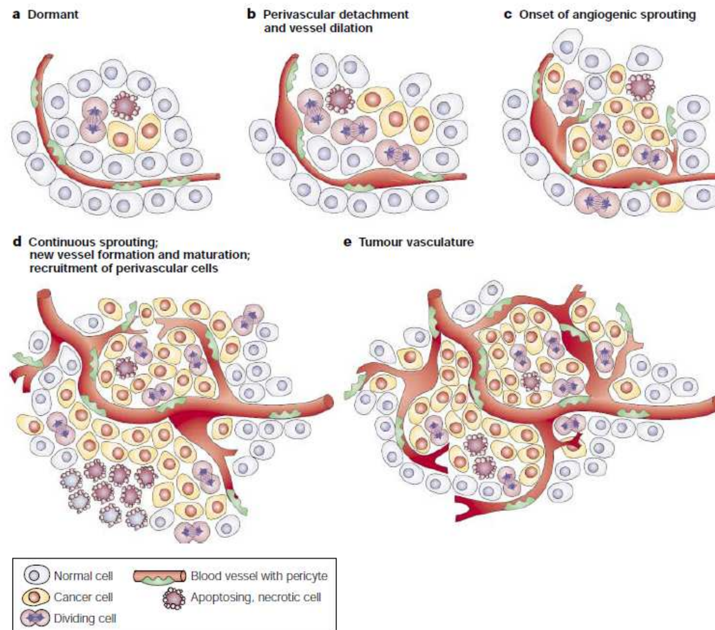


Fig.I.5. - **Model of Angiogenic Switch**: a) most tumors start as avascular nodules (dormant) until they reach a steady state level of proliferating and apoptotic cells. b) Perivascular detachment and vessel dilation occurs. c) Sprouting of blood vessels. d) New vessel formation and maturation. e) Blood vessel formation continues as long as the tumor grows, thereby providing nutrients. Adapted from Bergers et al, Nature 2002

Blood vessels are composed of basement membrane (BM) components, pericytes and endothelial cells. Endothelial cells are among the most quiescent and genetically stable cells of the body. During angiogenesis, endothelial cells can proliferate rapidly, being one of the main events that are required for the formation of new capillary blood vessel. Vascular endothelial cell proliferation is associated with degradation of BM, which leads to sprouting of pre existing micro vessels. These vessels invade the extra cellular

matrix, form tubes and finally the tips of the tubes connect to create loops that are capable of conducting the blood flow[25]. The vascular network that forms in tumors is often leaky and hemorrhagic due to the overproduction of VEGF and other soluble factors **FigI.6**

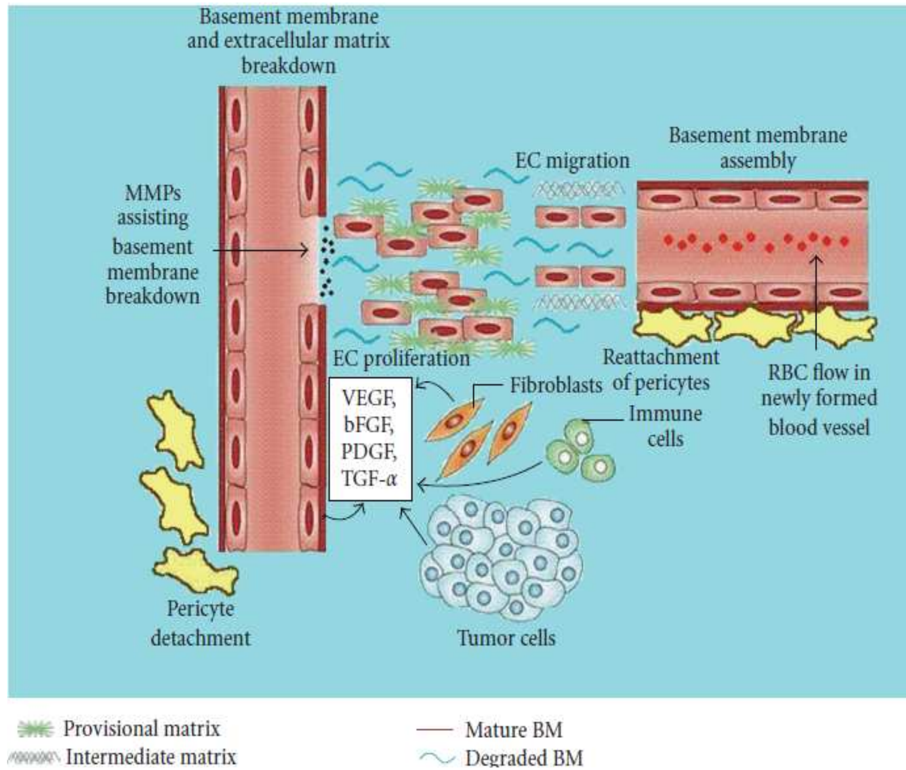


Fig.I.6. - Tumor influenced angiogenesis: the stepwise process of angiogenesis begins with the breakdown of ECM and BM followed by endothelial proliferation, migration and finally re-formation of blood vessel. Tumor cells secrete variety of factors which ensures that the new vessels formed are fed directly to the tumor tissues. Adapted from Herman et al, Journal of Oncology, 2010

I.2.1 Soluble Factors in regulating Angiogenesis

I.2.1.1 *Vascular Endothelial growth factor*

Vascular endothelial growth factor-A (VEGF-A) is a tumor-secreted cytokine with grave importance in both normal and tumor-associated angiogenesis and is the most potent pro-angiogenic protein described to date. It induces proliferation, sprouting and tube formation in endothelial cells [26, 27]. VEGF-A gene is located on the short arm of chromosome 6 and through alternative splicing is processed to yield four mature isoforms (VEGF121, VEGF165, VEGF189 and VEGF206 [28]. VEGF165 is the predominant isoforms and is over-expressed in a variety of solid tumors. VEGF189 is the most potent for vascularization of various cancers[29]. VEGF-A exert its biologic effect through interaction with cell surface receptors which includes VEGF receptor-1 (VGFR-1/Flt-1) and VEGFR-2/Flk-1 expressed on endothelial cells and neuropilin receptors (NP-1 and NP-2) expressed on neurons and vascular endothelium [30].When VEGF-A binds to the extracellular domain of the receptor, a cascade of downstream proteins are activated after the dimerization and autophosphorylation of the intracellular receptor tyrosine kinase domains. VEGFR-2 appeared to be the main receptor responsible for mediating pro-angiogenic effects of VEGF-A [27]. Deletion of VEGF-A resulted in vascular defects and cardiovascular abnormalities in mice [31]

Vascular endothelial growth factor-B (VEGF-B) is encoded by VEGF-B gene. It yields two polypeptide forms, VEGF-B167 and VEGF-B186 by alternate splicing [32]. The precise role of VEGF-B *in vivo* is not known but they might have a role in inflammatory angiogenesis in view of the results from knock out mice, that displayed reduced angiogenic responses in collagen-induced arthritis [33]. Silvestre et al demonstrated that VEGF-B promotes angiogenesis in association with activation of Akt and eNOS-related pathways in mice [34].

Other VEGF related molecules include VEGF-C and VEGF-D which have same structural similarity and less homology with VEGF-A [35]. Both growth factors stimulate angiogenesis *in vitro* and *in vivo* [36]

I.2.1.2 *Angiopoietins and Tie receptors*

Angiopoietins are a family of secreted protein growth factors that play a key role in angiogenesis and binds to Tie2 receptors [37]. Expression patterns of two Tie receptors, Tie1 and Tie2, are similar to those of VEGFRs [38]. Genetic studies also associate the Tie receptors with angiogenesis. Tie1 knock out mice die after they develop edema and hemorrhage. The vascular integrity is severely compromised in the knock out mice, indicating that Tie1 is necessary for the integrity and survival of endothelial cells during angiogenesis [39]. Tie2 deficient mouse died before birth and

displayed a reduction of endothelial cells in their blood vessels which showed an abnormal vasculature, lacking of branching and sprouting blood vessels, indicating the importance of Tie2 in these events [40].

Angiopoietin-1 (Ang-1) is the most studied angiopoietins and the mRNA is detected in embryonic days 9-11 in the myocardium and later in the mesenchyme surrounding blood vessels. In transgenic mice Ang-1 induced more abundant, highly branched and large blood vessels than in wild-type mice. The role of Ang-2 is more complicated than that of Ang-1. The use of Ang-2 inhibitors resulted in tumor inhibition in mice and corneal angiogenesis inhibition in rats [41].

I.2.1.3 *Fibroblast growth factor*

Another important set of proteins that mediates angiogenesis is fibroblast growth factor family (FGF). FGFs are soluble growth factors which has both acidic (aFGF) and basic (bFGF) variety. Both types are powerful inducers of EC migration, proliferation and micro vessel tube formation [22]. FGF is important in the remodeling of damaged blood vessels which can occur during both wound healing and tumor angiogenesis [42]. Murakami et al studied the function of FGF by inhibiting FGF receptors which led to the loss of function in the adherens and tight junctions, which then caused the loss of ECs, severe impairment of the endothelial barrier [43]

I.2.1.4 *Transforming Growth Factor-Beta*

Transforming Growth factor-beta (TGF-β) are family of homodimeric cytokines that help in controlling many different processes in the body, including angiogenesis. TGF-β are normally found in the ECM of many different cell types [44]. TGF-β has both pro and anti angiogenic properties in which low doses of TGF-β help initiating the angiogenic switch by upregulating angiogenic factors and proteinases and at high doses TGF-β inhibits EC growth, promotes basement membrane reformation, SMC differentiation and recruitment [37]. Genetic studies in mice has shown that loss of TGF-β leads to leaky vessels lacking structural integrity and to premature endothelial cell death [45]. Stimulation of angiogenesis through TGF-β is mostly via indirect mechanisms. TGF-β signals inflammatory mediators to the site of angiogenesis, where inflammatory cells release pro angiogenic factors such as VEGF, FGF, and PDGF [46].

I.2.1.5 *Interleukins*

Interleukins (ILs) are group of cytokines released by leukocytes and control a wide range of biological activities. They are mainly released by leucocytes but can be also synthesized by macrophages, endothelial cells and tumoral cells. Several interleukins have been shown to affect the growth of blood vessels [47]. Interleukin-1α (IL-1α) is a prototypical proinflammatory cytokine that has been shown to be strongly angiogenic in *in vivo* assays for measuring

angiogenesis [48]. IL-4 acts as a tumor inhibtor but its mechanism varies wih different tumor cells. IL-6 and IL-8 have also been implicated as inducers of angiogenesis in various cancers[49, 50]. Cells over secreting interleukins will rearrange the ECM which in turn enables the endothelial cells to leak out and makes branching of tubes.

I.2.1.6 ***Matix Metalloproteinases (MMPs)***

The degradation of basement membrane is an essential requirement for the formation of new vessels. Matrix metalloproteinases (MMPs) are a family of highly homologous Zn^{2+}-endopeptidases that cleave and remodel the extracellular matrix. There are two types, secreted type and membrane type MMPs [47]. The main members of this family comprise: collagenases (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-10 and MMP-11), and gelatinases (gelatinase A or MMP-2; gelatinase B or MMP-9), among others [47]. Recent studies by Bergers *et al* have shown that MMP-9 and to a lesser extent, MMP-2, are required for the mobilization of the sequestered VEGF and the initiation of tumor angiogenesis [48]. MMP-9 degrades type IV collagen which disrupts the organization of BM leading to the release of BM-bound VEGF. As BM undergoes MMP-mediated degradation and structural changes, cryptic domains of partially degraded collagens become exposed and promote angoiogenesis and tumor growth [49].

I.3 Angiogenesis in Pancreatic Cancer

Contribution of angiogenesis to PDAC progression has been poorly characterized, mainly because these tumors are poorly vascularized and therefore it has been assumed that angiogenesis does not play a relevant role in this pathology. However, recent studies suggest that pancreatic cancer can entail the substantial development of new blood vessels within the tumor tissue and that angiogenesis could be a key contributor to PDA malignancy [50]. Pancreatic microenvironment may also serve to promote tumor angiogenesis [51]. In one study by Fujioka *et al* immunohistologic datas of one hundred and four patients with pancreatic ductal adenocarcinoma were performed for VEGF, thymidine phosphorylase (TP), bFGF, CD34, p53 and Ki-67 and came to conclusion that angiogenesis in human pancreatic carcinoma is dependent on VEGF, TP and bFGF and p53 abnormality is likely to take part in VEGF related angiogenesis [50] (**Fig.I.7).**

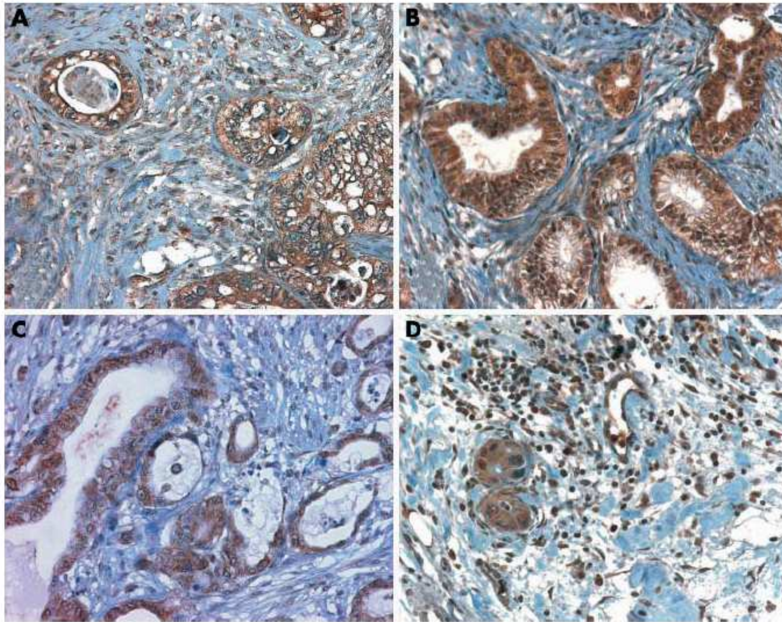


Fig.I.7.-Expression of proangiogenic growth factors in pancreatic ductal adenocarcinoma. Strong immunoreactivity for A) vascular endothelial growth factor-A (VEGF A), B) VEGF-C, C) basic fibroblast growth factor in the cytoplasm of the tumor cells D) positive inflammatory cells is visible in the stroma around the tumor cells and at the tumor pheriphery. Adapted from Esposito *et al*, J Clin Path, 2004

Morphological analysis of tumor specimens together with the immunohistochemical analysis allowed the characterization of inflammatory cells infiltrated in pancreatic adenocarcinoma, in which mast cells and macrophages (CD68) were identified. By means of double immunohistochemistry, most of the VEGF-A, VEGF-C, andf bFGF expressing cells were identified as mast cells and macrophages (**Fig.I.8**). The capacity of mast cells and macrophages to produce proangiogenic factors has been described previously [52, 53]

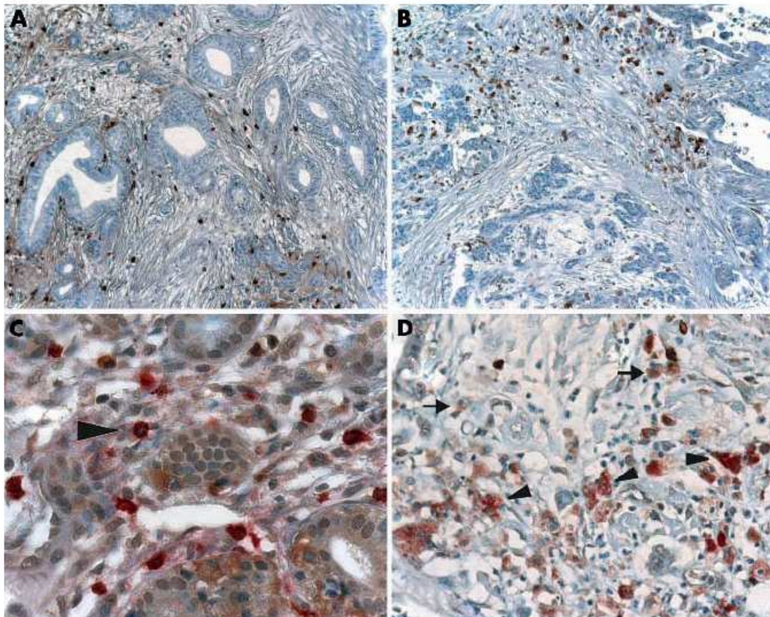


Fig.I.8. - Characterization of the inflammatory infiltrate in pancreatic ductal adenocarcinoma. A) Tryptase positive mast cells and B) CD68 positive macrophages are the most common inflammatory cells in the stroma of PDAC. Double immunohistochemistry shows that C) mast cells and D) macrophages (red staining) in PDAC. Adapted from Esposito *et al*, J Clin Path, 2004

The result of the study by Esposito *et al* showed that pancreatic cancer cells themselves are an important source of pro angiogenic factors [54]. They suggested that not only the cancer cells, but also the inflammatory cells, which expressed pro angiogenic growth factors and accumulate in cases with higher intratumorous microvessel density (IMD), influence the angiogenic property of pancreatic cancer. The number of VEGF-A positive inflammatory cells was higher in tumors with VEGF-A positive cancer cells. The mast cell specific proteases tryptase and chymase both expressed by pancreatic mast cells can promote angiogenesis [55] and in particular tryptase can specifically induce endothelial cell proliferation and capillary formation [56]

PDACs overexpress multiple additional mitogenic growth factors which are angiogenic in nature [57] (**Table 2**).

Growth Factors Activating Tyrosine Kinase Receptors	Receptor
VEGF-A	VEGFR-1 and VEGFR-2
VEGF-C	VEGFR-3
EGF, TGF-α, HB-EGF	EGF receptor
FGF-1, -2, -5	FGF receptors, types 1 and 2
PDGF B chain	PDGF receptors α and β
IGF-1	IGF-1 receptor
Hepatocyte growth factor	MET
Growth Factors that Activate Serine-Threonine Kinase Receptors	
TGF-β1, -2, -3	Type II TGF-β receptor
Pro-Angiogenic Chemokines	
IL-8	CXCR1 and CXCR2
Mip 3α	CCR6

Table.2 Angiogenic Growth Factors over expressed in Human Pancreatic Cancer and their cognate receptors.

VEGF-A is of crucial importance in promoting the growth and metastasis of pancreatic cancer cells in PDAC. It has been demonstrated that pancreatic cancer cells secrete biologically active VEGF-A [58] and cancer cells in PDAC as well as pancreatic cancer cell lines sometimes express VEGFR-1 or VEGFR-2 [59]. Adeno viral vectors carrying the sequences encoding soluble VEGFR-1 and VEGFR-2 or the VEGFR tyrosine kinase inhibitor PTK 787 inhibit the growth or metastasis of pancreatic cancers in mouse models [60]. These findings supported that VEGF –A plays a role in angiogenic process of PDAC and may exert direct effects on pancreatic cancer cells *in vivo*.

In addition to VEGF, other growth factors activate tyrosine kinase that are expressed in endothelial cells within the pancreatic tumor mass, such as EGFR [61]. The importance of tyrosine kinase receptors other than VEGFR in pancreatic cancer angiogenesis is described by the observation that inhibition of EGFR tyrosine kinase activity suppresses pancreatic cancer angiogenesis [17].

Other proangiogenic factors that are over expressed in PDAC include chemokines such as Mip3α and interleukin-8, which activate G-protein coupled receptors [62, 63]. The evidence for cross-talk between various angiogenic actors have been established like TGF-β1 and plasminogen activator inhibitor-1 (PAI-1) are over expressed in PDAC [64, 65], TGF-β1 induces PAI-1 expression in pancreatic cancer cells [66], and both TGF-β1 and PAI-1 can promote angiogenesis *in vivo* [67, 68]. The angiogenic potential of

TGF-βs may be enhanced by the presence of Smad4 mutations which are frequent in PDAC [69]. Since uPA and its receptors are overexpressed in PDAC it can transctivate EGFR and EGFR activation can induce the expression of VEGF and pro angiogenic chemokine IL-8 [70]. These observations suggest that multiple pathways interact to enhance angiogenesis in PDAC.

Pancreatic adenocarcinomas exhibit high levels of intratumoral microvessel density (IMD) which suggests tumor induced angiogenesis. Immunohistochemical studies by Kuehn et al found that IMD was increased in pancreatic cancer specimens over normal pancreas and chronic pancreatitis specimens[71]. Other researchers have found that high IMD in pancreatic cancer correlate with poor patient survival Karademir *et al., by* immunohistochemical analysis of 22 PDAC patients, found that tumor vascular surface density and number of microvessels in the stroma correlated with increased tumor proliferation, poor differentiation, larger tumor size and decreased patient survival [75]. These findings indicate that PDAC exhibit a high degree of IMD and, as has been demonstrated in other tumors such as breast, lung, prostate, cervix etc., high IMD can correlate with poorer prognosis and greater risk for metastasis.

I.3.1 *Hypoxia in Pancreatic Cancer*

Tumor hypoxia is a common feature of many cancers and it essentially occurs when the growth if the tumor outstrips the accompanying angiogenesis [76]. The master regulator of hypoxic response is the transcription factor hypoxia inducible factor-1 (HIF-1) [77]. It mediates the adaptive response to hypoxia by affecting the transcription of numerous hypoxia- inducible genes. It undergoes conformational changes in response to varying oxygen changes [78]. HIF-1 directly activates VEGF and VEGFR-1 transcription by binding to HRE, and plays an important role during normal growth and tumor formation [79]. Deletion of HIF-1α in endothelial cells disrupted an autocrine loop necessary for hypoxic induction of both VEGFR-1 and VEGFR-2 by VEGF signaling [80]. VEGFR-1 is directly upregulated by hypoxia via an HIF binding enhancer element located in the VEGFR-1 promoter, while the upregulation of VEGFR-2 is through post transcriptional regulation [81].

Hypoxia is clearly a driving force in pancreatic cancer and metastasis. The presence of significant hypoxia in pancreatic cancers has been long suspected in case the lesions characteristic of vascular appearance on X-ray computed tomography scan [82]. Koong *et al* first reported hypoxia in pancreatic cancer where they analysed the results from direct intratumoral needle oxygen measurements at the time of resection in seven patients with operable pancreatic cancers [83]. Several studies have indicated a

link between tumor hypoxia and VEGF production in pancreatic cancer. In this regard, Duffy *et al* reported that HIF-1 is activated in pancreatic cancer in response to low oxygen levels *in vitro* and *in vivo* [84]. Studies in human specimens of pancreatic cancer showed co localization of VEGF mRNA, which is produced in ductal cancer cells, and HIF-1α protein which was detectable in the cell nuclei of cancer cells. HIF-1α expression was upregulated in pancreatic cancer specimens [84]. Pancreatic cancer cell lines increased VEGF production under low oxygen levels by highly specific activation of HIF-1 DNA binding activity to the VEGF promoter. Cell lines with high constitutive levels of HIF-1α protein were found to produce higher basal levels of VEGF [84]. Study by Sun *et al* investigated the expression of HIF-1α, VEGF, Glut-1, CD34 and surviving in pancreatic tumors. These proteins were not detected in exocrine portion of all normal pancreatic samples. In contrast, in tumor cells HIF-1α was present in diffused pattern in the nucleus or cytoplasm and VEGF and Glut-1 were detected within the cytoplasm and cell membrane of the cancerous cells [85] (**Fig.I.9**).

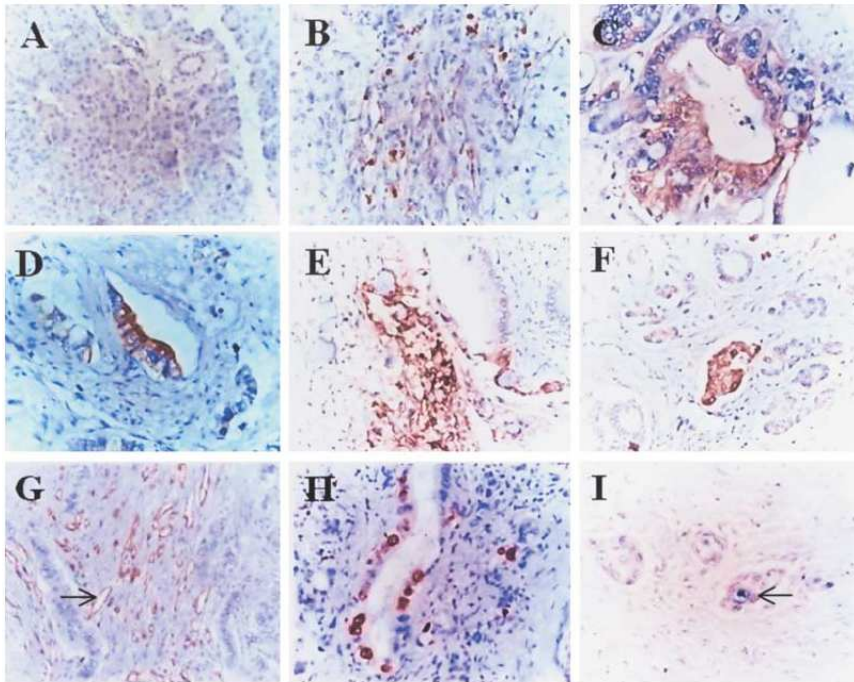


Fig.I.9.- Hypoxia in Pancreatic Cancer A) Negative expression of HIF-1α in normal pancreatic tissue B) Nucleus staining of HIF-1α in malignant cells C) Cytoplasmic staining of HIF-1α D) VEGF expression within cytoplasm and cell membrane of cancerous tissue E) Glut-1 expression in cytoplasm F) Survivin staining in the cytoplasm G) CD34 expression in vascular endothelial cells within the tumor (arrow shows one of the microvessels) H) Ki-67 positivity in the nucleus of malignant ductal cells I) An apoptotic cell (arrow). Adapted from Sun, et al.,Int J Oncol, 2007.

I.3.2 *Angiogenesis Inhibitors in PDAC*

The crucial role for angiogenesis in pancreatic cancer growth and metastasis comes from the effects of anti-angiogenic compounds on tumor growth, spread, and vascularity *in vitro* and *in vivo*. An angiogenic inhibitor fumagillin TNP- 470 decreased the growth and metastasis of pancreatic cancer [86, 87]. Studies by Hotz *et al* showed that treatment with TNP-470 in orthopic model of human

pancreatic cancer decreased tumor growth, IMD, and metastatic spread by anti angiogenic mechanisms versus anti tumor effects [88]. In a hamster model of metastatic pancreatic cancer the endogenous anti angiogenic compound angiostatin, a fragment of Plasminogen, has been shown to inhibit growth and neovascularisation of hepatic metastases [89].

Other experiments have targeted specific pro-angiogenic cytokines like VEGF-A to inhibit tumor growth. VEGF inhibition is being evaluated as a strategy for the prevention of angiogenesis and vascular leakage in malignant tissue and represents one possible strategy for improving survival in patients with advanced pancreatic cancer [27]. In one study by Hotz *et al* they used diphtheria toxin-VEGF fusion protein which significantly reduced the tumor volume, IMD, metastasis and increased animal survival *in vivo* [90]. Bruns *et al* used an anti-VEGFR-2 antibody (DC101) with or without gencitabine in an orthopic mouse model [91]. Treatment with DC101 alone and gencitabine alone both inhibited tumor growth, but the combination therapy yielded better result and produced endothelial cell apoptosis. These result suggested that VEGF targeted therapies may potentiate the effects of cytotoxic treatments in pancreatic cancer.

