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FERNANDO NÚÑEZ HERNÁNDEZ

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CReSA
Centre de Recerca en Sanitat Animal

IRTA
RESEARCH & TECHNOLOGY
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Universitat Autònoma de Barcelona

Director:	Tutor:	Author:
José Ignacio Núñez Garrote	**Joaquim Segalés i Coma**	**Fernando Núñez Hernández**

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Dr. José Ignacio Núñez Garrote, group leader at IRTA-CReSA as director and Dr. Joaquim Segalés i Coma, director of the center and professor at department of animal welfare and anatomy in Autonomous University of Barcelona as tutor

CERTIFY

That the thesis titled: "Identification and characterization of microRNAs in porcine circovirus type 2 and African swine fever firus infections *in vivo*" has been done by Fernando Núñez Hernández under their supervision.

In Bellaterra, July 21st, 2016

Dr. José Ignacio Núñez Garrote

Dr. Joaquim Segalés i Coma

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ABSTRACT

Four different studies were carried out in order to analyze the expression of porcine microRNAs (miRNAs) and viral miRNAs in two separate *in vivo* infections with porcine circovirus type 2 (PCV2) and African swine fever virus (ASFV). PCV2 is the essential etiological infectious agent of PCV2-systemic disease and has been associated with other swine diseases, all of them collectively known as porcine circovirus diseases. African swine fever is a re-expanding devastating viral disease, a highly lethal hemorrhagic disease that is currently threatening the pig industry worldwide. miRNAs are a new class of small non-coding RNAs that regulate gene expression post-transcriptionally. miRNAs play an increasing role in many biological processes. The study of miRNA-mediated host-pathogen interactions has emerged in the last decade due to the important role that miRNAs play in antiviral defense.

The objective of the first study was to identify the miRNA expression pattern in PCV2 subclinically infected and non-infected pigs. For this purpose an experimental PCV2 infection was carried out and small-RNA libraries were constructed from tonsil and mediastinal lymph node (MLN) of infected and non-infected pigs. High throughput sequencing determined differences in miRNA expression in MLN between infected and non-infected while, in tonsil, a very conserved pattern was observed. In MLN, miRNA 126-3p, miRNA 126-5p, let-7d-3p, mir-129a and mir-let-7b-3p were up-regulated whereas mir-193a-5p, mir-574-5p and mir-34a down-regulated. Prediction of functional analysis showed that these miRNAs can be involved in pathways related to immune system and in processes related to the pathogenesis of PCV2, although functional assays are needed to support these predictions. This was the first study on miRNA gene expression in pigs infected with PCV2 using a high throughput sequencing approach in which several host miRNAs were differentially expressed in response to PCV2 infection.

The objective of the second study was to explore if PCV2 can encode viral miRNAs. Viral miRNAs have been recently described and their number has increased in the past few years. From the constructed small RNA libraries, deep sequencing data revealed that PCV2 does not express miRNAs in an in vivo subclinical infection.

In the third study we have analyzed porcine miRNAs differentially expressed in spleen and submandibular lymph node of pigs experimentally infected with a virulent strain (E75) and its derived attenuated strain (E75CV1). Differences in porcine miRNA expression between these viruses were analyzed. Spleen presented a higher differential expression pattern in both comparisons and from the 9 DE miRNAs identified between two time points in infected animals with virulent strain and between infected animals with virulent strain and attenuated strain at the same time point, miR-451, miR-145-5p and miR-122 presented up-regulation at later times post-infection (pi) while miR-92a, miR-23a, miR-92b-3p, miR-126-5p, miR-23b and miR-92c presented down-regulation. These miRNAs are associated with cellular genes involved in pathways related to the immune response and virus-host interaction as well as with viral genes. This is, in our knowledge, the first description of miRNA regulation by AFSV in vivo.

In the fourth study we explored if ASFV can encode viral miRNAs. For this end, both previous strains E75 and E75CV1 were used in addition to samples from pigs infected with the attenuated strain and necropsied at 31 dpi and animals infected with the attenuated strain, re- inoculated at 31 dpi with virulent strain Ba71 and necropsied at 38 dpi. The deep sequencing from the small RNA libraries indicated that ASFV does not encode viral miRNAs in any of these conditions.

The study of the miRNAs expression will contribute to better understanding of PCV2 and ASFV pathogenesis, essential in the development of any strategy in order to control the diseases.

RESUMEN

Cuatro estudios diferentes fueron llevados a cabo con el fin de analizar la expresión de microRNAs (miRNAs) porcinos y miRNAs virales en dos infecciones *in vivo* con circovirus porcino tipo 2 (PCV2) y el virus de la peste porcina africana (VPPA). PCV2 es el agente causal de la enfermedad sistémica por circovirus porcino tipo 2 aunque también se ha asociado con otras enfermedades relativas al cerdo todas ellas conocidas colectivamente como enfermedades por circovirus porcino. miRNAs son una nueva clase de pequeños RNAs no codificantes que regulan la expresión génica a nivel post- transcripcional. Los miRNAs juegan un papel cada vez mayor en muchos procesos biológicos. El estudio de las interacciones entre los miRNAs, el hospedador y el patógeno ha aumentado en la última década debido al importante papel que juegan los miRNAs en la defensa frente a patógenos.

El objetivo del primer estudio fue identificar el patrón de expresión de miRNAs porcinos en cerdos infectados y no infectados con PCV2. Para ello, se llevó a cabo una infección experimental con PCV2 y se construyeron librerías de RNA de pequeño tamaño de tonsila y linfonodo mediastínico de animales infectados y no infectados. La secuenciación masiva mostró diferencias de expresión de miRNAs en linfonodo mediastínico mientras que en tonsila se observó un patrón mucho más conservado. En linfonodo mediastínico los miRNAs miR-126-3p, miR-126-5p, let-7d-3p, miR-129a y miR-let-7b-3p fueron encontrados sobre- expresados, mientras que miR-193a-5p, miR-574-5p y miR-34a estaban sub-expresados. La predicción de la función de estos miRNAs mostró su implicación en rutas relacionadas con la respuesta inmune y patogénesis de PCV2, sin embargo, son necesarios ensayos de funcionalidad que ayuden a afianzar estos resultados. Este ha sido el primer estudio de la expresión de miRNAs en animales infectados por PCV2, empleando técnicas de secuenciación masiva e identificando miRNAs diferencialmente expresados como respuesta a la infección. La segunda parte del estudio consistió en comprobar si PCV2 es capaz de expresar miRNAs virales, los cuáles son producidos por el

propio virus. El número de virus capaces de expresarlos ha incrementado en los últimos años.

A partir de las mismas librerías ya creadas, la secuenciación masiva mostró que PCV2 no codifica miRNAs virales.

En el tercer estudio se ha analizado la expresión de miRNAs porcinos en animales infectados con el virus de la peste porcina africana, la cual es una enfermedad viral devastadora que se está re- expandiendo y afectando a la industria porcina por todo el mundo. Se analizó la expresión de miRNAs en bazo y linfonodo submandibular de animales infectados con una cepa virulenta (E75) a distintos tiempos (3 y 7 días post- infección) y entre animales infectados con esta cepa virulenta y animales infectados con una cepa atenuada a un mismo tiempo (día 3 post- infección). A partir de muestras de ambos tejidos se realizaron librerías de ARN de pequeño tamaño y se observó una mayor diferencia de expresión en bazo en todas las comparaciones. De los 9 miRNAs diferencialmente expresados en la comparación de animales infectados con la cepa virulenta a distinto tiempo y entre animales infectados con la cepa virulenta y la atenuada a un mismo tiempo, miR-451, miR-145-5p y miR-122 se encontraron sobre-expresados mientras que miR-92a, miR-23a, miR-92b-3p, miR-126-5p, miR-23b y miR-92c se encontraron sub-expresados. Al mismo tiempo, se comprobó la relación de estos miRNAs diferencialmente expresados con rutas biológicas asociadas a la respuesta inmune y a las interacciones hospedador- patógeno.

En el cuarto estudio se investigó la capacidad del VPPA de expresar miRNAs virales, añadiendo dos nuevos casos de infección para aumentar el espectro del estudio con un animal infectado con la cepa atenuada y sacrificado a día 31 post-infección y dos animales infectados con la cepa atenuada, reinoculados a día 31 con una cepa virulenta, Ba71 y sacrificados a día 38. Sin embargo, la secuenciación masiva de las librerías de ARN de pequeño tamaño no pudo detectar la presencia de miRNAs virales en todas las condiciones que se llevaron a cabo.

El estudio de la expresión de miRNAs contribuirá a un mejor entendimiento de la patogénesis de la PPA, lo cual es esencial para el desarrollo de una estrategia para el control de la enfermedad.

ABBREVIATIONS

A

AAV: Adeno- associated virus

AD: Alzheimer's disease

ADV: Aujeszky's disease virus

AGO: Argonaute

ASF: African swine fever

ASFV: African swine fever virus

B

BCL2: B-cell CLL/lymphoma 2

BIM: Bcl-2-like protein 11

BKV: Polyomavirus BK

BLV: Bobine leukemia virus

C

CCD: Charge couple device

CN: Copy number

D

DE: Differentially expressed

DGCR8: DiGeorge syndrome critical region gene 8

DPI: Days post-infection/ inoculation

E

EBV: Epstein- Barr virus

EDTA: Ethylenediaminetetraacetic acid

ER: Endoplasmic reticulum

F

FC: Fold change

G

GO: Gene ontology

H

HAU: Hemagglutination units

hCMV: Human cytomegalovirus

HCV: Hepatitis C virus

HIV: Human immunodeficiency virus

I

IIF: Indirect immunofluorescence

IL: Interleukin

ISFET: Ion-sensitive field-effect transistors

ISH: *In situ* hybridization

J

JCV: John Cunningham virus

K

KEGG: Kyoto encyclopedia of genes and genomes

KSHV: Kaposi's associated Herpesvirus

L

LNA: Locked nucleic acid

M

MAPK: Mitogen- activated protein kinase

MDV1: Marek's Disease Virus 1

MGF: Multigenic family members

miRNA: microRNA

MLN: Mediastinal lymph node

mPy: Mouse polyomavirus

MRE: microRNA response elements

N

NCBI: National center for biotechnology information

NCLDV: Nucleocytoplasmic large DNA virus

ncRNAs: Non- coding RNAs

NGS: Next- generation sequencing

NK: Natural killer cells

P

PAZ: Piwi Argonaute and Zwille domain

PCV2: Porcine circovirus type 2

PCV2-SD: PCV2-systemic disease

PCVD: PCV disease

PD: Parkinson's disease

pDC: Plasmocitoid dendritic cells

PFV-1: primate foamy virus type 1

PI: Post- infection/ inoculation

PMWS: Postweaning multisystemic wasting syndrome

PPV: Porcine parvovirus

pre-miRNA: Precursor miRNA

pri-miRNA: Primary miRNA

PS: Polysaccharide

Q

qPCR: Quantitative real-time PCR

R

RIIIDs: RNAse III domains

RIN: RNA integrity number

RISC: Cytoplasmic RNA silencing complex

RLCV: Rhesus lymphocryptovirus

RTA: Master lytic switch protein

S

SGIV: Singapore grouper iridovirus

siRNA: Short interfering RNAs

SLE: Systemic lupus erythematosus

SV40: Simian vacuolating virus 40

T

Tag: T antigen

TCR: T cell receptor

TGEV: Transmissible gastroenteritis virus

TIR: Inverted repeats

TLRs: Toll- like receptors

TNF: Tumor necrosis factor

TRBP: Transactivation response RNA binding protein

Treg: Regulatory T cells

TUT: Terminal uridylyl transferase

U

UPR: Unfolded protein response

UTR: Untranslated region

X

XPO5: Exportin 5

PUBLICATIONS

The results presented in this thesis have been published or are in preparation for their submission in international scientific peer-reviewed journals:

Nunez-Hernandez, F., Perez, L. J., Munoz, M., Vera, G., Tomas, A., Egea, R., Cordoba, S., Segales, J., Sanchez, A. and Nunez, J. I. (2015). Identification of microRNAs in PCV2 subclinically infected pigs by high throughput sequencing. Vet Res **46**: 18.

Nunez-Hernandez, F., Perez, L. J., Vera, G., Cordoba, S., Segales, J., Sanchez, A. and Nunez, J. I. (2015). Evaluation of the capability of the PCV2 genome to encode miRNAs: lack of viral miRNA expression in an experimental infection. Vet Res **46**: 48.

Nunez-Hernandez, F., Perez, L. J., Munoz, M., Vera, G., Sanchez, A., Accensi, F., Rodríguez, F. and Nunez, J. I. Indetification of microRNAs in ASFV infected pigs with virulent and attenuated strains. Manuscript in preparation for submission.

Nunez-Hernandez, F., Munoz, M., Vera, G., Sanchez, A., Rodríguez, F. and Nunez, J. I. ASFV does not encode viral miRNAs in an experimental infection in pigs. Manuscript in preparation for submission.

1

INTRODUCTION

1.1. microRNAs: the beginning

In 1993 Rosalind C. Lee and pals were studying lin-4, an essential gene for the normal embryonic development of *Caenorhabditis elegans*. This gene regulates negatively the expression of lin-14 protein (Chalfie *et al.*, 1981) which appears in high levels in the nucleus of the cells in the larvae phase (L1) and decrease in the upcoming phase (L2). They found that lin-4 did not encode for any protein and had no conventional start – finish codons. When they analyzed this gene more deeply, they discovered two small RNA transcripts by Northern Blot and RNAse protection, a larger transcript (lin-4L) with 61 nt and a smaller one (lin-4S) with 22 nt (Figure 1). Both transcripts were complementary to a sequence in the 3' UTR of lin-14 mRNA. This homology was of only 10 nucleotides, and this fact had only been observed previously in RNA-RNA interactions, for example in interactions among spliceosomal RNAs and between spliceosomal RNAs and the pre-mRNAs (Datta and Weiner, 1991). It was also observed that lin-4s was much more abundant than lin-4L highlighting that the first one had the most important role in the binding and regulation of the lin-14 RNA in spite of its smaller size.

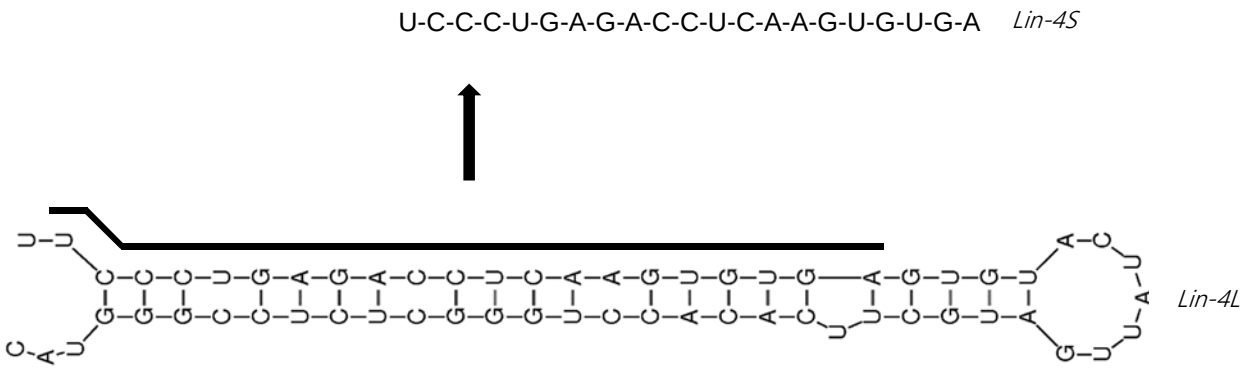


Figure 1. Lin-4L structure and its derived Lin-4S transcript, the first miRNA.

This was the first insight into the *world* of microRNAs (miRNAs). The interest to explore miRNAs has incredibly increased in the last few years as reflected in miRBase, the microRNA database, with 28 645 entries (www.mirbase.org). Since

then, several groups and studies have been giving light to this *darkness* that until this day surrounds the *ins and outs* of this such small but so important regulators that are present in all kind of living beings.

1.2. What are miRNAs?

All genomes usually are actively transcribed into RNA, but less than 2% of it is translated into proteins, the high majority of these transcripts are non- coding RNAs (ncRNAs). Within this group of RNAs there are three main sub-groups with approximately 20 to 35 nucleotides long: short interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs) and miRNAs. Lots of data remains unclear about these small regulators, so, what we know until today about them? And more specifically, what are exactly these miRNAs?. As Rosaline and Pals supposed, miRNAs are small single- stranded RNA molecules with regulatory functions. Their length ranges from 18 to 23 nucleotides. They are produced by several enzymatic reactions from their molecular predecessor, the primary miRNA (pri-miRNA), to their active form, the miRNA itself. These primal molecules, pri- miRNAs, have their origin depending on the genomic distribution of miRNAs genes. Different studies about this distribution throughout the genome reveal that these genes can appear in clusters transcribed as polycistronic primary transcripts, or within regions transcribed as independent units as intergenic regions, exon sequences of non- coding transcription units or intronic sequences of either protein coding or non- coding transcription units (Figure 2).

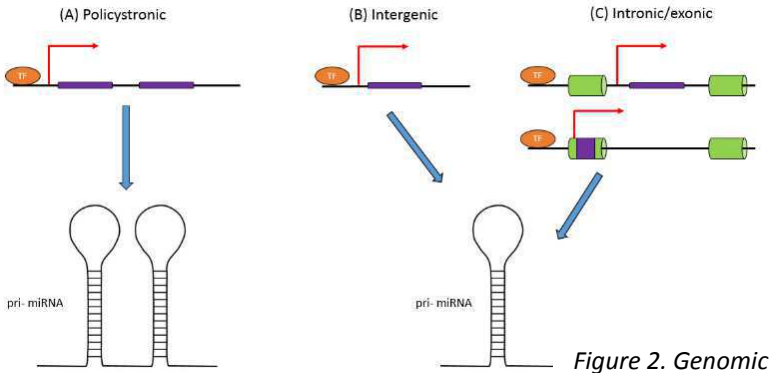


Figure 2. Genomic origin of miRNAs.

Independently of its genomic origin, the transcription, mediated by RNA polymerase II, produces long molecules of variable length (approximately 400 nt) called pri-miRNAs that share a common characteristic, a hairpin structure. All this pri-miRNAs have two complementary sequences that makes all the structure to bend over itself. This complementarity, as a rule, is not total, producing non-homologous regions intercalated between the homologous ones leads to the formation of a characteristic loop at the opposite end to the genome of both homologous sequences. After several cuts and modifications from the nucleus (including a shorter intermediate structure called pre- miRNA) to the cytoplasm of the individual cells, the real effector molecules are obtained, miRNAs.

The main function of this small RNAs within all the rest of regulation mechanisms present in an organism, is the regulation at a post- transcriptional level. They bind to a specific or specifics mRNAs by sequence homology, usually in the 3' untranslated regions.

This binding blocks the translation of the target mRNA or in other cases, leads to the destruction of this mRNA. This regulation in some cases can involve up to the 30% of the silencing of protein coding genes in an organism (Zhang *et al.*, 2007, Macfarlane and Murphy, 2010), fact that gives even more importance to the regulation task of this small RNAs in an organism.

The biogenesis of this small RNAs occurs in several modifications in which a considerable number of enzymes and enzymatic complexes are involved. The main routes and enzymes have been described but some key data are not clear to the present day.

1.2.1. Biogenesis

There are different routes that lead to the production of miRNAs but the most common one is the canonical biogenesis route although there are non- canonical routes that in specific situation produces miRNAs.

In the canonical pathway, miRNA genes are located in intergenic regions, being transcribed independently from exonic or intronic regions. These miRNA regions are transcribed by RNA polymerase II (Cai *et al.*, 2004, Lee *et al.*, 2004, Kim, 2005). As all molecular products from RNA polymerase II, the transcript obtained is capped and poly- adenylated. This primal transcription produces the previously mentioned pri- miRNAs. After the transcription, the `cropping´ phase occurs (first cleavage) near the end of the stem. It is carried out by Drosha, a class 2 ribonuclease III enzyme.

Drosha is a member of the ribonuclease III superfamily of double stranded RNA specific endoribonucleases and only acts in mammals and non- vertebrates. In this first cleavage is also involucrated DGCR8 microprocessor complex subunit (DiGeorge syndrome critical region gene 8), a cofactor that is essential for Drosha function (Macias *et al.*, 2002).

The binding site of Drosha is located in the C- terminal region of DGCR8. Drosha and DGCR8 integrate what is called the microprocessor complex, and both cannot act without the presence of the other one, demonstrating that both proteins are essential for the cleavage of pri-miRNAs. DGCR8 is the part of the complex that recognizes the pri-miRNA, the complex binds to the ssRNA – dsRNA junction and then, Drosha will interact with the stem. The processing center is located about 11 bp from the ssRNA region in the stem allowing Drosha to cleave in this approximate region. This cleavage produces the next-step-precursors called precursor miRNAs (pre-miRNAs) which have a 3' overhang in the known as passenger strand

This overhang allows a pre- miRNA classification including group I pre- miRNAs, with 2 nt overhang, and group II pre- miRNAs, with only 1 nt overhang. Pre- miRNAs

from this last group are rarely observed (Lee *et al.*, 2003). Normally, the 5' guide strand becomes the active RNA and the passenger strand is degraded but the alternative Drosha processing, that changes the stability of the miRNA ends, sometimes makes the passenger the active miRNA (Winter and Diederichs, 2013, Ha and Kim, 2014).

These pre-miRNAs are hairpin structured molecules of approximately 70 nt long are extracted from nucleus by Exportin- 5 (XPO5), a RanGTP-dependent dsRNA-binding protein (Bohnsack *et al.*, 2004). XPO5 is located in the nucleus and it binds cooperatively to the dsRNA and to the GTPase Ran in the active GTP- bound form. For the translocation from nucleus to cytoplasm, Ran- GTP is hydrolyzed to Ran-GDP what causes de disassembly of the complex and the pre- miRNA is released from its transporter.

Once in the cytoplasm, the pre- miRNA is loaded into the cytoplasmic RNA- induced silencing complex (RISC), this heterogeneous molecular complex can target almost any gene for silencing and its action starts when appears a dsRNA in the cytoplasm of an eukaryotic cell which will be processed into single stranded small regulatory RNAs, guided and linked to the complementary RNA target by base- pairing interaction. The target genes can be regulated by three routes: a) at a protein level, by repressing the translation, b) at a transcript level by mRNA degradation and c) at a genomic level by heterochromatin formation and DNA degradation (Pratt and MacRae, 2009). In the first step, the complex is called RISC- loading complex that is composed by DICER, the transactivation response RNA binding protein (TRBP) or the protein kinase RNA activator (PACT) (either can be involved, but with different effects in the miRNA obtainment), proteins of the argonaute family (AGO) and other proteins involved in the small RNAs loading onto AGO protein.

Dicer is a multi- domain RNAse III enzyme that plays a central role in the cleavage of the pre- miRNAs. Dicer has the ability to recognize dsRNA in order to obtain products with the precise size of miRNAs; this small RNAs that are generated have

approximately 22 base pairs from their open helical ends. Dicer has different domains, an helicase domain (HEL 1, 2, 3), DUF238 (with unknown function), the platform, the Piwi Argonaute and Zwille domain (PAZ) and the connection helix (Qin *et al.*, 2010, Sawh and Duchaine, 2012). Dicer domain PAZ recognizes the dsRNA and the platform domain, which works as a molecular ruler measures the correct size of the pre- miRNA with approximately 22 base pairs. The cleavage is carried out with the help of TRBP PACT, both dsRNA binding proteins. When the RNAse III cleavages the dsRNA it is loaded into the AGO protein with the cooperation of the heat shock cognate proteins 70 and 90 and conforming the complex called pre-RISC. Depending on the AGO protein involved in the process, the passenger strand can be unwounded or degraded (sometimes it has been observed that the passenger strand becomes active, but the exact mechanisms are still under investigation). When the dsRNA is unwounded, the RISC complex is complete with the AGO protein and the guide RNA within (miRISC), which will bind to target mRNA as the final active miRNA. Both will be guided to the target mRNA to carry out the miRNA's regulation function (Figure 5) (Lau *et al.*, 2012, Wilson *et al.*, 2015).

This is the most typical- canonical pathway of miRNAs biogenesis in vertebrates, but there are other non- canonical alternative mechanisms of biogenesis that have been discovered as: Drosha- DGCR8 independent pathway, the mirtron pathway, intronic canonical miRNAs biogenesis pathway, the non- canonical biogenesis of miRNAs from group II pre- miRNAs (tutase dependent) and the Dicer- bypass pathway (Kim *et al.*, 2009, Miyoshi *et al.*, 2010, Westholm and Lai, 2011, Heo *et al.*, 2012, Yoda *et al.*, 2013, Ha and Kim, 2014).

All these pathways show incredible flexibility in miRNAs biogenesis, but it is important note that, from of the total miRNAs produced in vertebrates, non-canonical pathways suppose only a 1% of the total and are restricted to very concrete miRNA cases.

1.2.2. IsomiRs

The high diversity, but at the same time, the high similarity of miRNAs led to the identification of the isomiRs. These are miRNAs which have different sequences that differ in one to three nucleotides, usually in the sequence ends and some times within the sequence. This sequence changes generally don't influence the specific function of these similar miRNAs, and all these sequences are considered as the same miRNA.

IsomiRs are produced in the same way as any miRNA, but at some point in the biogenesis, non- typical changes happen. These changes cause the apparition of different isomiRs isoforms. On one hand, those sequences with changes in their ends are classified as 5' isomiRs and 3' isomiRs depending on the modified end, and these changes can be templated, if they are similar to the pre- miRNA sequence or non- templated if not. On the other hand, we find the polymorphic isomiRs, those which have their changes within the sequence (Neilsen *et al.*, 2012) (Figure 3).