Overexpression of cyclooxygenase-2 (COX-2) is detected in 75% of resected pancreatic cancer and correlates with aggressive tumor biology [92]. COX-2 promotes tumor growth by up-regulating angiogenesis and invasiveness, and inhibiting apoptosis [93].

Celecoxib, a COX-2 specific inhibitor, has demonstrated anti-tumor activity against a variety of human cancers in animal models, including pancreatic cancer xenografts [94].

Epidermal growth factor (EGF) and its receptor EGFR are believed to be important in the control of angiogenesis. Pancreatic adenocarcinomas and dysplasias frequently overexpress receptor tyrosine kinases, such as EGFR [95]. In one analysis of 12 human pancreatic cell lines, 100% expressed EGFR.Cytoplasmic EGFR expression in human pancreatic cancer, particularly in the progression of pancreatic ductal adenocarcinoma, is associated with metastases ($P<.01$), as well as poor prognosis [16]. Blocking EGFR via the oral administration of a novel EGFR TKI, PKI166, has been shown to decrease VEGF expression and increase apoptosis of tumor associated endothelial cells in pancreatic cancer xenografts [17]. Blocking VEGF-induced angiogenesis, as well as EGFR and other growth factors, may lead to prolonged survival especially for those patients with advanced disease.

Sunitinib is a multitarget inhibitor of tyrosine kinase membrane receptors, with a potent antitumoral and antiangiogenic activity. In vivo studies have shown that it decreases microvascular density, prevents neovascularization and decreases lung metastasis in a Lewis carcinoma model [229]. Antitumoral effects of sunitinib has been also demonstrated in several xenograft models of colon, breast, lung, melanoma, glioblastoma and renal cancers [230] as well as clinical studies [231,232]. In fact, sunitinib has been

recently aproved by the FDA for the treatment of advanced and/or metastatic renal cell carcinoma and for malignant gastrointestinal stromal tumours [233] . In pancreatic cancer, sunitinib effects have been explored in patients with advanced tumors and in combination with gemcitabine [228]. Sunitinib effects together with gencitabine or radiotherapy have also been explored In animal models using subcutaneous xenografts of pancreatic tumoral cells [236].

I.4 Plasminogen System

The zymogen plasminogen is mainly produced by the liver and is present in the plasma as well as extravascularly in the interstitial fluids. It is converted locally to the active serine protease plasmin by proteolytic processing mediated by either of the two plasminogen activators, the serine proteases urokinase Plasminogen activator (uPA) and tissue type Plasminogen activator (tPA). Even though both uPA and tPA share a common plasminogen converting function they have a structural and functional differences. tPA mainly acts as a fibrin-dependent and intravascular activation enzyme involved in clot dissolution while uPA operates as a fibrin-independent, a cell surface receptor bound plasminogen activator and controls pericellular proteolysis [96] (**Fig.I.10**).

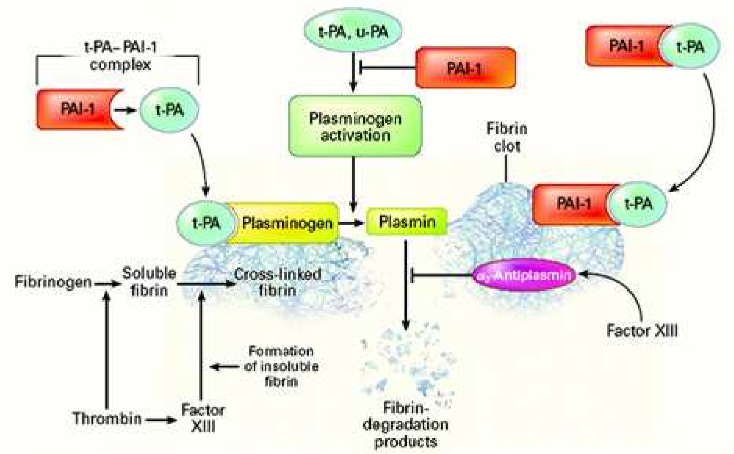


Fig.I.10. **- The Plasminogen system**. Adapted from Xavier Pi-Sunyer *et al*, Obesity, 2004

I.4.1 Components of Plasminogen system

I.4.1.1 *Plasminogen and Plasmin*

The precursor of the active protease plasmin is Plasminogen [97] and is produced by the liver. The plasma concentration of the Plasminogen is about 100-150 mg/L and the half life in circulation is about 2.8 days [98]. Plasminogen is a single chain glycoprotein of M_r about 90,000, consisting of 790 amino acid, and about 2% carbohydrate. The plasminogen gene, located on chromosome 6, spans 53.5 kb and consists of 19 exons [99]. Plasminogen is activated to the two-chain proteolytically active plasmin by tPA or uPA by cleavage of a single peptide bond, Arg_{560}-Val_{561} [100] . The two chains are held together by two disulphide bonds. There is a heavy (A) chain (M_r 60,000) and a light (B) chain (M_r 25,000).

The light chain contains the serine protease catalytic triad [101]. The heavy chain contains five kringles and mediates interactions of plasminogen with fibrinogen, inhibitors and cell-surface receptors (**Fig.I.11**).

Cell surface associated plasmin catalyses the breakdown of ECM and basement membrane molecules such as fibronectin, laminin, vitronectin, fibrin and collagen [102]. Latent forms of bFGF, TGF-β can also be activated by plasmin which on activation induces cell proliferation, angiogenesis etc.

Plasminogen deficient mice suffer multiple spontaneous thrombotic lesions, organ damage, high early morbidity and have impaired skin wound healing [103].

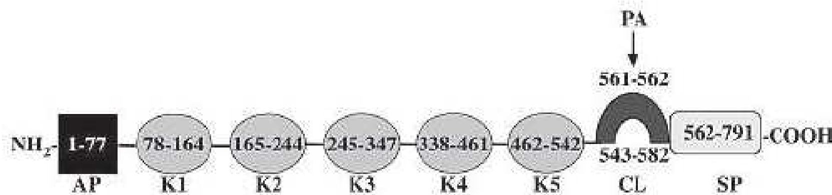


Fig.I.11.- **Plasminogen structure**. The 77-residue activation peptide (AP) is followed by 5 consecutive kringle (K) domains, with introns serving as the boundary determinants. These regions are also known to fold independently. The cleavage (activation) loop (CL) is depicted (residues 543–582), bounded by its gene introns. Plasminogen activators catalyze cleavage at the peptide bond of residues Arg561-Val562, leading to plasmin formation. Adapted from Castellino *et al*, Thromb Haemost 2005.

I.4.1.2 *The Urokinase-type Plasminogen activator*

The human single-copy gene uPA codes for a 53-kDa serine protease produced as a single chain protein (scuPA or pro-

uPA)[104]. When secreted pro-uPA is converted to the active two-chain form uPA by cleavage of the peptide bond K158-1159 by plasmin [105]. uPA contains one growth factor-like (EGF) domain and one kringle domain but no finger domain like tPA. The human uPA gene located on chromosome 10, spans 6.5 kb and has 11 exons. (**Fig.I.12**).

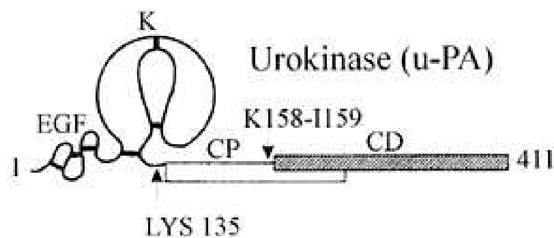


Fig.I.12: Schematic representation of Urokinase- type Plasminogen activator

The uPA present at the cell surface initiate a proteinase cascade which in turn leads to the activation of plasmin, the breakdown of the ECM and the release of cytokines, growth factors promoting cellular migration. Binding of uPA to its cellular receptor uPAR is important for the efficient activation of plasminogen at the cellular surface in several in vivo and in vitro systems [106]. In addition to supporting pericellular zymogen activation, the formation of the uPA-uPAR complex on the cell surface is nown to influence cell adhesion, migration, and chemotactic properties [107]. uPA/uPAR system was expected to contribute to cell associated proteolysis in biological process where cells are invasive. Several model systems indicated the ability of tumor cells to invade and metastasize can be downregulated by uPA inhibitors, anti-uPA antibodies, antisense uPAR expression and uPAR antagonists [108, 109].

Elevated levels of uPA/uPAR have been reported in numerous tumours, including pancreatic cancer. In PDAC patients concominant overexpression of uPA and uPAR was found to be associated with a shorter post operative survival compared with those patients in whom only uPA or its receptor were overexpressed [110]. In a study by Paciucci *et al*, the uPA/uPAR receptor proteolytic system has been reported to be involved in the hepatocyte growth factor (HGF) stimulated motility of pancreas cancer cells. HGF-induced cell motility is significantly reduced by inhibitors of uPA proteolytic activity, such as antibodies neutralising uPA activity , PAI-1 and amiloride [111]. Anti –uPA antibodies injected into mice together with tumor cells caused a significant inhibition of metastasis formation suggesting the involvement of this enzymatic system in tumor invasion and metastasis. Xue *et al* analysed mRNA expression of 15 genes from epidermal growth factor receptor, insulin-like growth factor (IGF), and uPA families were measured in 46 PDAC tissue samples using quantitative real-time reverse transcription-polymerase chain reaction. They came to conclusion that IGF, uPA/uPAR has the greater influence on survival in PDAC [112]

I.4.1.3 ***The Tissue- type Plasminogen activator***

The tissue type plasminogen activator (tPA) molecule is synthesized by endothelial cells [113] and released into the blood stream by

various stimuli such as stress, physical exercise and nicotinic acid. It is a 70-kDa protein secreted as a precursor in a single chain form [114]. Plasmin convert the precursor by cleaving the peptide bond Arg275-Ile276 to give an active two chain form held together by a single interchain disulphide bond. The tPA molecule contains 530 amino acids and has a molecular weight of about 67,000. The A-chain (M_r 38,000), is derived from the NH_2-terminal portion, and the B- chain (M_r 30,000) containing the active site from the COOH-terminal portion [115]. The A-chain contains four domains; a finger do- main, a growth factor-like domain and two kringle domains. The finger and kringle 2 domains are involved in binding to fibrin. The human tPA gene is located on chromosome 8 [114], spans 33 kb and has 14 exons (**Fig.I.13**). tPA exists as two different isoforms (type I and type II) that differ on their glycosylation, displaying species, cell and site specific patterns of this post translation modification [116]

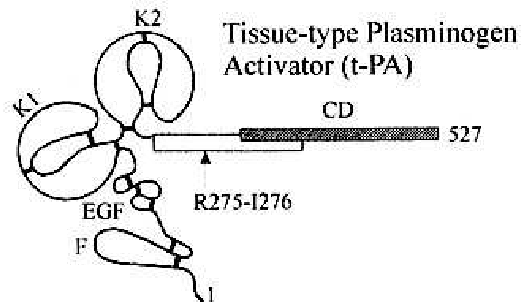


Fig.I.13: Schematic representation of Tissue- type Plasminogen activator

The main biological role of tPA is associated with fibrinolysis due to its high affinity for fibrin and activation by fibrin binding [117]. The finger domain present at the amino terminal enables tPA to have high affinity for fibrin making tPA a thrombolytic agent. Unlike uPA, tPA has got no specific receptors but it has a wide range of receptors including AnnexinA2 (AnxA2), α-enolase, HMG-1, LRP, NMDAR, etc.

I.4.1.4 ***The Plasminogen activator inhibitors***

The Plasminogen activator inhibitors (PAI)s are members of serine protease inhibitor super family (SERPIN) and are the most potent inhibitor of both tissue type (t-PA) and urokinase type plasminogen activator (u-PA) and thus regulate fibrinolysis as well as proteolysis, cell migration, and tumor cell invasiveness[118]. They are stimulated by cytokines, lipopolysaccharide, very low density lipoprotein, TGF- β-1 etc. In pathological conditions, increased PAI-1 levels mainly result from release by endothelial cells or tumor cells [119]. There are several inhibitors of plasminogen activators: PAI-1, PAI-2, PAI-3 and TAFI

I.4.1.4.1 ***Plasminogen activator inhibitor type-1***

Plasminogen activator inhibitor type-1 is a single chain 45-50 kDa glycoprotein secreted by many cell types and is the most important component of the PA system in the regulation of both physiologic

process and in the pathogenesis of many disorders including cancer [120]. It inhibits both uPA and tPA and also has high affinity for ECM proteins. PAI-1 not only binds to free uPA but also to uPAR – bound uPA [121]. uPA/PAI-1 complex interact with the transmembrane α2-macroglobulin receptor low-density lipoprotein (LDL) receptor- related protein (LRP), an endocytic receptor. By the combined action of uPAR ad LRP, the uPA/PAI-1 complex is internalized and degraded in lysosomes and the uPAR is recycled back to the cell surface [96, 122]. PAI-1 is believed to play a central role in cell adhesion mediated through integrins and the uPAR/uPA complexes [123]. Kwaan et al studied the action of PAI-1 in vascular smooth muscle cells and tumor cells and found out that PAI-1 inhibited apoptosis and contribute to tumor proliferation and to angiogenesis [124]. *In vivo* studies has shown that PAI-1 expression can be highly induced in both endothelial cells and activated platelets and it plays a role in inhibiting thrombolysis through rapid inhibition of tPA [125].

I.4.1.4.2 ***Plasminogen activator inhibitor type-2***

PAI-2 is a single chain protein of 47 kDA. PAI-2 is synthesized in the placenta and the monocytes as well as in the ovarian tumors and myeloid leukemic cells. PAI-2 also inhibits uPA and tPA [126]. PAI-2 exists in both secreted and cytosolic forms. In the secreted form PAI-2 participates in the control of tissue remodeling and fibrinolysis while in the cytosolic form it plays a role in intracellular

proteolysis involved in apoptosis and inflammation. In experimental tumor mouse models PAI-2 has shown to decrease the tumor growth and metastasis. The physiological outcomes obtained by the administration of exogenous PAI-2suggested that inhibition of extracellular uPA activity is the mechanism underlying the reduction in tumor size [127]

I.4.2 *The Plasminogen system in Cancer*

The plasminogen activator system has been linked to prognosis in a number of different clinical studies in various cancers. Many studies report that some of these molecules are associated with a poor prognosis presumably because of their ability to degrade the extracellular matrix (ECM) and thus allow tumor cells to spread to the rest of the body. Plasmin destroys components of tumor stroma, acts on components of the ECM like laminin, and activates latent matrix metalloproteases (MMP's) such as collegenase IV. Collagenase IV then cleaves collagen and other parts of the basal membrane and promotes metastasis[128]. Other than the degradation of the ECM, which is a prerequisite for cancer invasion and metastasis, uPA promotes angiogenesis and mitogenesis, modulates cell adhesion, stimulates proliferation and migration, and prevents apoptosis [129]. uPA binding to uPAR is capable of directing the mobility of the cell. According to Reuning *et al*, uPA is capable of triggering signal transduction pathways; the effects including mitogenic, chemotactic, migratory, and adhesive properties [130]. The binding of uPA to PAI-1 disrupts the normal

PAI-1-Vitronectin interaction, initiating a conformational change in PAI-1 that exposes a cryptic high-affinity binding site for LRP, allowing endocytosis to occur. Recycling of uPAR to the cell surface occurs following intracellular degradation of the complex. By binding to vitronectin, PAI-1 can modulate cellular adhesion and migration [131]. This effect on adhesion is one of the reasons why increased PAI-1 may be associated with poorer prognosis. According to Czekay *et al*, PAI-1 detaches cells from extracellular matrices by inactivating integrins, thereby facilitating the metastasis of cancer cells [132]. Chazaud *et al* reported that cells take advantage of high amounts of PAI-1 to migrate using a novel function of the uPA complex [133]. According to a study, uPAR is most often located in macrophage surrounding malignant epithelium, and this macrophage mediated proteolytic activity is involved in the invasion and spreading of cancerous cells [134]. In several reports concentrations of the uPA and PAI-1 complex showed stronger prognostic importance than uPA antigen or PAI-1 antigen individually. Plasminogen activator system has a greater role in breast cancer. According to Hansen *et al*, PAI-1 was an independent prognostic marker for recurrence-free survival in patients with primary breast cancer [135]. PAI-1 was also an independent prognostic marker in both low and high-risk breast cancer patients [136]. Cytosolic levels of uPA alone have been shown to be predictive of early relapse in primary breast cancer [137]. In gastric cancer, high levels of uPA and PAI-1 are significantly associated with decreased survival [138]. In colorectal carcinoma, increase in tumor and serum levels of uPA, uPAR, and

PAI-1 are associated with poor prognosis [139]. In adenocarcinoma of esophagus, uPA and PAI-1 levels were significantly elevated in cancerous tissues. In PDAC, PAI-2 seemed most important prognostic factor as strong PAI-2 expression was significantly associated with higher survival compared to lower levels of PAI-2 [64]. In endometrial and lung cancer uPA, uPAR, PAI-1 and PAI-2 levels were higher in tumor tissue than compared to the normal ones[140, 141].
tPA has also been related to tumor progression and the role of this protease in cancer and PDAC will be described in another section (see point I.4.4).

I.4.3 *The Plasminogen system and Angiogenesis*

The Plasminogen-plasmin system is involved in tumor angiogenesis in two aspects**.** First, along with tumor derived cytokines and VEGF, uPA and PAI-1 modulate endothelial cell proliferation. Second, plasmin and plasminogen activators proteolyse plasminogen and, acting with several cofactors, release one or more of the kringle structures. These kringles possess inhibitor effects on tumor angiogenesis, best exemplified by angiostatin. Plasmin, derived from uPA activation of plasminogen, can be reduced by plasmin reductase (phosoglycerate kinase) in the presence of co-factors, including glutathione and cysteine. The disulfide bonds between kringles are further proteolysed to form kringle peptides with potent inhibitory activity against the proliferation of microvascular endothelial cells [142, 143].

Studies by Pepper *et al* confirmed that when endothelial cells migrate they upregulate uPA, uPAR and PAI-1 [144]. A variety of angiogenic factors control the expression of these proteins in ECs, like VEGF and bFGF induces the expression of uPA, tPA, uPAR and PAI-1, while TGF-β down regulates uPA and enhances PAI-1 production [145]. Plasmin promotes tissue degradation and remodeling of the local extra cellular environment directly, by degrading ECM molecules and activating or releasing latent growth factors [146]. Plasmin also potentially activates pro–matrix metalloproteinases such as pro-MMP2 and pro-MMP9 [147] (**Fig.I.14**).

Hypoxia which is the major angiogenic stimulus has been reported to increase uPAR and PAI-1 expression in endothelial cells [148]. Regarding tumoral angiogenesis clinical studies have demonstrated that high uPA, uPAR and PAI-1 levels indicate a poor prognosis for the survival of patients suffering from wide variety of cancers [68, 149]. Microvessel density was markedly reduced in tumors cells transfected with mutant murine uPA that retained receptor binding but not proteolytic activity [150]. The endothelial cells with increased Plasminogen activator activity when compared to non transformed endothelial cells form cyst like structures when embedded into a fibrin gel. These findings implicated increased Plasminogen activator-plasmin-mediated proteolysis in aberrant vascular morphogenesis [151]. Injection of endothelial cells into uPA, tPA, PAI-1 or Plasminogen-deficient mice demonstrated that

tumor growth in vivo is dependent on the generation of uPA mediated plasmin. Angiogenic endothelial cells require uPA and plasmin to degrade ECM components and migrate [152]. O'Reilly and Stack *et al* demonstrated that angiostatin can be generated by limited proteolysis of Plasminogen by plasmin, uPA, tPA and MMP [143, 153]. Studies in PAI-1 deficient mice have revealed an absolute requirement for PAI-1 in tumor angiogenesis. The absence of PAI-1 markedly impaired tumor invasion and vascularization [154].

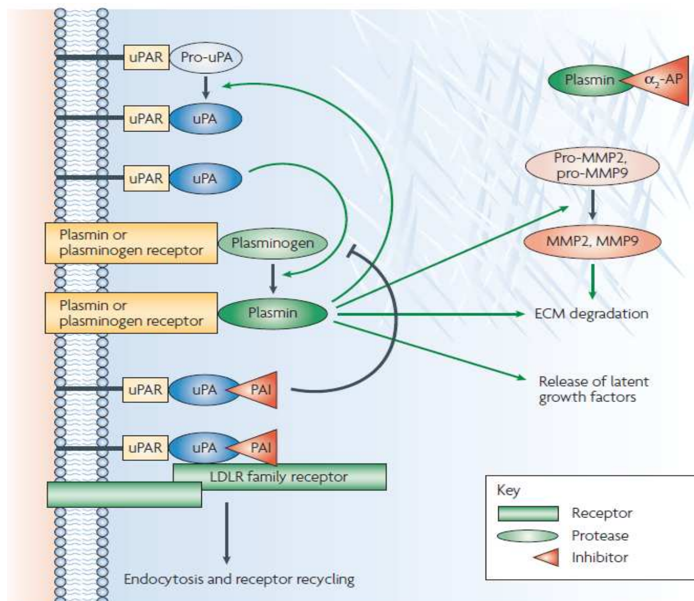


Fig.I.14.- Plasmin activating the angiogenic factors. Adapted from Croucher *et al*, Nat Rev Cancer, 2010

Some reports suggested that mice deficient in PAI-1, did not support invasion or angiogenesis of implanted tumors [96] indicatring that PAI-1 may have a dual role in angiogenesis, as an activator or an inhibitor, depending on the tumor's microenvironment and its level of PAI-1 . When tumors were

implanted in integrin α1-deficient mice (which express higher levels of matrix metalloproteinases and plasma angiostatin), reduced tumor angiogenesis was observed [155]. Plasma from these mice inhibited endothelial cell proliferation, and this inhibition was decreased by administering an angiostatin-neutralizing antibody or matrix metalloproteinase inhibitors. Mice injected with colon cancer cells that overexpressed t-PA had a markedly lower number of liver metastases and a higher survival rate than mice injected with untransfected colon cancer cells [156]. A study of tumor specimens taken from patients with melanoma or with breast cancer showed that tumors expressing a high level of t-PA were associated with better prognosis [157]. Thus, it has been hypothesized that human plasma may serve as a rich source of biologically important angiogenesis inhibitors [158]. Increasing the antiangiogenic activity of plasma proteins by proteolytic cleavage may alter the behavior of a malignant tumor and could have clinically useful implications.

I.4.4 *Tissue Plasminogen Activator in Cancer and PDAC*

Tissue Plasminogen activator (tPA) is a multifunctional protein that regulates a broad range of cellular functions like tissue remodeling neuronal plasticity as well as fibrinolysis. Many effects of tPA are mediated by its catalytic activity via plasmin generation. However, several studies have proven that tPA can also have catalytic-independent fucctions. Thus, studies Medina *et al* demonstrated that tPA can act as a cytokine triggering ERK pathway in hippocampal neurons mediating amyloid-induced neurotoxicity independent of its

catalytic activity [159]. More recently, our group has also found similar mechanisms in PDAC [237]. tPA is overexpressed in several cancers, including melanoma [160, 161], hepatocellular carcinoma[162], ovarian[163], uterine [164] and pancreatic ductal adenocarcinoma [165-167]. In pancreatic cancer tPA is overexpressed in 95% of pancreatic ductal adenocarcinoma, while it is absent in normal pancreas [165, 166].
tPA contributes to cell invasion in *in vitro* studies of pancreatic cancer [165] via its interaction with AnnexinA2[168]. tPA induces Erk phosphorylation and cell proliferation[168], involving AnnexinA2 and EGFR [169, 237].

The proteolytic activity of tPA involving MMP-9 and hb-EGF expression, which favor ECM degradation and subsequent invasion and tumor progression are reported. In athymic mice, pancreatic cancer cells with low tPA levels generate less proliferative and angiogenic tumors [168] The key contribution of tPA to pancreatic cancer progression has been highlighted by *in vivo* studies using the Ela-Myc mouse pancreatic cancer model. Mice with tPA-/- background showed an increase in survival, with less angiogenic and mitogenic pancreatic tumors [244]. This effect was clearly dependent on the tumor characteristics, being predominant in the ductal tumors resembling human PDAC, where tPA is found to be overexpressed.

AnnexinA2 is a tPA receptor and it has been very well characterize in ECs where it exerts an important physiological role enhancing

tPA fibrinolytic function. AnnexinA2 is overexpressed in 70% of cases of human pancreatic cancers [181a, 189a] and in ductal transgenic mice tumors, whereas its expression in normal pancreas is low and restricted to pancreatic islets [190]. Although this receptor has been clearly involved in tPA-mediated pancreatic cancer cell invasion, the fact that AnnexinA2 is the major tPA receptor in ECs and its important role in blood homeostasis, made it an appropriate target for PDAC therapy. In addition previous data showed that only 50% of tPA found in the cell membrane was associated to AnnexinA2 suggesting the presence of other tPA receptors. For this reason, our group performed a proteomic approach by pulling down human pancreatic cancer cell line extracts with tPA bound to sepharose [191]. Putative candidates were separated by 2D-electrophoresis and identified by peptide mass fingerprint. Results are summarized in the **Table 2.** Some of the candidates found had already been described such as enolase, cytokeratin 8 and 18 or tubulin, but others were described for the first time like Galectin-1 (Gal-1) and valosin containing protein. Due to its localization and previously reported functions, Gal-1 was choosen for further studies, as described later.

Protein name	Lysate	Raft	Reference	Best coverage	pI	Mass (kDa)	Localization	Function
Annexin A2	+	+	gi|16306978	48%	7.6	39	Membrane, cytoplasm, nucleus	Signal transduction; cell communication
Enolase[ψ]	+	+	gi|4503571	22%	7.0	47	Membrane, cytoplasm,	Metabolism
Galectin-1[ψ]	+		gi|42542978	51%	5.3	15	Membrane, cytoplasm, nucleus	Receptor binding; immune response
Cortactin[ψ]	+		gi|182087	35%	5.2	61	Cytoskeleton	Structural component
Cytokeratin 8[ψ]	+	+	gi|181573	33%	5.5	53	Cytoskeleton	Structural component
Cytokeratin 18	+	+	gi|30311	48%	5.3	47	Cytoskeleton	Structural component
Tubulin[ψ]	+	+	gi|2119276	29%	5.0	50	Cytoskeleton	Structural component
Vimentin	+	+	gi|2119204	54%	5.1	54	Cytoskeleton	Structural component
Actin	+	+	gi|3157976	38%	5.3	42	Cytoskeleton	Structural component
ARP3[ψ]	+	+	gi|5031573	32%	5.6	47	Cytoskeleton	Structural component
Cytokeratin 19	+		gi|24234699	49%	5.0	44	Cytoskeleton	Structural component
Enigma proteins with LIM and PDZ domains[ψ]	+		gi|13994151	39%	6.6	36	Cytoskeleton	Receptor signaling complex scaffold
Cystathionine-beta-synthase	+		gi|4557415	43%	6.2	61	Cytoplasm	Metabolism
Pyruvate kinase 3		+	gi|31416989	18%	7.9	58	Cytoplasm	Metabolism
Placental thrombin inhibitor	+		gi|20141722	53%	5.1	43	Cytoplasm	Protease inhibitor
Translin associated protein X	+		gi|6136057	60%	6.1	33	Cytoplasm	Transporter
Eukaryotic translation initiation factor 3	+		gi|4503513	37%	5.4	37	Cytoplasm	Translation regulation
CTP synthetase	+		gi|20981706	24%	6.0	67	Cytoplasm	Ligase; nucleotide and nucleic acid metabolism
Chaperonin (acute related morphine dependence protein)[ψ]	+		gi|4502643	28%	6.2	58	Cytoplasm	Chaperone activity; metabolism
Thioredoxin peroxidase[ψ]	+		gi|9955007	45%	5.4	22	Cytoplasm, nucleus	Peroxidase activity; metabolism
ERK 1[ψ]	+		gi|20986531	19%	6.5	42	Cytoplasm, nucleus	Kinase activity; signal transduction; cell communication
Valosin containing protein[ψ]	+		gi|6005942	47%	5.1	90	Cytoplasm, ER, nucleus	ATPase activity
Heterogeneous nuclear ribonucleoprotein H1	+		gi|5031753	37%	5.9	49	Nucleus	Ribonucleoprotein
ER-associated DnaJ protein 3	+		gi|18203497	33%	5.8	41	ER	Chaperone
DnaJ (Hsp40) homolog	+		gi|5453980	29%	5.8	58	ER	Chaperone
Eukaryotic translation elongation factor	+		gi|39644794	27%	6.3	50	ER	Translation regulator activity
Eukaryotic initiation factor 4B	+		gi|18146614	79%	5.4	69	Ribosome	Translation regulator activity; metabolism
PWP1-interacting protein 4	+		gi|14579002	33%	5.8	41	Mitochondria	DNA binding protein
Elongation factor Tu, mitochondrial precursor[ψ]	+		gi|34147630	51%	7.3	46	Mitochondria	Translation regulator activity
H+-transporting two-sector ATPase		+	gi|16359160	17%	9.2	59	Mitochondria	Ion channel activity; transport
Ubiquinol-cytochrome-c reductase		+	gi|731047	20%	6.9	53	Mitochondria	Catalytic activity; metabolism

a) Proteins present in PANC-1, but not in HUVEC, total cell lysates are marked with a superscript ψ. Cell localization and function of each protein were obtained from Human Protein Reference Database (http://www.hprd.org)

Table 2 The protein identification process yielded 31 tPA receptor candidates identified in gels of PANC-1 cell pull-downs, either from total lysates or from raft fractions.