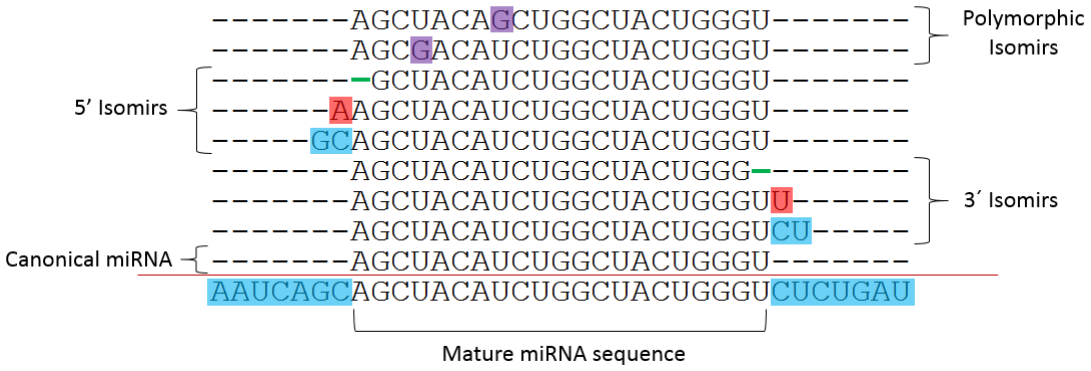


Figure 3. IsomiRs types.

Variations in Drosha and/ or Dicer processing is the most typical isomiR generation process, but other processing enzymes are also involved. It has been observed that variability is more abundant in the 3' end, it has been proposed that at the point where argonaute proteins act over the miRNA, the 5' end is buried within the MID domain, and the 3' end extends from the PAZ domain, been therefore, susceptible to the action of exoribonucleases. Nibbler, a 3'-5' exoribonuclease has been

identified as the cause of 3'trimming in isomiRs and the QDE-2-Interacting Protein (QIP) and the RNA exosome (3'-5' exoribonuclease activity) as the cause of 3' end degradation.

Also, nucleotidyl transferases are involved in the modifications of isomiRs, for example, Poly(A) RNA polymerase GLD2 (PAPD4) causes 3' adenylation, the terminal uridylyltransferase 4 (ZCCHC11), causes 3' uridylation, TUT1, causes 3' adenylation and uridylation and the adenosine deaminase acting on RNA (ADAR), adenosine to inosine editing.

Although 5' and polymorphic isomiRs are less abundant and sometimes rarely observed, they represent a significant proportion of the overall count of isomiRs. 5' isomiRs theoretically could target different mRNAs compared with canonically produced isomiRs due to the fact that changes in this end could modify the seed region and therefore the target sequence.

It has been observed that isomiR production could be modified depending of cellular stimuli, it is dynamic and regulatable, but in most cases, it is not subject to dramatic changes and this regulation is supposed to be limited (Cloonan *et al.*, 2011, Neilsen *et al.*, 2012).

1.3. miRNAs in action

When miRNAs' hairpin has been processed and loaded into the argonaute protein of RISC, the miRNAs pair by base- homology with mRNAs for a direct posttranscriptional repression. For the sequence recognition and binding it is a very important region within the miRNA, the seed region, a nucleotide interval in the 5' end region of the miRNA with high complementarity with the target mRNA.

1.3.1. The seed sequence

This region is defined between the 2nd and 8th nucleotide starting from the 5' end of the miRNA of 6 to 8 nucleotides long that is going to bind tightly to an homologous sequence in the 3' untranslated regions (UTR) of the mRNA, although it has also been observed that a small group of miRNAs can modulate expression by binding to 5' UTR and / or coding regions of some mRNAs (Lytle *et al.*, 2007, Orom *et al.*, 2008).

The seed starts at nucleotide 2 for at least a minimum length of 6 nucleotides called the core seed. This seed recognizes the target by Waston and Crick base pairing. The binding between both the seed and the mRNA can be diverse and classified in base to the number of homologous nucleotides in the binding, the start position from the 5' end and nucleotides that appear in the ends of this region (Bartel, 2009, Ellwanger *et al.*, 2011).

The union between the seed region and the mRNA, as previously described is perfect in the core of the seed having changes only in the flanking nucleotides, but in some cases miRNAs with internal mismatches and bulges can lead to mRNA processing repression, these are however very unusual facts and are poorly conserved (Friedman *et al.*, 2009).

It has been observed that most of all genuine target sites are covered by 7mer and 8mer, being 7mer with the highest recall and 8mer the most specific ones. 6mer seeds appeared not to be totally reliable and are associated with low repressive effects. Although the seed region is the most important part for recognition and binding, the rest of the miRNA sequence is also involved in this regulation usually by supplement pairing, enhancing binding specificity and affinity. These regions are known as 3'- supplementary sites, and the pairing is usually produced between nucleotides 13 – 16 of the miRNA and its UTR region of the mRNA. As observed in the seed pairing, this 3' pairing needs at least 3 to 4 Watson and Crick consecutive

pairs with no mismatches, bulges or wobbles. However, if the binding in the 3' end of the miRNA is because of mismatches or bulges in the seed region, the binding lengthens to 9 Watson and Crick pairs in the known as 3' compensatory site with similar functions to the supplementary site. This supplementation and compensation binding is atypical and doesn't enhance too much the recognition in comparison with those cases without them, having therefore, in most cases, a modest role (Lewis *et al.*, 2005).

1.3.2. miRNA action mechanisms

The miRNA- mRNA interaction has different outcomes depending on different factors, the most mentioned is the perfect base pairing between the seed and the mRNA target sequence.Also, the number of target sites for one miRNA and the relative position of them, the accessibility of the binding site, the adjacent sequences to the miRNA target site and the secondary structure of RNA can influence this hybridization (Finnegan and Matzke, 2003, Ohler *et al.*, 2004, Majoros and Ohler, 2007, Brodersen and Voinnet, 2009).

The miRNA action can be carried out at three different stages: Pretranslational, cotranslational and posttranslational, having variable results in the translational machinery and providing different potential mechanisms of miRNA- mediated downregulation:

A) Translation initiation mechanisms: mRNA decay, competing with cap structure or inhibition of close- loop mRNA formation.
B) Post- initiation mechanisms: Inhibition of ribosomal subunit binding, premature ribosome drop- off, slowed elongation or premature termination or cotranslational degradation of nascent protein
C) Posttranslational mechanism: Processing of target mRNA in the processing bodies (GW/P bodies).

Down-regulation by miRNAs is the most common activity related to this small RNAs, but recently, it has been discovered that in some cases, depending on the tissue and conditions, miRNAs can be involved also in posttranscriptional upregulation, ergo, miRNAs and their associated protein complexes can stimulate gene expression by direct and indirect mechanisms (Rehwinkel *et al.*, 2005, Behm-Ansmant *et al.*, 2006, Eulalio *et al.*, 2008, Mortensen *et al.*, 2011, Lee and Vasudevan, 2013, Inada and Makino, 2014, Valinezhad Orang *et al.*, 2014).

1.4. miRNAs biological involvement and disorders

miRNAs, as previously mentioned, are involved in a huge number of processes if not in almost all of them as more than 60% of all mRNAs are predicted to be under miRNA control (Bartel, 2009). Because of that, it is hard or almost impossible to mention their biological role in all the different processes. The most remarkable or important processes in which they are involved are: development, differentiation, proliferation, apoptosis, immune response and diseases.

1.4.1. Development and differentiation

miRNAs expression is spatiotemporal and tissue- and cell- specific suggesting that they are involved in tissue morphogenesis and cell differentiation. Specifically, it has been demonstrated that they regulate embryonic cell differentiation, limb development, adipogenesis, myogenesis, angiogenesis and hematopoiesis, neurogenesis and epithelial morphogenesis. Moreover, miRNAs are starting to have importance in the animal production field as they are involved in muscle and adipose tissues development with economic importance as they determine the meat quality. In different studies it has been observed that in pigs, polymorphisms in miR-1 affects the muscle fiber formation and depending on the breed and muscle

type, differentially expressed miRNAs have been identified (McDaneld *et al.*, 2009, Nielsen *et al.*, 2010, Zhou *et al.*, 2010). Similarly in the adipose tissue in pigs, where miRNAs affect the formation of the tissue with variations and differentially expressed miRNAs depending on the age, breed and tissue location as reported by (Li *et al.*, 2011, Chen *et al.*, 2012, Fatima and Morris, 2013). The huge number of combinations of miRNAs, different cells, tissues and development time- points, creates a very complex regulation machinery that makes their deeper analysis in this work. Some studies, however, have started scratching the surface in order to obtain further knowledge as how miRNAs influence the different biological processes (Kloosterman and Plasterk, 2006, Friedman and Jones, 2009, Shenoy and Blelloch, 2014).

1.4.2. Proliferation and Apoptosis

When talking of proliferation and apoptosis one must consider two different functions, namely, miRNAs with proproliferative/ antiapoptotic functions and miRNAs with antiproliferative/ proapoptotic functions (Hwang and Mendell, 2006). Both of them leads to one of the most interesting miRNAs implication, their participation in carcinogenic processes. The first time it was described that miRNAs were involved in tumorigenesis was in 2002 by Calin and colleagues in B cell chronic leukemia (Calin *et al.*, 2002). It has been observed that the most common alteration in cancer, pertaining to miRNAs, is the aberrant miRNA expression promoting reduced levels of mature miRNAs like miR-15a and miR-16-1. However, loss of determinate miRNAs that target anti- apoptotic proteins, such as BCL2, strongly suggests that these miRNAs act as tumor suppressive genes.

Studies have shown that disorders related to miRNAs of the let-7 and miR-34 families, which are necessary for the cell- cycle- exit, terminate the cell

differentiation and target many elements in growth control, increases the chance of cancer (Michael *et al.*, 2003).

But this is not the only activity of miRNAs related to cancer, they can also act as oncogens (oncomiRs). miR-21, the first miRNA known as "oncomiR" was almost always overexpressed in cancer. The study of miR-21 knockout animals has demonstrated tumor decrease, converting miR-21 in a promising therapeutic target for cancer. Also the miR-17-92 cluster has been found amplified in various tumor types including lung, colon and gastric cancer. In addittion, miR- 155 has been always a cancer-related miRNA. Its overexpression in early B- cells results in B- cell tumors but also promotes cell invasion in breast tumors (Stahlhut Espinosa and Slack, 2006, Yamakuchi *et al.*, 2008, Hermeking, 2012). Also alterations in the expression of complexes components of the miRNAs biogenesis can promote cancer, for example, has been observed that dicer is less expressed in tumors demonstrating that its loss promotes carcinogenic processes apparition. Many more miRNAs have already been identified and related to specific cancerous manifestations, the huge importance that cancer studies have reach, will undoubtedly increase the knowledge and number of identified miRNAs involved in these alterations and will help to a better understanding of their implications and importance (Ardekani and Naeini, 2010).

1.4.3. miRNAs, immune response and host- pathogens interactions

Apart from all the above actions, in these recent years miRNAs have also been studied as a regulator mechanism involved in the control of the immune response, diseases course and host- pathogens interactions. The immune system has many ways to be regulated, miRNAs have also recently been considered as an important regulator in this process. miRNAs control the strength and spatiotemporal dynamics of the immune responses. Immune system is controlled by the instructions given by

the immune signals and miRNAs are a very good tuner of these signals due to their capability of carrying out a fine regulation at a protein level.

A single miRNA is able to modulate more than one mRNA and the interactions between them, giving this single miRNA the capability of regulating a whole network. miRNAs can modulate the immune system signaling in four different ways like, linear, network, divergent and convergent (Figure 4).

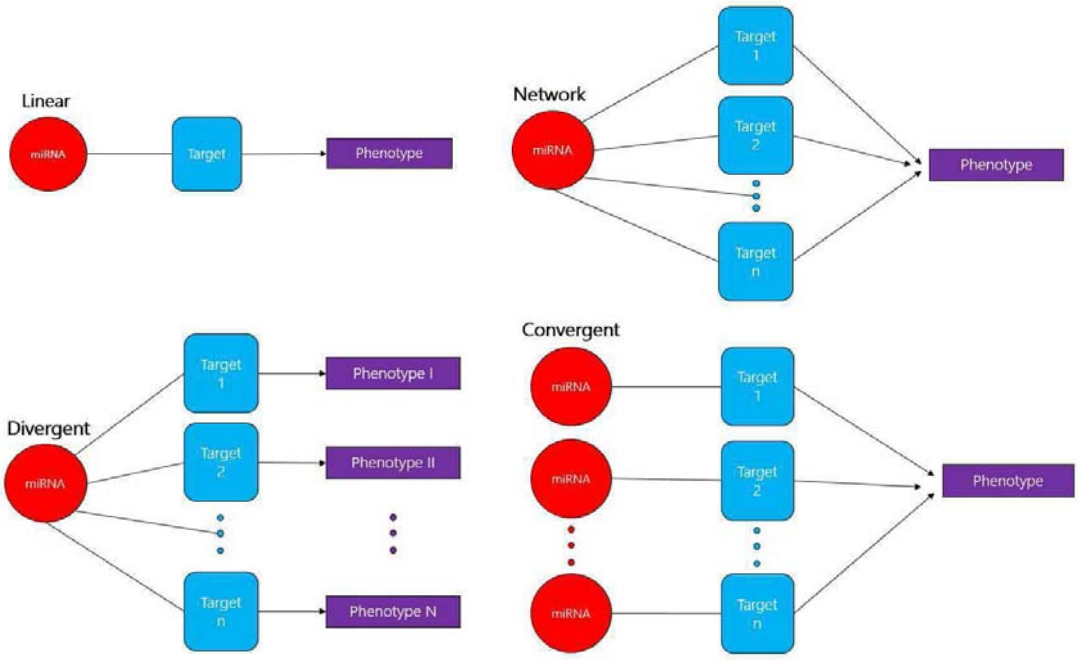


Figure 4. Different models of miRNAs modulation of immune system signaling.

In linear mode, miRNAs act over a single mRNA target (e.g. miR-150 controlling B-lymphocyte development by fine regulation of transcription factor c-Myb (Xiao *et al.*, 2007). In the divergent model, one single miRNA exert its control over multiple target mRNAs, these interaction are associated with different biological outcome (e.g. miR-155 regulates suppressor of cytokine signaling 1 (SOCS1) and proliferation of regulatory T cells (Treg) influencing class- switch recombination and affinity maturation). In the network model, one single miRNA regulates different target mRNAs but these interactions result in a specific biological response (e.g. miR-181a modulates T cell receptor signaling by regulation of different phosphatases such as

SH-2, DUSP5- 6 and PTPN-22). Finally, the convergent model different miRNAs regulates different targets but all the interactions converge in one single specific biological outcome (e.g. miR-146a and miR-155 targets STAT1 and SOCS1 respectively in order to modulate the development and function of Treg cells).

The first insight signaling that miRNAs could be involved in immune response appeared in 2004, when it was observed that miR-142, miR-181a and miR-223 had selective expression in immune cells (Chen *et al.*, 2004) and that miR-181a was expressed in the thymus and miR-223 in the bone marrow, indicating that they were involved in differentiation of pluripotent hematopoietic stem cells (HSCs) into the various blood cell lineages including B and T cells.

In immune cells, miRNAs may act as enhancers or inhibitors of the immune signaling, miRNAs can be turned on or off at a transcriptional level modulating this way the expression of positive or negative components of immune signaling pathways. Multiple miRNAs have been identified in different steps in the development of immune cells including lymphocytes (B and T cells) and myeloid cells (monocytes and neutrophils).

Recent studies have identified specific miRNAs such as miR-17~92 (miR 17~92 refers to a cluster including six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1), miR-155 and miR-181a with functions during T and B cells differentiation and miR-223 implicated in myeloid production is summarized in (Figure 5). However, many more miRNAs have been observed differentially expressed in the regulation of normal immune function and inflammation as reported by (Sonkoly *et al.*, 2008).

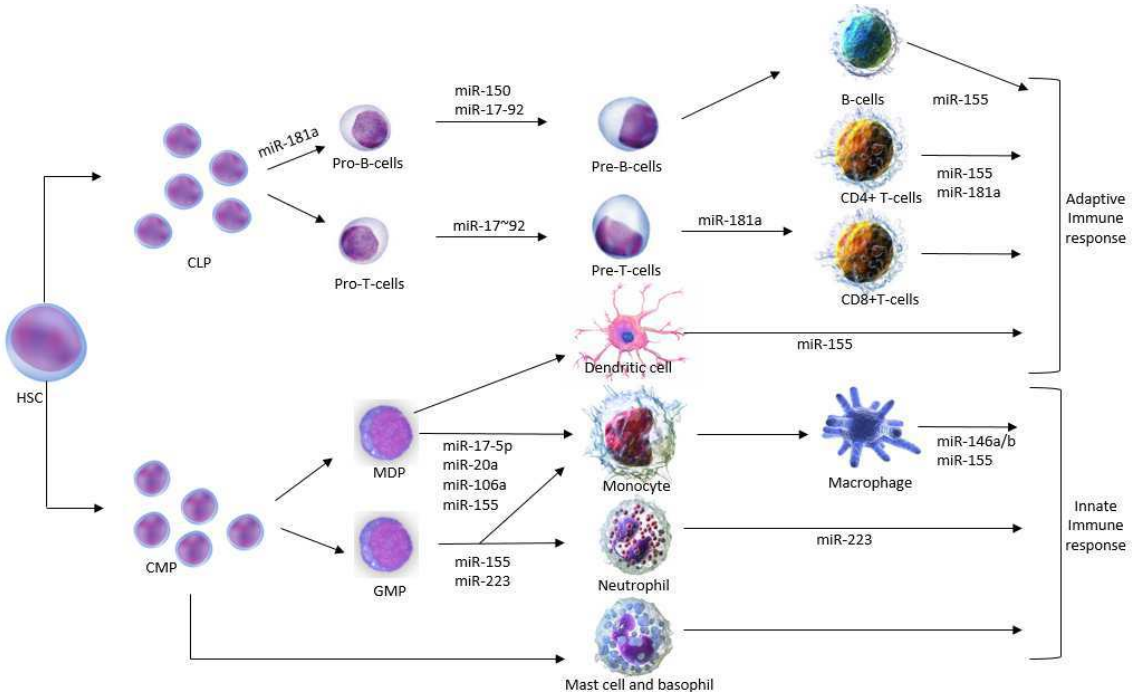


Figure 5. Identified miRNAs involved in the development of immune cells. HSC: hematopoietic stem cell. CLP: common lymphoid progenitor. CMP: common myeloid progenitor. GMP: granulocyte monocytic progenitor. MDP: myeloid dendritic progenitor.

The innate immune response is the first defense against external pathogens and it is predominantly carried out by macrophages, dendritic cells, monocytes and neutrophils. Macrophages and dendritic cells detect pathogens' presence by different pattern recognition receptors. Toll- like (TLR) and interleukin-1 receptors (TIRs) are the most known among them. It has been investigated that the activation of TIRs in addition to tumor necrosis factor α (TNFα) receptor carries a fast expression of different host miRNAs as miR-9, miR-146a and miR-155, which leads, within the affected signaling cascade, the regulation of the activation of myeloid cells. Activation in macrophages, monocytes and neutrophils of TIRs and TNFα receptors promotes the expression of the pro-inflammatory transcription factor nuclear factor (NF)kB and c-jun-N-terminal kinase (JNK) that increases inflammatory mediators TNFα, IL-1β and the levels of this three miRNAs, then, miR-146a targets IL-1 receptor activated kinase (IRAK1) and tumor receptor factor associated factor-6 (TRAF6), miR-9 targets NF-kB and miR-155 TGF-beta-activated kinase 1 and

MAP3K7-binding protein 2 (TAB2) to regulate the TIR induced pathways (Ma *et al.*, 2011).

Another recent study has demonstrated the implication of miR-223 in the differentiation of myeloid precursors into granulocytes such as neutrophils. It carries out its function by targeting ELF-1-like factor (MEF-2C) and the insulin- like growth factor receptor (IGFR), both targets promotes myeloid progenitor proliferation, because of that, miR-223 acts as a fine tuner of granulocyte production (Jia *et al.*, 2011).

The acquired or adaptive immune response requires the selective recognition and elimination of pathogens by T- cell receptors (TCRs) and antibodies expressed by T- lymphocytes and B- lymphocytes respectively. At this point, there are many controlled pathways as maturation, proliferation, differentiation and activation of lymphocytes. miR-17~92 cluster targets pro-apoptotic protein Bim and tumour suppressor PTEN, resulting in an increase of the pro-T to pre-T cell transition. miR-155 has been discovered as necessary for the differentiation of CD4+ Treg cells; transcriptional factor FoxP3, that is essential for this reaction, causes an increase in the levels of miR-155 which targets and downregulates SOCS1 that increases proliferation through IL-2 and STAT5 signaling pathways (Lu *et al.*, 2009). This miRNA is also required for the release of cytokines such as IL-2 and IFNγ. Other very important miRNA at this level is, miR-181a, involved in the production of CD4+ and CD8+ double positive T- cells. Its levels increase during T- cell maturation and modulates TCR signaling by down-regulation of different tyrosine phosphatases, Src homology 2 domain- containing protein- tyrosine phosphatase (SHP-2), protein-tyrosine phosphatase (PTPN-22) and the ERK- specific, dual specificity phosphatases (DUSP-5 and DUSP-6) reducing the threshold and increasing the strength of TCR signaling (Lindsay, 2008, Sonkoly *et al.*, 2008, Davidson-Moncada *et al.*, 2010).

All the previously described miRNAs are the best known related to the immune response, but all the immune related mechanisms need a deeper regulation by many other miRNAs, although a lot of them still remain unknown.

1.5. miRNAs and diseases

Expression profiles of miRNAs allow the identification of a normal or a pathological state and their deregulation expression, which can occur for several reasons, could lead to the apparition of diseases. This deregulation happens at different levels like: deletion of miRNA genes, mutations in the miRNA genes or in their targets, epigenetic silencing of the miRNA transcriptional units or defects in the miRNAs processing. The implication of miRNAs has been already assessed in multiple diseases. Cancer, as previously described, is one of the most important, but there have been many more revelations in recent years. Their involvement in neurodegenerative disorders as Alzheimer's (AD) and Parkinson's disease (PD), in cardiac diseases, skeletal muscle disorders, diabetes, obesity and immune diseases has been documented. Also, in bacterial infections such as those caused by *Helicobacter pylori*, *Salmonella enterica*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium species* or *Francisella tularensis* implication of miRNA has been speculated. Also, number of reports recently documented its involvement in viral infections (Eisenberg *et al.*, 2007, van Rooij and Olson, 2007, Lovis *et al.*, 2008, Tang *et al.*, 2009, Wang *et al.*, 2011, Eulalio *et al.*, 2012, Long *et al.*, 2012, Staedel and Darfeuille, 2013, Maqbool and Ul Hussain, 2014, Bao *et al.*, 2015, Sun *et al.*, 2015).

1.5.2. Viral diseases

During viral infection, cellular miRNA expression is altered. This alteration of the cellular environment depends on both host antiviral defenses and viral factors. Infected cells have many signaling mechanisms to sense and respond to virus infection and are capable of modifying their miRNA expression pattern as a response to viral infection, but, in the same way, viruses are capable of altering this pattern. It has been demonstrated that Epstein-Barr virus (EBV) induces the expression of miR-155 in type III latency B cells and miR-146a, a modulator of lymphocyte signaling pathways (Cameron *et al.*, 2008, Lu *et al.*, 2008, Linnstaedt *et al.*, 2010). Both miRNAs are also induced by bacterial lipopolysaccharide stimulation of monocytes. miR-146a targets LPS- activated components as TRAF6 and IRAK1 of the toll- like receptor signaling pathway for a negative feedback loop limiting innate immune responses. EBV LMP1 protein induces the expression of miR-146a leading to a reduction of several interferon-responsive genes. This protein also induces miR-29b expression that regulates T-cell leukemia gene 1 (TCL1), gene involved in cell survival and proliferation. Another virus, the oncogenic human papillomavirus encodes proteins E6 and E7 that inhibit p53 and Rb pathways altering cellular miRNAs controlled by these pathways (Honegger *et al.*, 2015). E6 down-regulates the expression of miR-34a, which is regulated by p53 leading to an alteration in the cell growth. Also, in human papillomavirus 16 (HPV-16) E6 down-regulates miR-218 which targets laminin 5 β3 (LAMB3) enhancing cell migration and tumorigenicity (Lo *et al.*, 2007, Cameron *et al.*, 2008). Human cytomegalovirus (HCMV) down-regulates miR-100 and miR-101 which regulate mTOR signaling pathway. This pathway control many cellular processes involved in growth, survival and metabolism.

Different studies in pigs have also demonstrated that viral infections alter the expression pattern of miRNAs in the host. As reported by (Li *et al.*, 2015) miR-143-3p was up-regulated in a highly-pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) infection, and this miRNA reduces cell proliferation

and induce apoptosis through down-regulating methyltransferase 3 alpha (DNMT3A). It was also observed that miR-22-5p was up-regulated in HP-PRRSV infection, this miRNA suppress the activity of NF-κB regulating the expression of nuclear receptor coactivator 1 (NCOA1). MiR-122 and miR-215 were down-regulated which enhance the replication of hepatitis C virus (HCV) in humans (Jopling *et al.*, 2005, Li *et al.*, 2015, Shrivastava *et al.*, 2015), so, the down-regulation of both miRNAs might be also an anti-viral strategy for pigs. In other studies, it has been observed that miR-181 and miR-130b inhibit virus replication by targeting viral genome (Guo *et al.*, 2013, Li *et al.*, 2015) while miR-24-3p promotes its replication by suppression of heme oxygenase-1 (HMOX1) (Xiao *et al.*, 2015).

Another infection with the transmissible gastroenteritis virus (TGEV) showed several differentially expressed (DE) miRNAs as a response to the virus. The analysis showed that these miRNAs target T cell receptor signaling pathway, which is involved in the regulation of T cell development, cytokine production and the activation of induced cell death, which is crucial for anti-virus activity. Also it was observed that 5 miRNAs target TGEV genome, ssc-miR-28–3p, ssc-miR-126–5p and ssc-miR-30b-5p target the 3' UTR of the TGEV genome and ssc-miR-2411 target the 5' UTR (Liu *et al.*, 2015).