I.5 Galectins

Galectins are a group of proteins that bind β-galactosides through evolutionarily conserved sequence elements of the carbohydrate recognition domain (CRD) [170, 171]. Despite the CRDs of all the galectins has affinity for N-acetyllactosamine disaccharide, each galectin can also recognize different modifications of this motif and display fine specificity for different ligands [224]. 15 galectins have been described in mammals of which 11 are expressed in humans and can be structurally clustered in three groups[172, 173]. Some of the galectins contain one CRD and are biologically active as monomer (Gal-1, 5, 7, 10, 11, 13, 14, and 15) and homodimers (Gal-4, 6, 8, 9, and 12) or chimerics (Gal-3) (**Fig.I.15**). Galectins can localized into multiple cell compartments in function of the status of the cells, being found in cytoplasm as well as in the nucleus [174]. Although galectins as a whole do not have the signal sequence required for protein secretion through the usual secretory pathway, some galectins are secreted and are found in the extracellular space [225].

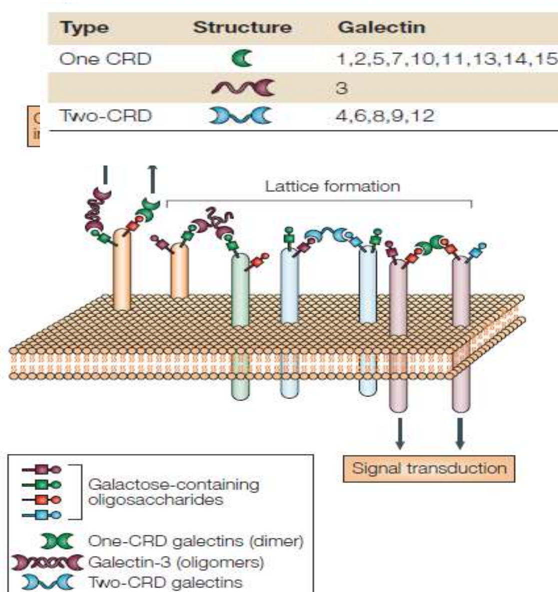


Fig.I.15. - **The galectin family**. Many galectins are either bivalent or multivalent with respect to their carbohydrate-binding activities. Adapted from Fu-Tong Liu *et al*, Nat Rev, 2005.

On the cellular level galectins are involved in mechanisms both inside the cell and extracellularly. Galectins have been proposed to play important roles in inflammation, immunity and cancer based on whole animal experiments and by their effects in tissue culture [175, 176]. Intracellularly, galectins are found in the cytosol and nucleus where they are involved in targeting exocytosis, cell activation and differentiation[177-179]. Galectins are also secreted by different
cells via the non-classical pathway and can attach to sugar structures on the cell surface [180]. By binding to N-glycosylated proteins Galectins may reside in solution as serum proteins or, on the cell surface, where they can modulate cell adhesion [181].

I.5.1 *Galectin Expression*

Some of the galectins are widely expressed in the body whereas others are only expressed in distinct compartments. Within the immune system, galectins are found in activated macrophages, activated B cells, dendritic cells and activated T cells [182]. Endothelial cells express several galectins. Gal-1 is expressed in human cultured endothelial cells, in aorta, umbilical vein and pulmonary artery, in vivo it was found in activated lymphoid tissue [183]. Galectin-3 expression has been observed in epithelial cells, macrophages, fibroblasts and activated T-cells [184]. Galectin-9

which was originally identified as an eosinophil specific chemo attractant is also expressed in HUVECs [185]. Galectin-8 is expressed in lung both in basal cells, ciliated bronchial cells, chondrocytes, serous cells of the bronchial glands, smooth muscle cells and endothelial cells [186]

I.5.2 *Galectin-1*

Galectin-1 (Gal-1), one of the most studied galectins is a symmetrical dimmer [170, 187, 188] of 14,5 kDa subunits involving two anti-parallel β–sheets[189]. Gal-1 has been shown to have a similar molecular structure to legume lectins, a structure consisting of a β–sandwich fold with a "jelly-roll" topology[190] Gal-1 is found in the cytoplasm, membrane, extracellular matrix and nucleus [191] and is implicated in several biological processes that are important in tumor progression, as modulation of cancer cell adhesion including cell–extracellular matrix and cell–cell interactions, cell migration, angiogenesis, and tumor–immune escape. For this reason it has been described as a promising cancer target [192, 193]. Gal-1 interaction with glycans is greatly enhanced when it is surface-bound on cell membranes or in ECM [197]. The structure reveals that there is one carbohydrate-binding site per monomer. The integrity of the dimer is maintained by the β-sheet interactions across the monomers and by formation of a hydrophobic core common to both [198].

I.5.3 *Galectin-1 in Cancer*

Gal-1 expression has been examined in several malignant tumors and its correlation with tumor invasiveness and lymph node metastasis was demonstrated in breast cancer , neuroblastoma, oral squamous cell carcinoma [194-196]. Gal-1 is one of the most important lectins to date participating in the malignant tumor development. With more data available on its expression in tumors it is already clear that Gal-1 is an important target for cancer diagnostics and treatment as previously mentioned. Gal-1 has been identified as a prognostic factor for tumor progression in many different neoplasm [199]. Increased expression of Gal-1 was observed in stroma of primary prostate carcinoma samples in comparison to stroma of normal prostate, and increased Gal-1 expression positively correlated with a poor prognosis of disease outcome [200, 201]. Gal-1 expressing lung tumors where shown to have poorer prognosis than non expressing ones by studies conducted by Szoke *et al* [203]. Different breast cancer cell lines express Gal-1 mRNA as it was shown by Lahm *et al* [204]. Several studies have reported that Gal-1 expression is upregulated in bladder cancer tissue in comparison to normal bladder tissue [205]. Most aggressive and undifferentiated anaplastic thyroid carcinomas consistently exhibited a marked increase of Gal-1 expression [207]. High Gal-1 mRNA levels in glioma tissues and glioma cell lines were also reported [208]. Galectin-1 is expressed in bone marrow samples of multiple myeloma patients [210].

I.5.4 *Galectin-1 in Pancreatic Cancer*

Pancreatic tumours overexpresses Gal-1 and Gal-3 proteins in comparison to normal pancreatic tissue [220]. Gal-1 was mainly detected in pancreatic cancer tissue stromal fibroblasts and extracellular matrix [220]. Gal-1 protein was upregulated in pancreatic ductal adenocarcinoma in comparison to normal pancreatic tissue [221]. By making changes in the ECM, Gal-1 could be involved in tumor progression in pancreatic cancer. Schaffert *et al* looked at different pancreatic cancer lines and human pancreatic carcinoma tissue and saw a uniform and strong overexpression of Gal-1 in pancreatic cancer cells compared to normal controls. The expression pattern of Gal-1 in pancreatic cancer tissues indicated that Gal-1 plays a role in the desmoplastic reaction that occurs around pancreatic cancer cells [220]. Harsha *et al* postulated that Gal-1 was overexpressed in the stroma of pancreatic cancer tissue and in the neoplastic ductal cells [222].

Our group has recently published a work showing that Gal-1 is highly expressed in PDA cells in culture, where it concentrates at the migration front, and in tissues, where it is expressed in epithelial cells and in the stroma. We also found that Gal-1 directly interacts with tPA and that the down-regulation of Gal-1 abolished the effects of tPA on ERK1/2 activation, cell proliferation and invasion, both in pancreatic and in tumor-derived fibroblasts. These findings identify a new molecular mechanism by which Gal-1/tPA

interaction contributes to PDA progression involving both transformed epithelial cells and tumoral stroma [243].

I.5.5 *Galectin-1 in Angiogenesis*

Gal-1 is also involved in tumor angiogenesis [209]. Endothelial cells of capillaries infiltrating to tumor stroma have strongly increased expression of Gal-1 in comparison to endothelial cells of capillaries in adjacent normal stroma. [202]. It is over expressed in endothelial cells of different human tumors. Previous studies have shown that Gal-1 is key in two mainstays of cancer such as proliferation and migration. Gal-1 support metastasis formation as it facilitates interactions between tumor cells and endothelial cells [211]. Thijssen *et al* found a direct involvement of Gal-1 in EC proliferation and migration *in vitro* and tumor angiogenesis *in vivo* [209]. Gal-1 has also been described as a novel hypoxia-regulated protein and a prognostic marker in head and neck squamous cell carcinoma. This study presented a new mechanism on how hypoxia can affect the malignant progression and therapeutic response of solid tumors by regulating the secretion of proteins that modulate immune privilege [212]. Expression knockdown of Gal-1 in cultured endothelial cells inhibits cell proliferation and migration [211]. The importance of Gal-1 in angiogenesis is illustrated in the zebra fish model where expression knockdown studies resulted in the impaired vascular guidance and growth of dysfunctional vessels [209]. Interaction with Gal-1 expressed by endothelial cells has been reported to play a role in tumor metastasis. In this regard, it

has been proposed that the dimeric nature of Gal-1 allows the crosslinking of integrins on the cell surface of tumoral cells to proteins on the ECM [217, 218], mediating tumoral cell-cell interactions or its interaction with endothelial cells and therefore facilitating tumor cell dispersion on the blood stream and establishment at distal sites during metastasis [202, 219].

OBJECTIVES

Previous data have shown a pro-angiogenic effect of tPA in *in vivo* models of PDAC [244, 168] although the molecular mechanisms are not well understood. The GENERAL AIM of this Thesis was to **elucidate the role of Tissue type Plasminogen Activator (tPA) in the Angiogenesis of Pancreatic Cancer**. To pursue this aim we have focused on the following **objectives**:

1) To analyze whether tPA-mediated angiogenesis is an INDIRECT effect through the positive regulation of pro-angiogenic molecules (VEGF, TGF-β, interleukins, MMPs) produced by pancreatic tumoral cells.
2) To analyze whether tPA-mediated angiogenesis is a DIRECT effect mediated by tPA direct activation of EC proliferation, migration and tubulogenesis, and whether these effects are tPA-catalytic activity dependent or independent.
3) To elucidate the molecular mechanisms involved in tPA pro-angiogenic effects: analysis of ERK1/2, AKT and JNK signaling pathways.
4) To investigate the role of cell membrane receptors (AnnexinA2, Galectin-1 and EGFR) in tPA- mediated endothelial cell signaling.
5) To investigate the mechanisms involved in tPA overexpression in pancreatic cancer (cytokines, hypoxia).

RESULTS

R.1.- *Effect of tPA on Angiogenic factors*

As mentioned in the Introduction, VEGF, TGF-β and different cytokines can stimulate angiogenesis. We analyzed whether tPA pro-angiogenic effects can be indirectly mediated by the induction of these pro-angiogenic molecules in tumoral cells and in ECs. Pancreatic cancer cell lines (RWP1 and SKPC1) and an endothelial cell line (H5V) were exposed to 20μg/ml of tPA for 24 hours. RNA was extracted and checked for the expression of VEGF, TGFβ-1, TGFβ-2, TGFβ-3, IL1-α and IL-8 by semi-quantitative PCR, using β-actin levels as loading control. The levels of these molecules remained unaltered after tPA treatment (**Fig. R.1**), indicating that tPA-proangiogenic effects are not mediated by them.

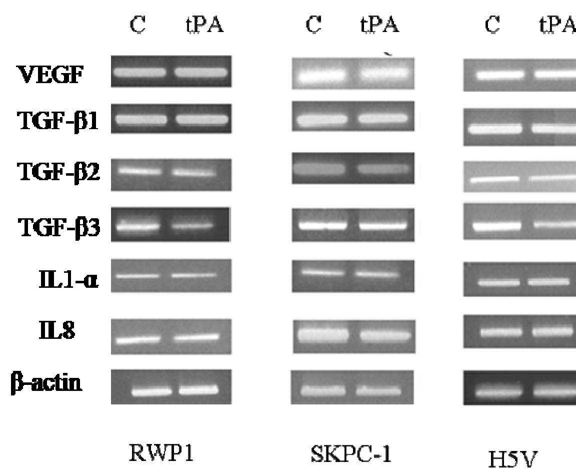


Fig.R.1.- Analysis by semi-quantitative RT-PCR of pro-angiogenesis molecules in pancreatic (RWP1, and SKPC-1) and endothelial (H5V) treated with tPA. Cells were exposed to tPA (20μg/ml) for 24 hours and then

analyzed by RT-PCR for expression of VEGF, TGFβ-1, -2 and -3, IL-1α and IL-8. Levels were normalized using β-actin as loading control.then analyzed by RT-PCR for expression of cytokines. The RNA levels were normalized with β-actin.

Matrix metalloproteinases (MMPs) family has also been reported to induce angiogenesis. In particular, emerging data have identified a role for gelatinases MMP-2 and MMP-9 in angiogenesis. Interestingly, it has been described that tPA treatment induced rapid dose-dependent upregulation in MMP-2 and MMP-9 in astrocytes [226] and that endogenous tPA enhances MMP-9 expression and proteolytic activation after ischemia reperfusion in rat brains [227]. We have then explored whether tPA can induce and/or activate MMP-2 and MMP-9 in pancreatic and endothelial cells. Pancreatic cancer cell lines (Hs766T, SKPC1, RWP1) and endothelial cells (HUVEC), were treated with 20μg/ml of tPA for 24 hours. After treatment the cell conditioned medium was analyzed by gelatin zymography. Expression of latent MMP-9 (molecular weight of 92 kDa) in pancreatic and HUVEC cells was only detected after tPA treatment. The active form of MMP-9 (which appears at 82 kDa) was only seen in HUVEC but not in the pancreatic cell lines, indicating that proteolytic MMP-9 activation by tPA is specific for endothelial cells (**Fig.R.2A**). Gelatin zymography showed only the latent form of MMP-2 (72 kDa), that was present at similar expression levels in both untreated and tPA-treated cells, indicating that tPA is not involved in MMP-2 regulation in pancreatic or endothelial cells (**Fig.R.2A**). Densitometric analysis of each cell line is shown in **Fig.R.2B**. MMP-2 and MMP-9 were also analyzed at the RNA level by semi quantitative PCR. In agreement with

protein data, MMP-2 RNA levels remained unaltered after tPA treatment in all cell lines. In contrast, MMP-9 RNA levels showed a slight increase only in HUVEC after tPA treatment and remained unaltered in pancreatic cells, suggesting that MM-9 increase obeserved after tPA-treatment in pancreatic cells should be mediated by post-transcriptional mechanisms (**Fig. R.2C**).

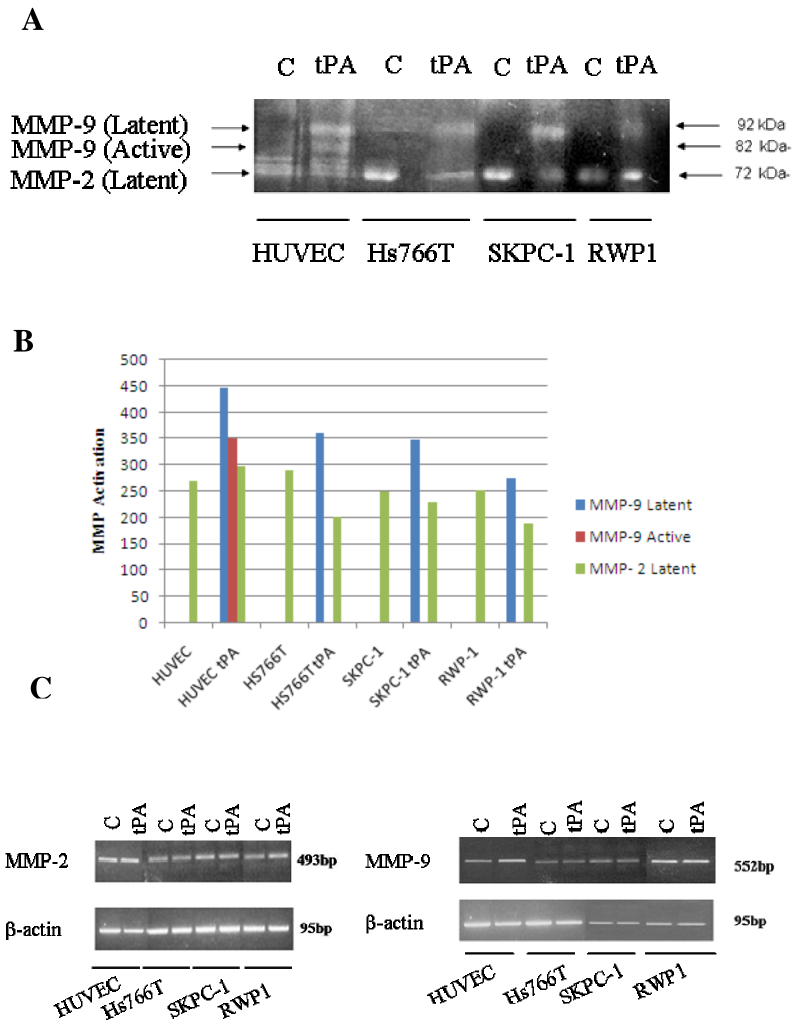


Fig R.2.- MMPs activity and expression was analyzed by gelatin zymography and RT-PCR analysis in pancreatic and endothelial cells after tPA treatment. A. Cell conditioned media were resolved in gelatin preimpregnated polyacrylamide gels. Gels were developed to show clear bands of gelatinolysis, correlating with the latent and active isoforms of MMP-2 and -9. B. Densitometry analysis of the zymogram indicating the activity of MMP 9 in HUVEC. C. RT-PCR analysis of MMP-2 and MMP-9 mRNA from HUVEC and PDAC cells.

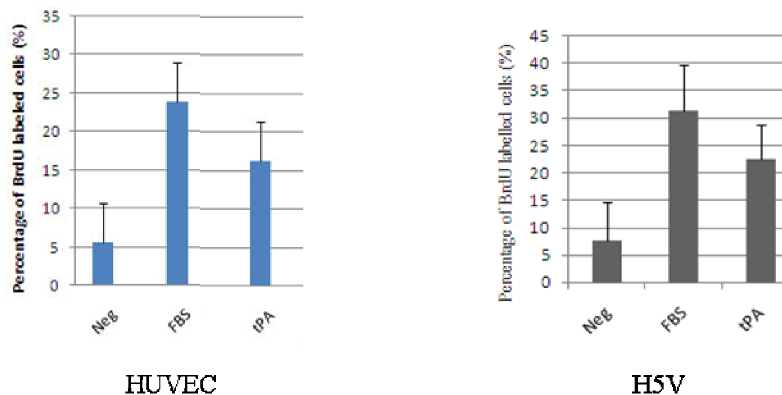
R.2.- *tPA induces endothelial cell Proliferation, Migration and Tubulogenesis*

In order to determine whether tPA can directly induce angiogenesis, two different endothelial cell lines (HUVEC and H5V) were treated with tPA (20 μg/ml) and functional studies of cell proliferation, migration and tubulogenesis were performed. The effects of tPA in EC proliferation were measured using BrdU incorporation after treatment with tPA (20μg/ml) for 24 h in serum-deprived cells. Quantification of BrDu labeled endothelial cells showed that tPA induces a high increase of cell proliferation both in HUVEC and H5V cells (**Fig. R.3A**). tPA effects in cell migration was analyzed using wound-healing assays. The endothelial monolayer was wounded with a sterile pipette tip, creating wounds of constant size, and wound closure was monitored over 24 hours. Treatment of cells with 20 μg of tPA resulted in a significant increase in the number of cells that migrated into the wound area (**Fig. R.3B**). Treatment with FBS was used as positive control in proliferation and migration tests. Finally, we analyzed tPA effects in tubulogenesis using the collagen sandwich assay. Endothelial cells cultured in the collagen sandwich model form tube-like structures, mimicking the major steps of vessel formation. In this model, ECs are grown between two collagen layers allowing a three

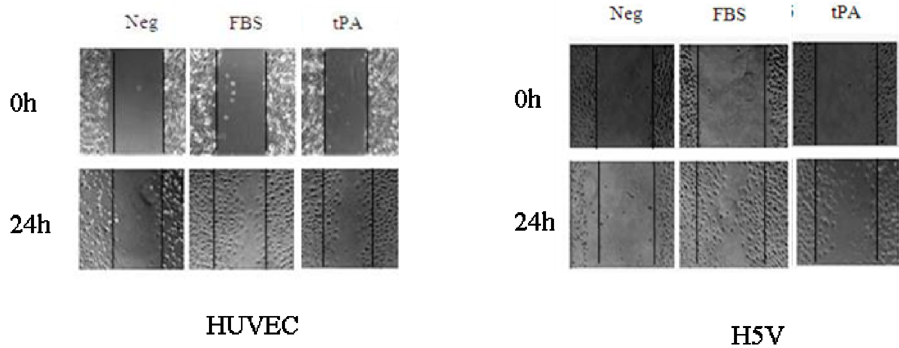
dimensional structure organization. The cells attach during the first hour and then, in the presence of angiogenic stimuli, migrate towards each other during the next 2-4 hours and form mature capillary-like tubes after 6-16 hours. HUVEC and H5V cells treated with tPA (0.5µg/ml) showed tube formation after 16 hours, indicating that tPA does promote tubule formation *in vitro* (**Fig.R.3C**). At 24-48 hours nearly all the cells were dead with complete destruction of tube structures (data not shown). VEGF (20µg/ml) treatment was used as positive control.

In order to test whether these effects were dependent on the catalytic activity of tPA, HUVEC and H5V cells were treated with a catalytically inactive mutant tPA (tPAS481A) and its effects in cell proliferation, migration and tubulogenesis was analyzed. tPAS481A increased EC proliferation and induced migration and tubulogenesis to the same extent than wild-type tPA, indicating that these pro-angiogenic tPA effects were directly mediated by tPA independently of its catalytic activity (data not shown).

A



B



C

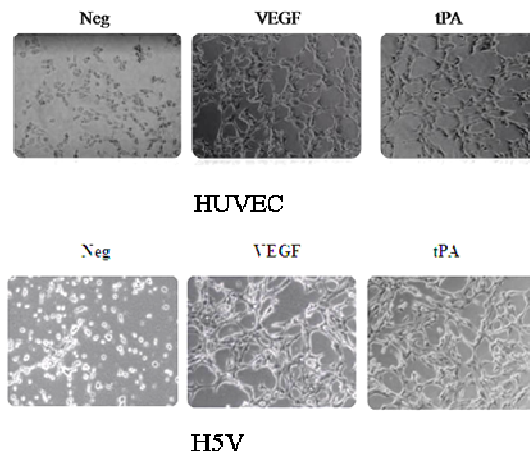
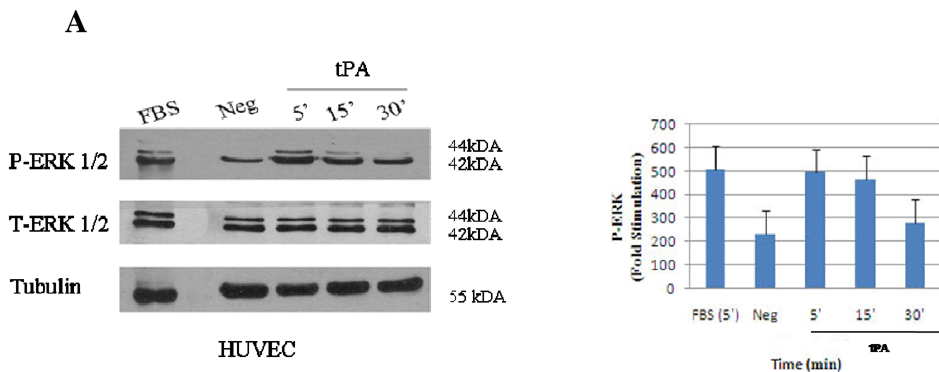


Fig.R.3.- tPA effects in endothelial cell proliferation, migration and tubulogenesis. A. Proliferation of HUVEC (left) and H5V (right) cells untreated (Neg), treated with 20 µg/ml of tPA (tPA) or with 5% FBS, as positive control (FBS), was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation. B. HUVEC and H5V cells were grown to confluence, deprived in medium without FBS for 3 days, wounded and allowed to migrate in the absence or presence of tPA (20 mg/ml). FBS was used as positive control. C. Tubule formation in collagen sandwich model of HUVEC and H5V cells untreated (Neg), treated with 0.5 µg/ml of tPA (tPA) or with VEGF (20 µg/ml, positive control).

R.3.- *tPA induces catalytic-independent activation of ERK1/2, AKT and JNK signalling pathways in ECs*.

tPA has been described to have a mitogenic role on variety of cell types like aortic smooth muscle cells, pancreatic cells and neurons and this effect has been related to ERK 1/2 signalling pathway activation. In addition, ERK 1/2 kinases have been shown to regulate endothelial cell survival, migration and proliferation, particularly in conjuction with growth factor signaling. To analyse whether tPA effects in ECs described in the previous section (R.2) could be mediated by ERK1/2 activation, we examined the ability of tPA to activate the ERK1/2 cascade in HUVEC and H5V endothelial cells. tPA induced an strong increase in ERK1/2 activation, detected by Western blot using antibodies against phospho-ERK1/2. This activation was transient, reaching it maximum at 5 minutes and was maintained for 15 minutes in HUVEC (**Fig. R.4A)** and H5V (**Fig.R.4 B**) cells. The levels of total ERK1/2 remained unchanged indicating a specific effect in ERK1/2 phosphorylation/activation. Tubulin levels were also analysed as loading control.