In a previous study, in an experimental infection with Aujeszky's disease virus (ADV), DE miRNAs were also identified and related to be involved in RIG-I-like receptor signaling pathway, responsible for detecting viral pathogens and also B and T cell receptor signaling pathways, which are key components for the activation of adaptive immunity and T lymphocytes that ensure an efficient response of the immune system. They also were related to Fc gamma R-mediated phagocytosis, which plays an important role in host-defense mechanisms through the uptake and destruction of infectious pathogens, and chemokine signaling pathway, which works on the inflammatory immune response (Timoneda *et al.*,

2014). Anselmo and colleagues demonstrated in other study carried out (Anselmo *et al.*, 2011), with ADV in cell culture, that differentially expressed miRNAs could play a role in nervous system development and function (since the primary sites of ADV latency are the sensory neurons of the trigeminal ganglia), cell growth, proliferation and death.

Likewise, in different influenza A (H1N2) infections, it has been also observed that several miRNAs that were identified as involved in the disease progress by targeting key genes from immunological and related pathways were DE (Skovgaard *et al.*, 2013, Brogaard *et al.*, 2016).

All these diseases have proven the capability of viruses to modify the normal pattern of miRNAs in the host, but at the same time the host can modify this pattern, host genome can also express miRNAs that specifically targets viral genome as an responser to the disease. One of the most known cases is HCV and miR-122. This miRNA targets two regions in the 5'UTR of the viral genome. This binding sites for miR-122 are conserved in all HCV genotypes and leads to a replication and viral protein synthesis enhancement (Roberts and Jopling, 2010).

In the case of human immunodeficiency virus 1 (HIV-1), several miRNAs have been observed that target directly the viral genome causing modifications in the replication and latency of the virus. miRNAs and their specific function over the virus are shown in (Table 1) (Houzet *et al.*, 2012, Swaminathan *et al.*, 2014).

miRNA	Target	Function
miR-29a	nef	Decrease HIV infectivity
miR-28	3' LTR	I) Latency in primary resting CD4+ T cells II) Restrict HIV replication in monocytes
miR-125b		
miR-150		
miR-223		
miR-382		
miR-138		

miR-133b	3′ LTR	Decreases HIV infectivity
miR-138		
miR-149		
miR-326		

Table 1. Host miRNA and their target and function over the HIV-1 viral genome.

In human papillomavirus (HPV), miR-125b targets cap protein region in the genome, but at the same time, HPV counteracts inhibiting the expression of this miRNA. Studies suggest that the binding of miR-125b reduces HPV replication (Ribeiro *et al.*, 2015).

miR-32 has been proven to effectively limit primate foamy virus type 1 (PFV-1) replication by binding to a poorly conserved region in the 3′ portion of the viral genome (Sarnow *et al.*, 2006).

In hepatitis B virus disease (HBV), hsa-miR-125a-5p interacts with the viral sequence and interferes with the viral translation, down-regulating the expression of the surface antigen (Russo and Potenza, 2011).

Finally, in vesicular stomatitis virus infections (VSV) , it has been detected that 2 miRNAs, miR-24 and miR-93 target the viral genome in the viral large protein (L protein) and phosphoprotein (P protein) genes, and their lack increases viral replication suggesting that they could play a role in the host interaction with the virus (Otsuka *et al.*, 2007).

Interestingly, all the miRNAs interaction between the infected cells and the virus does not end here, viruses have more abilities, they can express their own miRNAs to modulate their replication and to control specific host genes.

1.6. Viral miRNAs

It has been discovered that viruses, as other organisms, are capable expressing miRNAs. Was in 2004 when the first viral miRNA was found (Pfeffer *et al.*, 2004). Up to now, more than five hundreds of viral miRNAs have been identified in 29 viruses. Talking about viral miRNAs, mammalian viruses can be classified in four groups: herpesviruses, which encode multiple viral miRNAs; other nuclear DNA viruses, which encode one or two miRNAs; RNA viruses and cytoplasmic DNA viruses which do not encode any viral miRNA; and retroviruses, despite the controversy, HIV-1 which until this date has four identified viral miRNAs and the bovine leukemia virus (BLV) with 5 viral miRNAs as described in miRBase (Griffiths-Jones *et al.*, 2008, Cullen, 2010). The fact why some kind of viruses does not encode miRNAs is due to the lack of a nuclear phase that is necessary for the miRNA production but in the case of retroviruses, it has been proposed that they can express miRNAs through a non- canonical biogenesis route. Herpesvirus domain as those viruses which express a higher number of miRNAs and as those viruses with an average number of miRNAs encoded per virus (more than ten per virus). As previously mentioned, viruses most likely to encode miRNAs will have nuclear and DNA components to their lifecycle and have the ability to establish persistent infections.

Viral miRNAs can be classified in two groups: those which are analogs of host miRNAs and those which are viral specific. This leads to different models of viral miRNA function (Figure 6):

i) The host network model where viral miRNAs function as analogs of host miRNAs through seed sequence similarity, thereby targeting transcripts through the same docking sites as the mimicked host miRNAs.

ii) The primary target model where some viral miRNAs evolve to target only one or a few transcripts through novel sites not conserved for host miRNA functions.

iii) The convergent target model where host and viral miRNAs may target the same transcript through different docking sites.

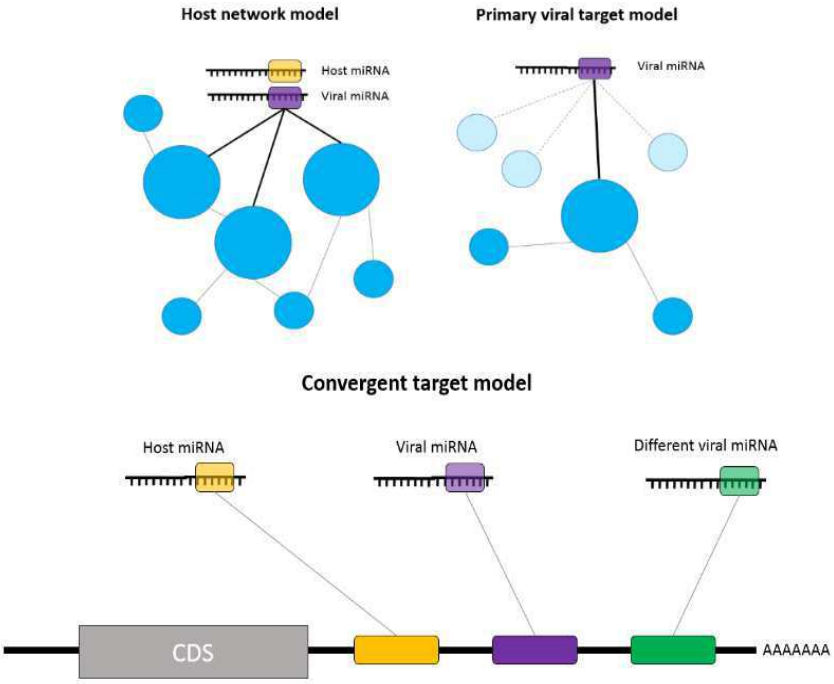


Figure 6. Models of viral miRNAs function.

As some viral proteins, miRNAs have evolved to mimic host effectors. Several viral miRNAs share the seed region with host miRNAs as previously described and it has been demonstrated in Kaposi's associated herpesvirus (KSHV), Marek's disease virus 1 (MDV1) and BLV which viral miRNAs negatively regulate transcripts via the same target docking sites as their equivalent host miRNAs. Viral miRNAs mimicking host miRNAs allow the virus to regulate several regulatory networks, for example inhibit apoptosis. It has been suggested that approximately 15 to 25% of viral miRNAs mimic host miRNAs with identical seed sequences. Based in what

is known until this day about viral miRNAs, although they are often detected during lytic infection, they have shown that their function is more focused in promoting persistent/ latent infections.

With all the data of the known viral miRNAs functions, these functions can be grouped in three categories: i) To prolong longevity of infected cells, ii) To evade the immune response and iii) To regulate host or viral genes to limit the lytic cycle.

Prolonging longevity of infected cells is a clear advantage to viruses that start persistent or latent infections in long-lived cells. A lot of host miRNAs are involved in the cellular homeostasis, and many of them regulate cell death. KSHV, MDV1 and EBV express miRNAs in order to prevent apoptosis by targeting pro-apoptotic host genes. In the case of EBV, its miR-BART5 targets the p53 upregulated modulator of apoptosis (PUMA), a pro- apoptotic host gene and members of the miRNA BART cluster target the Bcl-2-like protein 11 (BIM), another pro- apoptotic protein. Also, EBV BHRF1 miRNA and miR-M3 are involved in avoiding cellular apoptosis. In KSHV infection, different viral miRNAs target the tumor necrosis factor receptor superfamily member 12A (TWEAKR) and caspase 3 (CASP3). Three herpesviruses (HCMV, EBV and KSHV) have also shown to target pro- apoptotic gene bcl-2-associated transcription factor 1 (BclAF1) whit different target sites in BclAF1 mRNA (Choy *et al.*, 2008). Finally, more recently it has been demonstrated that KSHV miR variants processed from pre-miR-K10 inhibit TGF-β signaling by targeting TGF-β type II receptor (TβRII) to promote viral latency and contribute to malignant cellular transformation (Lei *et al.*, 2012). As recently observed, viral miRNAs also are associated with tumorigenesis, not as an advantage if not as a collateral effect when modifying cellular cycle, prevent cell death and avoid immune system. MDV1 and KSHV promote the apparition of lymphomas and both encode miRNAs homologous to miR-155 which misexpression alters lymphopoiesis promoting tumorigenesis (Faraoni *et al.*, 2009). Also, BLV encodes a miR-29 homolog which acts as an oncogene or tumor

suppressor depending on the context and is important to remark that other viruses as EBV, rhesus lymphocryptovirus (RLCV) and Marek's disease virus 2 (MDV2) encode miR-BART1-3p, miR-rL1-6-3p and miR-M21 which have miR-29 seed sequences (Pekarsky and Croce, 2010).

There are several non- structural proteins that act in order to evade immune response and viral miRNAs also can regulate this response. Some viral miRNAs have been associated in preventing the cytotoxic T cell response, natural killer cell response and, as previously described, apoptosis (Sullivan, 2008). Autoregulatory viral miRNAs could indirectly evade immune response by lowering the antigenicity of viral proteins or the overall amount of virus replication. This fact has been observed in the simian vacuolating virus 40 (SV40), a prototypic virus which encodes a miRNA (miR-S1) that is complementary to early viral transcripts and that is supposed to contribute to immune evasion by limiting viral antigen expression down- modulating the production of viral T antigen (TAg) (Grundhoff and Sullivan, 2011). This fact led to think that, as the genomic location of SV40 miR-S1 was conserved in other members of the polyomavirus family: John Cunningham virus (JCV) and polyomavirus BK (BKV), mouse polyomavirus (mPy), and the primate SA12 and Merkel cell virus (MCV), the same behavior would be observed, but until today in has not been demonstrated in an in vivo infection. Another study reported that thrombospondin 1 (THBS1) is down regulated by multiple KSHV miRNAs, including miR-K1, miR-K3-3p, miR-K6-3p, and miR-K11 (Samols *et al.*, 2007).This protein has been reported to function as a chemoattractant, involved in the recruitment of monocytes and T cells to sites of infection (Narizhneva *et al.*, 2005). Down-regulation of THBS1 by KSHV miRNAs may aid KSHV-infected cells in avoiding detection by the host immune system. More recently, the KSHV miR-K12-9 has been demonstrated to target two components of the TLR/IL-1R signaling cascade, IRAK1 and MYD88, to reduce inflammatory cytokine expression (Abend *et al.*, 2012). Another example of a virally regulated immunomodulator is the EBV miR-BHRF1-3 that targets

chemokine CXCL-11, a potent T-cell chemoattractant, which, if it was down regulated might allow infected cells to avoid T-cell detection and destruction (Xia *et al.*, 2008). For last, the HCMV miRNA, miR-UL112-1, was reported to inhibit expression of the MHC class I polypeptide-related sequence B (MICB), a stress-induced cell surface ligand recognized by the NKG2D receptor found primarily on natural killer cells down-regulation of MICB by miR-UL112-1 may allow HCMV to escape NK cells (Umbach and Cullen, 2009).

During latency, infected cells encode direct modulators of the immune response but also evade this response by expressing a limited number of proteins in order to reduce antigenicity. Studies of the KSHV latency system give the best examples of viral miRNAs regulating the latent/ lytic switch. KSHV K12-9-5p and miR-K-12-7-5p target the master lytic switch protein (RTA), miR-K12-1-5p directly targets IκBα which modulates the NF-κB pathway reducing lytic activation. KSHV miR-K12-3-5p also targets the Nuclear Factor I/B (NFIB), an activator of the RTA promoter (Lin *et al.*, 2011).

As described, most cellular targets of viral miRNAs identified are apoptosis regulators and immunomodulators. By interfering with apoptosis or evading host immune responses, viruses are able to prolong the life of the infected cell and maximize their own replication potential. Similarly to host miRNAs, it is necessary to determine which viral miRNAs possess biologically relevant activities. With some exceptions, viral miRNAs are not evolutionary conserved what could imply that they are a site of rapid evolution or maybe a speciation method. It is necessary to have a deeper investigation of those viruses which can express miRNAs to know why, even members of the same subfamilies do and do not encode miRNAs. This investigations will be very useful to better develop therapeutic methods taking into account viral miRNAs, because, as previously reported in the case of miR-122 in HCV, treatment, through the study of miRNAs

is possible and these studies give hope that targeting virus- encoded miRNAs may also be clinically viable (Kincaid and Sullivan, 2012).

1.7. Future and potential uses

miRNAs have a huge potential to act as biomarkers for the diagnosis or the prognosis of several diseases. Therefore, miRNAs can show their ability to act as novel therapeutic tools. Many of the current methods to modulate the levels of under- and over expressed miRNAs are adapted from existing gene therapy and antisense technology.

Recent data from the study of pharmacological modulation of miRNAs or miRNAs families in animal disease models has shown that miRNAs are viable targets for therapeutics. Some of the advantages for this fact reside in the short and often completely conserved sequences of miRNAs which makes them relatively easy to target therapeutically. In addition, miRNAs usually target many mRNAs in several cellular pathways in a disease state, which enables modulation of these networks via therapeutic targeting. Nowadays there are two ways to modulate miRNA activity: restoring the function of a miRNA by using synthetic double stranded miRNAs or viral vector based overexpression and inhibiting the function of a miRNA using chemically modified antimiR oligonucleotides (van Rooij and Kauppinen, 2014).

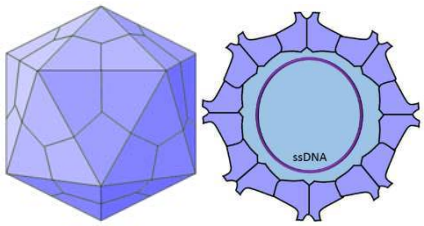
These mechanisms are allowing the first miRNA- based therapeutics (Table 2), many of them are still in a preclinical phase but in some cases as cancer or hepatitis C virus infection the development is in an advanced stage.

Company	miRNA	Disease	Formula
Regulus	Anti-miR-21, anti-miR-122, anti-miR-10b	Alport syndrome Hepatitis C NASH	Single stranded modified oligos

Santaris pharma	Miravirsen (anti-miR-21)	Hepatitis C	LNA-modified antimiR
miRgen	Anti-miR-208	Heart failure	LNA-modified antimiR
miRx	miR-34a	Liver cancer	Oligo+Nov340

Table 2. miRNA based treatments in development. NASH: Nonalcoholic steatohepatitis; LNA: Locked nucleid acid.

1.8. Porcine circovirus type 2 (PCV2)



Porcine circovirus type 2 (PCV2) belongs to the *Circoviridae* family, genus Circovirus, initially discovered in 1998 but retrospective investigations were able to trace PCV2 DNA and antigen back to 1962 (Jacobsen *et al.*, 2009). The viral particle contains a single-strand circular DNA genome of 1768-9 nucleotides (nt), enclosed within a non-enveloped icosahedral protein capsid with a diameter of 16- 18 nm. PCV2 is one of the smallest mammalian viruses encoding 11 potential reading frames, although expression has only been determined from 4 of them (Figure 7) . ORF1 encodes the non-structural replication-associated protein Rep and its truncated variant Rep' (Cheung, 2003) , ORF2 encodes the structural capsid protein Cap (Nawagitgul *et al.*, 2000), ORF3 encodes a non-structural protein with an uncertain function that is not necessary for the infection (Juhan *et al.*, 2010) although, it has been recently proposed to have apoptotic functions (Gao *et al.*, 2014)and for last, ORF 4, another non- structural protein for the infection which has been observed to play a role in suppressing caspase activity and regulating CD4+ and CD8+ T lymphocytes during PCV2 infection (He *et al.*, 2013). Cap and Rep/Rep' carry out the two most elementary

functions of a virus, copying and the successive packaging of the viral genome (Finsterbusch and Mankertz, 2009).

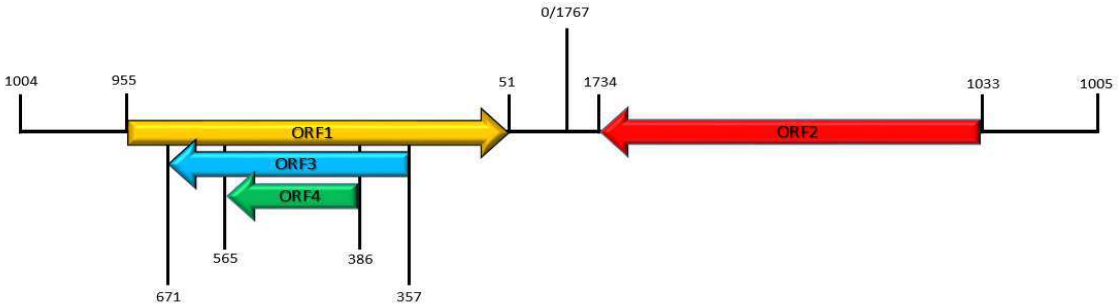


Figure 7. PCV2 genome distribution.

Four clades of the virus have been identified: PCV2a, PCV2b, PCV2c and PCV2d (mPCV2b) (Franzo *et al.*, 2015). By methods of neighbor- joining, maximum-likelihood, pairwise sequence comparison (PASC) and network analysis of the ORF2, classification of the different clades was possible. PCV2b is the most identified class followed by PCV2d (which levels are increasing nowadays), PCV2a and the less encountered one is PCV2c. PCV2d was initially identified in 1999 in samples collected in Switzerland and now is widespread in China and North America since 2012. During 2012–2013, 37% of all investigated PCV2 sequences from US pigs were classified as PCV2d and overall data analysis suggests an ongoing genotype shift from PCV2b towards PCV2d (Xiao *et al.*, 2015).

1.8.1. The disease, symptoms, histopathology and diagnosis

PCV2 is the etiological agent of PCV2-systemic disease (PCV2-SD), formerly known as postweaning multisystemic wasting syndrome, (PMWS) (Segales, 2012), an emerging disease in swine first described in 1991 (Rosell *et al.*, 1999) although recognizable disease outbreaks linked with PCV2 first appeared in the early 1990s and occurred almost simultaneously in France, United States and Canada. In subsequent years, PCV2-associated disease became one of the most important and

economically significant swine disease. PCV2 infection is widespread and its most frequent manifestation is by means of a subclinical infection. PCV2 is ubiquitous in swine livestock worldwide, but it has been demonstrated that PCV2 DNA load in serum is significantly higher in PCV2-SD affected pigs than in healthy pigs, which is considered an indicator of the disease (Fenaux *et al.*, 2000). PCV2-SD has a relatively high fatality rate among 5 to 12-week-old pigs.

To date, PCV2 infection is so widespread in the domestic pig population that there are few no seronegative farms as reflected in epidemiological studies (Larochelle *et al.*, 2003). On the other hand, PMWS prevalence is lower, from 4% to 30%, but with a 60% of morbidity that has been reported in some cases (Segales and Domingo, 2002). PCV2 infection can occur at any time during pig's life, but PMWS has been observed to affect animals from 8 to 16 weeks of age (Sibila *et al.*, 2004).

The first time PCV2 can be detected is at 7 days post infection (dpi), reaching a peak at days 14 -21 pi. At this time, PCV2 can be encountered at many organs but specially in lymphoid tissues. In infected pigs, PCV2 is mostly encountered in the cytoplasm of monocyte/ macrophage lineage cells where it can reaming for long periods of time with no active replication. In addition to these cells, PCV2 high loads can be found at epithelial cells from kidney and the respiratory tract, lymphocytes, hepatocytes, smooth muscle cells, enterocytes, pancreatic acinar and ductular cells (Rosell *et al.*, 1999, Sanchez *et al.*, 2004).

Specific immune response against PCV2 appears between the second and third week and the capability of the pig to show an adequate adaptive immune response against the virus seems as the determinant factor from being infected with PCV2 to develop PMWS. In subclinically infected pigs, the viremia in tissues is low with few alterations of the immune system although it has been observed long- lasting viremia in subclinically infected pigs with the presence of high PCV2 antibody titers (Allan *et al.*, 1999, Resendes *et al.*, 2004). On the other hand, the no presence of PCV2- specific antibodies and a poor humoral response leads to lymphoid lesions

and alterations in the immune response reaching the characteristic immunosuppressive status of PMWS (Meerts *et al.*, 2006). These facts concord with the observation of higher viral loads in sera and points of potential excretion between PMWS affected pigs and subclinically infected ones (Fort *et al.*, 2007). The duration of the viremia in subclinically infected pigs ranges from 5 to 21 weeks with some cases with high virus loads and PCV2 antibody levels (Sibila *et al.*, 2004).

It is not clear how pigs develop PMWS or remain in a subclinical infection status although it has been observed the progression to a clinical disease by inoculating PCV2 jointly with another pathogen as porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV) or *Mycoplasma hyopneumonie* (Allan *et al.*, 1999, Allan *et al.*, 2000, Opriessnig *et al.*, 2004). Also, PCV2 replication has been enhanced jointly with the development of PWMS by using immunostimulating products or vaccination (Krakowka *et al.*, 2001, Opriessnig *et al.*, 2003). All these conditions enhances PVC2 replication leading to the appearance of moderate to severe PMWS.

Genetic constitution of the pigs is also involved in the possibility to develop PMWS, certain breeds and genetic lines have shown to be more susceptible to develop the disease as reported by (Lopez-Soria *et al.*, 2004). Also, it has been identified one gene in chromosome 13, MyRIP, that encodes for a protein which is involved in exocytosis and cellular vesicles. The lack of expression of this gene confers susceptibility to PMWS (Karlskov-Mortensen *et al.*, 2008). But there are also virus-related factors that increases the susceptibility to develop PMWS as the infection with a wild type strain or another one with several cell culture passages, been the first one those which produce higher viremia and PCV2- related lesions. Also the genotype of the virus influences the progression of the infection, been PCV2b viruses which produce more PWMS cases compared with the rest of genotypes (Carman *et al.*, 2006).

The disease from a clinical point of view has been summarized as PCV disease (PCVD) or PCV-associated disease (PCVAD) depending on geographic origin. As previously described, the most recognized clinical PCVD manifestation is the PMWS, a systemic infection involving several organ systems. Clinical signs that are usually observed include enlarged lymph nodes and a decreased weight gain or wasting with many non-specific clinical signs such as dyspnea, a progressive loss of weight, anemia, tachypnea, diarrhea and jaundice. Other clinical signs include coughing, fever, central nervous signs, and sudden death (Harding, 2004). Respiratory disease is another typical clinical sign of the disease that usually affects pigs around 12 to 24 weeks of age. Affected pigs show signs of respiratory disease with fever and varying degrees of sneezing, coughing, nasal discharge, and respiratory distress as well as reduced weight gain. Also it can be observed enteric disease causing ileitis. Porcine dermatitis and nephropathy syndrome is also associated with PCV2 disease in non- vaccinated herds and is characterized by the appearance of skin lesions, fever and lethargy, and is commonly fatal. The last and less important is the reproductive failure that only appears in specific individuals with limited economic loss impact, the report of reproductive failure includes abortions, stillbirths, fetal mummification, and increased pre-weaning mortalities. In the study of PCV2-associated lesions in different organ systems, these are analyzed by in situ hybridization and immunohistochemistry. Microscopic lesions include lymphadenopathy, nephritis, pancreatitis, hepatitis and granulomatous interstitial pneumonia but as a multisystemic disease, affected organ systems and lesions vary depending on the cases (Opriessnig and Langohr, 2013). Typical lesions and related organs are summarized in (Figure 8):

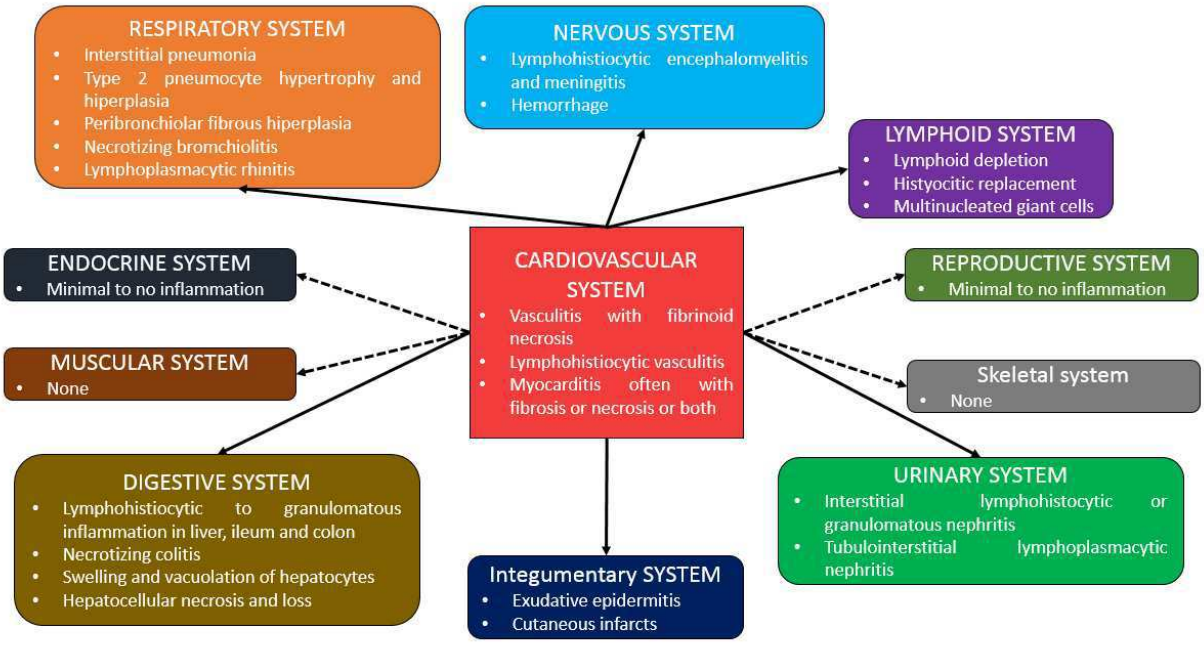


Figure 8. Microscopic lesions commonly encountered with porcine circovirus type 2 infection in different organ systems. Cardiovascular changes can be observed to varying degrees (indicated by regular or dashed arrows) in essentially all other organ systems.

The diagnosis of PMWS is more complex than it seems, the detection of PCV2 in serum or tissues from a pig, and the presence of clinical symptoms that can be related to PMWS are not enough to confirm a diagnosis. The fact that subclinical infections are common and clinical signs can be shared with other infectious and non- infectious diseases, makes diagnosis criteria to be based on specific aspects as the observation of clinical signs compatible with PMWS, referring to growth retardation and wasting, the presence of moderate to severe histopathological lesions with lymphocyte depletion and granulomatous inflammation and the finding of moderate to high quantity of PCV2 genome within lesions.

As the presence of subclinically infected pigs is rather high in PCV2 infected herds, it was necessary to found a way to distinguish between those pigs with a subclinical infection and PMWS cases, for that end (Olvera *et al.*, 2004) suggested that 10^7 DNA copies per ml is a potential threshold to differentiate between these two situations.

With this technique and serological tests as immunoperoxidase monolayer assay (IPMA) or seroneutralization it is possible to confirm o exclude the PMWS diagnosis.

The observation that PMWS affected pigs develop an immunodeficiency led to think that PCV2 could be able to modulate the immune system of the host. Several studies have supported this affirmation as PCV2 alters plasmocitoid dendritic cells (pDC) responsible of the production of IFNα and TNFα inhibiting their production and therefore affecting the maturation of these cells and the myeloid dendritic cells (mDC). As dendritic cells are such important in the innate defenses, PCV2 alters the capacity of the host to develop an appropriate immune response (Vincent *et al.*, 2007).

PMWS affected pigs present extensive lesions in lymphoid tissues accompanied by an important reduction in T and B lymphocytes levels, an increase of macrophage levels and a loss or redistribution of interfollicular dendritic cells (Chianini *et al.*, 2003). Lymphopenia and lymphoid depletion are, therefore, clear consequences of the PMWS affected pigs.

It has been demonstrated that PCV2 infected pigs generate PCV2 specific neutralizing antibodies (NA) which are developed between 10 to 28 dpi. Low titers of NA are related to a higher viral replication and development of PWMS (Meerts *et al.*, 2006) although in some cases it has been observed high levels of NA with high levels of viral titer (Fort *et al.*, 2007). This lead to think that the presence of PCV2 antibodies does not guarantee the total depletion of the virus and that other immune mechanisms are essential to face the disease.

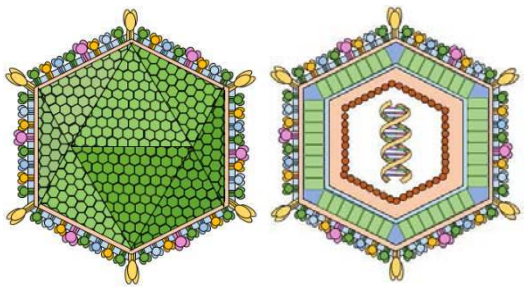
The detection of PVC2 is possible by isolation from serum or tissues of infected animals carrying out a conventional PCR amplifying a region of PCV2 ORF2. In addition, PVC2 antigens can be detected in histological sections by immunofluorescence or histochemical staining with virus- specific antibodies. Another very popular technics are the detection of PCV2- specific antibodies with serologic tests as virus neutralization assay or ELISA. Finally, as there is a strong

correlation between the severity of the histopathological lesions and the levels of PCV2 antigen and/ or nucleic acid it has been proposed different methods for diagnosis in order to avoid euthanasia. Different studies suggest the qPCR at different threshold levels as indicative of the disease diagnosis and detect the levels of PCV2.

1.8.2. Control and vaccination

Before the availability of PCV2 vaccines, the control to avoid PCV2-SD relied on the risk factor involved in the progression of the disease as proposed by (Madec *et al.*, 2001). The most important proposals from Madec includes the reduction of stress, limit pig-to-pig contact, optimize hygienic conditions and good nutrition. Nevertheless the best option to control the disease is the vaccination. Nowadays there are different commercial vaccines being the most used: Ingelvac CircoFlex® by Boehringer Ingelheim (Boehringer Ingelheim GmbH, Binger Strasse, Germany), with PCV2 cap protein as antigen, Circovac® by Merial (Merial Limited, Duluth, United States), a PCV2 inactivated vaccine, Suvaxyn® PCV2 One Dose by Fort Dodge (Fort Dodge Animal Health, Iowa, United States), an inactivated PCV1-2 chimera or Porcilis PCV® by Merck (Merck Sharp & Dohme, Kenilworth, United States) with PCV2 cap protein as antigen. PCV2 vaccination has been proven to be effective both passively acquired (sow vaccination) as actively induced (piglet vaccination).

1.9. African swine fever virus (ASFV)



African swine fever (ASF) is an infectious disease affecting domestic and wild pigs caused by ASF virus (ASFV) belonging to the *Asfarviridae* family, genus Asfivirus. ASF was

first described in 1921 in Kenya (Eustace Montgomery, 1921) spreading rapidly throughout other African countries. The disease is endemic in most of the sub-Saharan Africa and in Sardinia. In 1932 was the first time that the virus was isolated from pigs. ASFV entered Europe for the first time in 1957 in Portugal and in 1960 appeared in Spain from where the disease spread all over Europe. During 1970 to 1980 the disease appeared in different European countries and in America although it was eradicated in all these places but Sardinia where the disease is endemic (Giammarioli *et al.*, 2011). ASF remained in the continent until its eradication from Spain and Portugal in 1995. In 2007 the virus re-entered Europe through Georgia and since then it has spread to East-Europe, becoming a real threat for all Europe. Confirming the menace, four countries from the EU declared in 2014 some ASF outbreaks which number has increased in 2015. The main route for the entrance of the virus in disease- free countries has been and nowadays is (as in Georgia's case), the animal feeding with food leftovers and contaminated meat products with infectious virus (as it is highly resistant to physical and chemical agents inactivation) from countries with ASF (Plowright and Parker, 1967).

ASFV is an icosahedral, enveloped, dsDNA virus ranging from 170 to 193 kpb (de Villiers *et al.*, 2010). The viral particle, with a diameter of 200 nm, has three concentric wraps with an additional external wrap obtained when coming out by gemmation from the infected cell. The hexagonal capsid includes an electron-dense 80nm nucleus wrapped in a lipid envelope. Have been identified between 150 and 175 ORFs but half of them do not have any known function. Sixteen different genomes have been identified and fully sequenced from pigs, wild boars and ticks and 22 genotypes have been described. All of them have in their ends terminal inverted repeats (TIRs) and the multigenic family members (MGF) a very variable region between ASFV isolates (Yanez *et al.*, 1995) while the central region of the genome is very conserved (Figure 9). The MGFs are adjacent to the TIRs and there are at least 5 MGFs (MGF360, MGF530, MGF10, MGF300, MGF505). As these are the places of higher differences between the isolates, it could indicate that these regions are

related to the generation of antigenic variability, a possible evasion mechanism of the virus from the immune system. Thirty four structural proteins and more than 100 infection proteins have been identified in virus-infected macrophages and at least 20 are immunogenic and induce antibodies in natural infection. Some of these proteins are highly immunogenic as p72, p54 and p12 (involved in virus adhesion to the cell) and p32 (involved in virus entry). The virus infects immune system cells: monocytes and macrophages. In addition, it has been observed viral replication in endothelial cells, hepatocytes, renal tubular epithelial cells and neutrophils.

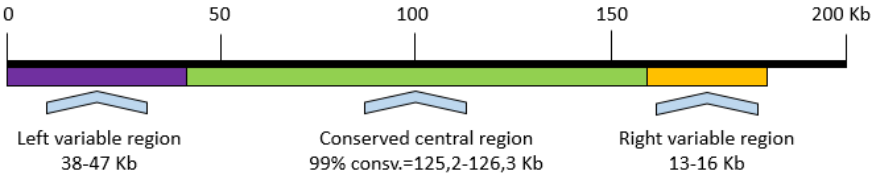


Figure 9. Location of variable and conserved regions in ASFV genome.

1.9.1. The disease, symptoms, histopathology and diagnosis

ASFV does not induce neutralizing antibodies and this is the reason of non serotype classification. ASFV infection produces an enormous specific antibody response, which is easily detected by laboratory diagnosis techniques from day seven post-infection. Specific antibodies remain for a long time, up to years, and are partly responsible for delaying the appearance of clinical signs and reducing viremia levels. However, these antibodies are not capable of completely neutralize ASFV. It has been demonstrated the presence of a non- neutralizable virus fraction (~10%) which could help to explain the incapability of the antibodies to fully protect against the virus or the persistent presence in chronic ASFV infected pigs (Carrillo *et al.*, 1994). Cellular immunity also plays an important role in immune protection against ASFV infection, specifically, cell activity of $CD8^+$ lymphocytes and natural killer cells (NK) (Oura *et al.*, 2005). Different studies have related NK cells activation with protection,

while animals with an hyper- activation of NK cells remained asymptomatic, other animals with less activation developed chronic lesions of ASF. Although it is not well understood how these NK cells act against the infection, they have an important role in the innate immunological response by secreting IFNα and IFNγ as they can inhibit viral replication *in vitro* (Esparza *et al.*, 1988, Leitao *et al.*, 2001). In other studies, it was observed the existence of specific cytotoxic T lymphocytes (CTLs) after the infection which recognized two structural proteins p32 and p72 (Alonso *et al.*, 1997, Leitao *et al.*, 2001). It has also been described the induction of lymphocytes in vitro when stimulated with ASFV and the secretion of IL-2 parallelly to the development of lymphokine activated killer cells (LAK) capable of lising infected macrophages. But the importance of the CTLs was perfectly observed *in vivo* by deletion of T-$CD8^+$ cells demonstrating the huge importance of these lymphocytes in the protection against ASFV (Scholl *et al.*, 1989, Oura *et al.*, 2005).

ASF associated symptoms are very variable depending on the isolate virulence, route, dose of exposure and the breed and physical condition of the pig. European domestic pigs and wild boar are equally susceptible to ASFV infection suffering and showing the clinical signs of the disease, while wild African pigs (*Phacochoerus*) do not present symptomatic forms. Isolates from African regions usually induce acute or peracute disease while isolates from European regions can induce acute/sub-acute, chronic or unapparent clinical picture. The disease is characterized by fever, hemorrhages and high mortality that leads to huge economical losses in affected areas. (Blome *et al.*, 2012, Guinat *et al.*, 2014). During the ASF acute forms, pigs usually present high temperature (40-42ºC), depression, lack of appetite, and sometimes respiratory disorders. In some cases there may be nasal hemorrhages, constipation and vomiting, and to a lesser extent, diarrhea with or without hemorrhagic discharge. Abortion frequently occurs in pregnant females. In acute cases, the disease leads to death in 90 to 100% of the affected animals between 4 - 10 dpi.

During the first stages of the infection, virus replication occurs in macrophages that allows the posterior dissemination of the virus to other cell types causing the typical hemorrhagic pathology of the disease. The virus causes damage in vascular endothelial cells what increases vascular permeability and alters homeostasis; this, at the end causes generalized hemorrhages (Gomez-Villamandos *et al.*, 1995, Oura *et al.*, 1998). These hemorrhages are not only caused because of the apoptosis of infected endothelial cells, it is thought that the most important cause is the fact that infected macrophages release different factors as TNFα, IL-1α and IL-6 (Gomez del Moral *et al.*, 1999, Carrasco *et al.*, 2002). Apart from the hemorrhages, lymphoid tissues destruction is other of the most typical lesions derived from the apoptosis of infected cells and the apoptosis of non- infected lymphocytes that contributes to the hemorrhagic pathology. Apoptosis of these lymphocytes is caused due to the liberation from infected and/ or activated macrophages of different soluble factors (Oura *et al.*, 1998, Salguero *et al.*, 2005).

Lesions are generally characterized by splenic lesions and hemorrhages in skin, lymph nodes and affected organs. Skin lesions are very common in ASF, with the appearance of hyperemia, petechias and cyanosis in extremities, ears, chest, abdomen and perineum. Other alterations caused by the virus include lymphopenia and thrombocytopenia.

Most used technics for ASFV detection and isolation are haemadsorption, direct immunofluorescence (DIF) and PCR (conventional and real- time). The serological techniques for detecting specific antibodies against ASFV are indirect immunofluorescence (IIF), ELISA and immunoblotting.

ASF transmission is produced by direct contact between infected animals and susceptible ones. Also, there are indirect transmission routes through contaminated pork, fomites, vehicles, people and biological vectors. Biological vector transmission through *Ornithodoros* spp. ticks is especially important. The first association of ASFV with a tick was made in 1963 by (Sanchez-Botija, 1963). The virus entry normally

happens through oronasal route, although there are other routes as intramuscular, subcutaneous and intravenous due to tick bite. The incubation period varies from 3 to 21 days depending on the isolate and the route of exposure. Primary replication happens in monocytes and macrophages of the lymph nodes nearest the entry site. Monocytes and macrophages from tonsil and mandibular lymph nodes are the first affected if the entry of the virus is oral. From these places, the virus spreads through blood circulation associated to red cell membranes or through lymphatic route. Viremia usually begins 2-3 to 8 dpi and, due to the absence of neutralizing antibodies, persists for a long time, even months. Second replication occurs when ASFV reaches the different organs, for example the lymph nodes, bone marrow, spleen, kidneys, lungs and liver (Sanchez-Vizcaino *et al.*, 2014). The main routes for the elimination of ASFV making susceptible to transmission to other animals are nasal secretions, saliva, feces, urine, conjunctival and genital exudate and bleeding wounds.

1.9.2 Disease control

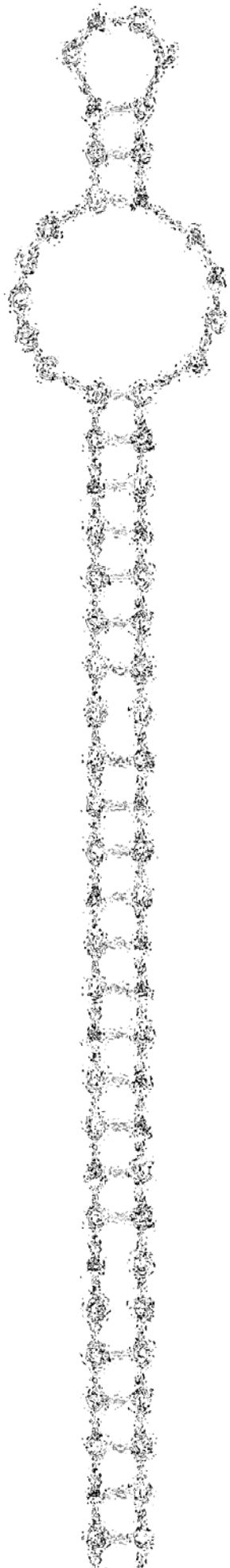
No commercial vaccine is available in spite of the efforts carried out to achieve it. Engineered live attenuated strain and cell culture adapted virus have been the two approaches more extended in order to obtain a candidate vaccine. Cell culture adaptation generally leads to attenuation, but the genomic changes responsible of the phenotypic modification and the mechanisms involved are not well understood.

All the attempts for vaccination with inactivated viruses by heat or chemical agents didn't give protective immunity in the animals, but the use of attenuated viruses have demonstrated the capability to obtain protection by the deletion of determinate genes related with the virulence. The use of attenuated viruses gives the possibility to obtain an effective vaccine against ASFV as recently discovered by (Paula-Monteagudo *et al.*, 2016) where the deletion of CD2v gene from a virulent

strain of ASFV causes its attenuation in vivo, which could be a good possibility for the vaccination against homologous and heterologous viruses. On the other hand there have been studies of vaccination with the hemagglutinin (HA) of the virus (which has huge homology with the leucocitary antigen CD2v) promoting the antibody and cellular response, but again, not reaching the total protection (Ruiz Gonzalvo *et al.*, 1986, Ruiz Gonzalvo *et al.*, 1986).

Lately it has been used genomic libraries of ASFV in order to characterize potential antigens that could help to obtain a successful vaccine against ASF (Kollnberger *et al.*, 2002).

The prevention for those free-of-virus regions is based on the application of *risk analysis* according to scientific methodology approved by the World Organisation for Animal Health (OIE).



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OBJECTIVES

miRNAs have become very popular molecules in the study of current landscape of diseases both in the knowledge of the course of the disease as their diagnosis and possible treatments. In this thesis we have investigated the involvement of miRNAs, both viral and host miRNAs, in two viral infections, one with PCV2 as an example of causative agent of an endemic disease and a second infection with ASFV as an example of causative agent of an exotic disease. Therefore, based on this premise, the objectives for this thesis were:

1- Investigate the expression pattern of miRNAs in experimentally PCV2 infected and non- infected pigs and analyze the biological functions of the differentially expressed miRNAs.

2- Evaluate the capability of PCV2 to express viral miRNAs.

3- Study the expression pattern of miRNAs in two different conditions in an experimental ASFV infection: (i) Animals inoculated with virulent strain E75 at 3 and 7 days post infection and (ii) Animals inoculated with E75 virulent strain and animals inoculated with E75CV1 attenuated strain at 3 days post infection and finally analyze the biological function of the differentially expressed miRNAs.

4- Explore the capability of ASFV to encode viral miRNAs.

3

MATERIALS AND METHODS

3.1. Viruses

For the experimental PCV2 infection, isolate Sp-10-4-54-13 from genotype b was used (accession number GU049342) (Fort *et al.*, 2010).

For the experimental ASFV, three different viruses were used. First, the España- 75 (E75) virulent strain (accession number FN557520), a very infectious and virulent strain with almost a 100% of mortality classified within p72 genotype I. This virus was isolate in an outbreak in Lerida in 1975 (de Villiers *et al.*, 2010). Secondly the E75CV1, an attenuated strain from E75 strain obtained by cellular passage in a stable line of monkey kidney fibroblasts, kidney of an adult male African green monkey (*Cercopithecus aethiops*) (Ruiz-Gonzalvo F, 1983). Finally, the Badajoz-71 virulent strain (Ba71), which has been adapted to grow in vero cells (Enjuanes *et al.*, 1976). This virus was isolated in Badajoz in 1971, it is highly virulent and it was the first ASF virus which genome was fully sequenced (Yanez *et al.*, 1995).

3.1. viral miRNAs prediction

For the analysis of the potential capability of both viruses to encode viral miRNAs, Vmir software was used (Grundhoff, 2011). PCV2 Sp-10-4-54-13, ASFV E75, ASFV Ba71 and ASFV Ba71V genomic sequences were introduced and the threshold was adjusted to a score equal or superior to 100. Resulting potential hairpins were annoted for sequence comparison with highthroughput sequencing results.

3.2. Animal infection

For the PCV2 *in vivo* infection , six 6-week-old Landrace x Large White pigs were used. Four of them were intranasally inoculated with a total dose of 7x104.8TCID50 of PCV2 isolate Sp-10-7-54-13, while two control pigs received PBS by the same

route. At 21 days post-inoculation (dpi), animals were euthanized. Samples from eight tissues: spleen, inguinal lymph node, kidney, tonsil, thymus, mediastinal lymph node (MLN), lung and mesenterical lymph node, were collected in duplicate and immediately frozen in liquid nitrogen and stored at -80ºC. A third sample was collected in formalin for histopathological studies. Tissue sections were stained with haematoxylin and eosin, besides, and in situ hybridization (ISH) technique was carried out in order to detect viral genome.

For the ASFV *in vivo* infection, eight 8-week-old Landrace x Large White pigs were used. Two pigs were intramuscularly inoculated with 10^4 HAU of the E75 virulent strain (accession number "FN557520") (Lacasta *et al.*, 2015). Five pigs were inoculated with same amount (10^4 HAU) of the E75CV1; an attenuated strain that resulted from adapting E75 to grow in the CV1 cell line. One additional pig was euthanatized at day 0 as healthy pig. One animal inoculated with the virulent strain and two with the attenuated strain were necropsied at 3 days post-infection (dpi). At 7 dpi, one animal inoculated with the virulent strain was necropsied. Three pigs inoculated with the attenuated strain were maintained till 31 dpi with no clinical signs. One pig was euthanized at 31 dpi and two pigs were re-inoculated with the Ba71 virulent strain and necropsied at 38 dpi.

All animal experiments were performed at CReSA facilities, and all procedures were carried out according to the guidelines of the institutional animal ethics committee of UAB, preserving the Spanish and European animal experimentation ethics law.

Submandibular lymph node and spleen samples were collected, firstly, in formalin for histopathological studies where tissue sections were stained with haematoxylin and eosin, in addition, an in situ hybridization (ISH) was performed to detect viral genome.

3.3. Tissue macerates

Samples from eight tissues (spleen, inguinal lymph node, kidney, tonsil, thymus, mediastinal lymph node (MLN), lung and mesenterical lymph node) collected in duplicate and immediately frozen in liquid nitrogen and stored at -80ºC from the in vivo infection with PCV2 were extracted and maintained in dry ice to avoid RNA degradation while the macerate of the samples was carried out. About 50 mg of each tissue samples were isolated from the total portion with the help of scalpel and scissors and introduced in 1.5 ml RNAse free tubes. Then, 0.5 ml of Trizol® (Thermo Fisher Scientific, Massachusetts, United States) were added and the tissues samples were homogenated with RNAse free pestles. Tubes were then full-filled to their max volume (1.5 ml) with Trizol® in order to be moved from the Biosafety Level 3 (BSL3) facilities and immediately conserved in dry ice until the subsequent storage at -80ºC. The surplus of tissue was also stored at -80ºC.

Samples from the two tissues obtained from the in vivo infection with ASFV (submandibular lymph node and spleen) and conserved at -80ºC, received the same treatment as the PCV2 infection samples for the macerates and extraction from BSL3.

3.4. Total RNA isolation

The total RNA extraction was done following the same methodology for both viruses. Samples were extracted from -80ºC storage and incubated for 5 minutes at room temperature. 0.2 ml of chloroform was added and the tubes vigorously shaken by hand. After 2-3 minutes at room temperature, samples were centrifuged at 12000 x g for 5 minutes at 4ºC. After centrifugation 3 phases appear in the tubes (RNA layer, protein layer and DNA layer). The upper aqueous phase corresponds to the total RNA fraction that was removed and pipetted to another tube, the rest was stored for further DNA extractions. About 5 to 10 µg of glycogen (acts as a carrier)

and 0.5 mL 100% isopropanol were added, the mixture was incubated at room temperature for 10 minutes, then was centrifuged at 12000 x g for 10 minutes at 4ºC. The supernatant was removed and the pellet washed with 1ml 75% ethanol, then vortexed and centrifuged at 7500 x g for 5 minutes at 4ºC. The supernatant was discarded and the pellet dried. 30 µl of RNAse free water were then added and the samples stored at -80ºC until next step.

3.5. DNA isolation

0.3 mL of 100% were added and tubes were incubated for 3 minutes at room temperature. Samples were centrifuged at 2000 x g for 5 minutes at 4ºC. The supernatant was discarded and DNA pellet was washed with 1 mL of 0.1M sodium citrate in 10% ethanol (pH 8.5). After an incubation for 30 minutes at room temperature samples were centrifuged at 2000 x g for 5 minutes at 4ºC. The supernatant was discarded and the pellet washed again. 2 mL 75% ethanol was added and incubated for 20 minutes at room temperature followed by another centrifugation at 2000 x g for 5 minutes at 4ºC. The supernatant was dircarded and the pellet was dried for 10 minutes. The pellet was resuspended with 0.3 mL of 8mM NaOH and centrifuged at 12000 x g for 10 minutes at 4ºC. The supernatant containing the DNA was transferred to new tubes.

3.6. Real- time PCR

From PCV2 infected tissues, stored at -80ºC in BSL3 facilities, a quantitative PCR (qPCR) was performed from DNA extracted from the eight tissues of all animals to quantify PCV2 load. Probe and primers used for the procedure were those designed by (Olvera *et al.*, 2004). The mix for the qPCR contained 900 nM primers PCV2F (5' CCAGGAGGGCGTTGTGACT 3') and PCV2R (5' CGCTACCGTTGGAGAAGGAA 3'), 150

µM probe, 0.4 µL IC kit, 12.5 µL Taqman Universal Master Mix and 2.5 µL template. Nanopure autoclaved water was added to final volume of 25 µL. The conditions for the amplification were 10 min at 95ºC, 2 min at 50ºC and 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Triplicates of each sample were used for the qPCR.

ASFV qPCR of the two stored tissues was carried out in order to quantify viral load. SYBR Green qPCR was the selected procedure with ROCK1 primer (5' CCTCGGCGAGCGCTTTATCAC 3') as forward primer and ROCK2 (5' GGAAACTCATTCACCAAATCCTT 3') as the reverse one that were designed to target a highly conserved region within the p72 ORF as described by (Zsak *et al.*, 2005). The mix for the qPCR contained 480 nM each primer, 12.5 µl Power SYBR® Green PCR Master Mix and 2.5 µl template. Nanopure autoclaved water was added to final volume of 25 µl. The conditions for the amplification were 10 min at 95ºC, 2 min at 50ºC and 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Triplicates of each sample were used for the qPCR. Triplicates of each sample were used for the qPCR.

3.7. RNA quality and integrity assay

The quality and integrity of both total RNA and small RNA from the samples were analyzed using the Agilent 2100 Bioanalyzer system (Agilent technologies, Santa Clara, United States). Each RNA was analyzed with its specific kit, for total RNA the Agilent RNA 6000 Nano Kit, and for small RNA, the Agilent Small RNA Kit.