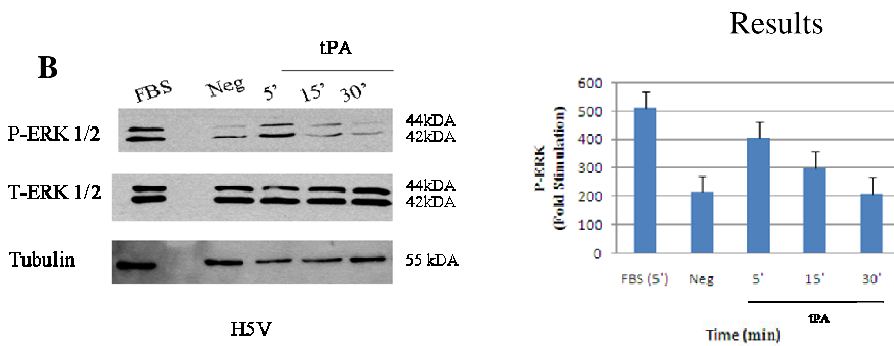
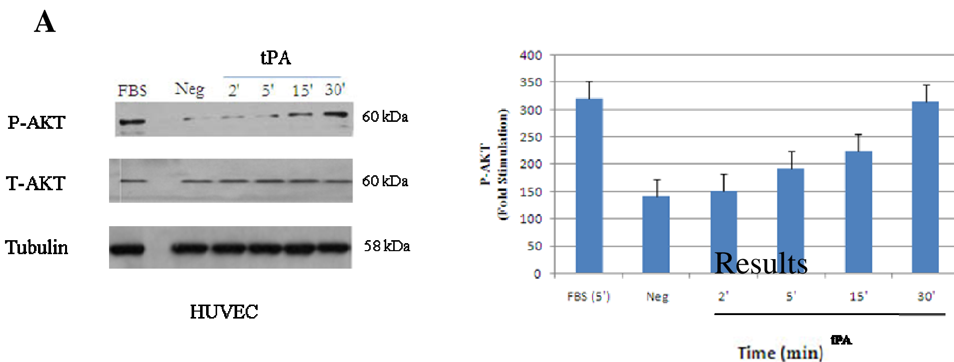


Fig R.4. - tPA induces ERK activation in HUVEC and H5V cells. A)Western blot analysis of phosphorylated extracellular signal-regulated kinase (p-ERK) and ERK in human umbilical vein endothelial cells (HUVEC) stimulated with tPA (20µg/ml) for 5, 15, and 30 min. (B) WB analysis of p-ERK in H5V endothelial cells treated with tPA (20µg/ml) for 5, 15 and 30 min. FBS (5%) treatment was used as positive control. Three independent experiments were performed and quantification is shown in the graph.

Recent report by An *et al* [242] demonstrated that tPA induces phosphorylation of AKT -a key pathway involved in cell survival- in the ischemic brain. To determine whether tPA can induce AKT activation in ECs, HUVEC and H5V were treated with tPA (20 µg/ml) and AKT activation was analyzed. tPA induced AKT phosphorylation in both HUVEC (**Fig.R.5A**) and H5V (**Fig.R.5B**) cells. AKT activation was detected at 15 minutes in HUVEC while in H5V it can be already observed at 2 minutes



B

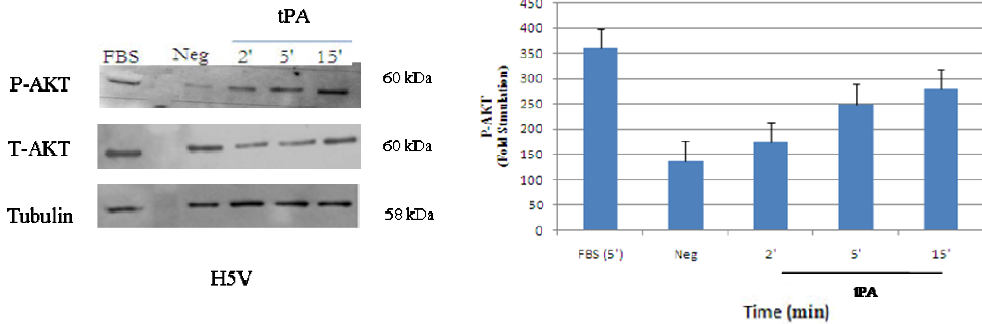
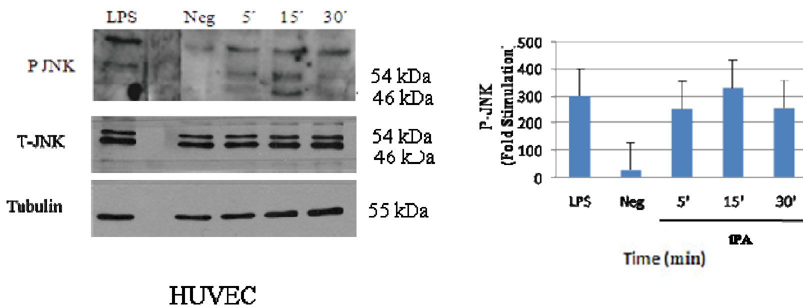


Fig.R.5.- tPA induces AKT activation in HUVEC and H5V cells. A. Western blot analysis of phosphorylated AKT (P-AKT) and total AKT (T-AKT) in HUVEC stimulated with tPA (20µg/ml) for 2, 5, 15, and 30 min. B. WB analysis of phosphorylated AKT (P-AKT) and total AKT (T-AKT) in H5V endothelial cells treated with tPA (20µg/ml) for 2, 5 and 15min. Three independent experiments were performed and quantification is shown in the graph

JNK signalling pathway has been also related to endothelial activation and angiogenesis, and recent data from our group has shown that tPA can activate JNK in microglial cells. We examined whether tPA can induce JNK activation in endothelial cells. Treatment with tPA induced JNK activation at 5 minutes and was maintained until 30 min in both HUVEC (**Fig. R.6A**) and H5V (**Fig.R.6B**) cells. Total levels of JNK and tubulin were also analyzed to rule out nonspecific effects in total protein levels.

A



B

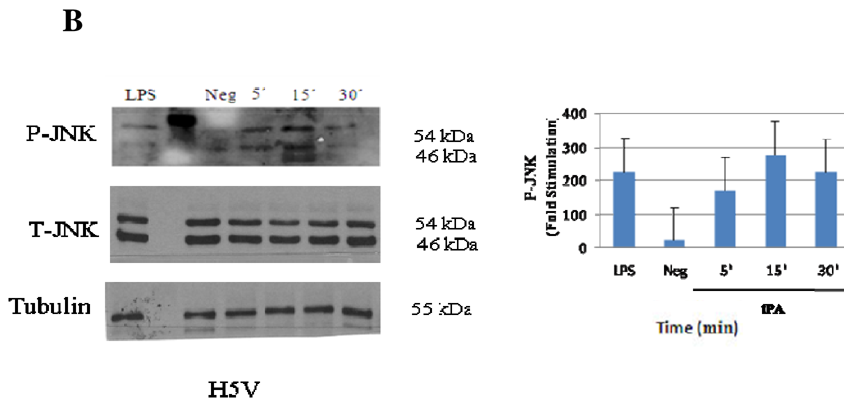


Fig.R.6.- tPA induces JNK activation in HUVEC and H5V cells. A. Western blot analysis of phosphorylated JNK (P-JNK) and total JNK (T-JNK) in HUVEC stimulated with tPA (20µg/ml) for 5, 15, and 30 min. B. WB analysis of phosphorylated JNK (P-JNK) and total JNK (T-JNK) in H5V endothelial cells treated with tPA (20µg/ml) for 5,15 and 30 min. Three independent experiments were performed and quantification is shown in the graph

Activation of signalling pathways by tPA can be mediated by its catalytic activity through activation of latent growth factors or directly by tPA in a catalytic independent way [234,237]. In order to discriminate whether tPA-mediated ERK1/2 activation in endothelial cells was catalytic dependent or independent, H5V cells were treated with tPAS481A and the activation of ERK1/2 was analyzed by Western blot. Catalytic inactive tPA was able to induce phosphorylation of ERK1/2 to similar extent than wild type tPA (**Fig. R.7**), indicating that tPA-mediated activation of this signalling pathways is independent of its catalytic activity.

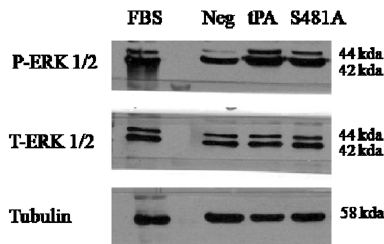
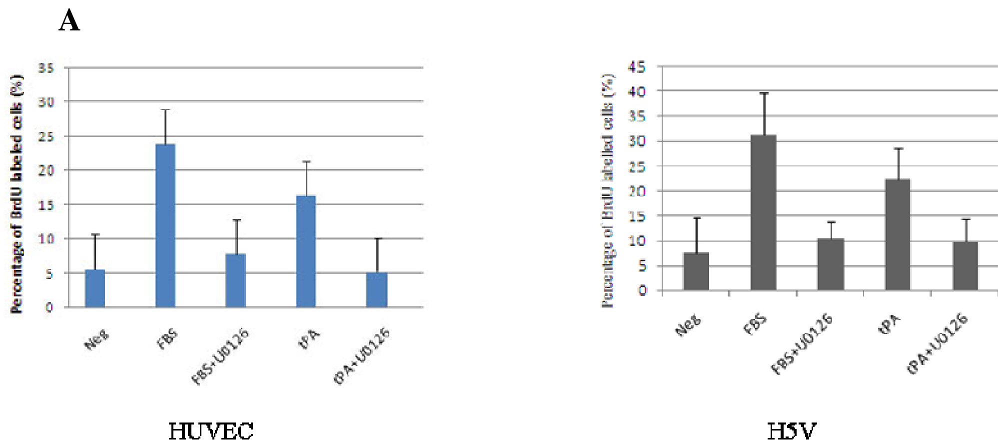


Fig.R.7.- tPA-mediated phosphorylation of ERK kinases in endothelial cells does not require its catalytic activity. Mutant tPA S481A induces ERK1/2 activation similar to wild type tPA, indicating that proteolytic activity of tPA is not required for the activation of ERK signalling pathway.

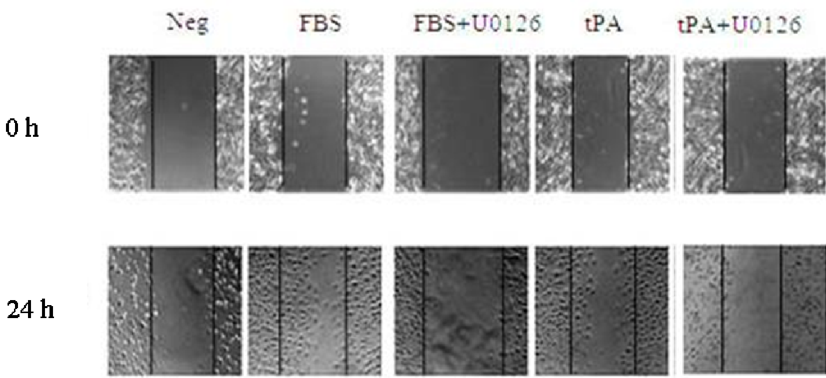
R.4. - *ERK1/2, AKT and JNK signalling pathways are responsible for tPA-mediated EC Proliferation, Migration and Tubulogenesis*

We next aimed to validate whether the intracellular signalling cascades triggered by tPA, in a catalytic independent way, are responsible for its effects in EC pro-angiogenic response. To address this point we analyzed HUVEC and H5V proliferation, migration and tubulogenesis after the treatment with tPA alone or in the presence of inhibitors for the different signalling pathways. Treatment of HUVEC and H5V cells with tPA in the presence of MEK/ERK1/2 inhibitor U0126 (10μM) resulted in a dramatic decrease in the cell proliferation, migration and tubulogenesis (**Fig.R.8**).

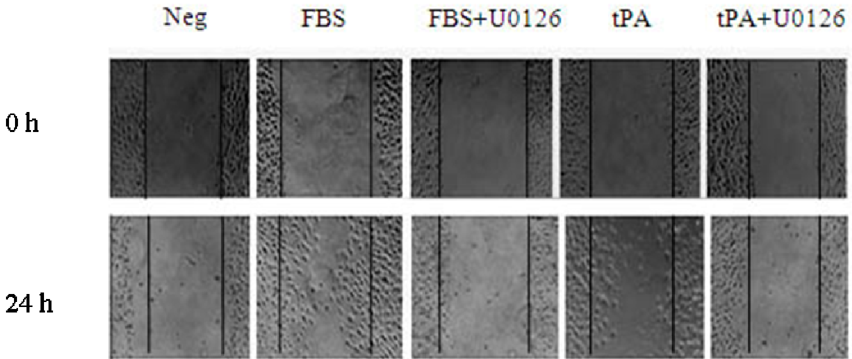
A



B

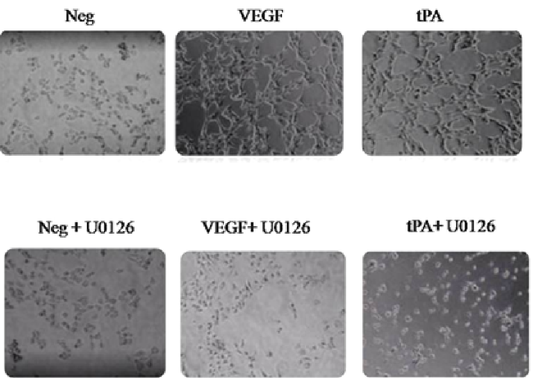


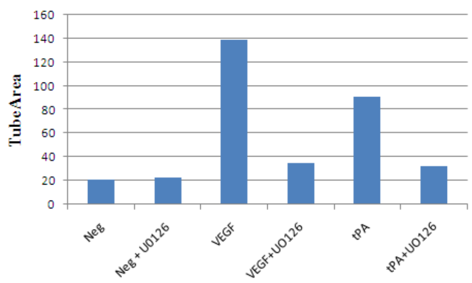
HUVEC



H5V

C





HUVEC

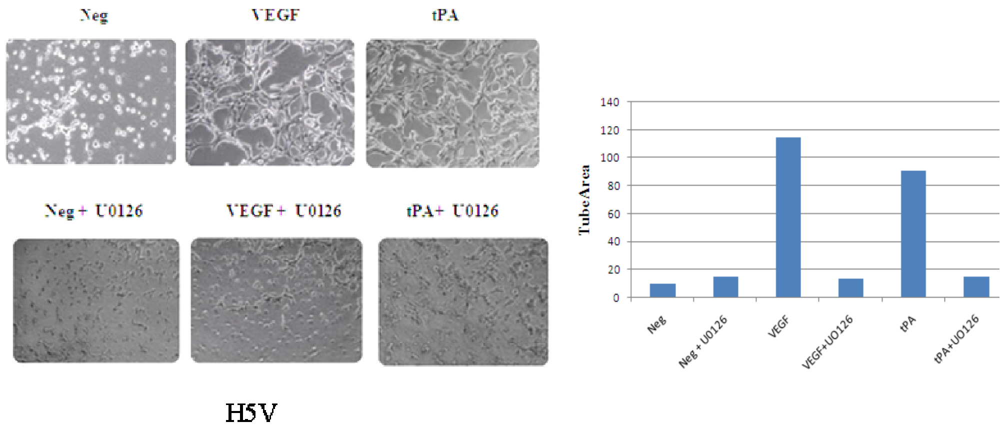
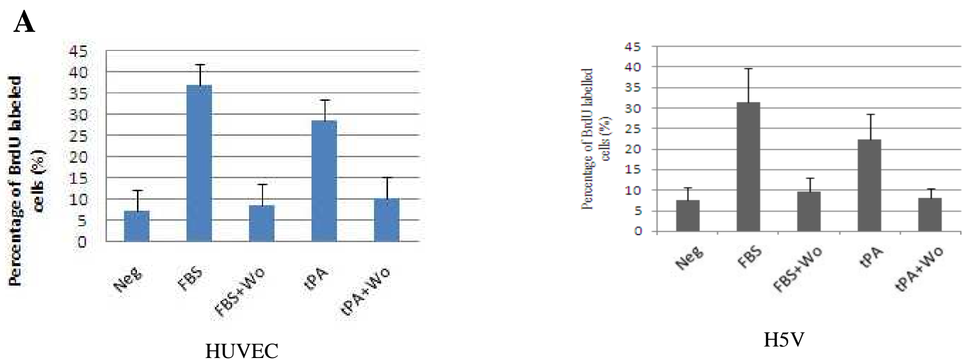


Fig.R.8. - Inhibition of ERK1/2 signalling pathway with U0186 abolishes tPA effects on endothelial cell proliferation, migration and tubulogenesis. A. tPA (20 µg/ml) treatment in the presence of 10µM U0126 (tPA+U0126) failed to increase HUVEC and H5V proliferation, measured by BrdU incorporation. B. tPA (20 µg/ml) treatment in the presence of 10µM U0126 (tPA+U0126) failed to induce HUVEC and H5V migration in a wound healing assay. C. tPA (0.5µg/ml) treatment in the presence of 10µM U0126 (tPA+U0126) failed to induce HUVEC and H5V tubulogenesis in a collagen sandwich assay.

Similar results were obtained after tPA treatment in the presence of wortmannin (10µM) – an inhibitor of the AKT pathway- (**Fig.R.9**) or in the presence of a JNK inhibitor, JNKI1 (JNK inhibitor-1) (20µM) (**Fig.R.10**). Altogether these data demonstrate that activation of ERK1/2, AKT and JNK by tPA are required to induce endothelial cell proliferation, migration and angiogenesis.



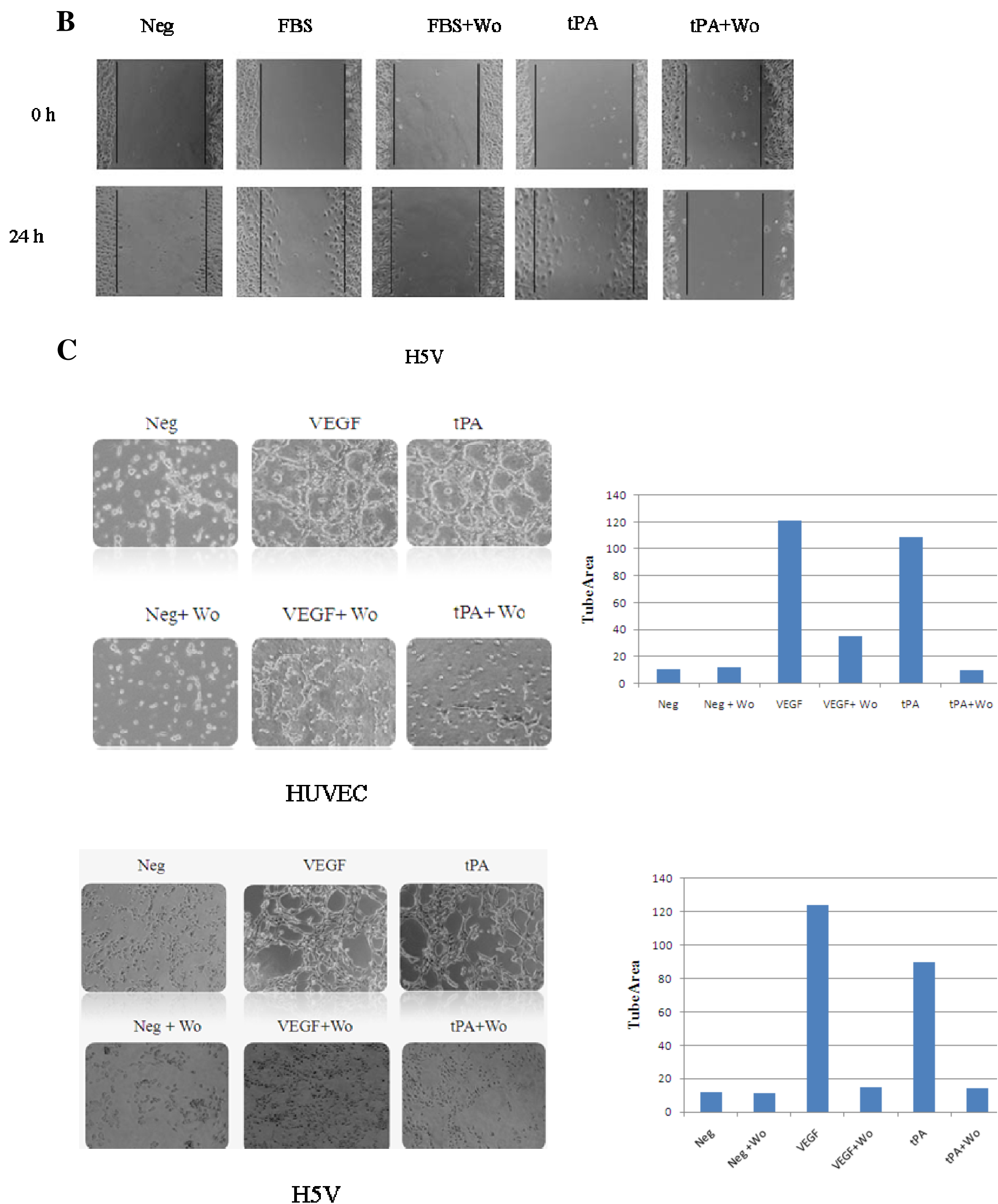
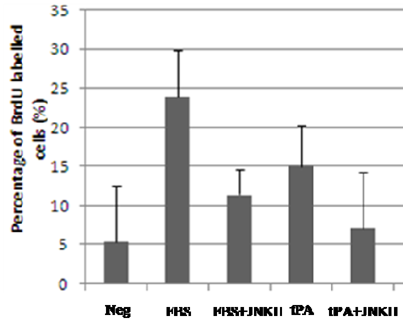


Fig.R. 9. - Inhibition of AKT signalling pathway with Wortmannin abolishes tPA effects on endothelial cell proliferation, migration and tubulogenesis. A. tPA (20 µg/ml) treatment in the presence of Wortmannin (10µM) (tPA+Wo) failed to increase HUVEC and H5V proliferation, measured by BrdU incorporation. B. tPA (20 µg/ml) treatment in the presence of Wortmannin

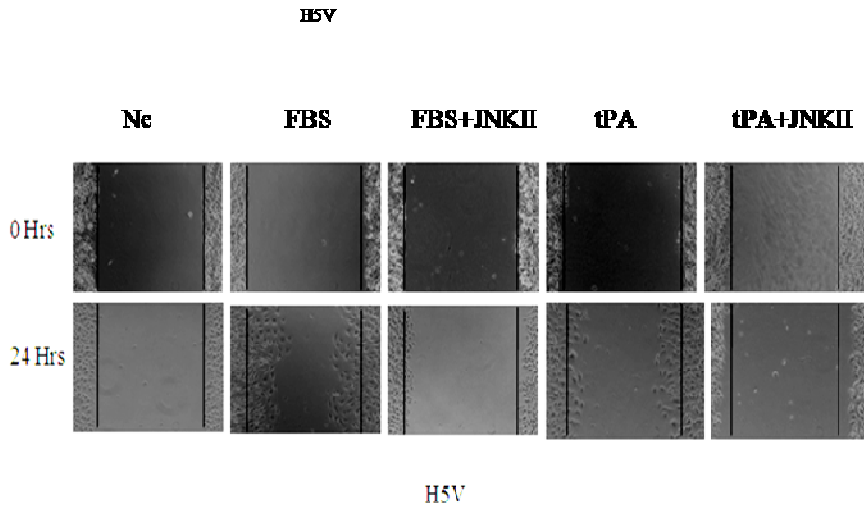
(10µM) (tPA+Wo) failed to induce HUVEC and H5V migration in a wound healing assay. C. tPA (0.5 µg/ml) treatment in the presence of Wortmannin (10µM) (tPA+Wo) failed to induce HUVEC and H5V tubulogenesis in a collagen sandwich assay.

A



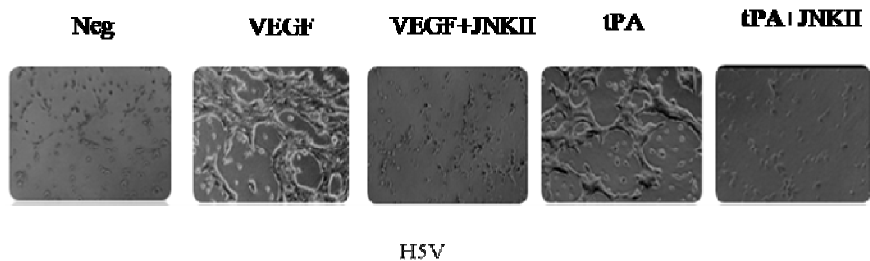
H5V

B



H5V

C



H5V

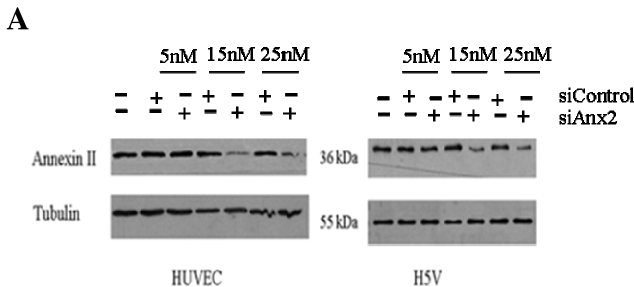
Fig. R.10.- Inhibition of JNK signalling pathway with JNKI1 abolishes tPA effects on endothelial cell proliferation, migration and tubulogenesis. A. tPA treatment in the presence of JNKI1 (20µM) (tPA+ JNKI1) failed to increase H5V proliferation, measured by BrdU incorporation. B. tPA treatment in the presence of JNKI1 (tPA+ JNKI1) failed to induce H5V migration in a wound healing assay. C. tPA treatment in the presence of JNKI1 (tPA+ JNKI1) failed to induce H5V tubulogenesis in a collagen sandwich assay.

R.5. – *AnnexinA2, EGFR and Galectin-1 are involved in tPA-mediated signalling activation in endothelial cells*

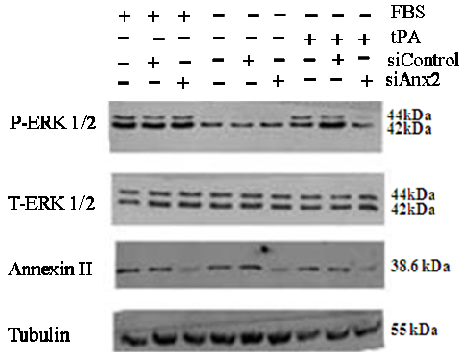
tPA-mediated activation of signalling pathways requires its interaction with cell surface receptors that would mediate intracellular signal transduction. Several receptors have been found to be involved in tPA-mediated triggering of intracellular cascades in different cell types [159, 243]. In our group we has previously identified that ERK1/2 activation by tPA in pancreatic cancer cells requires AnnexinA2 (AnxA2), EGFR [237] and Galectin-1 (Gal1) receptors [243]. We therefore decided to study the involvement of these cell membrane receptors in tPA signalling in endothelial cells. AnxA2 has been well characterized as tPA receptor in endothelial and promyelocytic leukemia cells. We next addressed the question whether the tPA binding to AnxA2 is required for the activation of ERK signaling pathway in endothelial cells, using siRNA technology. Double stranded siRNA corresponding to nucleotides 1-22 of AnxA2 (siAnxA2) at various concentrations (5nM to 25nM) was transfected into HUVEC and H5V cells. At 15nM we observed a 70% reduction in total cellular levels of AnxA2 proteina and this concentration was choosen for further experiments (**Fig.R.11A**). The levels of tubulin remained unaffected indicating the specificity

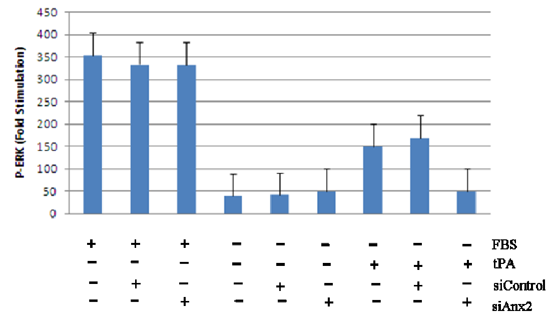
of siAnxA2 oligos. Treatment with double stranded oligonucleotide targeting an irrelevant transcript (siControl) was used as negative control. Downregulation of AnxA2 expression resulted in a complete abolishment of tPA-induced ERK1/2 activation in both HUVEC and H5V cells. As expected, transfection with siControl had no effect on activation of ERK1/2, indicating the specificity of siAnxA2 (**Fig.R.11B**). In contrast, FBS-mediated ERK1/2 activation remained unchanged by either siAnxA2 or siControl treatment, indicating the specificity of AnxA2 down regulation in the tPA-induced ERK1/2 signaling (**Fig.R.11B**). Contribution of AnxA2 to tPA-mediated JNK activation in endothelial cells was also analyzed. H5V cells were transfected with siRNA for AnxA2 (siAnxA2) or an irrelevant transcript (siControl) and JNK activation after tPA-treatment was studied by Western blot. AnxA2 downregulation led to an inhibition of JNK activation after tPA treatment, as shown in **Fig.R.11C**. Altogether these data indicate that AnxA2 is required for ERK1/2 and JNK activation induced by tPA in endothelial cells.