For total RNA and small RNA assays, chip gel was be prepared. The gel matrix provided was centrifuged at 10000 x g in an spin tube for 15 minutes at room temperature to be filtered. Then, 1 µl of dye was added to 65 µl of filtered gel for total RNA and 2 µl of dye in 40 µl of filtered gel for small RNA. The mix was centrifuged at 13000 x g for 10 minutes. In both cases, 9 µl of gel mix were loaded into its well in the chip, and with the priming station, with the plunger at 1 mL and holding it down for 60 seconds, the gel was loaded into the chip. Then 9 µL of gel-

dye and RNA conditioning solution were loaded into its wells and 5 µl of RNA marker was also loaded in its position. For last, 1 µL of ladder and the samples, previously incubated at 55-60ºC to avoid double stranded formations, were loaded into the rest of empty wells in the chip. The chip was vortexed at 1400 rpm in the IKA vortexer for 1 minute. When the software was ready and the chip was in the analyzer, the program was run and the resulting data for both total RNA and small RNA could be analyzed.

All these results are accompanied by RNA area, RNA concentration, rRNA ratios and the RNA Integrity Number (RIN), this last value gives an idea of the integrity of the RNA with a numeric classification from 1 to 10, been approximately a RIN 10 for intact RNA, RIN 5 for partially degraded RNA and RIN 3 for degraded RNA.

All these data is especially useful to know the quality, quantity and integrity of all your RNA samples prior to the creation of the small RNA libraries (Schroeder *et al.*, 2006).

3.8. Small RNA library creation

3.8.1. Polyacrylamide gel electrophoresis (PAGE)

The enrichment of the small RNA fraction from total RNA is the first step in the library creation. For this procedure, the small RNA fraction was recovered from PAGE (Bio- Rad, California, United States). All components, prior to start, were cleaned with RNAse ZAP (Thermo Fisher Scientific, Massachusetts, United States) and rinsed with RNAse free water, and the glasses, in the contact-with the sample-side, with acetone and 100% ethanol. Then, the 12% denaturing (7M Urea) acrylamide gel was prepared in a 50 mL falcon tube with 3.60 gr urea (Sigma-Aldrich, St. Louis, United States), 750 µL TBE 10X (Thermo Fisher Scientific, Massachusetts, United States), 2 mL RNAse free water (Thermo Fisher Scientific,

Massachusetts, United States), 2.25 mL of acrylamide solution 40% (Sigma- Aldrich, St. Louis, United States), 7.5 µL of Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, St. Louis, United States) and 32.5 µL of ammonium persulfate (APS) (Sigma-Aldrich, St. Louis, United States). The mixture was loaded into the support with the combs and was left polymerizing for 1 hour. With the bucket filled with TBE 1X, the gel was filled with Gel loading buffer II (Thermo Fisher Scientific, Massachusetts, United States) for a pre- run at 200V. Then, the sample was prepared with 25 µL of the RNA and 25 µL of gel loading buffer II and at the same time, for the ladder,6 µL of spike with 6 µL of gel loading buffer II. Both preparations were heated at 70ºC for 10 minutes, and then, loaded into the gel and run at 200V.

Finished the run, the gel was revealed with 27 mL water RNAse free, 3 mL TBE 1X and 3 µL GelStar (Lonza Inc. & Lonza America Inc., New Jersey, United States) for 10 minutes into darkness and visualized under UV light. The bands corresponding with the size of the spike were cut with a scalpel. Each band for each sample were crushed with RNAse free pestles in 200 µL RNAse free water and incubated at 70ºC for 10 minutes. The samples were placed into Performa® DTR Gel Filtration Cartridges (Edge BioSystems, Maryland, United States) and centrifuged for 3 minutes at 2900 rpm and 4ºC twice for purification. Each sample then was preserved with 1.5 µL glycogen (20 mg/ mL), 25 µL sodium acetate (3M) and 900 µL cold 100% ethanol. Samples were stored at -20ºC or -80ºC to precipitate.

3.8.2. 3' ligation

After precipitation, samples were centrifuged at 13000 rpm for 15 minutes at 4ºC. The supernatant was discarded, the pellet dried, and then, resuspended in 6.5 µL RNAse free water. The small RNA was ligated with 0.5 µL 3' linker (50µM) (5' rAppCTGTAGGCACCATCAAT/3ddC/ 3'), with 1 µL T4 RNA ligase (1U/µL) (Thermo Fisher Scientific, Massachusetts, United States) in absence of ATP, 1 µL bovine serum

albumin (20mg/ mL) (Thermo Fisher Scientific, Massachusetts, United States), 1 µL 10X ligation buffer (Thermo Fisher Scientific, Massachusetts, United States) and 6.5 µL sample to a total volume of 10 µL. The mixture was incubated at 37ºC for 2 hours. After the incubation, 90 µL RNAse free water, 1.5 µL glycogen, 10 µL sodium acetate and 250 µL cold 100% ethanol were added for a 30 minutes incubation at -80ºC. Then, samples were centrifuged at 13000 rpm for 15 minutes at 4ºC in order to discard the supernatant and dry the pellet which was resuspended in 5 µL RNAse free water. All samples were heated at 70ºC for 10 minutes in order to denature prior to be analyzed by PAGE as previously described. Gel was stained and the 3' ligation products purified as in the previous step.

3.8.3. 5' ligation

Samples coming from precipitation at -80ºC were centrifuged at 13000 rpm for 15 minutes at 4ºC, supernatant was discarded and the pellet was resuspended in 7.5 µL RNAse free water. For 5' ligation reaction, 0.5 µL 5' linker (50 µM) (5' TGGAATrUrCrUrCrGrGrGrCrArCrCrArArGrGrU 3'), 1 µL T4 RNA ligase (Thermo Fisher Scientific, Massachusetts, United States), 1 µL 10X Buffer ligation (Thermo Fisher Scientific, Massachusetts, United States) and 7.5 µL sample were added. Reaction was incubated for 2 hours at 37ºC and 90 µL RNAse free water was added. Glycogen, sodium acetate and cold 100% ethanol were incorporated prior to freezing at -80ºC.

3.8.4. RT

Samples extracted from freezing were centrifuged at 13000 rpm for 15 minutes at 4ºC, supernatant was discarded and the pellet was dried prior to resuspension with 11 µL RNAse free water. In a first step, 1µL dNTPs (10 mM each), 1 µL RT primer (10

μM) (5'-GATTGATGGTGCCTACAG -3') and 11 μL sample were prepared for a 5 minutes at 65ºC incubation. To this 13 μL preparation, 4 μL 5X RT buffer, 1 μL 1,4-Dithiothreitol (DTT) (1mM), 1 μL RNA inhibitor (20u/μL) and 1 μL SuperScript® III Reverse Transcriptase (200u/μL) (Thermo Fisher Scientific, Massachusetts, United States) were added to a final volume of 20 μL. RT reaction was carried out at 55ºC for 90 minutes and 70ºC for 15 minutes.

3.8.5. PCR

PCR was performed from the obtained cDNA using Expand™ High Fidelity PCR System. Reaction was prepared with 10 μL 10X Expand High Fidelity Buffer (15 mM $MgCl_2$), 1 μL dNTPs (10 μM each), 1 μL common Primer B (10 μM), 0.5 Expand High Fidelity Enzyme (3.5 u/μL) and 32.5 μL water to a total volume of 45 μL to which 1μL sample- specific primer A and 4 μL cDNA were added. PCR conditions were 94ºC for 3 min, 25 cycles at 94ºC for 30 s, 57ºC for 45 s and 72ºC for 1 min followed by 71ºC for 7 min. PCR products were validated in a 3.5% agarose gel. PCR prodcuts were purified from agarose gels.

Different primers were used in each study depending on the Highthroughput sequencing method used, GS FLX+ System (Roche Diagnostics, Basel, Switzerland) for PCV2 study and Ion Torrent (Thermo Fisher Scientific, Massachusetts, United States) for ASFV one. Primers A were sample- specific, each primer had one different label in order to identify each sample after sequencing but also contained the specific sequence for each sequencing method. Primers B were common for all the samples in the study and also contained the identifier for the sequencing (Figure 1).

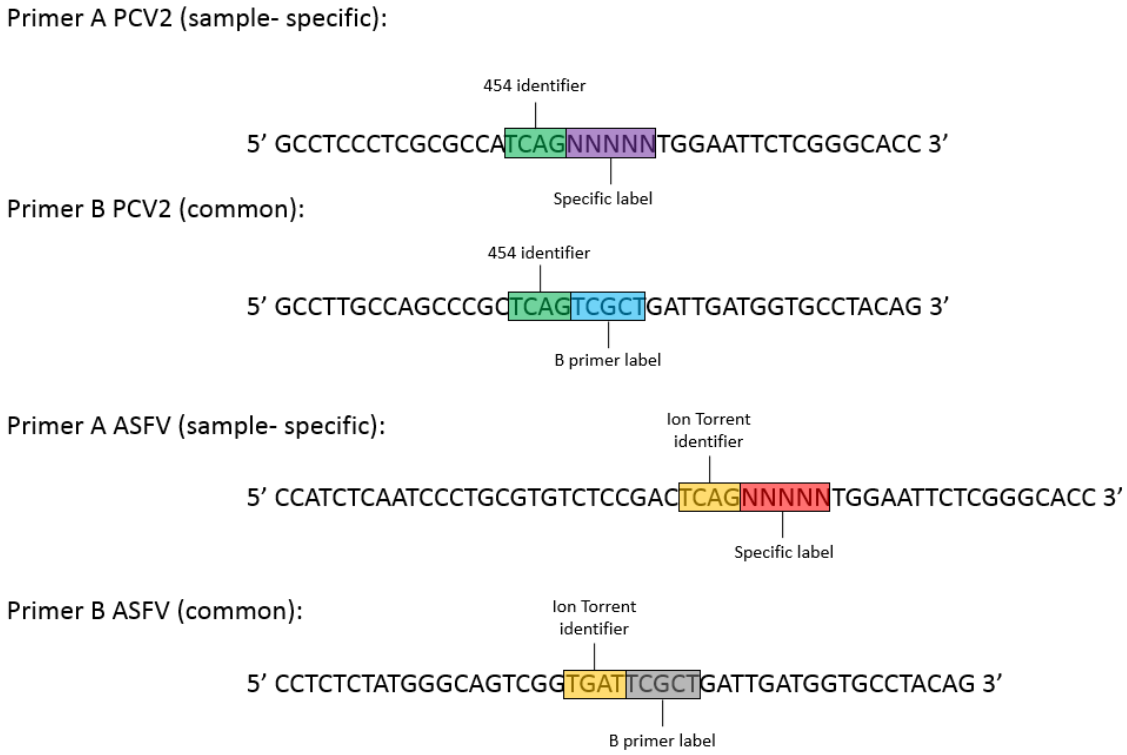
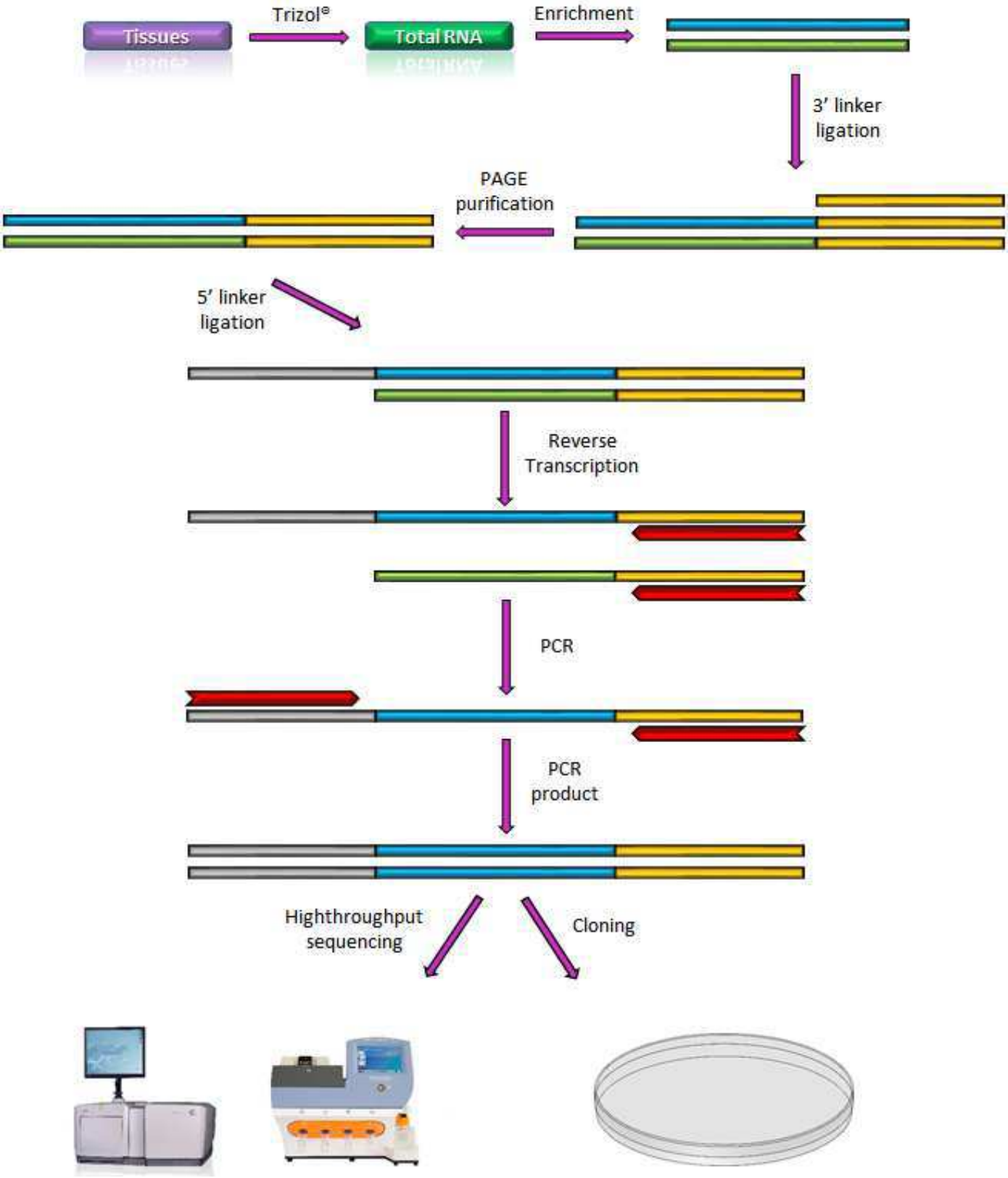


Figure 1. PCR primers structure with 454/ Ion torrent identifiers and specific labels highlighted.

3.8.6 Library creation summary



3.8.6. PCR product purification

The PCR products obtained were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Wisconsin, united States) following the protocol offered by the manufacturer.

All samples were loaded in a 3% agarose gel to be checked and quantified. The quantification was done by band density, for this end, PCR product bands were compared with DNA Molecular Weight Marker VI (Sigma- Aldrich, St. Louis, United States). Another quantification was also done using QubitTM fluorometer, Quant-ITTM (InvitrogenTM, Carlsbad,178 USA), prepared to a $1{\cdot}10^{11}$ DNA molecules/ µL and equimolecular pooled.

3.9. Cloning

3.9.1. Plasmid ligation

Prior to highthroughput sequencing, small RNAs libraries were cloned in order to check the correct development of the procedure. For the cloning step, pGEM®-T Easy Vector System (Promega Corporation, Wisconsin, United States) was used according with the manufacturer protocol.

3.9.2. Plasmid transformation and plating

For plasmid transformation One Shot® TOP10 Chemically Competent E. coli (Thermo Fisher Scientific, Massachusetts, United States) was used. Positive colonies were selected in plates with LB medium, ampicillin, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and X- Gal.

3.9.3. Colonies PCR and LB growth

Those colonies that were correctly transformed (white ones) were collected in order to carry out a colonies PCR and LB Broth medium growth. The colonies PCR was done using AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific, Massachusetts, United States). To the extracted colonies with a pipette tip, 2.5 µL GeneAmp 10X PCR buffer, 1.5 µL $MgCl_2$ (25mM), 0.5 µL dNTPs (10 µM each), 0.5 µL primers mir4 (5' TGGAATTCTCGGGCACC 3') and mir5 (5' GATTGATGGTGCCTACAG 3') (10 µM each), 0.25 µL AmpliTaq Gold (5u/ µL) and water to a total volume of 25 µL were added. PCR conditions were 7 minutes at 94ºC, 30 cycles of 30 seconds at 94ºC, 45 seconds at 55ºC and 1 minute at 72ºC followed by 7 minutes at 72ºC. PCR products were loaded in a 3, 5% agarose gel. PCR products were purified using the QIAquick PCR Purification Kit (Quiagen, Hilden, Germany) following the protocol offered by the manufacturer.

At the same time, those colonies correctly transformed were collected in tubes with 1 mL LB Broth with ampicillin (Sigma- Aldrich, St. Louis, United States). Tubes were incubated overnight in the orbital shaker at 37ºC and 225 rpm.

Plasmid DNA was extracted with the Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Wisconsin, United States). All the procedure was done following the protocol offered by the manufacturer

3.9.4. Sanger sequencing

For the sequencing reaction BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, United States) was used but with changes in their protocol. For the reaction, 0.5 µL Terminator ready reaction mix, 1.5 µL 5X BigDye sequencing buffer, 0.35 µL primer pgemRev (5' TGTGGAATTGTGAGCGGATA 3') or pgemFor (5' GTTTTCCCAGTCACGACGTT 3') (10 µM each), 2 µL PCR product and water to a 10 µL final volume were added. Used

PCR conditions were 1 minute at 96ºC, 25 cycles of 10 seconds at 96ºC, 5 seconds at 50ºC and 4 minutes at 60ºC.

For precipitation 1 µL ethylenediaminetetraacetic acid (EDTA) (125 mM), 1 µL sodium acetate (3M) and 25 µL 100% ethanol were mixed and added to the 10 µL of PCR product. Everything was incubated for 15 minutes at room temperature and then, centrifuged for 10 minutes at 4ºC and 13000 rpm. Supernatant was discarded and 35 µL 100% ethanol added and vortexed prior to centrifugation at 4ºC for 15 minutes and 13000 rpm. Supernatant was discarded again and the pellet, when dried, was resuspended with 10 µL formamide (≥ 99.5%) (Sigma- Aldrich, St. Louis, United States) and incubated for 10 minutes. The plate with the samples was sequenced with the ABI PRISM® 3700 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, United States).

3.9.5. Sanger sequencing results analysis

With the raw data from the ABI PRISM® 3700 Genetic Analyzer, sequences were cleaned removing the adaptors located at both sides from the sequence of interest. When we had our putative miRNA, sequences were blasted against miRBase (The microRNA database) and National Center for Biotechnology Information (NCBI) Blast Database to identify if they corresponded to microRNAs, pig sequences or viral sequences. All this processing allowed us to do a pre- analysis from our samples prior to star with the highthroughput sequencing.

3.10. Highthroughput sequencing

For PCV2 study, the sequencing was done using the GS FLX+ System (Roche Diagnostics, Basel, Switzerland) a pyrosequencing method where light emission is detected by a charge coupled device (CCD) camera, the registered emission

depends on each inserted nucleotide. For the ASFV study, the sequencing method was Ion Torrent (Thermo Fisher Scientific, Massachusetts, United States) which works with an electrochemical detection system called ion-sensitive field-effect transistors (ISFET) which can detect ions as they are released by DNA polymerase during sequencing by DNA synthesis.

3.11. *In silico* analysis

3.11.1. Highthroughput sequencing data manipulation

Raw data from the sequencing was manipulated in order to obtain suitable sequences to keep working. In first place, adaptors were recognized and then the primers were eliminated from the reads in order to have clean sequences. From all these sequences only were maintained those between 15 and 29 nucleotides.

3.11.2. miRNAs database blast

Normally, all discovered miRNAs by the different groups in this study field, updates their new sequences to miRBase, the microRNA database. MiRBase, in its version 21, contains 28645 miRNAs entries. Specifically, there are 411 identified mature miRNAs in Sus scrofa and 502 viral miRNAs (Griffiths-Jones, 2006, Griffiths-Jones, 2010).

After the cropping phase and the selection of those sequences ranging between 15 and 29 nucleotides, these sequences were compared to all available miRNA sequences (miRBase v20) using local Blast. Parameters were set to 100% identity and up to 4 mismatches allowed at the end of the sequences to solve the miRNA variability on 3' and 5' ends. All this data was normalized and compared, in the case of PCV2, between infected and non- infected pigs and in the case of ASFV between infected pigs with E75 virulent strain at different time points (3 and 7 dpi) and

between pigs infected with E75 virulent strain and E75CV1 attenuated strain at 3 dpi. This comparison led to the identification of differentially expressed miRNAs in these different situations.

In addition, from both infections, reads were compared with the corresponding viral genome in order to identify viral miRNAs candidates.

3.11.3. Target genes prediction

When identified the differentially expressed miRNAs, those with more reads and with a fold change superior to 5 were introduced in Diana microT-CDS v5.0 software to search for their target genes. The 5.0 version of the program works with Ensembl (v69) and miRBase (v18) and contains target genes for human, Drosophila melanogaster and Caenorhabditis elegans miRNAs. Selected differentially expressed miRNAs were blasted as human miRNAs supposing sequence conservation with Sus scrofa. Software were used selecting a threshold of 0.7. For each miRNA, target Ensemble Gene Id, miTG score (Target prediction score) and other method predictions were obtained, and for each target the software also gives information of related bibliography, microRNA response elements (MRE) information, chromosomal position, conservation and graphical representation (Maragkakis *et al.*, 2009, Paraskevopoulou *et al.*, 2013).

3.11.4. Biological pathways analysis

The WebGestalt (WEB-based GEne SeT AnaLysis Toolkit) algorithm was used in order to search for those biological pathways in which, target genes for each of our miRNAs, are involved (Zhang *et al.*, 2005, Wang *et al.*, 2013). This search was done based on the Kyoto Encyclopedia of Genes and Genomes (KEGG). Parameters of the software were adjusted as follows: Hypergeometric statistical method, Benjamini &

Hochberg multiple test adjustment, 0.5 as significance level and 2 as minimum number of genes for a category. With KEGG results we were specially focused on immune system pathways, but also, other pathways that could be involved in other aspects related to infections produced by PCV2 or ASFV as could be mTor signaling pathway or MAPK signaling pathway.

3.11.5. Viral genome target prediction

MiRanda software, an algorithm for finding genomic targets for microRNAs, was used in order to identify potential targets in the viral genome, the program was used with the following parameters: -sc 140 -en 20. Each selected miRNA was compared with viral genome (Enright *et al.*, 2003).

3.11.6. Gene regulatory network creation

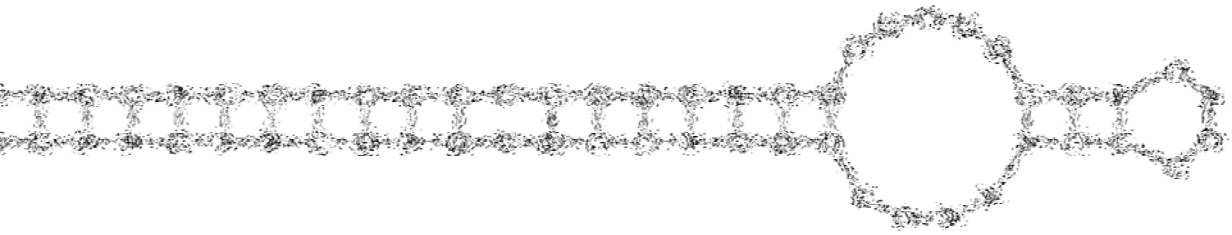
Cytoscape software was used in order to recreate and visually present all the interactions between miRNAs, target genes and biological pathways. Cytoscape is an open source software platform for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles and other state data (Shannon *et al.*, 2003).

3.11.7. Folding analysis

MFold, a RNA and DNA folding server was used in order to analyze the candidates obtained from the highthroughput sequencing to become viral miRNAs. This application allows the analysis of the appropriate secondary structure of these candidates and the free minimum energy of their folding (Zuker, 2003).

4

RESULTS



4.1

IDENTIFICATION OF MIRNAS IN PCV2 SUBCLINICALLY INFECTED PIGS BY HIGHTHROUGHPUT SEQUENCING

4.1.1. Animal infection

In this first study, as previously described, animals were inoculated with the Sp-10-4-54-13 from genotype b strain of PCV2. Until 21 dpi no animals developed clinical signs.

PCV2 infection was confirmed in inoculated animals by qPCR in 6 out of 8 tissues: Spleen, inguinal lymph node, tonsil, mediastinal lymph node, lung and mesenteric lymph node, confirming the subclinical nature of the infection (Table 1). No amplification was observed in non-infected animals or in kidney and thymus of tested animals.

	Animal Number					
Tissue	1	2	3	4	5	6
Spleen	*	*	$3,2x10^2$	$2,9x10^2$	$7,2x10^1$	$4x10^2$
Inguinal ln	*	*	$1,2x10^4$	$3,3x10^2$	$1,7x10^3$	$8,9x10^2$
Kidney	*	*	*	*	*	*
Tonsil	*	*	$5,2x10^1$	$1,8x10^3$	$2,1x10^3$	$8 x 10^2$
Thymus	*	*	*	*	*	*
Mediastinal ln	*	*	$8,2x10^3$	$1,9x10^4$	$3,8x10^4$	$4,6x10^4$
Lung	*	*	$6x10^1$	$2,1x10^2$	$1,1x10^2$	$9,2x10^1$
Mesenteric ln	*	*	$2,5x10^2$	$7,6x10^2$	$7,2x10^1$	$1,1x10^3$

Table1. PCV2 infected and non infected tissues qPCR

PCV2 detection by in situ hybridization was negative for all samples tested. No histological lesions were observed at necropsy. MLN and tonsil were selected for small RNA library construction because of their higher viral load and because tonsil is the primary replication site.

4.1.2. miRNA sequence annotation

A total of 10 small RNA libraries were constructed and sequenced. From the total reads obtained (1 106 437), after trimming the adaptors sequences, and selecting inserts ranging from 15 to 29 nt, a total of 796 710 reads were obtained (Table 2). Reads were aligned to the miRBase database (v20), not allowing any changes inside but allowing a maximum of four changes in the sequence extremes due to the insight variability of isomirs. Finally, 562 483 reads (4700 unique sequences) were aligned to miRBase. A total of 508 miRNAs were described.

Total reads	1 106 437
Trimmed, not empty reads, ranging from 15 to 29 nt	796 710
Aligned to miRBase	562 483
Unique sequences	4700
Number of miRNAs	508

Table 2. Reads obtained from the 10 small RNAs libraries by highthroughput sequencing

4.1.3. Differential expression analysis

miRNAs were considered differentially expressed (DE) when fold change (FC) difference between infected and non-infected animals was higher than 5 and when the up- or down-regulation was conserved for all animals per group. From the 119 miRNAs highly expressed (>80 reads) in MLN, 8 miRNAs were DE, five up-regulated (mir-126-3p, mir-126-5p, let-7d-3p, mir-129a, mir-let- 7b-3p) and three down-regulated (mir-193a-5p, mir- 574-5p and mir-34a) (Table 3). In tonsil, no miRNA was DE from the 115 miRNAs with more than 80 reads.

miRNA	Inf/ non inf	Reads
ssc-miR-126-5p	6,28	18 794
ssc-miR-126-3p	7,51	17 050
ssc-miR-193a-5p	-54,29	3139
ssc-let-7d-3p	9,3	770
hsa-miR-574-5p	-19,9	310
hsa-miR-34a	-5,34	165
ssc-miR-129a	8,28	115
hsa-let-7b-3p	6,5	83

Table 3. Differentially expressed microRNAs in mediastinal lymph node

4.1.4. Target prediction and functional analysis

Diana micro-T was employed to identify putative targets for eight selected DE miRNAs. A list of 5502 target genes was identified (see Additional file 1). A gene ontology (GO) enrichment analysis was used to identify the functions of the target genes (Table 4). No significant related pathways were found for mir-let-7d-3p and sscmiR-126-3p. Some pathways related to viral infection process and immune response were found to be significant, such as the T cell receptor signalling pathway, Fc gamma R-mediated phagocytosis and the Fc epsilon RI signalling pathway. The prediction of putative targets in viral genome indicated that two of the DE miRNAs targeted the PCV2 genome: mir-let-7d-3p and mir-129a, both targeting the Cap gene.

miRNA	Genome pathway
ssc-miR-126-3p	-
ssc-miR-126-5p	T cell receptor signalling pathway

	Natural killer cell mediated cytotoxicity
	B cell receptor signalling pathway
	Fc epsilon RI signalling pathway
	Chemokine signalling pathway
	mTor signalling pathway*
	MAPK signalling pathway*
ssc-miR-193a-5p	Cytosolic DNA-sensing pathway
ssc-let-7d-3p	-
ssc-miR-34a	Fc gamma R-mediated phagocytosis
	Hematopoietic cell lineage
	B cell receptor signalling pathway
	Fc epsilon RI signalling pathway
	T cell receptor signalling pathway
	Natural killer cell mediated cytotoxicity
	Leukocyte transendothelial migration
	MAPK signalling pathway*
hsa-miR-574-5p	T cell receptor signalling pathway
	MAPK signalling pathway*
hsa-let-7b-3p	T cell receptor signalling pathway
	Chemokine signalling pathway
	Fc gamma R-mediated phagocytosis
	Leukocyte transendothelial migration
	NOD-like receptor signalling pathway
	Hematopoietic cell lineage
	B cell receptor signalling pathway
	Toll-like receptor signalling pathway
	MAPK signalling pathway*
ssc-miR-129a	Fc epsilon RI signalling pathway

	B cell receptor signalling pathway
	Chemokine signalling pathway
	MAPK signalling pathway*

*Table 4. Genome pathways predicted for selected miRNAs from Kyoto Encyclopedia of Genes and Genomes, related to the immune system and PCV2 pathogenesis. *Signal transduction*

4.2

EVALUATION OF THE CAPABILITY OF THE PCV2 GENOME TO ENCODE MIRNAS IN AN EXPERIMENTAL INFECTION

4.2.1. Viral miRNAs prediction

For the second study, the expression of miRNAs in subclinically PCV2 infected pigs was analyzed using high throughput sequencing. Firstly, in silico prediction was carried out in order to check if the PCV2 genome encodes possible miRNA precursors. The Vmir prediction algorithm (Grundhoff, 2011) was used to predict the possible presence of hairpin structures in the PCV2 genome compatible with the existence of miRNAs. Computational prediction of viral miRNAs indicated that 41 miRNA candidates could be identified, 16 of them with a score among 100-150 and two with a score >150 (Figure 1). In order to explore whether these candidates were present in the virus, next-generation sequencing (NGS) of small RNAs was carried out from tonsil and mediastinal lymph node of animals subclinically infected with PCV2.

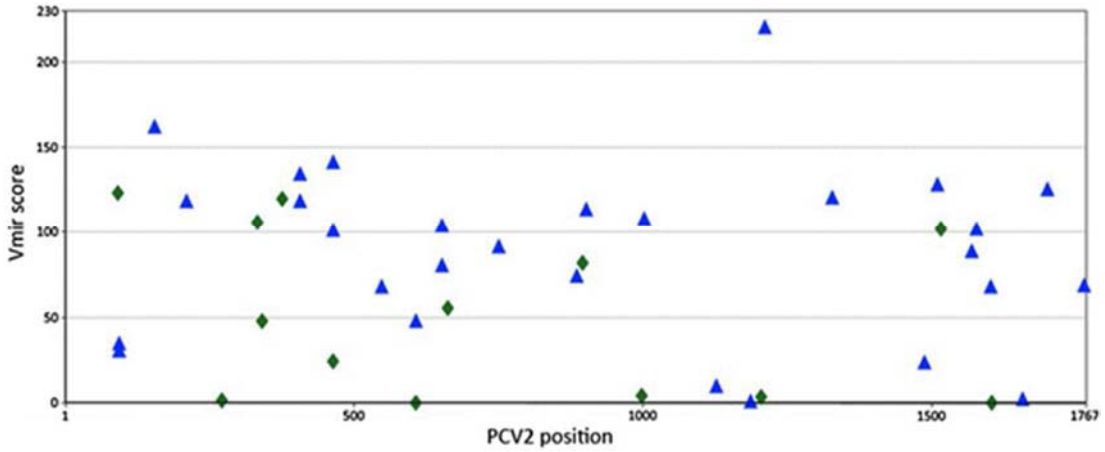


Figure 1. Hairpin structures predicted for PCV2 genome of Sp-10-7-54-13 isolate (accession number GU049342) by using Vmir with default parameters. Green diamonds and blue triangles indicate stem-loop structures in direct or reverse orientation, respectively.

4.2.2. Blast analysis

The analysis of the reads that aligned to the *Sus scrofa* genome are reported elsewhere because these sequences constitute porcine miRNAs (Nunez-Hernandez *et al.*, 2015). For viral miRNA discovery, sequences were blasted to the PCV2 isolate Sp-10-7-54-13 genome (NCBI Reference Sequence: GU049342) considering only

sequences with 100% of alignment and identity (perfect match). In order to search potential viral miRNAs, a blast against the viral genome was done with an increased number of mismatches in the extremes due to miRNA variability (isomiRs) (Morin *et al.*, 2008, Ebhardt *et al.*, 2009). The presence of isomiRs entails differences in length and point mutations in both extremes, with the 3'-terminus having a higher proportion of such mutations. In some cases, a blast was done allowing internal variations with a <100% of alignment in order to consider the variability of the viral genome (Firth *et al.*, 2009). Also, sequences were blasted to the output of Vmir hairpin PCV2 structures.

4.2.3. Candidate analysis

No hits were found in the viral PCV2 genome sequence (GU049342) with a 100% homology. One candidate of 18 nt with 58 copies was identified when allowing <100% of alignment and identity in the ORF2, positions 1189 to 1206. In accordance with the Vmir prediction, these positions were included in the pre-miRNA candidate MD18, (the hairpin with the highest score), presenting one internal mismatch, and 94.4% sequence identity. The secondary structure and the minimum free energy of the pre-miRNA candidate was analyzed with MFold software (Zuker, 2003) (Figure 2).

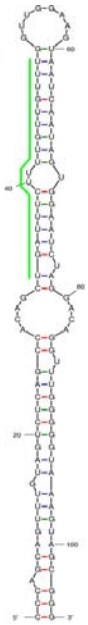
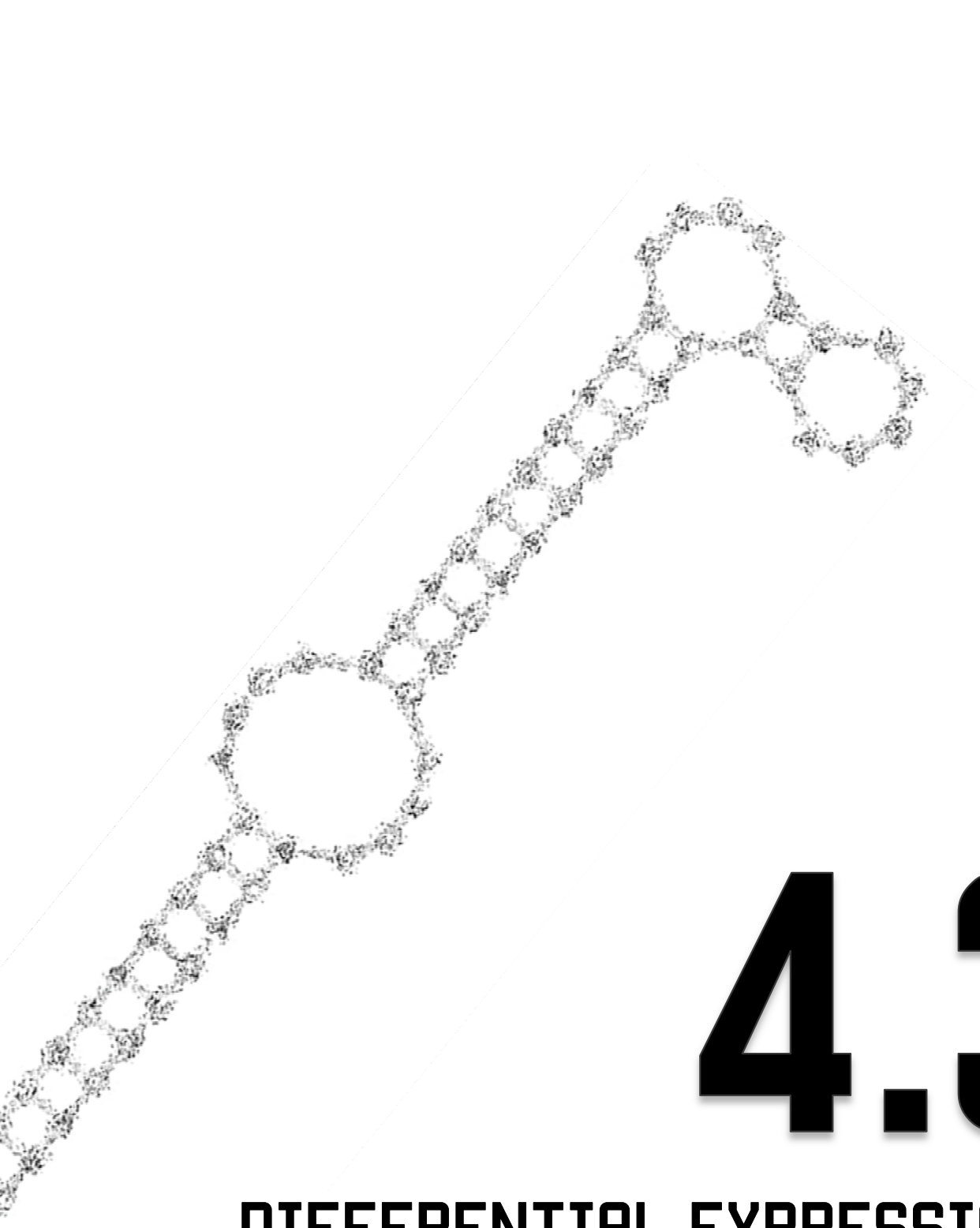


Figure 2. Folding structure using MFold of pre-miRNA candidate predicted by Vmir. The minimum free energy (ΔG) calculated was -26.60 Kcal/mol. Position of the miRNA candidate sequence detected by next-generation sequencing is indicated into the pre-miRNA structure with a green line.

As this candidate presented a point mutation, and due to the described variability of the virus, the corresponding viral DNA fragment from the mediastinal lymph node of an infected animal was amplified and Sanger sequenced. Because the exact candidate sequence was not found in the viral sequence (94.4% sequence identity), it was not considered a miRNA encoded by the virus. The miRNA candidate sequence was compared to the porcine genome Sscrofa10.2 (GCA_000003025.4) using blast and showed 100% sequence identity with the ssc-miR-29a hairpin precursor located in chromosome 18. Also, the 18 nt miRNA candidate sequence was compared to the miRBase (v.21) showing 100% sequence identity with miR-29a-5p. The alignment of this region was carried out including all PCV2 sequences available in the databases (Figure 3). miR-29a-5p has been described in many species like human, bovine, mouse, but it has not been described in pigs, where the precursor miR-29 has been included in miRBase along with the mature ssc-miR-29a-3p. From the total reads obtained in this study, 1276 sequences of 22 nt, comprising the 18 nt of the candidate, blasted to the miR-29a-5p. From all of the above data, miR-29a-5p can be considered as a miRNA encoded by the porcine genome and the 18 nt candidate as one of the miR-29a-5p isomiRs.

miRNA/PCV2 strains	Nucleotide Sequence			aa	No of isolates
	1188	1202	1209	173	
mir-29a-5p	ACTGATTTCTTTTGGTGTTCAG[a]				
PCV2 Sp-10-7-54-13	G	T	TG	N	977
PCV2 A/2002	G	C	TG	S	6
PCV2 Henan-1	G	A	TG	I	1
(-)[b]	G		TG	T	0

Figure 3. Alignment of miR-29a-5p with positions 1188-1209 of the Sp-10-7-54-13 sequence and all PCV2 available sequences in the EMBL database. A representative of each sequence is included in the alignment. The number of isolates with the same sequence is shown. The derived amino acid (aa position 173) from nucleotide change at position 1202 is indicated. [a]U has been replaced by T to facilitate the understanding of the figure. [b]No viral sequence was found in the database with this sequence.



4.3

DIFFERENTIAL EXPRESSION OF MIRNAS IN PIGS INFECTED WITH ATTENUATED AND VIRULENT STRAINS OF AFRICAN SWINE FEVER VIRUS

4.3.1. Animal infection

Pigs inoculated with the E75CV1 attenuated strain developed no clinical signs while pigs inoculated with E75 virulent strain developed typical African swine fever clinical signs. Three animals were necropsied at 3 dpi as early times of infection while one pig inoculated with the virulent strain was euthanized at 7 dpi because of the development of severe clinical signs. Therefore, 7 dpi was considered as late times of infection. Classical histopathological lesions were found in tissues of animals with clinical signs. DNA from attenuated virus was not detected at 3 dpi by qPCR. One animal inoculated with the attenuated strain was maintained until 31 dpi showing either no viremia and no clinical signs of acute ASF except fever episodes at 7 dpi that reached undetectable levels by 28 dpi. Ct values in tissue samples are indicated in table 1. RNAs from submandibular lymph node (SLN) and spleen were used to prepare small RNA libraries.

	Animal number				
Tissue	1	2	3	4	5
SLN	31,99	27,81	--	--	--
Spleen	27,71	24,6	--	--	--

Table 1. Ct values from ASFV inoculated pigs with E75 virulent strain (animals 1 and 2), E75CV1 attenuated strain (animals 3 and 4) and healthy pig (animal 5) from SLN and spleen.

4.3.2. miRNA sequence annotation

A total of 8 small RNA libraries from two tissues of the infected animals were constructed and sequenced with Ion Torrent PGM sequencer. From the 301 802 total reads obtained, after trimming the adaptor sequences, the number of inserts comprising 15 to 29 nt were 105 156. Sequences were alignment to miRBase database (v.21) and a total of 66 599 were aligned, corresponding to 366 entries in miRBase.

4.3.3. Analysis of the miRNA expression in animals infected with the virulent ASFV at different times post-infection

The normalized count of sequencing reads (reads/total sequencing tags in the library) could be used to quantify miRNA expression levels among early times post-infection (3 dpi) and late times post-inoculation (7 dpi) in both analyzed tissues. miRNAs were considered differentially expressed (DE) with a fold change (FC) ≥ 5 or when a miRNA was not expressed in one condition.

From the 14 miRNAs expressed at higher levels (>80 reads) in spleen, 9 miRNAs (64,3%) were DE, two were up-regulated at 7 dpi when compared with 3 dpi (miR-451 and miR-145-5p) and 6 down-regulated (table 2). miR-122 was not expressed at 3 dpi. Interestingly, miR-451 and miR-145-5p were the most represented miRNAs with more than two and one thousand counts among all miRNAs, respectively. Among the down regulated miRNAs, miR-126-5p presented the highest FC differences with 225 FC. In SLN, from the 25 miRNAs more expressed (>80), six miRNAs (24%) were DE, two up-regulated at 7 dpi when compared with 3 dpi (miR-181a and miR-145-5p) and 4 down-regulated (table 3). miR-126-5p was, in SLN, the miRNA with the highest fold change (41,84 FC), as has been shown in spleen.

A more conserved miRNA expression pattern was observed in SLN than in spleen.

miRNA	Virulent 3-7 dpi spleen	Reads
bta-miR-451	46,15	2305
ssc-miR-145-5p	12,39	1212
ssc-miR-92a	-21,02	445
ssc-miR-23a	-7,92	285
ssc-miR-92b-3p	-13,79	271
hsa-miR-25-3p	-2,10	203
ssc-miR-126-5p	-224,86	202
ssc-miR-23b	-16,74	152
ssc-miR-26a	-1,61	151
ssc-miR-122	106/0	106
ssc-miR-125b	-3,29	96
ssc-miR-21	1,83	92

ssc-miR-181a	1,06	88
bfl-miR-92c	-13,87	86

Table 2. Differences in the expression of miRNAs in spleen between virulent ASFV infected animals at 3 and 7 dpi from the most represented miRNAs (CN >80). miRNAs were considered differential expressed when FC was higher than 5 between 7 dpi and 3 dpi samples of infected pigs or when a miRNA was not expressed in one condition.

miRNA	Virulent 3-7 dpi SLN	Reads
ssc-miR-126-3p	-9,32	1892
ssc-miR-23a	-2,49	1576
ssc-miR-126-5p	-41,84	622
ssc-miR-23b	-5,14	586
ssc-miR-26a	-1,06	530
ssc-miR-125b	1,54	447
ssc-miR-92a	-1,05	440
ssc-miR-21	4,69	431
hsa-let-7b-5p	2,67	423
hsa-miR-25-3p	-1,05	412
ssc-miR-92b-3p	2,30	312
ssc-miR-15b	1,32	284
ssc-miR-150	1,29	257
ssc-miR-99a	-1,54	199
ssc-miR-10b	-2,07	180
ssc-miR-30e-5p	-2,80	174
ssc-miR-30d	-7,11	161
ssc-miR-100	-1,29	137
ssc-miR-181a	5,36	131
ssc-miR-16	4,20	116
ssc-miR-99b	-2,97	115
ssc-miR-378	-2,92	113
bfl-miR-92c	1,00	109
ssc-miR-145-5p	5,87	95
hsa-miR-500a-5p	-4,09	94

Table 3. Differences in the expression of miRNAs in SLN between virulent ASVF infected animals at 3 and 7 dpi from the most represented miRNA (CN >80). miRNAs were considered differential expressed when FC was higher than 5 between 7 dpi and 3 dpi in samples of infected pigs.

4.3.4. Analysis of the miRNA expression in attenuated and virulent infected animals at early times post-infection

The normalized count of sequencing reads (reads/total sequencing tags in the library) could be used to quantify miRNA expression levels among virulent and attenuated infected pigs at early times post-infection (3 dpi). miRNAs were considered differentially expressed with a fold changes ≥ 5 and when the up- or down-regulation was conserved for both animals infected with E75CV1 strain.

From the 22 miRNAs more expressed (>80 reads) in spleen, 7 miRNAs (31,8%) were DE, four up-regulated in virulent infected animal (miR-92a, miR-126-5p, miR-92c and miR-30e-5p) and 3 down-regulated (miR-125b, miR-451 and miR-125a) (table 4). In SLN, from the 33 miRNAs more expressed (>80), three miRNAs (9,1%) were DE, two up-regulated in virulent infected animal (miR-30e-5p and miR-500a-5p) and one down-regulated (miR-125a) (table 5). miR-303-5p and miR-125a presented the same regulation in both tissues analyzed, up and down regulated, respectively.

In this case, the miRNA expression seems to be also more conserved in SLN than in spleen at that time post-infection

miRNA	Virulent/ Attenuated spleen	Reads
ssc-miR-125b	-6,64	1496
ssc-miR-23a	-1,42	1266
ssc-miR-23b	-1,31	642
ssc-miR-92a	13,06	508
ssc-miR-92b-3p	3,29	478
bta-miR-451	-5,80	446
ssc-miR-125a	-7,59	440
ssc-miR-145-5p	-2,63	418
ssc-miR-126-5p	12,42	250
hsa-miR-25-3p	3,14	213
ssc-miR-99a	-2,05	211
ssc-miR-378	-2,14	194
hsa-let-7b-5p	1,06	172
ssc-miR-126-3p	2,03	162

ssc-miR-100	-3,03	145
ssc-miR-30d	2,04	130
ssc-miR-26a	2,86	129
ssc-miR-99b	-1,85	127
ssc-miR-21	-1,59	93
bfl-miR-92c	13,58	90
ssc-miR-30e-5p	5,70	90
ssc-miR-150	-1,02	84

Table 4. Differences in the expression of miRNAs in spleen between virulent and attenuated ASFV infected animals at 3 dpi from the most represented miRNA (CN >80). miRNAs were considered differential expressed when FC was higher than 5 between E75 and E75CV1 at 3 dpi in samples of infected pigs.

miRNA	Virulent/ Attenuated SLN	Reads
ssc-miR-126-3p	1,55	2601
ssc-miR-23a	1,17	2424
ssc-miR-125b	-2,55	1030
ssc-miR-92b-3p	-4,41	869
ssc-miR-23b	1,39	853
ssc-miR-126-5p	1,89	847
ssc-miR-92a	-1,10	646
ssc-miR-26a	2,49	614
hsa-let-7b-5p	-1,21	568
hsa-miR-25-3p	1,68	509
ssc-miR-21	1,03	452
ssc-miR-150	1,06	355
ssc-miR-15b	1,66	333
ssc-miR-99a	-1,34	332
ssc-miR-125a	-6,91	291
ssc-miR-10b	1,17	261
ssc-miR-100	-1,42	238
ssc-miR-30d	1,79	223
ssc-miR-30e-5p	6,83	183
ssc-miR-99b	1,02	182
ssc-miR-191	-2,32	164
bfl-miR-92c	1,19	145
ssc-miR-145-5p	-2,48	140
ssc-miR-378	2,51	133

ssc-miR-181a	1,05	131
ssc-miR-16	1,16	115
ssc-miR-204	1,09	115
ssc-miR-218	1,28	103
hsa-miR-500a-5p	5,74	101
hsa-miR-29c-5p	-1,09	96
ssc-miR-374a-5p	2,09	89
ssc-miR-186	2,57	84
efu-miR-126	-1,34	82

Table 5. Differences in the expression of miRNAs in SLN between virulent and attenuated ASFV infected animals at 3 dpi from the most represented miRNA (CN >80). miRNAs were considered differential expressed when FC was higher than 5 between E75 and E75CV1 at 3 dpi in samples of infected pigs.

4.3.5. miRNA target prediction and biological pathways analysis

Target predictions were carried out for miRNAs DE. Ten miRNAs were selected for target prediction according to their highest representation by tissue and conditions: miR-23a, miR-30e-5p, miR-92a, miR-122, miR-125b, miR-126-5p, miR-145-5p, miR-125a, miR-451 and 126-3p. From these ten miRNAs a total of 8 774 target genes were identified. Cellular target genes were functionally analyzed through KEGG pathways database (Table 6). For 7 miRNAs: miR-23a, miR-30e-5p, miR-92a, miR-122, miR-125b, miR-126-5p and miR-125a, significant pathways were found related to immune response like B and T cell receptor signaling pathway, natural killer cell mediated cytotoxicity or Fc gamma R-mediated phagocytosis and with some processes related to the pathogenesis and virus-host interaction, like desencapsidation, apoptosis inhibition, autophagy or host DNA damage response. For miR-451, miR-126-3p and miR-145-5p, no pathways were identified, although some of the target genes have been related to ASFV infection.

miRNA	Pathways
23a	Endocytosis
	Regulation of actin cytoskeleton
	RIG-I-like receptor signaling pathway
	Leukocyte transendothelial migration
	Fc gamma R-mediated phagocytosis
	Protein processing in endoplasmic reticulum
	NOD-like receptor signaling pathway
	Cell adhesion molecules (CAMs)
	Chemokine signaling pathway
	Apoptosis
	T cell receptor signaling pathway
	Hematopoietic cell lineage
	Phagosome
	Fc epsilon RI signaling pathway
30e-5p	Regulation of actin cytoskeleton
	Natural killer cell mediated cytotoxicity
	T cell receptor signaling pathway
	B cell receptor signaling pathway
	Focal adhesion
	Protein processing in endoplasmic reticulum
	Endocytosis
	Fc epsilon RI signaling pathway
	Apoptosis
	Regulation of autophagy
	Fc gamma R-mediated phagocytosis
	Leukocyte transendothelial migration
	Cell adhesion molecules (CAMs)[a]
	Chemokine signaling pathway
	mTOR signaling pathway[a]
	Toll-like receptor signaling pathway
	RIG-I-like receptor signaling pathway
92a	Focal adhesion
	Regulation of actin cytoskeleton
	Endocytosis
	Fc gamma R-mediated phagocytosis
	Cell adhesion molecules (CAMs)
	Leukocyte transendothelial migration
	Chemokine signaling pathway
	B cell receptor signaling pathway
	T cell receptor signaling pathway

122	Antigen processing and presentation
	Endocytosis
	T cell receptor signaling pathway
125b	Toll-like receptor signaling pathway
	Endocytosis
	Apoptosis
	Chemokine signaling pathway
	Regulation of actin cytoskeleton
	Fc epsilon RI signaling pathway
	NOD-like receptor signaling pathway
	Cell adhesion molecules (CAMs)
	T cell receptor signaling pathway
	Natural killer cell mediated cytotoxicity
	Hematopoietic cell lineage
126-5p	Focal adhesion
	Regulation of actin cytoskeleton
	T cell receptor signaling pathway
	Protein processing in endoplasmic reticulum
	Natural killer cell mediated cytotoxicity
	B cell receptor signaling pathway
	Fc epsilon RI signaling pathway
	Chemokine signaling pathway
	Apoptosis
	Endocytosis
	Regulation of autophagy
145-5p	Regulation of autophagy
	Protein processing in endoplasmic reticulum
125a	Toll-like receptor signaling pathway
	Endocytosis
	Apoptosis
	Chemokine signaling pathway
	Antigen processing and presentation
	Regulation of actin cytoskeleton
	Fc epsilon RI signaling pathway
	NOD-like receptor signaling pathway
	Hematopoietic cell lineage
451	-
126-3p	-

Table 5. Genome pathways predicted for selected miRNAs from Kyoto Encyclopedia of Genes and Genomes, related to the immune system and ASFV pathogenesis. [a] Signal transduction.