A

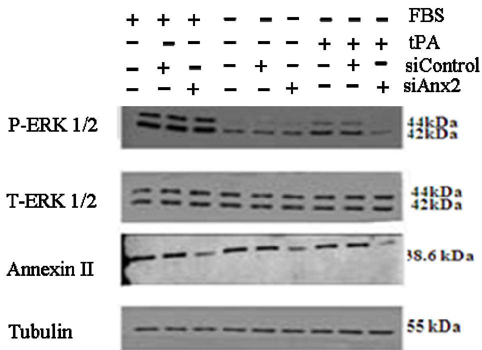


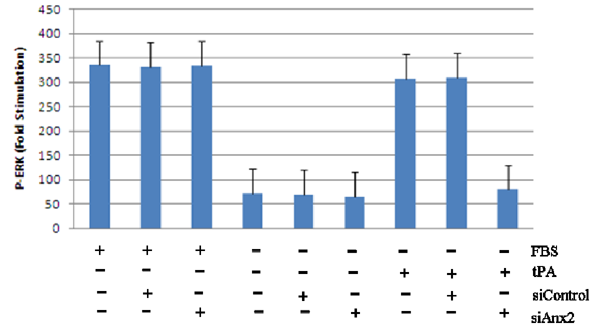
B





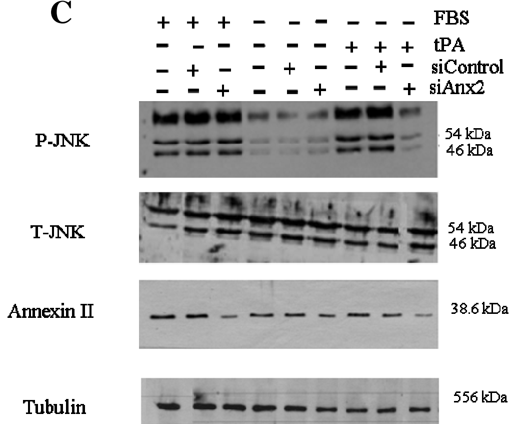
HUVEC

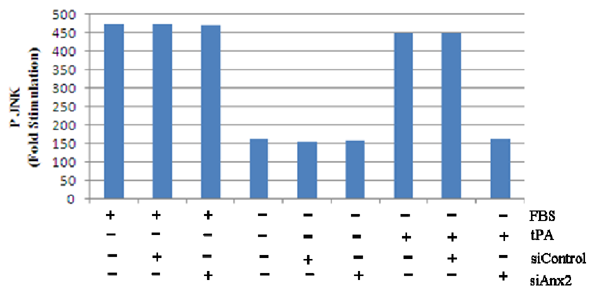




H5V

C





H5V

Fig. R.11.- AnxA2 is required for tPA-induced ERK1/2 and JNK signaling in HUVEC and H5V cells. A. Inhibition of AnxA2 expression using siRNA in HUVEC and H5V cells. The cells were transiently transfected with siRNA against AnxA2 (siRNA) or an irrelevant target (siControl). Different concentrations of siRNA and siControl were used. Tubulin levels in cell lysates were assayed as a control. **B.** Depletion of AnxA2 using siRNA blocks tPA-induced ERK1/2 activation. HUVEC or H5V cells, untransfected (C) or transfected with siAnxA2 or siControl, were treated with or without tPA (20µg/ml) for 5 minutes or with FBS for 5 minutes. ERK1/2 activation was detected by WB using antibodies against phosphorylated ERK1/2. Three separate experiments were performed and quantification of reduction in tPA-mediated ERK1/2 activation by silencing of AnxA2 is shown in the graph. **C.** Depletion of AnxA2 using siRNA blocks tPA induced JNK activation. H5V cells, untransfected or transfected with siAnxA2 or siControl, were treated with or without tPA (20µg/ml) for 5 minutes or with FBS for 5 minutes. JNK activation was detected by WB using antibodies against phosphorylated JNK. Three separate experiments were performed and quantification of reduction in tPA-mediated JNK activation by silencing of AnxA2 is shown in the graph.

EGFR integrates signals from various types of cytokines/growth factors, is classically related to ERK1 /2 signal pathway activation [235], and is highly expressed in ECs. We next aimed to explore the role of EGFR in tPA-mediated signalling activation in ECs. For this purpose, endothelial cells (HUVEC and H5V) were grown to 50% in serum deprived medium and then treated with FBS (5%, positive control), tPA (20µg/ml) or EGF (1ng/ml) in the presence or absence of the selective EGFR chemical inhibitor Gefitinib (10 µM) and the effects ERK 1/2 phosphorylation were determined. ERK1/2 activation was completely abolished in the presence of Gefitinib, indicating the involvement of EGFR in ERK1/2 activation induced by tPA (**Fig. R.12**). Activation of ERK1/2 by EGF treatment was used to check the specificity of the Gefitinib inhibitor, showing that EGF in the presence of Gefitinib failed to induce ERK1/2 phosphorylation, as expected (**Fig. R.12**).

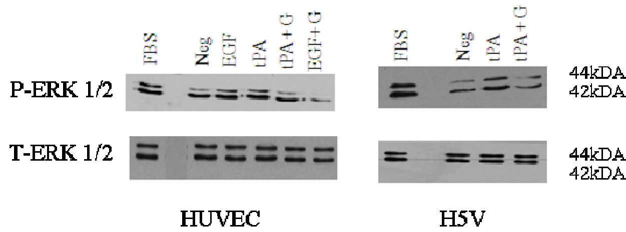


Fig.R.12.- EGFR is required for tPA-induced ERK1/2 signaling in HUVEC and H5V cells. ECs were treated with 20 µg/ml tPA for 15 minutes in the absence or in the presence of the EGFR inhibitor Gefitinib (10 µM). Cells were collected and lysates were analyzed by Western blotting using antibodies against phosphorylated (P-ERK1/2) and total ERK1/2 (T-ERK1/2). Treatment with FBS and EGF were used as positive control for ERK1/2 activation and Gefitinib effects, respectively. Inhibition of EGFR results in abolishment of tPA signaling.

Gal-1 has been recently found to be a receptor for tPA and down-regulation of Gal-1 by siRNA confirmed the involvement of Gal-1/tPA interaction in ERK1/2 activation, cell proliferation, and invasion in pancreatic and fibroblastic cells [243]. Taking into account that Ecs express Gal-1, we decided to explore whether this receptor could be involved in the activation of ERK1/2 induced by tPA in endothelial cells. HUVEC and H5V cells were transfected with si RNA against Gal-1 (siGal1) or an irrelevant transcript (siControl). Western blot analysis using anti-Gal-1 antibody confirmed the down regulation of Gal-1 and the optimal concentration of oligonucleotides in HUVEC (25 nM) and H5V cells (1 nM). The levels of tubulin remained unaffected indicating the specificity of Gal-1 siRNA. As expected, the treatment with double stranded oligonucleotide targeting an irrelevant transcript

(siControl) did not affect the Gal-1 levels. Downregulation of Gal-1 completely abolished ERK1/2 activation by tPA in both HUVEC and H5V cells whereas transfection with siControl had no effect on activation of ERK1/2 (**Fig.R.13**). In contrast FBS-induced ERK1/2 activation and total levels of ERK1/2 were unaffected by either siGal1 or siControl treatment indicating the specificity of Gal-1 knock down in tPA-induced ERK 1/2 signaling (**Fig.R.13**).

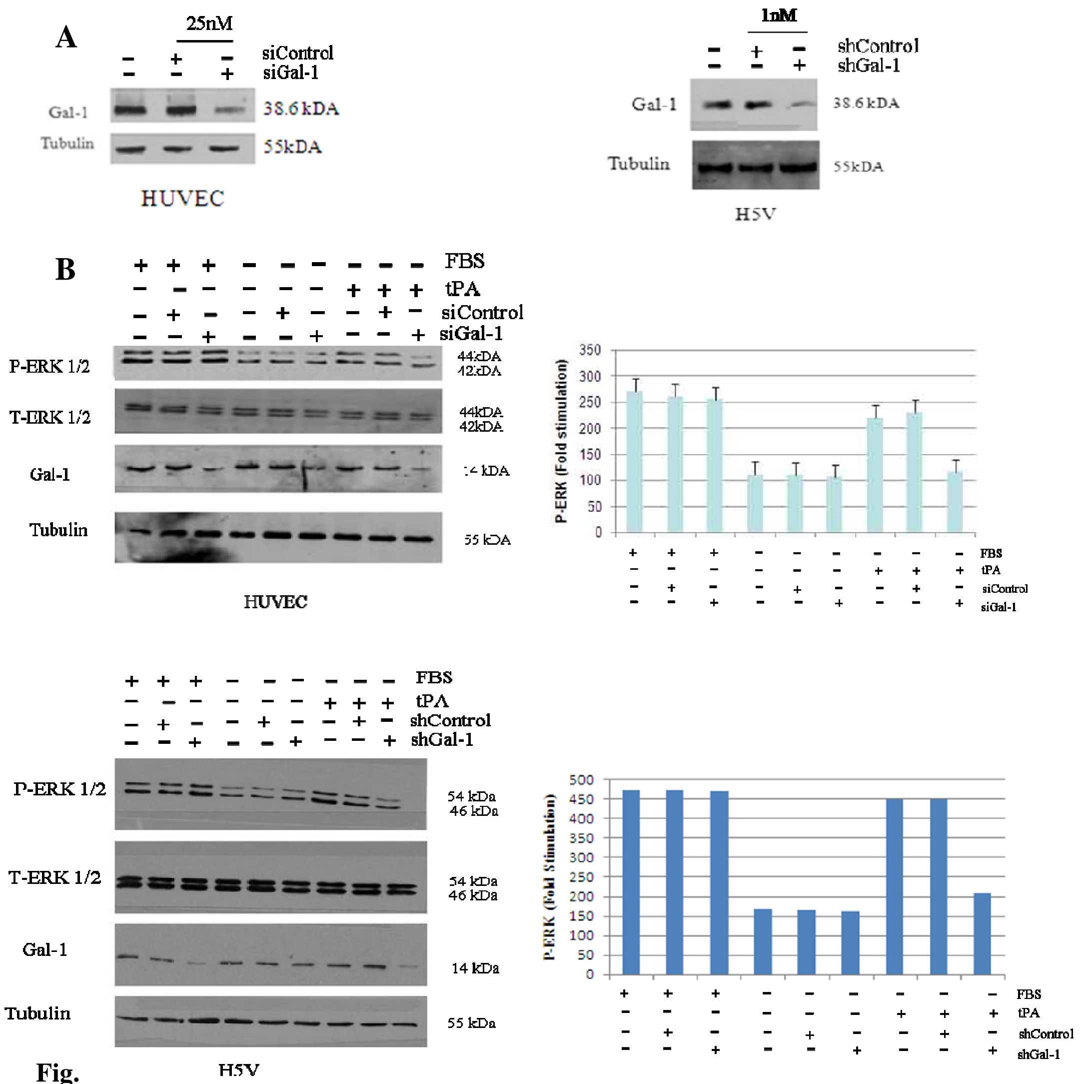


Fig.

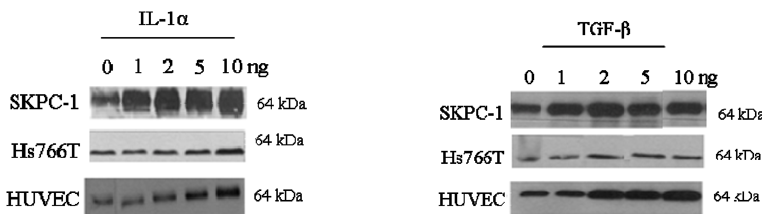
R.13.- Gal-1 is necessary to induce ERK 1/2 activation in endothelial cells. A. Inhibition of Gal-1 expression using siRNA in HUVEC and H5V cells. The cells were transiently transfected with siRNA against Gal-1 (siRNA)(25nM, HUVEC; 1nM, H5V) or an irrelevant target (siControl). **B.** Depletion of Gal-1 using siRNA blocks tPA-induced ERK1/2 phosphorylation in HUVEC and H5V cells . Ecs, untransfected (C), transfected with an irrelevant siRNA (siControl) or with Gal1 siRNA (siGal1),were treated with tPA (20µg/ml) for 5 minutes or with FBS for 5 minutes. ERK1/2 activation was detected by WB using antibodies against phosphorylated ERK. Three separate experiments were performed and quantification of reduction in tPA-mediated ERK1/2 activation by silencing of Gal-1 is shown in the graph.

R.6. – *TGF-β and IL-1a induce tPA in PDAC and HUVEC cells*

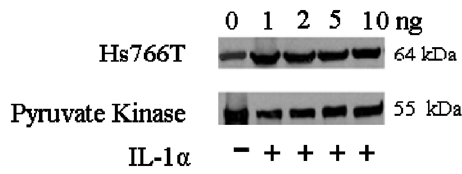
The data described until now show the role of tPA as stimulator of angiogenesis. These results together with the overexpression of tPA in PDAC prompted us to analyze which factors could be responsible for tPA overexpression in PDAC and their involvement in tPA-mediated angiogenesis in this tumor. IL-1α and TGF-β are known to be important stimulators in the induction of angiogenesis in pancreatic cancer and has been reported to be produced by different cell lines derived from PDAC and by tumoral stroma. We checked whether IL-1α and TGF-β can upregulate tPA expression in PDAC cell lines as well as in HUVEC cells. For this purpose pancreatic cancer cells (SKPC-1 and Hs766T) and HUVEC were exposed to different concentrations of recombinant IL-1 α and TGF- β (1, 2, 5 and 10 ng/ml for 24 h, and tPA protein expression levels in the conditioned medium (as tPA is a secreted protein) was determined by Western blot analysis. Treatment with IL-1α or TGF induced an increase in tPA expression in a dose dependent manner in all cells (SKPC-1, Hs766T and HUVEC) (**Fig.R.14A**). Combination of IL1-α and TGF-β treatment resulted in a synergestic response in the

induction of tPA expression in Hs766T cells (**Fig.R.14B**). Induction of tPA expression by IL-1 α, TGF-β or both cytokines was also determined at the RNA level using RT-qPCR. **Fig. R.14C** show that treatment of pancreatic cancer cells or HUVEC with IL-1 α, TGF-β or combined treatment (IL-1 α + TGF-β) resulted in a dose-dependent increase of tPA mRNA levels, indicating that the induction of tPA expression by IL-1 α and TGF-β is at the transcriptional level.

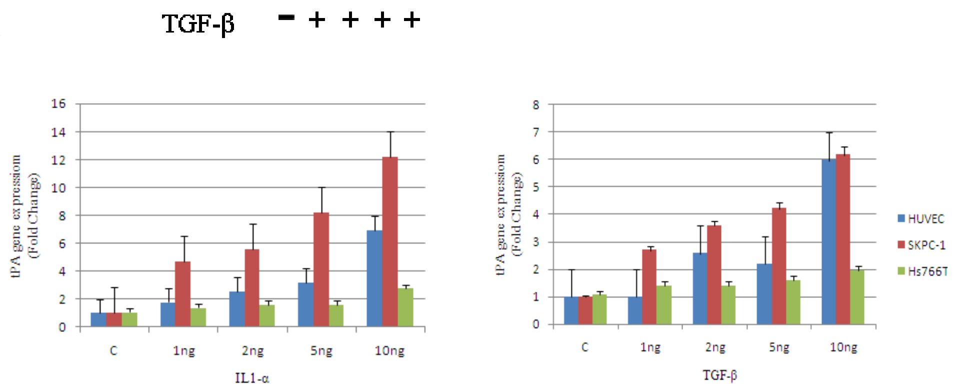
A



B



C



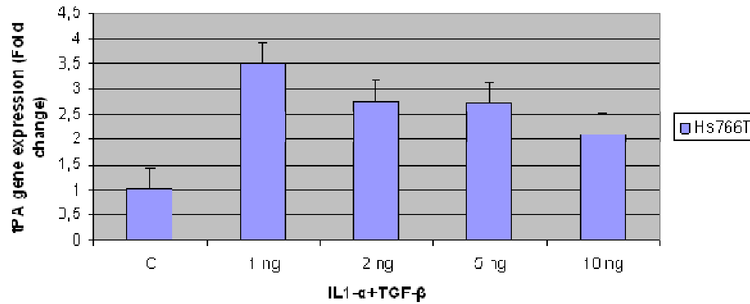
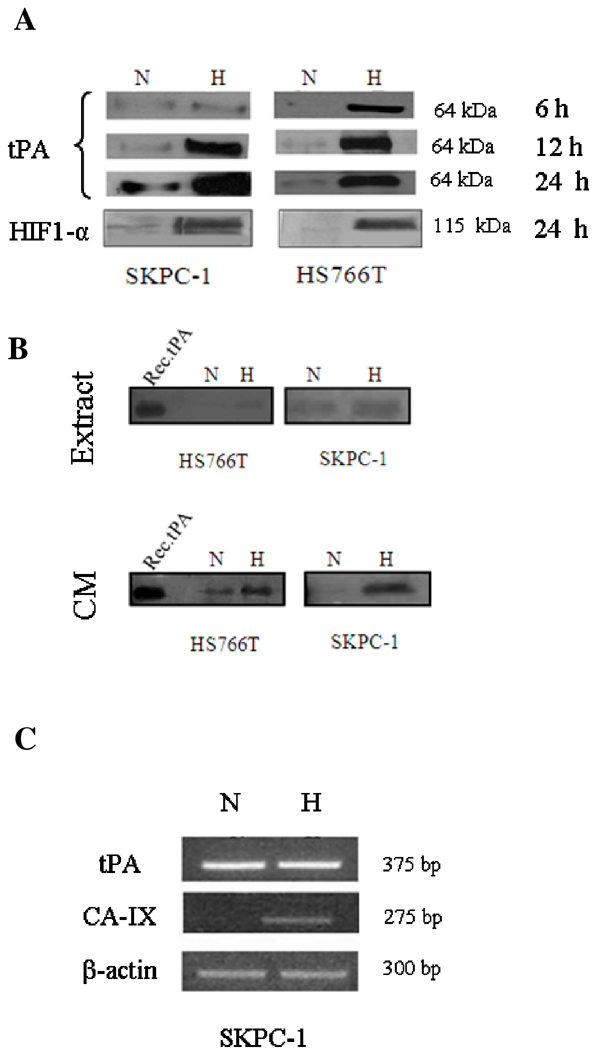


Fig.R.14. - Effect of IL1-α and TGF-β in tPA induction in pancreatic cancer cells and HUVEC A. Dose dependent stimulation of tPA protein by IL1-α and TGF-β in PDAC cells (SKPC-1 and Hs766T) and HUVEC. **B.** Combined treatment with IL1-α a and TGF-β at various concentrations in HUVEC cells lead to a sinergistic effect in tPA induction. **C.** tPA mRNA expression levels were analysed by RT-qPCR after treatment with IL1-α, TGF-β or combined IL1-α+TGF- β in HUVEC and PDAC cells. Levels were normalised with β-actin.

R.7. – *Hypoxia induces tPA in PDAC cells*

Hypoxia has been reported to be fundamental for the angiogenic process and is one of the hallmarks of many cancers, including PDAC. We checked whether tPA expression in pancreatic cancer cells is upregulated after hypoxic conditions both at protein and RNA levels. SKPC-1 and Hs766T were incubated under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions and the levels of tPA in conditioned media after 6, 12 and 24 hours were analyzed by Western blot (**Fig R. 15A**), zymography (**Fig.R.15B**), semiquantitative PCR (**Fig. R.15C**) and RT-qPCR (**Fig. R.15D**). At the protein level, three fold more tPA was detected in the conditioned media by hypoxic cells than by normoxic cells at 6, 12 and 24 hours (Fig. 14A). Caesin zymography analysis confirmed that tPA activity in the conditioned media.was more in hypoxic than in normoxic conditions (**Fig.R.15B**). In contrast, tPA mRNA levels remained unaltered comparing hypoxia versus normoxia conditions.

These data suggest that tPA induction by hypoxia should be regulated post transcriptionally.



D

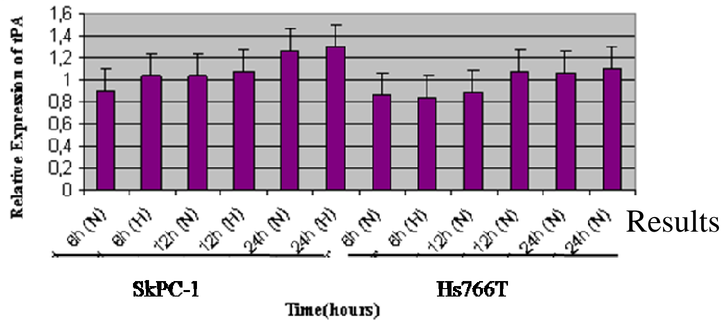


Fig.R.15.- Effect of hypoxia in tPA expression in PDAC cell lines. tPA was induced when SKPC-1 or Hs766T cells were grown in hypoxia (1% oxygen). **A.** Western blot analysis of tPA in conditioned media from cells grown in normoxic or hypoxic conditions during 6, 12 and 24 hours. **B.** tPA activity was determined by caesin zymography of conditioned media from cells grown in normoxic or hypoxic conditions during 24 hours to check the activity of tPA during hypoxia in cell extract and conditioned medium. **C.** Analysis of tPA mRNA by semiquantitative PCR after 24 hours of cell culture in normoxic or hypoxic conditions. **D.** Analysis of tPA mRNA by RT-qPCR after 6, 12 and 24 h of cell culture in normoxic or hypoxic conditions.

DISCUSSION

D.1 tPA can have an indirect pro-angiogenic effect by inducing MMP-9 expression in tumoral pancreatic and endothelial cells

Tumor growth is dependent upon angiogenesis and it is a prognostic indicator for several malignancies. Angiogenesis consists mainly of two components: increased EC migration and increased EC proliferation [22]. Previous data from *in vivo* and *in vitro* studies have shown that tPA can exert pro-angiogenic effects [168, 244], although the molecular mechanisms have not been characterized. In addition the role of catalytic activity/plasmin in tPA-mediated angiogenesis is controversial. Studies have implicated tPA to be important during angiogenesis through its ability to activate plasminogen although some have suggested that plasminogen activator is not essential for angiogenesis [144] [152]. Due to the proteolytic potential, tPA has generally been considered to be beneficial in the pathogenesis of fibrotic lesions, leading to increased matrix degradation and decreased matrix accumulation the events necessary for angiogenesis [156]. Indeed, tPA contributes to the vascular remodeling process, as supported by the observation that it increases blood brain barrier permeability which is associated with angiogenesis. Here, we provide evidences for both indirect – catalytic-mediated- and direct effects of tPA in angiogenesis. We have seen that tPA can induce pro-angiogenic molecules that will be in turn responsible for increased angiogenesis. In our experiments by semiquantitative PCR analysis of pro-angiogenic cytokines

VEGF, TGF-β (1, 2 and 3), IL1 and IL8 treatment with tPA of pancreatic tumoral cells or endothelial cells does not induce the expression of these molecules. In contrast, analysis of MMP-9 after tPA treatment of pancreatic tumoral cells and ECs showed an increase in expression and activity, that can be explained by proteolytic processing by tPA/plasmin. MMP-9 belongs to the matrix metalloproteinases and, in endothelium, it has been reported that degradation of native type IV collagen by MMP-9 was a prerequisite for angiogenesis [239]. Induction of MMP-9 by tPA has been already reported in other cellular systems. In rat kidney interstitial fibroblasts, MMP-9 gene expression is induced by tPA through a molecular mechanism involving tPA interaction with the cell membrane receptor LRP [245]. The involvement of LRP receptor was also reported in the induction of MMP-9 by tPA in microglial [246] and endothelial cells [238]. Our data provide evidence that tPA mediates both MPP-9 expression and activation (proteolytic processing). Induction of MMP-9 expression by tPA was found in both tumoral pancreatic and ECs. In contrast, in our gelatin zymography experiments to check MMP activation, we found latent and active forms of MMP-9 in HUVEC whereas in pancreatic cell lines only latent form was seen after treatment with tPA. These data indicate that tPA is able to induce MMP-9 expression both in pancreatic and endothelial cells, but this metalloproteinase is only active in endothelial cells. The involvement of tPA catalytic activity in this event remains to be explored. Interestingly, when we checked the levels of MMP-9 mRNA expression by semiquantitative PCR analysis we found an

increase of mRNA level in HUVEC cells treated with tPA but in pancreatic cells mRNA levels remain unchanged in comparison to untreated cells. These data suggest that tPA-induced MMP-9 expression is differentially controlled in pancreatic and endothelial cells, in pancreatic cells should be mediated by postranscriptional mechanisms (probably at the translational level, as no protein is detected in untreated cells) whereas in endothelial should be mediated by transcriptional activation. In this regard, taking into account the already reported involvement of LRP in tPA-induced MMP-9 expression reported in other cells described above, is tempting to speculate that in endothelial cells tPA increase MMP-9 gene expression by binding to LRP and activation of signal transduction pathways leading to MMP-9 transcriptional activation.

In addition to MMP-9, tPA has also been reported to induce a rapid dose-dependent upregulation in MMP-2 [247]. However, we found that tPA-treated endothelial or pancreatic tumoral cells do not show any change in MMP-2 expresion and/or activation, in comparison to untreated cells. These results indicate that tPA specifically induce MMP-9 in our cell lines.

D.2 tPA induces endothelial cell proliferation, migration and tubulogenesis by activation of ERK1/2, AKT and JNK signaling pathways and independently of tPA proteolytic activity

The direct role of tPA in angiogenesis has been demonstrated by its effects in endothelial cell proliferation, migration and tubulogenesis. tPA has mitogenic effect on variety of cell types including pancreatic cells. Schwalm et al reported that mitogenic effects of tPA on smooth muscle cells require activation of SRC and ERK1/2 and are independent from its catalytic activity [228]. Indeed, it has been reported that tPA may function as a cytokine that binds to a cell membrane receptor, triggering a cascade of intracellular signal transduction like ERK pathway that ultimately leads to the transactivation of specific genes in the nucleus. ERK1/2 pathway plays a major role in the control of cell proliferation and migration. We have examined the capacity of tPA to activate ERK1/2 pathway in endothelial cells. Both HUVEC and H5V endothelial cells when treated with tPA activated ERK1/2 pathway in a time dependent manner. tPA induced an early increase in phospho-ERK1/2 levels (5 to 15 minutes after its addition to cells) and this activation was maintained for 15 minutes in HUVEC and H5V cells. We also found that ERK1/2 activation by tPA is independent of its catalytic activity, as treatment with mutant tPAS481A has the same effects in EC proliferation, migration and tubulogenesis. These data, together with the fact that EC treated with tPAS481A display similar proliferation, migration and tubulogenesis than those treated with wild type tPA, suggest that

tPA acts as a cytokine also in endothelial cells. The key role of ERK1/2 activation in tPA effects in endothelial cell proliferation and migration has been demonstrated using U0126 MEK inhibitor, that blocks the induction in cell proliferation and migration in endothelial cells treated with tPA. We have also tested the role of tPA-mediated ERK1/2 activation in tube formation in ECs in 3-D collagen gels. Our experiments with endothelial cells cultured on collagen sandwich showed that tPA treatment can induce the cells to form tubular structures resembling or mimicking the blood vessel formation. Combination of tPA with the pharmacological inhibitor U0126 showed that tubule formation was completely abolished indicating the relevance of ERK1/2 pathway in this process. Previous data have shown that ECs stimulated with angiogenic factors (bFGF, VEGF, and PMA) can activate of ERK1/2, Akt, and PKC leading to increased migration but this is, at least to our knowledge, the first report for the involvement of tPA-induced ERK activation in tubulogenesis. Altoghether these experiments confirmed the role of ERK1/2 pathway in migration, proliferation and tubule formation of endothelial cells in response to tPA.

The lipid kinase phosphoinositide 3-OH kinase (PI3K) and its downstream target AKT, also known as protein kinase B (PKB), are crucial effectors in oncogenic signaling induced by various receptor-tyrosine kinases [241]. PI3K/AKT signaling components are frequently altered in a variety of human malignancies and effects of PI3K/ AKT signaling on proliferation, survival and resistance to apoptosis, angiogenesis and cell motility in

gastrointestinal cancers are well known [242]. In addition, activation of PI3K/ AKT by various growth factors, the modulation of downstream targets by AKT-induced phosphorylation as well as novel treatment strategies targeting this pathway in gastrointestinal tumors are widely studied. Activation of the AKT kinase orchestrates a number of signaling pathways potentially involved in angiogenesis. The multiple downstream substrates of AKT not only converge to prevent the induction of apoptosis but may also interfere with numerous biological functions of the endothelial monolayer, which contribute to vascular remodeling and vessel integrity during the angiogenic process [231]. In our experiments we analzyed whether AKT pathway is induced in response to tPA. We found that AKT pathway was induced at 15 min and sustained till 30 min in HUVEC, while in H5V it was induced at 2 min and sustained till 30 min. Importantly, the effects of tPA in the induction of EC proliferation, migration and tubulogenesis were reduced to control levels when tPA was added in the presence of wortmanninm, a pharmacological inhibitor of AKT pathway. These data demonstrate that AKT signalling pathway is required for these events.

The mitogen-activated protein kinase c-jun N-terminal kinase (JNK) has a controversial role in the process of angiogenesis, with previous evidence supporting JNK as both a positive and negative regulator of blood vessel growth [232]. Phosphorylated JNK was observed in cultured endothelial cells, and levels were constant regardless of extracellular matrix composition. Inhibition of JNK

attenuated sprout growth in 3D capillary sprout cultures [233], and reduced endothelial cell proliferation and migration in vitro [234]. JNK inhibition and siRNA knockdown of c-jun (a downstream target of JNK) decreased protein levels of the transcription factor Egr-1, a regulator of genes involved in proliferation and migration [232]. In addition, c-Jun silencing decreased both MMP-2 and membrane type-1 (MT1)-MMP mRNA in endothelial cells, implicating both JNK and c-jun as activators of proteolysis [232]. Taken together, these results provide evidence that JNK, and its downstream target c-jun, positively regulate angiogenesis via activation of endothelial cell proliferation, migration and proteolysis. We have found that tPA induces JNK signalling pathway in ECs and, in our proliferation and migration assays, addition of a JNK inhibitor along with tPA decreased the proliferative and migrative rates of endothelial cells thereby underlying the importance of this pathway in tPA-induced mitotic and migratory effects. JNK pathway has been also related to functional network formation in 3-D Matrigel in human lung microvascular endothelial cells [233] and in TGF-β mediated capillary tube formation in bovine aortic endothelial cells in a fibrin sandwich 3-D assay [234]. In our tubulogenesis assay in H5V cells we observed that a JNK inhibitor, JNKI-1, along with tPA diminshed the tubule formation. These results, toghether with those previously mentioned in proliferation and migration, highlight the importance of the AKT signalling pathway in tPA-induced angiogenesis.

D.3 Annexin A2, EGFR and Galectin-1 are involved in binding of tPA to initiate the activation of signaling pathways in endothelial cells.

tPA is a secreted protein and therefore the initiation of signaling pathways by tPA requires its binding to plasma membrane receptors that would be responsible for intracellular signal transduction. AnxA2 has been described as a tPA receptor in endothelial and promyelocytic leukemia cells. In pancreatic cancer, it has been described to interact with tPA and to mediate *in vitro* invasion. Interestingly, our group has previously identified that tPA binding to AnxA2 is required for the activation of the ERK1/2 signalling pathway in pancreas and that EGFR is also necessary for this signalling activation [237]. EGFR integrates signals from various types of growth factors classically related to ERK1/2 signal pathway activation and is over expressed in PDAC [240]. A role for AnxA2 or EGFR in the tPA-mediated signaling pathways activation have not been reported in endothelial cells. Using siRNA approach we have shown that the receptor AnxA2 is involved in EC tPA-induced ERK1/2 and JNK signaling. As shown in Fig. R.10 B and C, whereas the transfection with an irrelevant siRNA had no effect on the activation of ERK1/2 or JNK, transfection with siAnxA2 resulted in a complete abolishment of tPA-induced ERK1/2 and JNK phosphorylation. The specificity of AnxA2 interference in tPA-induced signaling in ECs was proven by the fact that activation of ERK1/2 and JNK by FBS and total levels of ERK1/2 were

unaffected by the treatment with siAnxA2. Similarly, tPA treatment in the presence of an EGFR chemical inhibitor, Gefitinib, led to levels of phospho-ERK1/2 similar to basal levels. These data indicate that both AnxA2 and EGFR are required for ERK1/2 activation induced by tPA in ECs.

The work from Roda et al indentified that tPA exerts mitogenic effects on pancreatic cells through ERK1/2 activation via Gal-1 receptor [249]. In their experiments they showed that inhibition of Gal-1 expression using siGal-1 RNA markedly decreases tPA induced ERK1/2 activation and proliferation, confirming the crucial role of Gal-1 in tPA induced mitogenic process in pancreatic cancer cells. In our study we checked whether Gal-1 attenuation will affect the ERK1/2 and JNK signaling in endothelial cells treated with tPA. We found that downregulation of Gal-1 by siRNA transfection in HUVEC and H5V cells totally abolished the ERK1/2 signaling induced by tPA. These results indicated that Gal-1 is necessary for triggering ERK1/2 activation in tPA induced angiogenesis. One possible explanation for the activation of ERK1/2 via Gal-1 receptor might be the action of upstream element H-Ras, as it has been reported that Gal-1 can bind H-RasGTP promoting its membrane anchorage [248].

Altogether these data demonstrate that AnxA2, EGFR and Gal-1 are required for ERK1/2 activation induced by tPA in ECs. Future work will be required to determine whether EGFR and/or Gal-1 are also involved in the activation of JNK and AKT signaling pathways induce by tPA in endothelial cells, as well as the role of AnxA2 in AKT activation.

D.4 Cytokines (IL-1α and TGFβ) induce tPA expression in Pancreatic cancer and Endothelial cell lines.

Interleukin-1α (IL-1α) is a multifunctional pro-inflammatory cytokine which induces activation of immediate early transcription factors and genes that promote the survival and proliferation of cells [240]. It has been reported that IL-1α enhanced adhesion molecule expression and metastatic potential in PDAC cell lines via IL-1 receptor type1. Work by Matsuo *et al* has identified that highly liver metastatic PDAC cells enhanced angiogenesis through the secretion of IL-1α [240]. In one study by Lian *et al* they found out that IL-1α and TGF-β influenced the secretion of tPA from epidermal keratinocytes [226]. TGFβ is a potent stimulator of angiogenesis and the importance of TGF-βs as pro-angiogenic factors in PDAC is underscored by the recent observation that expression of a soluble TβRII in pancreatic cancer cells interferes with TGF-β actions, attenuates tumor growth and metastasis, and suppresses tumor angiogenesis [227]. In our experiments we decided to check whether IL-1α and TGFβ can induce the expression of tPA in PDAC and HUVEC cell lines. Our results showed that tPA protein and mRNA levels were highly enhanced in dose dependent manner on treatment with IL-1α and TGFβ (Fig. R.14C). Moreover, we found a synergistic response in the induction of tPA mRNA and protein when TGF-β and IL-1 α were combined together for 24 hours in HUVEC. These data indicate that cytokines produced by tumoral cells or the stroma (ECs, activated fibroblasts, immune

cells) can induce tPA expression in both tumoral and ECs leading to an angiogenesis stimulation.

D.5 Hypoxia induces tPA expression in Pancreatic cancer cell lines.

A hypoxic microenvironment is a characteristic of many solid tumors including pancreatic cancer [76]. Hypoxia is a known inducer of many potent angiogenic factors like VEGF, EGF, FGF which stimulate the initiation of angiogenesis in many types of cancer. The prototypical angiogenic cytokine increased by hypoxia is vascular VEGF and its receptor flt-1; however, other angiogenic factors like angiopoietin-2, placental growth factor, and platelet-derived growth factor B (PDGF-B) are also regulated by hypoxia [79]. Another angiogenic cytokine, basic fibroblast growth factor (bFGF), exerts enhanced proliferative activity on endothelial cells during hypoxia due to an increase of low-affinity heparan sulfate bFGF binding sites. Hypoxia also induces a hypoxia-inducible factor-1 (HIF-1α)-dependent bFGF autocrine loop that drives angiogenesis in human endothelial cell [78]. The study by Sun *et* al showed that 70.7% of PDAC cells showed positive HIF-1α staining [78]. Buchler et al demonstrated that hypoxia induced HIF-1α dramatically transactivated VEGF gene expression in pancreatic cancer cells in vitro and explored co-localisation of VEGF mRNA and HIF-1α protein in the same ductal cancer cells [84]. In our study we tried to see if tPA can be induced in hypoxic conditions in PDAC cells thereby enhancing the prospect of angiogenesis. Two PDAC cell lines (SKPC-1 and Hs766T) were subjected to hypoxic conditions in time dependent manner and conditioned medium was collected to check the levels of secreted tPA protein. Using

Western blot analysis and casein zymography (Fig. R.15) we found that tPA expression and activity was enhanced in a time-dependent manner during hypoxia. To determine whether the cells were in hypoxic conditions the levels of HIF-1α proteins were also checked by Western blotting. tPA activity was more prominently seen from conditioned media than from the cell extract thereby underling the fact that tPA is secreted and is active during hypoxic conditions. Interestingly, the levels of tPA mRNA in PDAC cells remained unchanged during both normoxia and hypoxia conditions . These data suggest that tPA overexpression during hypoxia is mediated by posttranscriptional mechanisms (i.e. RNA translation, protein stability, etc.) although additional experiments are required to further characterize the specific molecular mechanisms involved in this regulation.

In the light of our results regarding the role of inflammatory cytokines and hypoxia in tPA induction by pancreatic and endothelial cells, and considering our data about the mechanisms involved in tPA pro-angiogenic effects, we propose the following model: pancreatic tumoral cells and tumor microenviroment (stroma), that will be in hypoxic conditions as a consequence of tumor growth, will produce pro-angiogenic molecules like IL-1α and TGFβ to create an angiogenic network in order to compensate hypoxia. All these factors will induce tPA overexpression in both tumoral and endothelial cells, which in turn is able to induce angiogenesis both directly promoting EC proliferation, migration and tubulogenesis, or indirectly by MMP-9 upregulation. In this

way pro-angiogenic stimuli and tPA will collaborate in a feedback loop to promote tumoral angiogenesis **(Fig.D.1)**

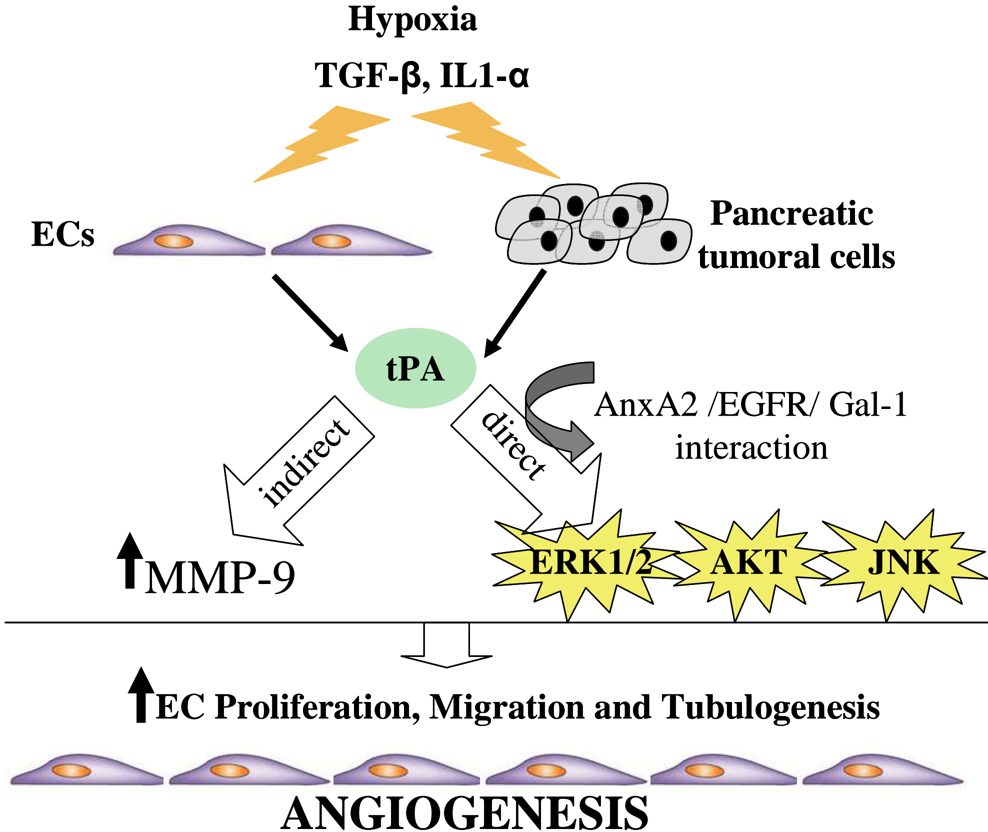


Fig D.1 Proposed model for tPA angiogenic role in PDAC. Hypoxia and TGF-β/ IL-1α cytokines induce tPA overexpression in pancreatic tumoral cells and endothelial cells (ECs). tPA can then induce angiogenesis both indirectly, through MMP-9 induction and activation, or directly by interaction with EC cell membrane receptors (AnxA2, EGFR, Gal-1) and activation of intracellular signaling pathways (ERK1/2, AKT and JNK) that will lead to increased EC proliferation, migration and tubulogenesis.

CONCLUSIONS

1. The regulation of pro-angiogenic molecules like VEGF, TGF-β, IL-1α and IL-8 are not mediated by tPA, but the activation of the angiogenic molecule MMP-9 is mediated by tPA. These results indicating that MMP-9 upregulation can indirectly mediate tPA effects in angiogenesis.
2. tPA induce directly and in a catalytic-independent way endothelial cell proliferation, migration and tubule formation.
3. tPA induces activation of ERK1/2, AKT and JNK signaling pathways in endothelial cells and this activation is necessary to induce proliferation, migration and tubule formation .
4. AnnexinA2, EGFR and Galectin-1 cell membrane receptors are involved in tPA- mediated endothelial cell signaling
5. Inflammatory cytokines (TGF-β and IL-1α) and hypoxia induce tPA overexpression in pancreatic cancer and endothelial cells.

Experimental Procedures

E.P.1 Cell Line Culture

All cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) except for HUVEC cell lines which were grown in Minimal Essential Medium (MEM, GIBCO BRL). Media were supplemented with glucose 4.5g/L (Life Techonologies), glutamine 2mM, penicillin 56 U/ml,streptomycin 56 µg/L and 10% fetal bovine serum (FBS;Gibco). Cells were maintained in at 37ºC in a humid atmosphere containing 5% Co_2 and 95% air.

Cell Line	Orgin
HUVEC	Human Umbilical Cord
H5V	Mouse Heart Blood Vessel
SKPC-1	Human Pancreatic Carcinoma
Hs766T	Human Pancreatic Carcinoma
RWP1	Human Pancreatic Carcinoma

Table E.P.1. Cell lines used during the development of this study

H5V cells and HUVEC cells are endothelial cells which were used in this study . SKPC-1, Hs766T and RWP1 are the cancer cell lines derived from pancreatic ductal adenocarcinoma.

E.P.2 Cell Treatments

To analyse signaling pathway activation and proliferation, cell were grown to confluence in complete medium and starved for 2 to 3 days using culture medium without FBS or growth supplements. tPA (Actilyse; Boehringer Ingelheim, Barcelona, Spain) (0.5,2, and 20µg/ml) was then added for variable periods of time. U0126 (10µmol/L) inhibitors of mitogen-activated protei kinase (MEK1/2) were added 1 hour before tPA treatment. JNK peptide inhibitor I (JIP)(20µmol/L), an inhibitor of JNK pathway was also added 1 hour prior the treatment with tPA. Wortmannin (10µmol/L), an inhibitor of AKT pathway is added 6 hours prior exposure to tPA. Gefitinib, a specific inhibitor of EGFR was added 1 hour prior exposure to tPA. S481A (Loxo, Germany), a noncatalytic tPA (20µg/ml) is added in endothelial cells for migration and wound healing experiments.

E.P.3 siRNA oligonucleotide transfection

For HUVEC, siRNA knockdown was performed using synthetic double stranded RNA purchased from Xeragon (Huntsville,AL). The sequence used to target AnxA2 was 5’-AUGUCUACUGUUCACGAAAUCCGC- 3’ (siAnxA2) and an unrelated oligonucleotide (siControl) recognizing an irrelevant transcript was used as a negative control. The concentration of 15nm was used for further experiments. For H5V the cells were transfected with siRNA SMARTpool® against AnxA2 (5’-GCUTGAUCTUUGAAUGTTCAUTC-3’) or with non-targeting

siRNA pool® (siControl) (Dharmacon) using the lipofectamine system (Invitrogen). For Gal-1 siRNA the following oligonucleotides were used human 5'-AUUCTCCUTGGUUTACUGTAAA-3' (siGAL-1) and an untreated oligonucleotide (siControl) were used.

The cells are transfected with siRNA by the following protocol.

- Cells ($1x10^6$) were seeded in 60 mm culture dishes and kept in culture 16-24h at 37^oC, 5% CO2.
- 15 nM of siRNA was mixed in 22.82 µl of Opti-MEM reduced-serum (Gibco-BRL) and left for 5 minutes at RT. At the same time, 2 µl of Plus (Invitrogen) were added dropwise to 40.82 µl of Opti-MEM and siRNA. Both mixes were pooled in one tube and left for 20 minutes at RT.
- At the same time mix 1 µl of Lipofectamine and Opti-MEM upto 25µl.
- Add this mixture to the wells containing Opti-MEM+siRNA+Plus. Mix well and waited for 20 min at RT.
- Media was removed from cells and 200 µl of Opti-MEM were added. 50 µl of transfecting mixture were also added and incubated at 37^oC, 5% CO2 for 3-5 hours
- Media was changed for Fresh DMEM+10% FBS and cells were cultured for 24 hours at 37oC, 5% CO2.
- Protein extraction and total RNA isolation were performed afterwards

E.P.4 shRNA oligonucleotide transfection

The cells are transfected with shRNA by the following protocol. shGal-1 oligonucleotide is used for the experiments along with shControl.

- Cells ($1x10^6$) were seeded in 60 mm culture dishes and kept in culture 16-24h at 37°C, 5% CO2.
- 1ng of shRNA (shGal-1) was mixed in 22.82 µl of Opti-MEM reduced-serum (Gibco-BRL) and left for 5 minutes at RT. At the same time, 2 µl of Plus (Invitrogen) were added dropwise to 40.82 µl of Opti-MEM and siRNA. Both mixes were pooled in one tube and left for 20 minutes at RT.
- At the same time mix 1 µl of Lipofectamine and Opti-MEM upto 25µl.
- Add this mixture to the wells containing Opti-MEM+siRNA+Plus. Mix well and waited for 20 min at RT.
- Media was removed from cells and 200 µl of Opti-MEM were added. 50 µl of transfecting mixture were also added and incubated at 37oC, 5% CO2 for 3-5 hours
- Media was changed for fresh DMEM+10% FBS and cells were cultured for 24 hours at 37oC, 5% CO2.
- After 24 hours the fresh DMEM medium with FBS along with puromycin (1.5µg/ml) were added and incubated for 48 hours.
- After 48 hours the medium is changed and again puromycin is added and incubated for 24 hours at 37°C.

- After 24 hours the cells are lysed and western blot performed.

E.P.5 Total RNA Isolation

RNA was obtained by using GenElute™ Mammalian Total RNA Miniprep Kit (RTN70, SIGMA-ALDRICH). The protocol in use was the one supplied by the manufacturer:

- Medium was thoroughly removed and cells were washed with PBS, twice.
- PBS was removed and 500 µl of Lysis Solution/2-Mercaptoethanol were added. Culture dish was rocked to completely cover the cells and rested for 1 minute at RT.
- Cell lysate was scraped and pipetted into a GenElute Filtration Column to remove cell debris and shear DNA. Column is centrifuged at maximum speed (12,000-16,000 x g) for 2 minutes. Column was discarded.
- 500 µl of 70% ethanol solution was added to the filtered lysate and mixed thoroughly. Solution is loaded into a GenElute Binding Column and centrifuged at maximum speed for 30 seconds.
- Column was retained and returned to a collection tube. 500 µl of Wash Solution 1 was added into the column and centrifuged at maximum speed for 30 seconds.
- Flow-through liquid was discarded and column was applied in a new collection tube. 500 µl of Wash Solution 2 were

added to the column and it was centrifuged at maximum speed for 30 seconds.

- Flow-through was discarded and 500 µl of Wash Solution 2 were added to the column again and it was centrifuged at maximum speed for 2 minutes.
- Flow-through was again discarded and centrifuged 1 extra minute to eliminate residual ethanol.
- Column was inserted in a new collection tube and 50 µl of Elution Buffer were added and centrifuged at maximum speed for 1 minute.
- Eluted RNA was quantified with a Nanodrop spectrophotometer (Thermo-Scientific) at 260 nm and stored at -80°C.

E.P.6 Semiquantitative RT-PCR

To perform semiquantitative RT-PCRs, commercial Transcriptor First Strand cDNA Synthesis Kit (04 896 866 001, Roche Applied Science) was used to obtain cDNA product from RNA extracts. The protocol in use was the following:

- Template-primer mix was prepared after quantifying RNA samples

Component	**Volume**	**Final Concentration**
RNA+ Elution buffer	11µl	1
Random Hexamer Primers (600pmol/µl)	2 µl	60Mm
Total Volume	13 µl	

Table E.P. 2 RNA template-primer mix

- Template-primer mix was denatured by heating for 10 minutes at 65°C in a T1 thermal block cycler (050-901, biometra) with a heated lid to minimize evaporation, in order to ensure denaturation of secondary structures. Solution was immediately cool on ice.
- The rest of the components were added to the template-primer mix tube in the following order:

Component	**Volume**	**Final Concentration**
Ranscriptor ReverseTranscriptase Reaction Buffer (5x)	4µl	8 mM MgCl2
Protector RNase Inhibitor (40 U/µl)	0.5 µl	20 U
Deoxynucleotide Mix (10 mM each)	2 µl	1 mM each
Transcriptor Reverse	0.5 µl	10 U

Transcriptase (20 U/µl)		
Final Volume	20 µl	

Table E.P. 2: Second mix of the RT assay.

- The RT reaction was performed in a thermal block cycler:
  - 10 minutes at 25°C.
  - 60 minutes at 50°C.
  - 5 minutes at 85°C.

Reaction was stopped by placing tubes on ice. Standard semiquantitative PCR was performed using cDNA samples, which were previously normalised. The following mix was prepared:

Component	**Volume**	**Final Concentration**
BIOTAQ Buffer (10x)	2.5 µl	1x
MgCl2 (50 mM)	1 µl	2 mM
Deoxynucleotide Mix (10 mM each)	0.5 µl	200 µM
Primer Forward (10 µM)	1.5 µl	0.6 µM
Primer Reverse (10 µM)	1.5 µl	0.6 µM
BIOTAQ polymerase (5U/µl)	0.25	1.25 U
cDNA	Variable	100-400 ng
H_2O	Variable	
Final Volume	25 µl	

Table E.P.3 Semiquantitative RT-PCR mix.

And the following PCR reaction was designed:

PCR Step	Temperature	Time
1. Initial denaturation	94°C	5-10 minutes
2. Denaturation	94°C	30 seconds
3. Annealing	55-63°C (depending on Tm)	30 seconds
4. Extension	68-72°C	1-2 minutes
Number of cycles (from 2 to 4)	**20-35 cycles (depending on abundance of transcript)**	
5. Last extension	68-72°C	10 minutes
6. End	4°C	∞

Table E.P.4 PCR conditions for semiquantitative RT-PCR

The following pairs of oligonucleotides were used

Primers	Sequences
VEGF	F 5'-CCAGCAGAAAGAGGAAAGAGGTAG-3' R 5'-CCCCAAAAGCAGGTCACTCAC-3'
TGF-β1	F 5'-GCCCTGGACACCAACTATTGC-3' R 5'-GCTGCACTTGCAGGAGCGCAC-3'
TGF-β2	F 5'-TACAGACCCTACTTCAG-3' R 5'- AAATCTTGCTTCTAGTT-3'

TGF-β3	F 5'-GCACTTGCAAAGGGCTC-3' R5'-TTGGCATAGTATTCCGA-3'
IL1-α	F 5'-GAGCATGTACTGAGCCCT-3' R 5'-AGAGTGGTCTCATGGTTGTC-3'
IL8	F 5'-ATGACTTCCAAGCTGGCCGT-3' R 5'-TCCTTGGCAAAACTGCACCT-3'
B-actin	F 5'-TCATTATGCCGAGGATTTGGA-3' R 5'-GCCTCCCATCTCCTTCATGAC-3'

Table E.P.4: Primers used for semiquantitative RT-PCR experiments.

Products were visualized by ethidium bromide staining using agarose electrophoresis.

E.P.7 Quantitative Real-Time PCR

Real-Time PCR was performed using SYBR® Green PCR Master Mix (4312704, Applied Biosystems), working on a 96-well plate without cover. cDNA samples were obtained from RNA extracts of treated cells using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and normalised to a specific concentration (25 ng/µl).

The following mixes were prepared

cDNA – SYBR Green mix	Volume	Concentration
cDNA	2 µl	5 ng/µl
SYBR® Green PCR Master Mix (2x)	5 µl	1x

Table E.P.5 cDNA-SYBR Green mix.