4.3.6. Gene regulatory network

DE miRNAs were analyzed with miRanda algorithm in order to identify the possible target in the ASFV genome. For the miRNA expression in animals infected with the virulent ASFV at different dpi, a total of 25 interactions have been found for the seven DE miRNAs and the annotated 165 genes in ASFV genome (Figure 1). Also, 42 interactions have been identified with the porcine genes involved in virus-host interactions as: apoptosis, chemokine receptors, endoplasmic reticulum stress, transcription factors, autophagy, ATM pathway, virus entry, immune response and replication.

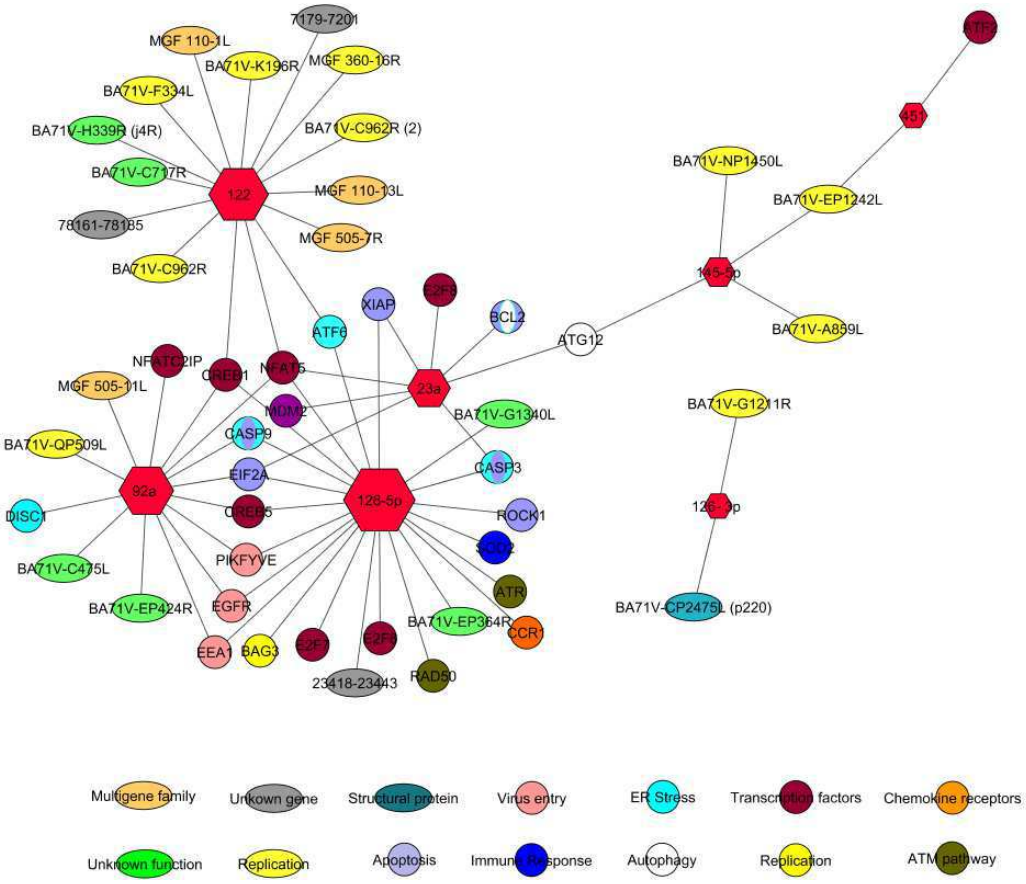


Figure 1. Network of DE miRNAs interactions in animals infected with the virulent strain at different time points with ASFV genes and porcine genes involved in virus- host interactions. Hexagons size is proportional to the miRNAs number of interactions. Circles indicate cellular genes and ovals indicate viral genes. Colors indicate the main/s pathway/s in which they are involved or the described function of the viral genes.

For the miRNA expression in attenuated and virulent infected animals at early times post-infection, 17 interactions have been found for the five DE miRNAs and the annotated genes in ASFV genome (Figure 2) and 46 interactions have been identified with the porcine genes involved in virus-host interaction, like: chemokine receptors, endoplasmic reticulum stress, apoptosis, transcription factors, autophagy, virus entry and P300 coactivator protein.

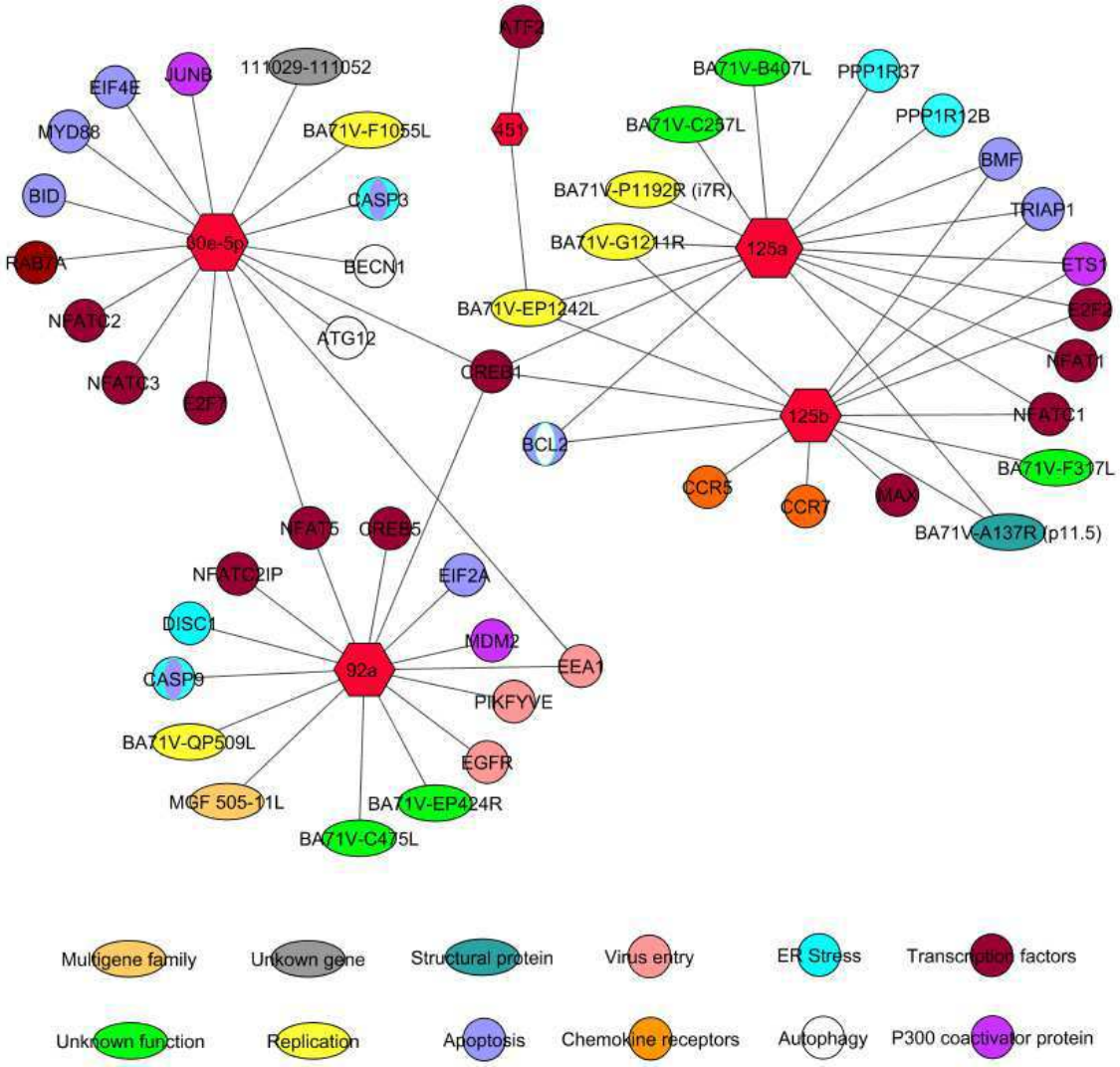


Figure 2. Network of DE miRNAs interactions in animals infected with virulent strain and animals infected with attenuated strain at 3 dpi with ASFV genes and porcine genes involved in virus- host interactions. Hexagons size is proportional to the miRNAs number of interactions. Circles indicate cellular genes and ovals indicate viral genes. Colors indicate the main/s pathway/s in which they are involved or the described function of the viral genes.

In a global approach, considering all DE miRNAs by ASFV infection condition, 37 interactions have been found between 12 DE miRNAs included and the annotated 165 genes in ASFV genome (Figure 3). Also, 76 interactions have been identified with the porcine genes involved in virus-host interaction as previously described for both conditions.

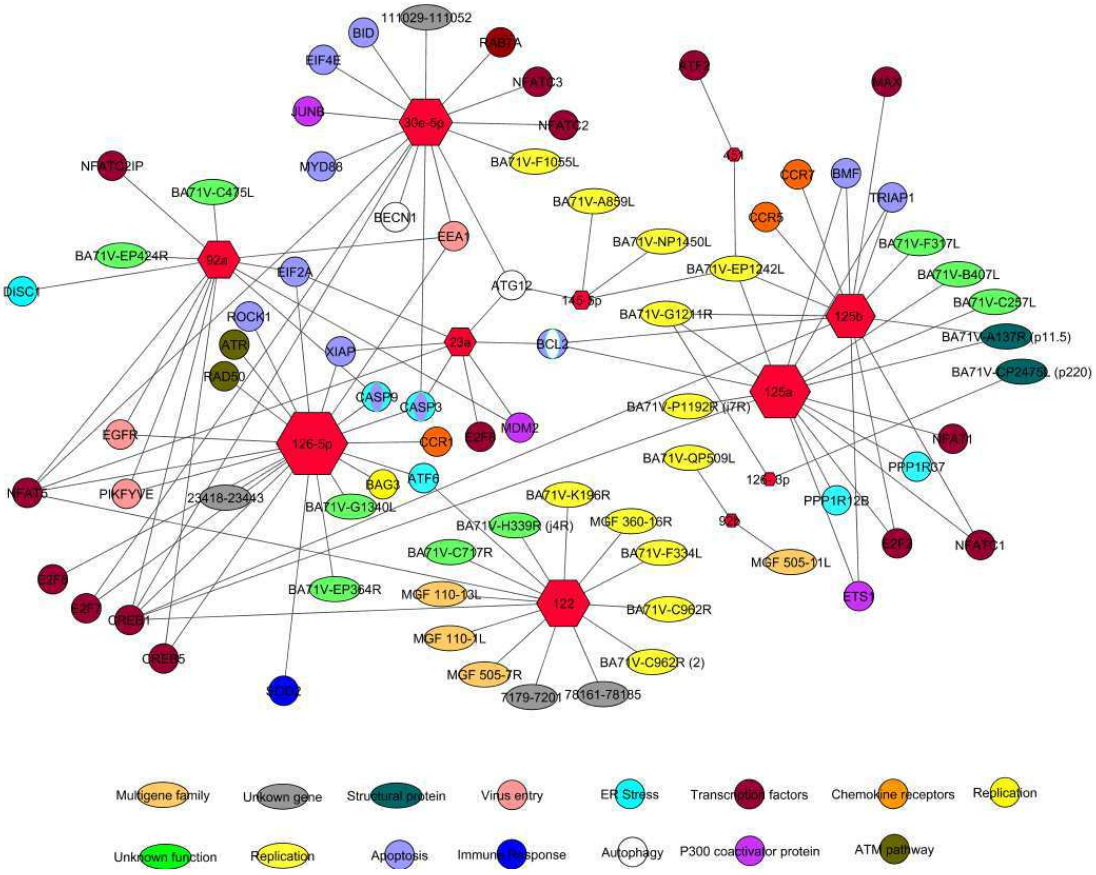
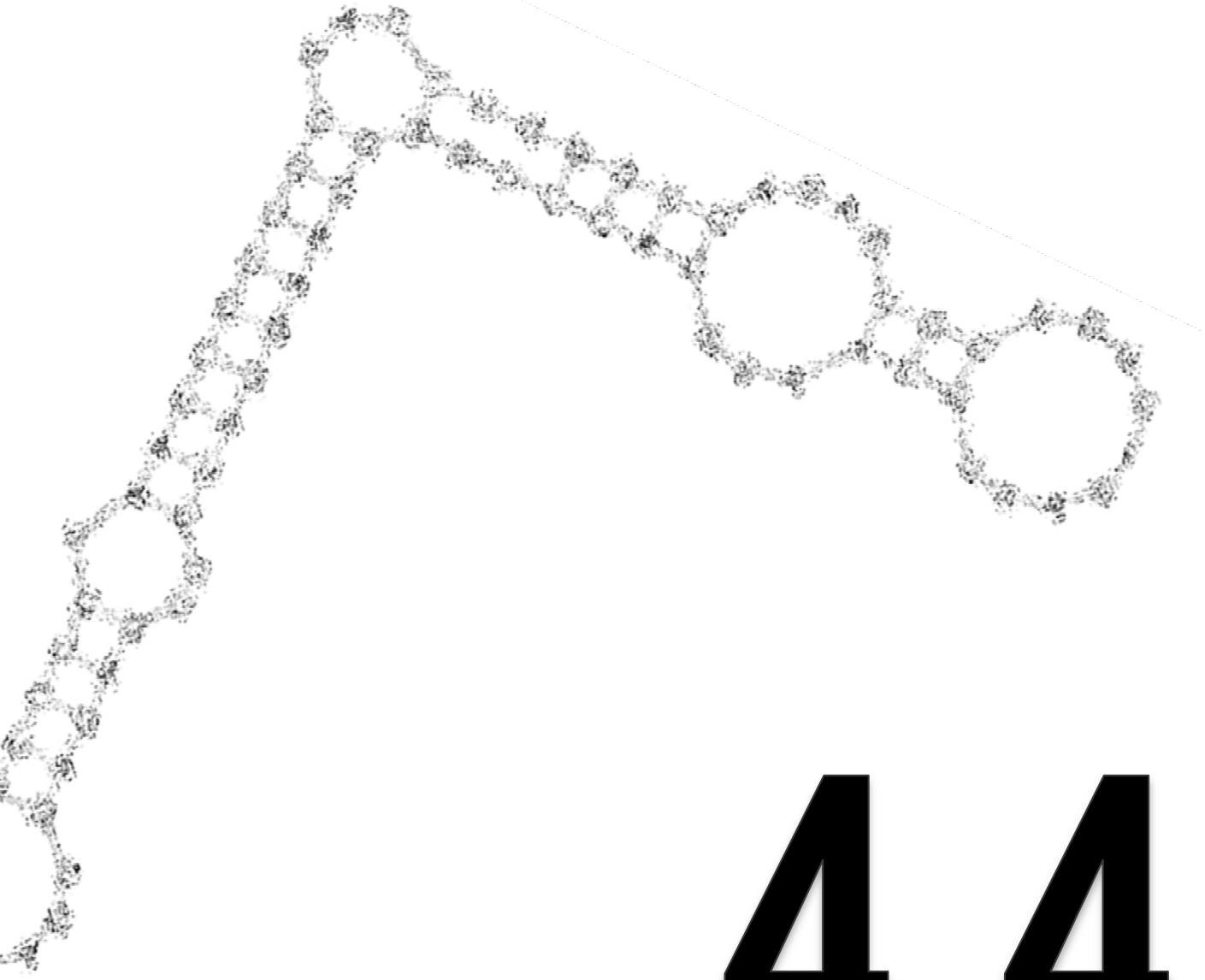


Figure 3. Global miRNAs network interaction. Related DE miRNAs in both approaches with cellular target genes involved in ASFV infection and with viral genes. Hexagons size is proportional to the miRNAs number of interactions. Circles indicate cellular genes and ovals indicate viral genes. Colors indicate the main/s pathway/s in which they are involved or the escribed function of the viral genes.



4.4

ANALYSIS OF THE CAPABILITY OF ASFV TO EXPRESS VIRAL MIRNAS IN AN EXPERIMENTAL INFECTION

The possible expression of viral miRNAs in ASFV infected pigs was analyzed by highthroughput sequencing in three conditions: i) Animals infected with virulent strain E75 necropsied at 3 and 7 dpi, ii) Animals infected with the attenuated strain E75CV1 and necropsied at 3 and 31 dpi, iii) Animals infected with the attenuated strain E75CV1 and re- inoculated at 31 dpi with the virulent strain Ba71 and necropsied at 38 dpi. As negative control, small RNA libraries were sequenced from a non- infected animal at 0 dpi.

4.4.1. Computational viral miRNAs prediction

Firstly, *in silico* prediction was carried out in order to examine if the ASFV genomes of E75 and Ba71 encode possible miRNA precursors (E75CV1 prediction was not done due to the lack of its sequence availability in the databases). The Vmir prediction algorithm was used to predict the possible presence of hairpin structures in the E75 (accession number FN557520), Ba71 (accession number KP055815). Also it was included the prediction with Vero highly adapted Ba71V strain genome (accession number NC_001659). Computational prediction by using Vmir of viral miRNAs, setting as conditions a hairpin size from 50 to 100 nt and a window count of 15, in E75 strain indicated that 415 miRNA candidates were identified with a score higher than 100 (Figure 1) and in the case of Ba71, 391 candidates with a score higher than 100 were identified (Figure 2). In order to explore whether these candidates were present in the virus, next-generation sequencing (NGS) of small RNAs was carried out from spleen and SLN of animals infected with virulent strain, attenuated strain and also animals non- infected in the conditions previously described.

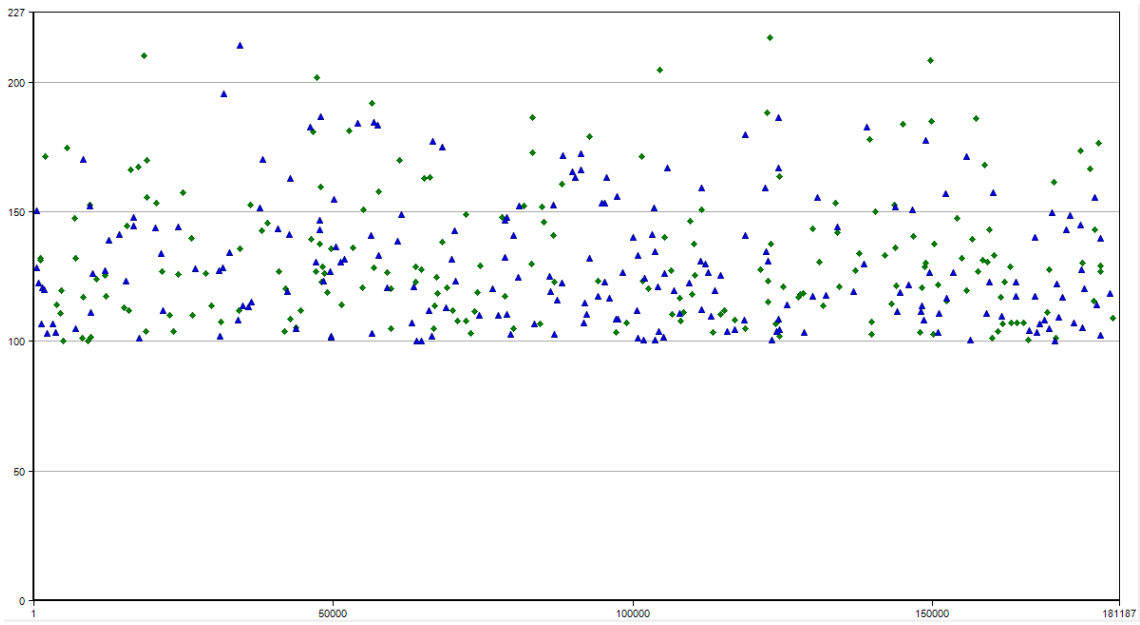


Figure 1. Hairpin structures predicted for ASFV genome of E75 isolate (accession number FN557520) by using Vmir with score higher than 100, hairpin size from 50 to 100 and window count of 15. Green diamonds and blue triangles indicate stem-loop structures in direct or reverse orientation, respectively.

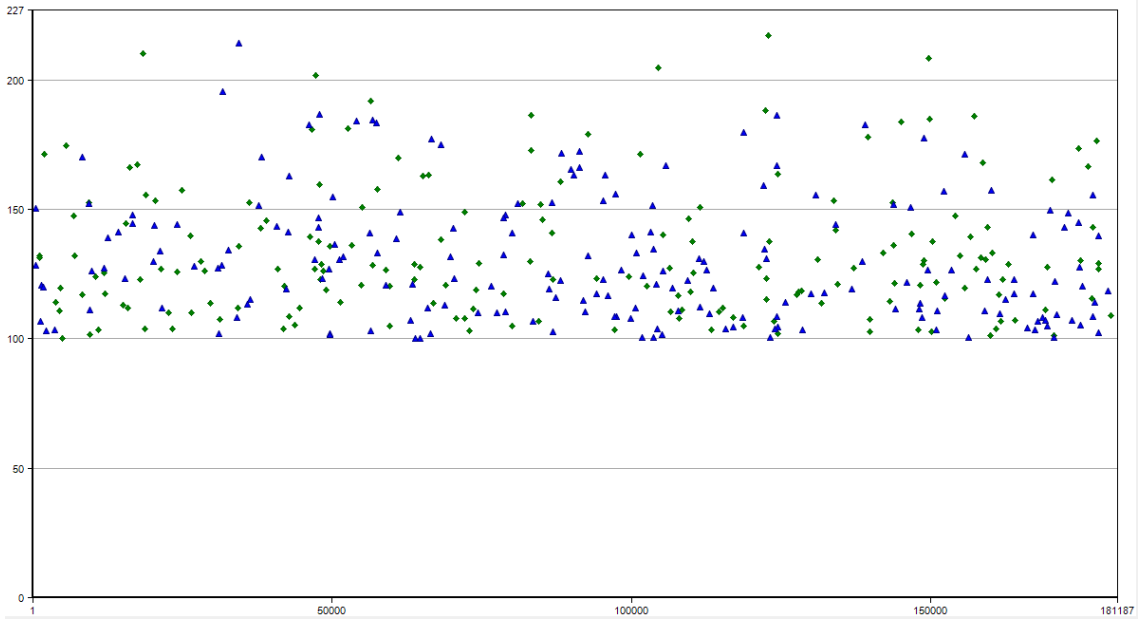


Figure 2. Hairpin structures predicted for ASFV genome of Ba71 isolate (accession number KP055815) by using Vmir with score higher than 100, hairpin size from 50 to 100 and window count of 15. Green diamonds and blue triangles indicate stem-loop structures in direct or reverse orientation, respectively.

4.4.2. Experimental infection

For this study, one animal was inoculated with the attenuated strain E75CV1 and sacrificed at 31 dpi with no clinical signs or lesions and two more animals were inoculated with the attenuated strain and re-inoculated with the virulent strain Ba71 at 31 dpi and sacrificed at 38 dpi; re-inoculated animals showed the typical clinical signs and lesions of ASFV infection.

Quantitative PCR was performed for all the samples where animals inoculated with the E75CV1 attenuated strain and negative control animal showed no amplification while animals re- inoculated with Ba71 virulent strain showed amplification (Table 1).

	Animal number			
Tissue	5	6	7	8
Spleen	*	*	24,84	27,3
SLN	*	*	25,17	28,11

Table1. Ct values from negative control animal scarified at 0 dpi (5), animal inoculated with E75CV1 attenuated strain sacrificed at 31 dpi (6), and animals re-inoculated with Ba71 virulent strain at 31 dpi and sacrificed at 38 dpi (7 and 8).

4.4.3. Sequencing and blast analysis

Eight small RNA libraries were constructed from spleen and SLN from the four animals and sequenced with Ion torrent in addition to the eight sequenced libraries from the previous study.

Highthroughput sequencing provided 272 967 reads from the eight new libraries which in addition to eight previously sequenced libraries with 301 802 reads provided a total of 574 769 reads from the sixteen libraries (Table 2). When trimmed to filter sequences ranging from 15 to 29 nucleotides, a total of 169 511 reads were obtained. For viral miRNA discovery, sequences were blasted to the E75 and Ba71 isolates considering only sequences with 100% of alignment and identity (perfect

match) rendering 9 hits with the ASFV genome (Table 2). In order to search potential viral miRNAs, a blast against the viral genome was done with an increased number of mismatches in the extremes due to miRNA variability (isomiRs) rendering 31 hits. This processing allowed the identification of 40 viral genome hits with 8 unique sequences when allowing 100% homology (Tables 2 and 3) and 23 unique sequences when allowing <100% homology (Tables 2 and 4). From these sequences, 26 candidates were obtained when grouped by isomirs and 6 candidates when CN was ≥ 2.