Primer – H_2O mix	**Volume**	**Concentration**
Primer Forward (10 µM)	0.3 µl	300 nM
Primer Reverse (10 µM	0.3 µl	300 nM
H_2O	2.4 µl	

Table E.P.6 Primer-H_2O mix.

Both mixes were combined in each well and the following PCR reaction was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems).

PCR Step	**Temperature**	**Time**
1	50°C	2 min
2	95°C	10 sec
3	95°C	15 sec
4	60°C	1 min
Number of cycles (from 3. to 4.)	**40 cycles**	

Table E.P.7 PCR program for Real-Time PCR experiments.

The following pairs of oligonucleotides were used

Primers	Sequences
TGFβ-1	F 5'-GCCCTGGACACCAACTATTGC-3' R 5'-GCTGCACTTGCAGGAGCGCAC-3'
IL1-α	F 5'-GAGCATGTACTGAGCCCT-3' R 5'-AGAGTGGTCTCATGGTTGTC-3'
tPA	F 5'-GGTGGCCCAGGCGGCCTCTG -3' R 5'- GCTGGCCGGCCTGGCCCG -3'
HPRT	F 5'-TCATTATGCCGAGGATTTGGA-3' R 5'-GCCTCCCATCTCCTTCATGAC -3'

Table E.P.8 Primer-H2O mix

Data was analysed using SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems).

E.P.8 Protein extraction

We treated the cells to obtain nuclear and total protein extracts. The following lysing buffers were used

Lysis Buffer A	Concentration
Sucrose	0.25 M
HEPES pH 7.5	10 mM
CaCl2	3 mM
NaCl	10 mM
NP-40	0.25%
PMSF	1 mM

DTT	1 mM

Table E.P.8 Lysis Buffer A composition

RIPA Buffer	**Concentration**
Sodium deoxycholate	0.5 %
Tris-HCl pH 8.0	50 mM
NaCl	150 mM
SDS	0.1 %
NP-40	1.0 %
EDTA	2 mM
PMSF	2 mM
DTT	1 mM
c0mplete cocktail	1x

Table E.P.9 Ripa Buffer composition

To obtain nuclear protein extracts, the following protocol was used:

- Medium was removed from cell dish and cells were washed with PBS, twice. 1 ml of PBS was added and cells were scraped, pipetted into an eppendorf tube on ice and centrifuged at 3,000 rpm for 5 minutes.

- Supernatant was discarded and pellet was resuspended with 1 ml of buffer A, and was incubated for 10 minutes on ice. Afterwards, cells were centrifuged at maximum speed for 1 minute at 4°C. This step was repeated twice.

- After three steps of washing cells with buffer A, a volume of RIPA buffer between 50-150 µl was added to the pellet of cells, and it was incubated for 30 minutes on ice. Finally, it was centrifuged at maximum speed for 15 minutes at 4°C.

Supernatant was stored as it contained the nuclear protein fraction.

- To obtain total protein extracts, cells were washed with PBS, scraped and centrifuged at 3,000 rpm for 5 minutes as in the above protocol. Supernatant was discarded and a volume of RIPA buffer between 50-150 µl was added to the pellet of cells and incubated for 30 minutes on ice. Cells were centrifuged at maximum speed for 15 minutes at 4°C.

Protein extracts were stored at -80°C.

E.P.9 Western blot

The following buffers were used to perform western blot assay

Separating gel solution	Concentration
Tris-HCl pH 8.8	375 mM
Bis-acrylamide	8 %
SDS	0.1 %
APS	0.1 %
Temed	

Experimental Procedures

Table E.P.10 Separating gel solution composition

Stacking gel solution	Concentration
Tris-HCl pH 6.8	125 mM
Bis-acrylamide	4 %
SDS	0.1 %
APS	0.1 %
Temed	

Table E.P.11 Stacking gel solution composition

Electrophoresis buffer	Concentration
Tris base	25 mM
Glycine	190 mM
SDS	0.1 %

Table E.P.12 Electrophoresis buffer composition

Transferance buffer	Concentration
Tris pH 7.5	200 mM
Glycine	1.5 M
Methanol	20%

Table E.P.13 Transferance buffer composition

Experimental Procedures

Blocking buffer	Concentration
Tween-20	0.1 %

Milk	5 %

Table E.P.14 Blocking buffer composition

Antibody dilution buffer	**Concentration**
Tween-20	0.1 %
BSA	0.3 %
Sodium azide	20%

Table E.P.15 Antibody dilution buffer composition

Proteins were normalized to a specific quantity and separated by vertical SDS-polyacrylamide gel electrophoresis using mini-protean III system (BioRad) at 100V for 2 hours at RT. Afterwards, proteins were transferred to Westran™ Polyvinylidine Fluoride (PVDF) Clear Signal membranes (10485287, Whatman). Blocking buffer was added to the membranes for 30 minutes to improve the sensitivity of the assay by reducing background interference and improving the signal to noise ratio..After washing excess of blocking buffer, membranes were immunoblotted with the following primary antibodies at 4°C:

Marker	**Antibody**	**Origin**	**Dilution**
tPA	Mouse	Actilyse	1:500
Gal-1	Rabbit	Gabeus	1:500
P-ERK	Rabbit	Cell Signaling	1:1000
T-ERK	Rabbit	Cell Signaling	1:1000
P-AKT	Rabbit	Cell Signaling	1:1000

T-AKT	Rabbit	Cell Signaling	1:1000
JNK	Rabbit	Cell Signaling	1:1000
P-JNK	Rabbit	Cell Signaling	1:1000
Annexin A2	Mouse	BD labs	1:1000
HIF-1 α	Mouse	Abcam	1:250
Pyruvate Kinase	Rabbit	Sigma	1:5000
Tubulin	Mouse	Sigma	1:10000

Table E.P.15. Antibodies used in western blotting assays

After washing excess of primary antibodies, membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit, K4003; anti-mouse, K4001, DakoCytomation) for 1 hour at RT. Membranes were extensively washed and Pierce ECL Western Blotting Substrate (32106, Thermo-Scientific) was used to detect HRP signal.

E.P.10 Casein Zymography

Casein zymography was performed to evaluate the activity of tPA in conditioned medium and cell extract. Aliquotes of cell lysates and medium conditioned by cells were analysed by gelatin zymography. Cells were lysed in SDS sample buffer and the condtioned medium was then concentrated using centrifugal filter units (Millipore , Ireland), treated with sample buffer (1X) and then loaded in 10% acrylamide gels. After electrophoresis gels were

washed with 2.5 % Triton X-100 to remove SDS. The gels are then washed with H2O to remove triton.

Preparation of Casein Gel

10 ml of gel solution
5 ml agarose (2% in H2O)
2.5 ml milk (8 % milk in PBS)
2.5 ml Tris-cl pH 7.6 (1M)

Agarose solution is boiled and the remaining solutions are added. The whole solution is stirred well until the temperature becomes below 45ºC. Then 1mg/ml of plasminogen is added to the solution and immediately pour in the glass plate. After the casien gel has been set on the top of it place the acrylamide gel and incubated at 37ºC overnight.

E.P.11 Gelatin Zymography

To measure the production and activity of gelatinases in the conditioned media from cultures receiving tPA treatments, gelatin zymography was performed .Briefly, 8 to 10 µL of each supernatant of the conditioned medium was used. The media supernatants were mixed with an equal volume of 1X SDS-PAGE sample buffer without boiling or reduction. Samples were fractionated in an 8% polyacrylamide gel containing gelatin (0.5 mg/mL). After electrophoresis, gels were washed with 2.5% Triton X-100 to

remove SDS and renature the MMP species in the gels. The gels are then incubated in the developing buffer (1 M Glycine, pH 8) overnight. The gels were stained with 0.25% Coomassie brilliant blue R-250 in 40% isopropanol for 2 hours and destained with 7% acetic acid. Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

E.P.12 Tubule Formation Assay (Collagen Sandwich Model)

Preparation of Collagen matrix

Collagen from tail of rat (4.05 mg/ml) (BD Bioscience, USA) working concentration -1.2mg/ ml
M199 medium (Gibco BRL, USA)
0.1 N NaOH
300 ml of the collagen matrix solution is prepared of which 195 µl of M199 , 90µl Collagen, 15 µl of NaOH are prepared.
The collagen matrix solution is poured in 24 well plate and allowed to polymerise for 1 hour at 37 ºC. By the time the collagen is been set , endothelial cells are trypsinized and 1.2 x 10^5 are seeded on the top of the collagen matrix and incubated for 2 h at 37 ºC. The plates are washed with M199 medium to remove the unattached cells. The collagen matrix is again poured on the plate and then set for 1 hour at 37 ºC. M199 medium is added with or without tPA (0.5 µg/ml), VEGF (20 µg/ml) and incubated for 16 hours at 37 ºC.

The tubules formed are then photographed in phase contrast microscopy (Leica 776, USA). The tube area is measured by using Image J software

E.P.13 Proliferation Assay

5-bromo-2'-deoxyuridine (BrdU) (Abcam) is incorporated into nascently synthesized DNA and provides a measurement of cell proliferation. The cells ($1x10^4$) were seeded into the cover glass in the wells and at 60% confluence they are serum deprived for 48 hours and then treated with various concentration of the molecule of interest for 24 hours. After 24 hours the following conditions are been performed

- The cells are fixed with 4% paraformaldehyde for 5 minutes at RT.
- Following fixation wash in 0.01M Phosphate Buffered Saline (pH 7.4) with 0.1% TritonX100 (3x 5min)
- Incubate in HCl (4N) for 10min on ice to break open the DNA structure of the labelled cells.
- This is followed by HCl (2N) for 10 min at RT before moving them to an incubator for 20 min at 37°C.
- Samples are then washed in in 0.01M Phosphate Buffered Saline (pH 7.4) with 0.1% TritonX100 (3x 5min) at RT.
- Incubate in 0.01M Phosphate Buffered Saline (pH 7.4) + 0.1% TritonX100 + Glycine (1M) + 5% Bovine serum

albumin (1hr) prior to incubating overnight (RT) with anti-BrdU or a combination of anti-BrdU and other antibodies.

- Following the incubation overnight wash in 0.01M Phosphate Buffered Saline (pH 7.4) with 0.1% TritonX100 (3x 5min)
- Samples can then be treated with a variety of secondary antibodies to visualise the anti-BrdU labelled cells including HRP conjugated secondaries with diaminobenzidine(DAB) or fluorescent conjugated antibodies such as AlexaFlour 488 or 533.
- The cells were also stained for nucleus using Hoest (0.25µg/ml)
- The cells were photographed in Flourecsent microscope (Leica,USA)

E.P.14 Migration Assay

In vitro wound-healing assay was used to assess cell motility in two dimensions. Endothelial cells ($1x10^5$) were plated overnight to achieve a subconfluent cell layer in 24-well plates. A scratch was made on the cell layer with a sterile micropipette tip, and cultures were washed twice with serum-free medium to remove floating cells. Cells were then treted in culture medium containing with or without 5% FBS, with 20 µg/ml tPA , 20 µg/ml, U0126 (10mM), JIP (20 mM), Wortmannin (10mM) etc. The cells after they are denuded is incubated at 37 ºC for 24 hours. Wound healing was visualized by comparing photographs taken at the time of addition

of factors and 24 h later, by a Nikon DS-5M Camera System mounted on a phase-contrast Leica microscope. The distance traveled by the cells was determined by visually comparing the area of the wound covered. Three experiments were done in quadruplicates.

References

1. Jemal, A., et al., *Cancer statistics, 2009.* CA Cancer J Clin, 2009. **59**(4): p. 225-49.
2. Alexakis, N., et al., *Current standards of surgery for pancreatic cancer.* Br J Surg, 2004. **91**(11): p. 1410-27.
3. Chu, G.C., et al., *Stromal biology of pancreatic cancer.* J Cell Biochem, 2007. **101**(4): p. 887-907.
4. Hruban RH, Adsay NV, bores-Saavedra J, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol.* 2001; 25:579–586
5. Andea A, Sarkar F, Adsay VN. Clinicopathological correlates of pancreatic intraepithelial neoplasia: a comparative analysis of 82 cases with and 152 cases without pancreatic ductal adenocarcinoma. *Mod Pathol.* 2003;16**:**996–1006.
6. Everhart, J. and D. Wright, *Diabetes mellitus as a risk factor for pancreatic cancer. A meta-analysis.* Jama, 1995. **273**(20): p. 1605-9.
7. Gapstur, S.M., et al., *Abnormal glucose metabolism and pancreatic cancer mortality.* Jama, 2000. **283**(19): p. 2552-8.
8. Jones, S., et al., *Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science, 2008. **321**(5897): p. 1801-6.
9. Mahadevan, D. and D.D. Von Hoff, *Tumor-stroma interactions in pancreatic ductal adenocarcinoma.* Mol Cancer Ther, 2007. **6**(4): p. 1186-97.
10. Maitra, A., et al., *Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray.* Mod Pathol, 2003. **16**(9): p. 902-12.
11. Rozenblum, E., et al., *Tumor-suppressive pathways in pancreatic carcinoma.* Cancer Res, 1997. **57**(9): p. 1731-4.
12. Hirano, T., et al., *Dominant negative MEKK1 inhibits survival of pancreatic cancer cells.* Oncogene, 2002. **21**(38): p. 5923-8.

13. Fleming, J.B., et al., *Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: justification for K-ras-directed therapy.* Mol Cancer Res, 2005. **3**(7): p. 413-23.
14. Wilentz, R.E., et al., *Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression.* Cancer Res, 2000. **60**(7): p. 2002-6.
15. Lemoine, N.R., et al., *Ki-ras oncogene activation in preinvasive pancreatic cancer.* Gastroenterology, 1992. **102**(1): p. 230-6.
16. Ueda, S., et al., *The correlation between cytoplasmic overexpression of epidermal growth factor receptor and tumor aggressiveness: poor prognosis in patients with pancreatic ductal adenocarcinoma.* Pancreas, 2004. **29**(1): p. e1-8.
17. Bruns, C.J., et al., *Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma.* Cancer Res, 2000. **60**(11): p. 2926-35.
18. Moore, M.J., et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* J Clin Oncol, 2007. **25**(15): p. 1960-6.
19. Lee, C.J., J. Dosch, and D.M. Simeone, *Pancreatic cancer stem cells.* J Clin Oncol, 2008. **26**(17): p. 2806-12.
20. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.
21. Kalluri, R., *Discovery of type IV collagen non-collagenous domains as novel integrin ligands and endogenous inhibitors of angiogenesis.* Cold Spring Harb Symp Quant Biol, 2002. **67**: p. 255-66.
22. Papetti, M. and I.M. Herman, *Mechanisms of normal and tumor-derived angiogenesis.* Am J Physiol Cell Physiol, 2002. **282**(5): p. C947-70.
23. Li C, Heidt DG, Dalerba P et al. Identification of pancreatic cancer stem cells. Cancer Res. 2007; 67:1030-1037.
24. Hermann PC, Huber SL, Herrler T et al. Distinct populations of cancer stem cells determine tumor growth and metastatic

activity in human pancreatic cancer. Cell Stem Cell 2007;1:313 -323.

25. Folkman, J. and P.A. D'Amore, *Blood vessel formation: what is its molecular basis?* Cell, 1996. **87**(7): p. 1153-5.
26. Rini, B.I. and E.J. Small, *Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma.* J Clin Oncol, 2005. **23**(5): p. 1028-43.
27. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nat Med, 2003. **9**(6): p. 669-76.
28. Tischer, E., et al., *The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing.* J Biol Chem, 1991. **266**(18): p. 11947-54.
29. Nishi, M., et al., *Cell binding isoforms of vascular endothelial growth factor-A (VEGF189) contribute to blood flow-distant metastasis of pulmonary adenocarcinoma.* Int J Oncol, 2005. **26**(6): p. 1517-24.
30. Dvorak, H.F., *Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy.* J Clin Oncol, 2002. **20**(21): p. 4368-80.
31. Carmeliet, P., et al., *Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.* Nature, 1996. **380**(6573): p. 435-9.
32. Silins, G., et al., *Analysis of the promoter region of the human VEGF-related factor gene.* Biochem Biophys Res Commun, 1997. **230**(2): p. 413-8.
33. Mould, A.W., et al., *Vegfb gene knockout mice display reduced pathology and synovial angiogenesis in both antigen-induced and collagen-induced models of arthritis.* Arthritis Rheum, 2003. **48**(9): p. 2660-9.
34. Silvestre, J.S., et al., *Vascular endothelial growth factor-B promotes in vivo angiogenesis.* Circ Res, 2003. **93**(2): p. 114-23.
35. Robinson, C.J. and S.E. Stringer, *The splice variants of vascular endothelial growth factor (VEGF) and their receptors.* J Cell Sci, 2001. **114**(Pt 5): p. 853-65.
36. Cao, Y., et al., *Vascular endothelial growth factor C induces angiogenesis in vivo.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14389-94.

37. Carmeliet, P., *Angiogenesis in health and disease.* Nat Med, 2003. **9**(6): p. 653-60.
38. Yancopoulos, G.D., M. Klagsbrun, and J. Folkman, *Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border.* Cell, 1998. **93**(5): p. 661-4.
39. Puri, M.C., et al., *The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells.* Embo J, 1995. **14**(23): p. 5884-91.
40. Dumont, D.J., et al., *tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors.* Oncogene, 1992. **7**(8): p. 1471-80.
41. Davis, S., et al., *Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning.* Cell, 1996. **87**(7): p. 1161-9.
42. Miller, D.L., et al., *Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice.* Mol Cell Biol, 2000. **20**(6): p. 2260-8.
43. Murakami, M., et al., *The FGF system has a key role in regulating vascular integrity.* J Clin Invest, 2008. **118**(10): p. 3355-66.
44. Massague, J., *The transforming growth factor-beta family.* Annu Rev Cell Biol, 1990. **6**: p. 597-641.
45. van den Driesche, S., C.L. Mummery, and C.J. Westermann, *Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis.* Cardiovasc Res, 2003. **58**(1): p. 20-31.
46. Falcone, D.J., et al., *Transforming growth factor-beta 1 stimulates macrophage urokinase expression and release of matrix-bound basic fibroblast growth factor.* J Cell Physiol, 1993. **155**(3): p. 595-605.
47. Pepper, M.S., *Extracellular proteolysis and angiogenesis.* Thromb Haemost, 2001. **86**(1): p. 346-55.
48. Bergers, G., et al., *Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis.* Nat Cell Biol, 2000. **2**(10): p. 737-44.
49. Xu, J., et al., *Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo.* J Cell Biol, 2001. **154**(5): p. 1069-79.

50. Liekens, S., E. De Clercq, and J. Neyts, *Angiogenesis: regulators and clinical applications.* Biochem Pharmacol, 2001. **61**(3): p. 253-70.
51. Tsuzuki, Y., et al., *Pancreas microenvironment promotes VEGF expression and tumor growth: novel window models for pancreatic tumor angiogenesis and microcirculation.* Lab Invest, 2001. **81**(10): p. 1439-51.
52. Moller, A., et al., *Human mast cells produce IL-8.* J Immunol, 1993. **151**(6): p. 3261-6.
53. Leibovich, S.J., et al., *Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha.* Nature, 1987. **329**(6140): p. 630-2.
54. Esposito, I., et al., *Inflammatory cells contribute to the generation of an angiogenic phenotype in pancreatic ductal adenocarcinoma.* J Clin Pathol, 2004. **57**(6): p. 630-6.
55. Taipale, J., et al., *Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells.* J Biol Chem, 1995. **270**(9): p. 4689-96.
56. Blair, R.J., et al., *Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor.* J Clin Invest, 1997. **99**(11): p. 2691-700.
57. Korc, M., *Role of growth factors in pancreatic cancer.* Surg Oncol Clin N Am, 1998. **7**(1): p. 25-41.
58. Luo, J., et al., *Pancreatic cancer cell-derived vascular endothelial growth factor is biologically active in vitro and enhances tumorigenicity in vivo.* Int J Cancer, 2001. **92**(3): p. 361-9.
59. Itakura, J., et al., *Concomitant over-expression of vascular endothelial growth factor and its receptors in pancreatic cancer.* Int J Cancer, 2000. **85**(1): p. 27-34.
60. Solorzano, C.C., et al., *Inhibition of growth and metastasis of human pancreatic cancer growing in nude mice by PTK 787/ZK222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases.* Cancer Biother Radiopharm, 2001. **16**(5): p. 359-70.
61. Korc, M., et al., *Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth*

factor and transforming growth factor alpha. J Clin Invest, 1992. **90**(4): p. 1352-60.

62. Kleeff, J., et al., *Detection and localization of Mip-3alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer.* Int J Cancer, 1999. **81**(4): p. 650-7.
63. Shi, Q., et al., *Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic.* Clin Cancer Res, 1999. **5**(11): p. 3711-21.
64. Takeuchi, Y., et al., *Expression of plasminogen activators and their inhibitors in human pancreatic carcinoma: immunohistochemical study.* Am J Gastroenterol, 1993. **88**(11): p. 1928-33.
65. Kleeff, J., et al., *Overexpression of Smad2 and colocalization with TGF-beta1 in human pancreatic cancer.* Dig Dis Sci, 1999. **44**(9): p. 1793-802.
66. Kleeff, J. and M. Korc, *Up-regulation of transforming growth factor (TGF)-beta receptors by TGF-beta1 in COLO-357 cells.* J Biol Chem, 1998. **273**(13): p. 7495-500.
67. Yang, E.Y. and H.L. Moses, *Transforming growth factor beta 1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane.* J Cell Biol, 1990. **111**(2): p. 731-41.
68. Andreasen, P.A., R. Egelund, and H.H. Petersen, *The plasminogen activation system in tumor growth, invasion, and metastasis.* Cell Mol Life Sci, 2000. **57**(1): p. 25-40.
69. Schwarte-Waldhoff, I., et al., *Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9624-9.
70. Hirata, A., et al., *ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase.* Cancer Res, 2002. **62**(9): p. 2554-60.
71. Kuehn, R., et al., *Angiogenesis, angiogenic growth factors, and cell adhesion molecules are upregulated in chronic pancreatic diseases: angiogenesis in chronic pancreatitis and in pancreatic cancer.* Pancreas, 1999. **18**(1): p. 96-103.
72. Niedergethmann, M., et al., *Angiogenesis and cathepsin expression are prognostic factors in pancreatic*

adenocarcinoma after curative resection. Int J Pancreatol, 2000. **28**(1): p. 31-9.

73. Ikeda, N., et al., *Prognostic significance of angiogenesis in human pancreatic cancer.* Br J Cancer, 1999. **79**(9-10): p. 1553-63.
74. Linder, S., et al., *Pattern of distribution and prognostic value of angiogenesis in pancreatic duct carcinoma: a semiquantitative immunohistochemical study of 45 patients.* Pancreas, 2001. **22**(3): p. 240-7.
75. Karademir, S., et al., *Tumor angiogenesis as a prognostic predictor in pancreatic cancer.* J Hepatobiliary Pancreat Surg, 2000. **7**(5): p. 489-95.
76. Folkman, J., *What is the evidence that tumors are angiogenesis dependent?* J Natl Cancer Inst, 1990. **82**(1): p. 4-6.
77. Semenza, G.L., *Hypoxia-inducible factor 1: master regulator of O2 homeostasis.* Curr Opin Genet Dev, 1998. **8**(5): p. 588-94.
78. Bruick, R.K. and S.L. McKnight, *A conserved family of prolyl-4-hydroxylases that modify HIF.* Science, 2001. **294**(5545): p. 1337-40.
79. Forsythe, J.A., et al., *Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1.* Mol Cell Biol, 1996. **16**(9): p. 4604-13.
80. Tang, N., et al., *Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis.* Cancer Cell, 2004. **6**(5): p. 485-95.
81. Gerber, H.P., et al., *Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia.* J Biol Chem, 1997. **272**(38): p. 23659-67.
82. Megibow, A.J., *Pancreatic adenocarcinoma: designing the examination to evaluate the clinical questions.* Radiology, 1992. **183**(2): p. 297-303.
83. Koong, A.C., et al., *Pancreatic tumors show high levels of hypoxia.* Int J Radiat Oncol Biol Phys, 2000. **48**(4): p. 919-22.
84. Buchler, P., et al., *Hypoxia-inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic cancer.* Pancreas, 2003. **26**(1): p. 56-64.

85. Sun, H.C., et al., *Expression of hypoxia-inducible factor-1 alpha and associated proteins in pancreatic ductal adenocarcinoma and their impact on prognosis.* Int J Oncol, 2007. **30**(6): p. 1359-67.
86. Shishido, T., et al., *Inhibition of liver metastasis of human pancreatic carcinoma by angiogenesis inhibitor TNP-470 in combination with cisplatin.* Jpn J Cancer Res, 1998. **89**(9): p. 963-9.
87. Kato, H., et al., *Anti-angiogenic treatment for peritoneal dissemination of pancreas adenocarcinoma: a study using TNP-470.* Jpn J Cancer Res, 2001. **92**(1): p. 67-73.
88. Hotz, H.G., et al., *Angiogenesis inhibitor TNP-470 reduces human pancreatic cancer growth.* J Gastrointest Surg, 2001. **5**(2): p. 131-8.
89. Yanagi, K., M. Onda, and E. Uchida, *Effect of angiostatin on liver metastasis of pancreatic cancer in hamsters.* Jpn J Cancer Res, 2000. **91**(7): p. 723-30.
90. Hotz, H.G., et al., *Specific targeting of tumor vasculature by diphtheria toxin-vascular endothelial growth factor fusion protein reduces angiogenesis and growth of pancreatic cancer.* J Gastrointest Surg, 2002. **6**(2): p. 159-66; discussion 166.
91. Bruns, C.J., et al., *Effect of the vascular endothelial growth factor receptor-2 antibody DC101 plus gemcitabine on growth, metastasis and angiogenesis of human pancreatic cancer growing orthotopically in nude mice.* Int J Cancer, 2002. **102**(2): p. 101-8.
92. Wang, H.X. and Q.K. Chen, *[Expression and significance of cyclooxygenase-2 in human pancreatic carcinomas].* Ai Zheng, 2003. **22**(6): p. 649-52.
93. Zhou, X.C., et al., *[Effects of rofecoxib on angiogenesis of pancreatic cancer xenograft in nude mice].* Ai Zheng, 2004. **23**(4): p. 376-80.
94. Raut, C.P., et al., *Celecoxib inhibits angiogenesis by inducing endothelial cell apoptosis in human pancreatic tumor xenografts.* Cancer Biol Ther, 2004. **3**(12): p. 1217-24.
95. Oikawa, T., et al., *Frequent expression of genes for receptor tyrosine kinases and their ligands in human pancreatic cancer cells.* Int J Pancreatol, 1995. **18**(1): p. 15-23.

96. Andreasen, P.A., et al., *The urokinase-type plasminogen activator system in cancer metastasis: a review.* Int J Cancer, 1997. **72**(1): p. 1-22.
97. Petersen, T.E., et al., *Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system.* J Biol Chem, 1990. **265**(11): p. 6104-11.
98. Collen, D., et al., *Metabolism of plasminogen in healthy subjects: effect of tranexamic acid.* J Clin Invest, 1972. **51**(6): p. 1310-8.
99. Magnaghi, P., et al., *Molecular characterisation of the human apo(a)-plasminogen gene family clustered on the telomeric region of chromosome 6 (6q26-27).* Hum Mol Genet, 1994. **3**(3): p. 437-42.
100. Robbins, K.C., et al., *The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin.* J Biol Chem, 1967. **242**(10): p. 2333-42.
101. Wiman, B., *Primary structure of the B-chain of human plasmin.* Eur J Biochem, 1977. **76**(1): p. 129-37.
102. Liotta, L.A., et al., *Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane.* Cancer Res, 1981. **41**(11 Pt 1): p. 4629-36.
103. Romer, J., et al., *Impaired wound healing in mice with a disrupted plasminogen gene.* Nat Med, 1996. **2**(3): p. 287-92.
104. Gunzler, W.A., et al., *The primary structure of high molecular mass urokinase from human urine. The complete amino acid sequence of the A chain.* Hoppe Seylers Z Physiol Chem, 1982. **363**(10): p. 1155-65.
105. Dano, K., et al., *Plasminogen activators, tissue degradation, and cancer.* Adv Cancer Res, 1985. **44**: p. 139-266.
106. Estreicher, A., et al., *Characterization of the cellular binding site for the urokinase-type plasminogen activator.* J Biol Chem, 1989. **264**(2): p. 1180-9.
107. Myohanen, H. and A. Vaheri, *Regulation and interactions in the activation of cell-associated plasminogen.* Cell Mol Life Sci, 2004. **61**(22): p. 2840-58.
108. Mignatti, P., E. Robbins, and D.B. Rifkin, *Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade.* Cell, 1986. **47**(4): p. 487-98.

109. Cajot, J.F., et al., *Mouse L cells expressing human prourokinase-type plasminogen activator: effects on extracellular matrix degradation and invasion.* J Cell Biol, 1989. **109**(2): p. 915-25.

110. Cantero, D., et al., *Enhanced expression of urokinase plasminogen activator and its receptor in pancreatic carcinoma.* Br J Cancer, 1997. **75**(3): p. 388-95.

111. Paciucci, R., et al., *Activation of the urokinase plasminogen activator/urokinase plasminogen activator receptor system and redistribution of E-cadherin are associated with hepatocyte growth factor-induced motility of pancreas tumor cells overexpressing Met.* Am J Pathol, 1998. **153**(1): p. 201-12.

112. Xue, A., et al., *Prognostic significance of growth factors and the urokinase-type plasminogen activator system in pancreatic ductal adenocarcinoma.* Pancreas, 2008. **36**(2): p. 160-7.

113. Loskutoff, D.J., et al., *Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells.* Proc Natl Acad Sci U S A, 1983. **80**(10): p. 2956-60.

114. Pennica, D., et al., *Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli.* Nature, 1983. **301**(5897): p. 214-21.

115. Wallen, P., N. Bergsdorf, and M. Ranby, *Purification and identification of two structural variants of porcine tissue plasminogen activator by affinity adsorption on fibrin.* Biochim Biophys Acta, 1982. **719**(2): p. 318-28.

116. Ichinose, A., K. Takio, and K. Fujikawa, *Localization of the binding site of tissue-type plasminogen activator to fibrin.* J Clin Invest, 1986. **78**(1): p. 163-9.

117. Lijnen, H.R. and D. Collen, *Interaction of plasminogen activators and inhibitors with plasminogen and fibrin.* Semin Thromb Hemost, 1982. **8**(1): p. 2-10.

118. Loskutoff, D.J., *A slice of PAI.* J Clin Invest, 1993. **92**(6): p. 2563.

119. Kruithof, E.K., *Plasminogen activator inhibitors--a review.* Enzyme, 1988. **40**(2-3): p. 113-21.

120. Stefansson, S., et al., *Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling.* Curr Pharm Des, 2003. **9**(19): p. 1545-64.

121. Cubellis, M.V., et al., *Accessibility of receptor-bound urokinase to type-1 plasminogen activator inhibitor.* Proc Natl Acad Sci U S A, 1989. **86**(13): p. 4828-32.
122. Blasi, F., *The urokinase receptor. A cell surface, regulated chemokine.* Apmis, 1999. **107**(1): p. 96-101.
123. Loskutoff, D.J., et al., *Regulation of cell adhesion by PAI-1.* Apmis, 1999. **107**(1): p. 54-61.
124. Kwaan, H.C., et al., *Plasminogen activator inhibitor 1 may promote tumour growth through inhibition of apoptosis.* Br J Cancer, 2000. **82**(10): p. 1702-8.
125. Huber, K., *Plasminogen activator inhibitor type-1 (part one): basic mechanisms, regulation, and role for thromboembolic disease.* J Thromb Thrombolysis, 2001. **11**(3): p. 183-93.
126. Kruithof, E.K., M.S. Baker, and C.L. Bunn, *Biological and clinical aspects of plasminogen activator inhibitor type 2.* Blood, 1995. **86**(11): p. 4007-24.
127. Fernandez-Soria, V., et al., *Adenovirus E1A orchestrates the urokinase-plasminogen activator system and upregulates PAI-2 expression, supporting a tumor suppressor effect.* Int J Oncol, 2006. **28**(1): p. 143-8.
128. Gershtein, E.S. and N.E. Kushlinskii, *Urokinase and tissue plasminogen activators and their inhibitor PAI-1 in human tumors.* Bull Exp Biol Med, 2001. **131**(1): p. 67-72.
129. Duffy, M.J., *Urokinase-type plasminogen activator: a potent marker of metastatic potential in human cancers.* Biochem Soc Trans, 2002. **30**(2): p. 207-10.
130. Reuning, U., et al., *Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis (review).* Int J Oncol, 1998. **13**(5): p. 893-906.
131. Duffy, M.J., *Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies.* Clin Chem, 2002. **48**(8): p. 1194-7.
132. Czekay, R.P., et al., *Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins.* J Cell Biol, 2003. **160**(5): p. 781-91.
133. Chazaud, B., et al., *Promigratory effect of plasminogen activator inhibitor-1 on invasive breast cancer cell populations.* Am J Pathol, 2002. **160**(1): p. 237-46.

134. Torre, E.A. and R.A. Fulco, *Tumor-associated urokinase-type plasminogen activator: significance in breast cancer.* Eur J Gynaecol Oncol, 1996. **17**(4): p. 315-8.
135. Hansen, S., et al., *Independent prognostic value of angiogenesis and the level of plasminogen activator inhibitor type 1 in breast cancer patients.* Br J Cancer, 2003. **88**(1): p. 102-8.
136. Grondahl-Hansen, J., et al., *Plasminogen activator inhibitor type 1 in cytosolic tumor extracts predicts prognosis in low-risk breast cancer patients.* Clin Cancer Res, 1997. **3**(2): p. 233-9.
137. Dazzi, C., et al., *A high cytosol value of urokinase-type plasminogen activator (uPA) may be predictive of early relapse in primary breast cancer.* Cancer Invest, 2003. **21**(2): p. 208-16.
138. Nekarda, H., et al., *Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer.* Cancer Res, 1994. **54**(11): p. 2900-7.
139. Berger, D.H., *Plasmin/plasminogen system in colorectal cancer.* World J Surg, 2002. **26**(7): p. 767-71.
140. Salden, M., et al., *The urokinase-type plasminogen activator system in resected non-small-cell lung cancer. Rotterdam Oncology Thoracic Study Group.* Ann Oncol, 2000. **11**(3): p. 327-32.
141. Taponeco, F., et al., *Expression and prognostic significance of urokinase and plasminogen activator inhibitor type-1 in endometrial hyperplasia and cancer.* J Exp Clin Cancer Res, 2001. **20**(2): p. 239-46.
142. O'Reilly, M.S., et al., *Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma.* Cell, 1994. **79**(2): p. 315-28.
143. Gately, S., et al., *The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin.* Proc Natl Acad Sci U S A, 1997. **94**(20): p. 10868-72.
144. Pepper, M.S., et al., *Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells.* J Cell Biol, 1987. **105**(6 Pt 1): p. 2535-41.

145. Carmeliet, P. and D. Collen, *Transgenic mouse models in angiogenesis and cardiovascular disease.* J Pathol, 2000. **190**(3): p. 387-405.
146. Ranson, M. and N.M. Andronicos, *Plasminogen binding and cancer: promises and pitfalls.* Front Biosci, 2003. **8**: p. s294-304.
147. Ra, H.J. and W.C. Parks, *Control of matrix metalloproteinase catalytic activity.* Matrix Biol, 2007. **26**(8): p. 587-96.
148. Uchiyama, T., et al., *Hypoxia induces transcription of the plasminogen activator inhibitor-1 gene through genistein-sensitive tyrosine kinase pathways in vascular endothelial cells.* Arterioscler Thromb Vasc Biol, 2000. **20**(4): p. 1155-61.
149. Stephens, R.W., et al., *The urokinase plasminogen activator system as a target for prognostic studies in breast cancer.* Breast Cancer Res Treat, 1998. **52**(1-3): p. 99-111.
150. Evans, C.P., et al., *Inhibition of prostate cancer neovascularization and growth by urokinase-plasminogen activator receptor blockade.* Cancer Res, 1997. **57**(16): p. 3594-9.
151. Montesano, R., et al., *Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene.* Cell, 1990. **62**(3): p. 435-45.
152. Sabapathy, K.T., et al., *Polyoma middle T-induced vascular tumor formation: the role of the plasminogen activator/plasmin system.* J Cell Biol, 1997. **137**(4): p. 953-63.
153. Stack, M.S., et al., *Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation.* Biochem J, 1999. **340 (Pt 1)**: p. 77-84.
154. Bajou, K., et al., *The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies.* J Cell Biol, 2001. **152**(4): p. 777-84.
155. Pozzi, A., et al., *Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization.* Proc Natl Acad Sci U S A, 2000. **97**(5): p. 2202-7.

156. Hayashi, S., et al., *Inhibitory effect on the establishment of hepatic metastasis by transduction of the tissue plasminogen activator gene to murine colon cancer.* Cancer Gene Ther, 1999. **6**(4): p. 380-4.
157. Ferrier, C.M., et al., *High tPA-expression in primary melanoma of the limb correlates with good prognosis.* Br J Cancer, 2000. **83**(10): p. 1351-9.
158. Browder, T., J. Folkman, and S. Pirie-Shepherd, *The hemostatic system as a regulator of angiogenesis.* J Biol Chem, 2000. **275**(3): p. 1521-4.
159. Medina, M.G., et al., *Tissue plasminogen activator mediates amyloid-induced neurotoxicity via Erk1/2 activation.* Embo J, 2005. **24**(9): p. 1706-16.
160. Stack, M.S., R.D. Gray, and S.V. Pizzo, *Modulation of murine B16F10 melanoma plasminogen activator production by a synthetic peptide derived from the laminin A chain.* Cancer Res, 1993. **53**(9): p. 1998-2004.
161. Mimura, K., et al., *Fibrinolysis activity promotes tumor invasiveness of B16 melanoma cell lines through a reconstituted gel matrix.* Invasion Metastasis, 1992. **12**(1): p. 24-34.
162. De Petro, G., et al., *Expression of urokinase-type plasminogen activator (u-PA), u-PA receptor, and tissue-type PA messenger RNAs in human hepatocellular carcinoma.* Cancer Res, 1998. **58**(10): p. 2234-9.
163. Fishman, D.A., et al., *Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells.* Cancer, 1997. **80**(8): p. 1457-63.
164. Saito, K., et al., *The concentration of tissue plasminogen activator and urokinase in plasma and tissues of patients with ovarian and uterine tumors.* Thromb Res, 1990. **58**(4): p. 355-66.
165. Paciucci, R., et al., *The plasminogen activator system in pancreas cancer: role of t-PA in the invasive potential in vitro.* Oncogene, 1998. **16**(5): p. 625-33.
166. Paciucci, R., et al., *Isolation of tissue-type plasminogen activator, cathepsin H, and non-specific cross-reacting antigen from SK-PC-1 pancreas cancer cells using subtractive hybridization.* FEBS Lett, 1996. **385**(1-2): p. 72-6.

167. Ryu, B., et al., *Relationships and differentially expressed genes among pancreatic cancers examined by large-scale serial analysis of gene expression.* Cancer Res, 2002. **62**(3): p. 819-26.
168. Diaz, V.M., et al., *Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro.* Gut, 2004. **53**(7): p. 993-1000.
169. Hurtado, M., et al., *Activation of the epidermal growth factor signalling pathway by tissue plasminogen activator in pancreas cancer cells.* Gut, 2007. **56**(9): p. 1266-74.
170. Barondes, S.H., et al., *Galectins: a family of animal beta-galactoside-binding lectins.* Cell, 1994. **76**(4): p. 597-8.
171. Houzelstein, D., et al., *Phylogenetic analysis of the vertebrate galectin family.* Mol Biol Evol, 2004. **21**(7): p. 1177-87.
172. Cooper, D.N., *Galectinomics: finding themes in complexity.* Biochim Biophys Acta, 2002. **1572**(2-3): p. 209-31.
173. Leffler, H., et al., *Introduction to galectins.* Glycoconj J, 2004. **19**(7-9): p. 433-40.
174. Danguy, A., I. Camby, and R. Kiss, *Galectins and cancer.* Biochim Biophys Acta, 2002. **1572**(2-3): p. 285-93.
175. Almkvist, J. and A. Karlsson, *Galectins as inflammatory mediators.* Glycoconj J, 2004. **19**(7-9): p. 575-81.
176. Dumic, J., S. Dabelic, and M. Flogel, *Galectin-3: an open-ended story.* Biochim Biophys Acta, 2006. **1760**(4): p. 616-35.
177. Delacour, D., et al., *Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells.* J Cell Biol, 2005. **169**(3): p. 491-501.
178. Delacour, D., et al., *Loss of galectin-3 impairs membrane polarisation of mouse enterocytes in vivo.* J Cell Sci, 2008. **121**(Pt 4): p. 458-65.
179. Rabinovich, G.A., et al., *An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer.* Scand J Immunol, 2007. **66**(2-3): p. 143-58.
180. Baptiste, T.A., et al., *Mechano-transduction mediated secretion and uptake of galectin-3 in breast carcinoma cells:*

implications in the extracellular functions of the lectin. Exp Cell Res, 2007. **313**(4): p. 652-64.

181. Paciucci, R., et al., *The plasminogen activator system in pancreas cancer: role of t-PA in the invasive potential in vitro.* Oncogene, 1998. **16**(5): p. 625-33
181. Stanley, P., *A method to the madness of N-glycan complexity?* Cell, 2007. **129**(1): p. 27-9.
182. Rabinovich, G.A., et al., *Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?* Trends Immunol, 2002. **23**(6): p. 313-20.
183. Thijssen, V.L., et al., *Galectins in the tumor endothelium: opportunities for combined cancer therapy.* Blood, 2007. **110**(8): p. 2819-27.
184. Rabinovich, G.A., N. Rubinstein, and M.A. Toscano, *Role of galectins in inflammatory and immunomodulatory processes.* Biochim Biophys Acta, 2002. **1572**(2-3): p. 274-84.
185. Lahm, H., et al., *Tumor galectinology: insights into the complex network of a family of endogenous lectins.* Glycoconj J, 2004. **20**(4): p. 227-38.
186. Caulet-Maugendre, S., et al., *Immunohistochemical expression of the intracellular component of galectin-8 in squamous cell metaplasia of the bronchial epithelium in neoplastic and benign processes.* Pathol Res Pract, 2001. **197**(12): p. 797-801.
187. Liao, D.I., et al., *Structure of S-lectin, a developmentally regulated vertebrate beta-galactoside-binding protein.* Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1428-32.
188. Morris, S., et al., *Quaternary solution structures of galectins-1, -3, and -7.* Glycobiology, 2004. **14**(3): p. 293-300.
189. Vishwanatha, J.K., et al., *Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers.* Carcinogenesis, 1993. **14**(12): p. 2575
189. Lopez-Lucendo, M.F., et al., *Growth-regulatory human galectin-1: crystallographic characterisation of the structural changes induced by single-site mutations and their impact on the thermodynamics of ligand binding.* J Mol Biol, 2004. **343**(4): p. 957-70.

190. Bourne, Y., et al., *Crosslinking of mammalian lectin (galectin-1) by complex biantennary saccharides.* Nat Struct Biol, 1994. **1**(12): p. 863-70.
191. Wang, J.L., et al., *Nucleocytoplasmic lectins.* Biochim Biophys Acta, 2004. **1673**(1-2): p. 75-93.
192. Allen, H.J., et al., *Role of galaptin in ovarian carcinoma adhesion to extracellular matrix in vitro.* J Cell Biochem, 1990. **43**(1): p. 43-57.
193. Scott, K. and J. Zhang, *Partial identification by site-directed mutagenesis of a cell growth inhibitory site on the human galectin-1 molecule.* BMC Cell Biol, 2002. **3**: p. 3.
194. Ahmad, N., et al., *Thermodynamic binding studies of bivalent oligosaccharides to galectin-1, galectin-3, and the carbohydrate recognition domain of galectin-3.* Glycobiology, 2004. **14**(9): p. 817-25.
195. Schwarz, F.P., et al., *Thermodynamics of bovine spleen galectin-1 binding to disaccharides: correlation with structure and its effect on oligomerization at the denaturation temperature.* Biochemistry, 1998. **37**(17): p. 5867-77.
196. Leppanen, A., et al., *Dimeric galectin-1 binds with high affinity to alpha2,3-sialylated and non-sialylated terminal N-acetyllactosamine units on surface-bound extended glycans.* J Biol Chem, 2005. **280**(7): p. 5549-62.
197. He, J. and L.G. Baum, *Presentation of galectin-1 by extracellular matrix triggers T cell death.* J Biol Chem, 2004. **279**(6): p. 4705-12.
198. Outenreath, R.L. and A.L. Jones, *Influence of an endogenous lectin substrate on cultured dorsal root ganglion cells.* J Neurocytol, 1992. **21**(11): p. 788-95.
199. Demydenko, D. and I. Berest, *Expression of galectin-1 in malignant tumors.* Exp Oncol, 2009. **31**(2): p. 74-9.
200. Chiariotti, L., et al., *Expression of galectin-1 in normal human thyroid gland and in differentiated and poorly differentiated thyroid tumors.* Int J Cancer, 1995. **64**(3): p. 171-5.
201. van den Brule, F.A., D. Waltregny, and V. Castronovo, *Increased expression of galectin-1 in carcinoma-associated stroma predicts poor outcome in prostate carcinoma patients.* J Pathol, 2001. **193**(1): p. 80-7.

202. Clausse, N., et al., *Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion.* Angiogenesis, 1999. **3**(4): p. 317-25.
203. Szoke, T., et al., *Prognostic significance of endogenous adhesion/growth-regulatory lectins in lung cancer.* Oncology, 2005. **69**(2): p. 167-74.
204. Lahm, H., et al., *Comprehensive galectin fingerprinting in a panel of 61 human tumor cell lines by RT-PCR and its implications for diagnostic and therapeutic procedures.* J Cancer Res Clin Oncol, 2001. **127**(6): p. 375-86.
205. Yamaoka, K., et al., *Expression of galectin-1 mRNA correlates with the malignant potential of human gliomas and expression of antisense galectin-1 inhibits the growth of 9 glioma cells.* J Neurosci Res, 2000. **59**(6): p. 722-30.
206. Perillo, N.L., et al., *Apoptosis of T cells mediated by galectin-1.* Nature, 1995. **378**(6558): p. 736-9.
207. Rabinovich, G.A., et al., *Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1.* Immunology, 1999. **97**(1): p. 100-6.
208. Grassadonia, A., et al., *90K (Mac-2 BP) and galectins in tumor progression and metastasis.* Glycoconj J, 2004. **19**(7-9): p. 551-6.
209. Thijssen, V.L., et al., *Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy.* Proc Natl Acad Sci U S A, 2006. **103**(43): p. 15975-80.
210. Abroun, S., et al., *Galectin-1 supports the survival of CD45RA(-) primary myeloma cells in vitro.* Br J Haematol, 2008. **142**(5): p. 754-65.
211. Lotan, R., et al., *Expression of galectins on microvessel endothelial cells and their involvement in tumour cell adhesion.* Glycoconj J, 1994. **11**(5): p. 462-8.
212. Le, Q.T., et al., *Galectin-1: a link between tumor hypoxia and tumor immune privilege.* J Clin Oncol, 2005. **23**(35): p. 8932-41.
213. Sanford, G.L. and S. Harris-Hooker, *Stimulation of vascular cell proliferation by beta-galactoside specific lectins.* Faseb J, 1990. **4**(11): p. 2912-8.
214. Maeda, N., et al., *Stimulation of proliferation of rat hepatic stellate cells by galectin-1 and galectin-3 through different*

intracellular signaling pathways. J Biol Chem, 2003. **278**(21): p. 18938-44.

215. Andersen, H., et al., *A proteome study of secreted prostatic factors affecting osteoblastic activity: galectin-1 is involved in differentiation of human bone marrow stromal cells.* J Bone Miner Res, 2003. **18**(2): p. 195-203.

216. Adams, L., G.K. Scott, and C.S. Weinberg, *Biphasic modulation of cell growth by recombinant human galectin-1.* Biochim Biophys Acta, 1996. **1312**(2): p. 137-44.

217. Moiseeva, E.P., et al., *Galectin 1 modulates attachment, spreading and migration of cultured vascular smooth muscle cells via interactions with cellular receptors and components of extracellular matrix.* J Vasc Res, 1999. **36**(1): p. 47-58.

218. van den Brule, F., et al., *Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin.* Lab Invest, 2003. **83**(3): p. 377-86.

219. Glinsky, V.V., et al., *Effects of Thomsen-Friedenreich antigen-specific peptide P-30 on beta-galactoside-mediated homotypic aggregation and adhesion to the endothelium of MDA-MB-435 human breast carcinoma cells.* Cancer Res, 2000. **60**(10): p. 2584-8.

220. Berberat, P.O., et al., *Comparative analysis of galectins in primary tumors and tumor metastasis in human pancreatic cancer.* J Histochem Cytochem, 2001. **49**(4): p. 539-49.

221. Chung, J.C., et al., *Proteomic analysis to identify biomarker proteins in pancreatic ductal adenocarcinoma.* ANZ J Surg, 2008. **78**(4): p. 245-51.

222. Harsha, H.C., et al., *A compendium of potential biomarkers of pancreatic cancer.* PLoS Med, 2009. **6**(4): p. e1000046.

223. Hernández-Muñoz I., et al.,*Pancreatic ductal denocarcinoma: cellular origin, signaling pathways and stroma contribution.* Pancreatology. 2008;**8**(4-5):462-9

224. Ahmad, N., et al*., Thermodynamic binding studies of bivalent oligosaccharides to Gal-1, galectin-3, and the carbohydrate recognition domain of galectin-3*. Glycobiology, **14**, 817–825.

225. Camby I et al. Glycobiology vol. 16 no. 11 pp. 137R–157R, 2006

226. Lee., S.R., et,al., *Induction of matrix metalloproteinase, cytokines and chemokines in rat cortical astrocytes exposed to plasminogen activators*. Neuroscience letters,2007.**417**(1) :p. 1-5.

227. Tang.,J., et al., *Endogenous tissue plasminogen activator increases hemorrhagic transformation induced by heparin after ischemia reperfusion in rat brains*. Neurol Res, 2010 **32**(5):p.541-6.

228. Javle.,M., et al., *Recent advances in gastrointestinal oncology—updates and insights from the 2009 annual meeting of the American society of clinical oncology*. J.Hematol Oncol,2010 **23**(3) :p.11

229. Hammers.,J.H., et al., *Reversible epithelial to mesenchymal transistion and acquired resistance to sunitinib in patients with renal cell carcinoma: evidence from a xenograft study*. Mol Cancer Ther. 2010. **9**(6): p: 1525-1535.

230. Bouard.,S., et al., *Antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastoma.* Neuro Oncol.2007.9(4): p.412-423.

231. Chow and Eckhardt.,*Sunitinib: From Rational Design to Clinical Efficacy*. J Clin Oncol. 2007.25: p. 884-896.

232. Socinski.,A.M., et al., *Multicenter, Phase II trial of sunitinib in previously treated, advanced non small cell lung cancer*. J Clin Oncol.2008.**26(**4):p. 650-656.

233. Faivre.,S., et al., *Safety and efficacy of sunitinib in patients with advanced hepatocellular carcinoma: an open-label, multicentre,phase II study*. The Lancet Oncology. 2009. **10** (8):p. 794-800.

234. Roda.,O., et al., *A proteomic approach to the identification of new tPA receptors in pancreatic cancer cells*. Proteomics 2006,6,S-36-S341

235. Hackel.,P.O., et al., *Epidermal growth factor receptors: critical mediators of multiple receptor pathways*. Curr Opin Cell Biol 1999,11: 184-189

236. Casneuf., V.J., et al., *Antiangiogenic versus cytotoxic therapeutic approaches in a mouse model of pancreatic cancer: an experimental study with a multitarget tyrosine kinase inhibitor (sunitinib), gemcitabine and radiotherapy*. Oncol Rep. 2009 Jul;**22** (1):105-13.

237. Ortiz-Zapater, E., et al., *Tissue plasminogen activator induces pancreatic cancer cell proliferation by a non-catalytic mechanism that requires extracellular signal-regulated kinase 1/2 activation through epidermal growth factor receptor and annexin A2.* Am J Pathol, 2007. **170**(5): p. 1573-84.

238. Zhao et al, *Depletion of Tissue Plasminogen Activator Attenuates Lung Ischemia-Reperfusion injury* . Am.J.Phy, 2011.

239. Genersch, E., et al*., Sustained ERK phoshorylation is necessary but not sufficient for MMP-9 regulation in endothelial cells: involvement of Ras-dependent and independent pathways*. J.Cell Sci,2000. **113** (23):p(4319-30).

240. Matsuo,Y., *Interleukin-1α Secreted by Pancreatic Cancer Cells Promotes Angiogenesis and Its Therapeutic Implications* J.Surg.Res, 2009. **153**(15): p. 274-281

241. Monreal,F.M., *Is Tissue type plasminogen a neuromodulator?* Mol.Cell.Neuro,2004. **25** (4): 594-601

242. Li., et al, *Macrophage migration inhibitory factor regulates proliferation of gastric cancer cells via the PI3K/Akt pathway*. World J Gastroenterol. 2009 November 28; **15**(44): 5541–5548.

242. An, J., et al., *Tissue-type plasminogen activator and the low-density lipoprotein receptor-related protein induce Akt*

phosphorylation in the ischemic brain. Blood, 2008. **112**(7): p. 2787-94

243. Roda O, Ortiz-Zapater E, Martínez-Bosch N, Gutiérrez-Gallego R, Vila-Perelló M, Ampurdanés C, Gabius HJ, André S, Andreu D, Real FX and Navarro P. *Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer.* Gastroenterology, 2009. **136**(4):1379-1390.
244. Aguilar S, Corominas JM, Malats N, Dufresne M, Real FX and Navarro P. The tissue plasminogen activator system in murine exocrine pancreas cancer:selective expression in ductal tumors and contribution to cancer progression. Am. J. Pathol., 2004. **165**(4): 1129-1139.
245. Hu K et al. Tissue-type plasminogen activator acts as a cytokine that triggers intracellular signal transductiona nd induces matrix metalloproteinase-9 gene expression. J. Biol. Chem. 2006. **281**: 2120-2127.
246. Zhang C et al. Microglial low-density lipoprotein receptor-related protein 1 mediates the effect of tissue-type plasminogen activator on matrix metalloproteinase-9 activity in the ischemic brain. J Cereb Blood Flow Metab. 2009. 29(12):1946-54
247. Lee SR., et al., Neurosci Lett. 2007 Apr 24;417(1):1-5. Epub 2007 Jan 11.

248. Paz,A.,et al., *Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation*. *Oncogene* **20**, 7486-7493 (2001)