Total reads	574 769
Trimmed not- empty reads (15-29 nt)	169 511
Blast ASFV 100%	9
Unique sequences 100%	8
Blast ASFV <100%	31
Unique sequences <100%	23
Total unique sequences	31
Viral miRNA candidates[a]	26
Viral miRNA candidates CN≥2	6
Candidate + Mfold structure/ ASFV miRNAs	-

Table 2. Summary of the highthroughput reads, blast and candidates for viral miRNAs discovery. [a] Viral candidates CN after sequence association by isomirs.

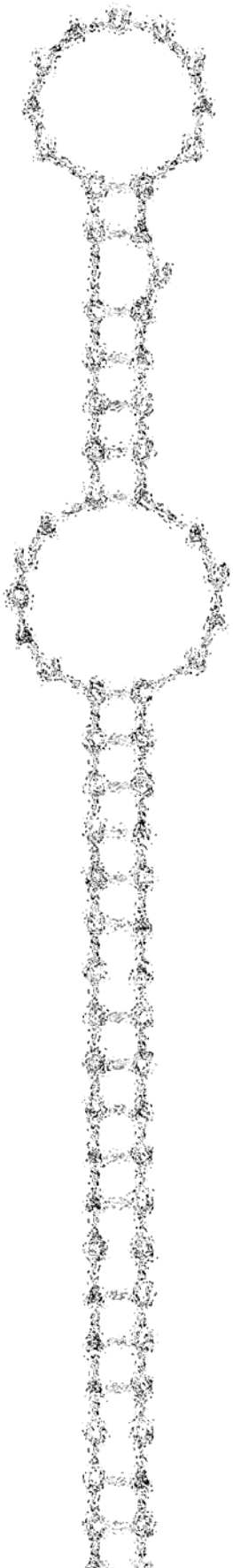
Candidates sequences blast vs ASFV =100%	Candidates CN
GAGACCAAGAACCTGGGCA	2
ATGTGCCCTGGTCCCGTAGGAGTG	1
CGTCTGGGAGAAATGGAGCA	1
CACCGTGTGGATGCCCAGGG	1
GAGCTCGTGACCATTGAA	1
GGTTCACTGGTGTCCATGAT	1
ATGATCGTAGCATTTGGTGTA	1
GGCGAGTCACTTGGTTTGCT	1

Table 3. Candidates obtained with a 100% of homology with the ASFV genome and their CN.

Candidates sequences blast vs ASFV <100%	Isomirs CN	Candidate CN
TCTCCCAACCTGTACAGT TCTCCCAACCTGTACAGGT CTCCCAACCTGTACAGT	4 1 3	8
TGAGGTAGTAGGTTGTGT TGAGGTAGTAGGTTGTGTG	2 2	4
CGCTGCGGGTGTGGTGGGCC CGCTGCGGGTGTGGTGG	1 1	2
TGTGCAAATCTATGCAAAAC	2	2
GGTAGTAGGTTGTGTGGTT GGTAGTAGGTTGTGTGGAT	1 1	2
AGGGCACCAAGGTAAC	1	1
ACGCCCTGGGGCCTATGAG	1	1
TCGCTGCGGGTGTGGTGGG	1	1
CCGCTGCGGGATGAAC	1	1
GGGAAAATCCACGGCCCTGC	1	1
TGTATGGAGGCCCTTTTT	1	1
CTCCTGGGGCCGCACTCTC	1	1
TCGTCCCAACCTGTACAGT	1	1
TTCACTGCCCTGATCCTCT	1	1
GCGACCCGTCAAGTCCAACAG	1	1
GTCGGAGCCTGAGCCGGGAG	1	1
CCCCCACACAGTTTGAC	1	1
TCTCCCAATCCTTGCCAGT	1	1

Table 4. Candidates obtained with less than 100% of homology with the ASFV genome and their CN grouped by isomirs.

Finally, sequences were blasted to the output of Vmir hairpins from E75, Ba71 and Ba71V. However, after the analysis of the complementarity of the candidates with the Vmir prediction, and the analysis with Mfold of their secondary structure and the free minimum energy of their folding, none of the candidates could be considered as a viral miRNA by failing to fulfill the necessary requirements.



5

DISCUSSION

5.1. Identification of miRNAs in PCV2 subclinically infected pigs by highthroughput sequencing

miRNAs can be an important factor playing a role in virus/host interaction and in the immune response involved in the pathogenesis of PCV2 infection, in both subclinical and clinical scenarios. This work is the first study on miRNAs gene expression in pigs infected with PCV2 and ASFV using a deep sequencing approach.

In the experimental infection carried out in PCV2 infected pigs, the lack of clinical signs, histological lesions and viral detection by ISH and the low viral load detected by qPCR is in agreement with previous studies where a subclinical infection has been developed (Tomas *et al.*, 2010).

Taking into account the comparison of the most expressed miRNAs in both tissues (CN > 80), 74.8% of the miRNAs were common. This is in concordance with the lymphoid origin of both tissues. When comparing the tissues analyzed, the expression profile was affected differentially by the PCV2 infection. The miRNA expression was affected notably in MLN, whereas in tonsil, the miRNA expression pattern was less affected. A possible factor affecting this expression is the difference in viral load. However, differences in cytokine expression have been described between tonsil and others lymph nodes (Darwich *et al.*, 2003) although the basis of this differential expression is not clear. Thus, the conserved miRNA expression in tonsil could be associated to this different cytokine expression. On the other hand, tonsil has been suggested as a primary replication site for PCV2 (Nawagitgul *et al.*, 2000). Because all animals were euthanized at day 21, the expression of miRNA could be altered by the differences in the period of time when the tonsil and MLN were infected (Goher *et al.*, 2013).

Differential expression of miRNAs in MLN is high due to the PCV2 infection; from the most represented miRNAs in both tissues (CN > 80): mir-126-3p, mir-126-5p, mir-let-7d-3p, miR-129-2-3p and mir-let-7b-3p were up-regulated in MLN of PCV2

infected animals, while miR-193a-5p, miR-574-5p and miR-34a-5p were down-regulated.

miRNAs DE could regulate genes that would be involved in the immune response, such as T cell receptor signaling pathways, natural killer cell mediated cytotoxicity, the B cell receptor signaling pathway, the chemokine signaling pathway, Fc gamma R- mediated phagocytosis, leukocyte transendothelial migration, the cytosolic DNA sensing pathway, the NOD-like receptor signaling pathway, hematopoietic cell lineage and also related to Fc epsilon R-I signaling pathways.

miR-126-5p is the most represented miRNA DE in MLN and has been involved in the intracellular expression of Toll-like receptors (TLRs) 7 and 9 by plasmacytoid dendritic cells, producing (anti-viral) type I interferons (Ferretti and La Cava, 2014), thus, mir-126-5p can modulate the physiopathology of the immune response against PCV2. The extracellular signal-regulated kinase (ERK) signaling pathway, one of the three mitogen-activated protein kinase (MAPK), has been shown to be involved in the induction of autophagy by PCV2 (Zhu *et al.*, 2012) and the inhibition of ERK signaling pathways increases PCV2 replication (Wei and Liu, 2009). In our functional analysis, miR-126-5p was associated with the MAPK signaling pathway, therefore, this miRNA could be involved in both processes.

TFPI (tissue factor pathway inhibitor) is one of the genes targeted by miR-126-5p. Comparison with a previous study (Tomas *et al.*, 2010) in which gene expression in response to PCV2 infection was analyzed by microarray hybridization, showed a TFPI gene down-regulation in MLN. This decreased expression could inhibit migration and cell proliferation and thus, could be involved in the inflammatory process developed in pigs with PCV2-SD (Fernandes *et al.*, 2009). On the other hand, in a coxsackievirus infection, miR-126 targets SPRED1, LRP6, and WRCH1 genes, mediating cross-talk between the ERK1/2 and Wnt/β-catenin pathways, enhancing viral replication and contributing to the viral cytopathogenicity (Ye *et al.*, 2013).

Target prediction analysis indicated that miR-126-3p was potentially able to regulate only 20 genes but no related pathways were found. Interestingly, one of the targeted genes is the previously mentioned SPRED1, shared with miR-126-5p.

miR-let-7b-3p was up-regulated in MLN and has been shown to be able to potentially regulate a great number of genes, thereby several immunological pathways have been found for this miRNA. Besides this, this miRNA has also been associated with the MAPK signaling pathway. Target prediction analysis showed that miR-let-7b-3p can regulate the expression of the AP1S3 gene, which is involved in the modulation of Toll-like receptor 3 (TLR 3) signaling (Setta-Kaffetzi *et al.*, 2014).

mir-let-7d-3p was up-regulated in MLN, although 13 genes were found as potentially regulated by this miRNA, no related immunological pathways were found. miR-193a-5p was down-regulated in mediastinal lymph node, and could potentially regulate genes related to the cytosolic DNA- sensing pathway.

miR-34a-5p is down-regulated in MLN and this regulatory role was predicted to be involved in several immunological pathways and the MAPK signaling pathway. In this case, target prediction analysis revealed that miR-34a-5p targets KLRK1, killer cell lectin-like receptor subfamily K, member 1, which binds to a diverse family of ligands that include MHC class I chain-related A and B proteins and UL-16 binding proteins, where ligand-receptor interactions can result in the activation of NK and T cells (Fernandes *et al.*, 2009). It also regulates ARHGP6, a member of the Rho family that participates in endocytosis processes. PCV2 internalization is produced mainly by endocytosis, mainly through Rho- GTPase mediated dynamin- independent pathway (Tomas *et al.*, 2010).

miR-574-5p was down-regulated in MLN and was predicted to be involved in the regulation of the T cell receptor signaling and MAPK signaling pathways. miR-129-2-3p was down-regulated in MLN, predicted pathways in which this miRNA was involved were the Fc epsilon RI signaling, the MAPK signaling, the B cell receptor signaling, and chemokine signaling pathways. This miRNA has previously been

described as targeting transcription factor SOX4 which has a critical function in normal B-cell ontogenesis (Koens *et al.*, 2013).

Some of the predictions described in this work could be involved in the manifestation of the pathogenesis of the subclinically infected animals, but at this point, we want to indicate that further functional assays are needed to confirm the computational predictions of miRNAs target genes and the regulation of the pathways described.

The effect of host miRNAs on viral gene expression was analyzed using miRanda. Thus, mir-126-3p and mir-126-5p, the most represented DE miRNAs, showed no targets in the viral genome, while mir-let-7d-3p and mir-129a presented targets in the Cap gene of PCV2.

Both miRNAs are up-regulated in MLN and can affect viral replication as has been seen for others viruses where several miRNAs can down-regulate the expression of viral proteins (Cullen, 2010, Yan *et al.*, 2014). Nevertheless, the role of host miRNAs in the regulation of the viral replication is controversial as has been revealed in an extensive study (Bogerd *et al.*, 2014). The authors showed that many human viruses are refractory to inhibition by host miRNAs due to miRNA driven viral evolution. Thus, experimental assays are needed to clarify if PCV2 is regulated or not by porcine miRNAs.

The identification of the DE miRNAs and the prediction analysis regarding the functions of these miRNAs in an in vivo PCV2 infection in its natural host has been described in this study. The two tissues analyzed presented a different behavior in their miRNA expression pattern in response to the infection.

5.2. Evaluation of the capability of the PCV2 genome to encode miRNAs: Lack of viral miRNA expression in an experimental infection

The study of the capability of PCV2 to encode viral miRNAs is the first one trying to identify their expression. As PCV2 is a ssDNA virus with a nuclear phase in its replicating cycle (Tischer *et al.*, 1995) and this step is considered essential for the production of viral miRNAs, considering that the first steps in miRNA maturation take place in the cellular nucleus (Cullen, 2010), there was the possibility that it was able to encode them. Nevertheless, high throughput sequencing has failed to identify any miRNA encoded by the viral genome in the natural host after an experimental infection. Thus, PCV2 may increase the list of DNA viruses not encoding miRNAs, as Cowpox virus (Skalsky and Cullen, 2010). Notwithstanding, the capacity of PCV2 to encode miRNAs has to be evaluated in cell culture, in a different clinical form or at a different time points.

Only one miRNA candidate was initially identified, but the posterior analysis indicated that it was a host miRNA, miR-29a-5p. The homology of the viral sequence with miR-29a-5p, with only one internal mismatch, led us to analyze with more detail if miR-29a-5p can regulate the expression of the Cap protein. A miR-29a-5p target prediction was evaluated using Miranda software (Enright *et al.*, 2003) in order to explore if the Cap gen constitutes a target. As expected, a predicted target was identified in ORF2, with a low free energy of -21.65 Kcal/mol and a high complementarity score of 187. The alignment of the region with all PCV2 isolates available in the database indicated that A, C and T, but not G, has been found at position 1202 respecting Sp-10-7-54-13. All substitutions lead to a non synonymous change. The lack of the presence of G at position 1202 could be due to restriction in the protein conformation or due to the pressure exerted by miR-29a-5p in order to avoid regulation by this miRNA. If this is a consequence of viral evolution as (Bogerd *et al.*, 2014) proposed, it needs further investigation. How viruses can evolve

to avoid the inhibition by host miRNAs is a critical question. Some authors indicate that this evolution allows viruses to replicate without being targeted by host miRNAs by encoded viral miRNAs or modifying the expression of host miRNAs (Cullen, 2013). On the other hand, the secondary structure of MD18 could avoid the regulation by miR-29a-5p due to the inaccessibility of the target sequence as has been proposed for HIV (Whisnant *et al.*, 2013).

If PCV2 can express miRNAs in vitro has to be determined, but in its natural host, in a subclinical infection, NGS failed to identify viral miRNAs. The exploration of the possible capacity of PCV2 to encode miRNAs could contribute to the understanding of the pathogenesis of PCV2, especially for the candidate with highest score identified by the in silico prediction. In addition, further studies on the similarity of this candidate with miR-29a-5p, and its significance, could shed light on how miRNAs affect viral evolution (Guo and Steitz, 2014).

5.3. Differential expression of miRNAs in pigs infected with attenuated and virulent strains of African swine fever virus

In the third study, changes in the miRNA expression associated to ASFV infection in pigs have been investigated. The study has been carried out in order to deciphering the role of the miRNAs in the ASFV infection and to increase the knowledge of the viral pathogenesis as this information may contribute in search of new targets to control the disease. This study has been carried out in two ways, analyzing differences in expression at different times post-infection with a virulent strain and analyzing the expression pattern induced by virulent and attenuated strains at early times post-infection.

Animals inoculated with virulent strain E75 developed clinical signs, while animals inoculated with the attenuated strain E75CV1 developed no clinical signs and no viral genome was detected in the selected tissues, as expected for this isolate. This is in agreement with the reported data for these two strains (Lacasta *et al.*, 2015).

We focused on the differential expression of miRNAs in pigs infected with the virulent strain at different time points or in pigs infected with virus strains of different virulence.

In the comparison of the expression at different times in an infection with the virulent strain, in spleen, more than a half of the miRNAs with the highest copy number were DE (64,3%), two were up-regulated at 7 dpi (miR-451 and miR-145-5p), while the rest were down-regulated. In submandibular lymph node, a minor proportion (24%) of the miRNAs were DE. This matches with the differential transcription and protein profile observed in gastrohepatical lymph node at these two times post-infection (Lacasta *et al.*, 2015).

Regarding the differential expression pattern between the virulent E75 and the attenuated E75CV1 at early times post-infection (3 dpi), again, a higher differential

expression have been detected in spleen while a more conserved pattern was observed in submandibular lymph node. In this case, 31,8% (7 out of 22) of the more represented miRNAs (CN>80) were DE. In submandibular lymph node, the percentage decrease till 9,1% (3 out of 33) of the miRNAs with the same CN (>80).

In both analyzed tissues, in the two comparisons carried out, the expression of the miRNA in spleen has been more affected than in submandibular lymph node by the ASFV. Although both tissues can be considered as target for virus replication, some features as the different vascularization, anatomical organization and function could be involved in this differential regulation.

In submandibular lymph node, the four DE miRNAs more represented, ssc-miR-126-3p, ssc-miR-126-5p, ssc-miR-23b and ssc-miR-30d were down-regulated at 7 dpi. miRNAs DE could be regulating genes involved in the regulation of the immune response including: hematopoietic cell lineage, complement and coagulation cascades, platelet activation, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, cytosolic DNA-sensing pathway, natural killer cell mediated cytotoxicity, antigen processing and presentation, T cell receptor signaling pathway, B cell receptor signaling pathway, Fc epsilon RI signaling pathway, Fc gamma R-mediated phagocytosis, leukocyte transendothelial migration, intestinal immune network for IgA production and chemokine signaling pathway.

miR-451 was the most represented miRNA in the spleen of the virulent infected animals and also, is DE between 3 and 7 dpi, presenting up-regulation 7 dpi. At 3 dpi, miR-451, was also DE between animals infected with the virulent strain and animals infected with the attenuated strain. Target prediction analysis showed that this miRNA was potentially able to interact with 37 genes, but no significant pathways related to immune response or pathways involved in ASFV-host interaction have been identified.

miR-145-5p was up-regulated at 7 dpi in the spleen of the animal infected with the virulent strain. As miR-451, no pathways related to immune response have been predicted for this miRNA, among the 293 target genes identified. Nevertheless, one pathway related to ASFV-cell interaction, regulation of autophagy through ATG12, has been found.. ASFV does not induce autophagy, but inhibits it persistently throughout the infectious cycle to facilitate replication and to avoid virus clearance (Alonso *et al.*, 2013).

miR-92a was DE at different times in spleen of infected animals with the virulent strain E75. Its expression was down-regulated at 7 dpi. In the spleen of animals infected with the virulent strain and animals infected with the attenuated strain at 3 dpi was also DE, where the expression is up-regulated by the virulent strain. Target prediction showed 1192 regulated genes and the pathway analysis revealed that was involved in five pathways related to immune response, B and T cell receptor signaling pathways, Fc gamma R-mediated phagocytosis, chemokine signaling pathway and leukocyte transendothelial migration. Other pathways associated are endocytosis and regulation of actin cytoskeleton. The participation of actin in the endocytosis process has been proposed as stimulator of the entry of large viruses, like vesicular stomatitis virus (Cureton *et al.*, 2009), therefore, we anticipate that miR-92a could be involved in the entry process of ASFV.

Another miRNA with differences in its expression pattern in spleen between 3 dpi and 7 dpi was miR-23a. This miRNA was down-regulated at 7 dpi pi. A high number of significant pathways were found associated to miR-23a target genes, some of them related to the immune response, such as RIG-I-like receptor signaling pathway leukocyte transendothelial migration, Fc gamma R-mediated phagocytosis, NOD-like receptor signaling pathway, Chemokine signaling pathway, T cell receptor signaling pathway, Hematopoietic cell lineage and Fc epsilon RI signaling pathway. On the other hand, pathways related to the entry of the virus, like endocytosis and regulation of actin cytoskeleton, as well as other related to the apoptotic machinery.

Among the target genes predicted, Bcl2 and Caspase 3 were two of the most significative genes involved in apoptosis (Liang *et al.*, 2002).Also, this miRNA regulates eIF2a which is involved in ER stress process (B'Chir *et al.*, 2013). Thereby, miR-23a can play a role in the apoptosis induced by ASFV and in the immune response against the virus.

Compared with 3 dpi, miR-126-3p was down-regulated in submandibular lymph node at 7 dpi after infection with the virulent E75 strain. Only 20 genes were identified as possibly regulated by miR-126-3p, and no related immunological pathways were detected.

miR-126-5p was down-regulated in submandibular lymph node at 7 dpi after infection with the virulent E75 strain, compared with 3 dpi, and could regulate genes related to the immune response and many aspects of the virus-host interaction like entry, apoptosis, regulation of autophagy, ER stress or chemokine receptors.

miR-125b was DE in spleen at 3 dpi between virulent and attenuated strains, being down-regulated by the virulent E75 strain. miR-125b has been shown to interact with a high number of genes, thus, some pathways related to immune response have been identified for this miRNA. In addition to this, it has also been associated with pathways involved in the ASFV-cell interaction such as endocytosis, regulation of actin cytoskeleton and apoptosis (Alonso *et al.*, 2013). Target prediction analysis showed that miR-125b can interact with Bcl2.

miR-125a was down-regulated by the virulent E75 strain at 3 dpi compared with attenuated E75CV1 in both spleen and submandibular lymph node. Its regulatory role was predicted to be involved in several immunological pathways as well as in pathways related to the virus-host interaction like, endocytosis and apoptosis. This miRNA also targets viral gene P1192R, which codifies for a type II DNA topoisomerase that is essential for viral replication and/or transcriptional events (Coelho *et al.*, 2015). In addition, this topoisomerase has been proposed as a

potential target to control the disease by using poisons and inhibitors against this enzyme (Coelho *et al.*, 2016).

miR-30e-5p was up-regulated in submandibular lymph node and spleen at 3 dpi after infection with the virulent E75 strain, compared with the attenuated E75CV1. Target prediction indicated that miR-30e-5p can potentially regulate a great number of genes, and can be involved in many immunological pathways. Likewise, can be related with most of the pathways implicated in virus-cell interaction. miR-30e-5p and miR-125a are up and down-regulated, respectively, at 3 dpi after infection with the virulent E75 strain, compared with their expression in the attenuated E75CV1 infection in both analyzed tissues, spleen and SLN, indicating that both miRNAs can be related with the differences in virulence.

miR-122 was not expressed in spleen at 3 dpi of animal infected with the virulent E75. At 7 dpi, their expression was notably high. A small number of pathways related with immune response has been observed for this miRNA and it has only been associated with endocytosis pathway.

The most expressed miRNA in spleen, ssc-miR-451, is not involved in any pathway related to the regulation of the immune response while miR-145-5p, the second most expressed, is related to autophagy and protein processing in endoplasmic reticulum (RE). Curiously, both miRNAs are predicted to interact with viral gene EP1242L, which encodes for a DNA-dependent RNA polymerase involved in transcription (Yanez *et al.*, 1993) suggesting an important role of this viral gene with its regulatory miRNAs in virus-host interaction.

miR-92a is DE in spleen between early and late between the two types of strain which expression is decreased at 7 dpi.And it is involved in four common pathways related to the immune response: T cell receptor signaling pathway, Fc gamma R-mediated phagocytosis, chemokine signaling pathway and leukocyte transendothelial migration.

Thus, with all this analysis we can conclude that ASFV modifies the miRNAs expression pattern involved in the immune response and course of the disease during the infection.

From the gene network analysis we conclude that some miRNAs like miR-451 and miR-145-5p, which are the most represented DE miRNAs in spleen, and are highly up-regulated at 7 dpi, are associated with the viral gene 1242L. This gene, also regulated by miR-125a and miR-125b, is involved in RNA transcription and processing (Iyer *et al.*, 2006). ASFV genome transcription is carried out without using the host RNA polymerase II. The described gene function for this gene is RNA polymerase subunit 2 making it a candidate to explore as a target to regulate the viral replication.

The A179L is the homologous viral gene of the apoptosis inhibitor gen Bcl2 (Hernaez *et al.*, 2013). A179L, is not regulated by the three miRNAs that interact with Bcl2 (miR-23a, miR-125a and miR-125b), in spite of the inclusion of the highly conserved domains with the cellular Bcl2. A179L presence could constitute a mechanism to avoid this inhibition and participate in the apoptosis induced by ASFV.

miR-92a interacts with the three genes identified to be involved in ASFV entry, EEA1, EGFR and PIKFYVE (Cuesta-Geijo *et al.*, 2012, Sanchez *et al.*, 2012) both in the virulent infection at 3 and 7 dpi and between attenuated and virulent at 3 dpi. Interestingly, this miRNA was found in much higher levels (thirteen times) in spleen of the animal inoculated with the virulent strain at 3 dpi compared to spleens of animals inoculated with the attenuated strain. This difference of expression could be involved in the differences in the dynamics of virus infection depending on its virulence and also, this results concords with the recent study where the diminution of PIKFYVE decreases infectivity and viral production (Munoz-Moreno *et al.*, 2015).

Target prediction for miR-122 revealed that it interacts with 12 different viral genes classified in multigene family, replication, genes with unknown function and unknown genes. This miRNA interacts with MGF 360-16R and it has recently

described that this viral multigene family component modulates host innate responses by determining the tropism, virulence and suppression of type I IFN response (Munoz-Moreno *et al.*, 2015). In addition, miR-122, together with miR-126-5p, targets ATG6 which is activated by ASFV. The virus uses the ER as a site of replication and this process of activation of ATF6 can trigger ER stress and the unfolded protein response (UPR) of the host cell (Galindo *et al.*, 2012). This miRNA is the DE miRNA with the highest number of viral target gene and none of these are regulated by other miRNA. On the other hand, miR-122 is not express in spleen from an infected animal with the virulent E75 strain at 3 dpi, while at 7 dpi its expression was notably increased. In addition, it is well known that miR-122 play a key role in Hepatitis C virus infection (Luna *et al.*, 2015). Accordingly, this miRNA could be involved in the regulation of the "success of the ASFV infection".

To our knowledge, this is the first time that a deep sequencing approach has been used to study miRNA gene expression in pigs infected with ASFV. Although a higher number of sequences and animals should be necessary to assess a more accurate vision of the relation between miRNAs with porcine target genes and ASFV genes to decipher the role of miRNAs in the ASFV infection.Nevertheless we have been able to identify differentially expressed porcine miRNAs at different times post-infection, between different strains with differences in virulence, their target genes and the pathways related to the disease in which they are involved. This knowledge will contribute to clarify host- pathogen interactions, the mechanisms of the viral infection and the development of the disease.

5.4. African swine fever virus can not encode miRNAs in an experimental infection

In the last study, the capability of E75, Ba71 and E75CV1 isolates of ASFV to encode viral miRNAs by using highthroughput sequencing was explored. This is the first approach to identify if ASFV encodes miRNAs.

The genomic characteristics of ASFV fit with the requirements of a virus to be able to encode viral miRNAs because it is a DNA virus with a nuclear phase (Van Etten, 2009, Ballester *et al.*, 2011) and the *in silico* prediction carried out in this work found several potential hairpins to be virus- encoded miRNAs. In addition, 16 viral miRNAs have been identified in Singapore grouper iridovirus (SGIV) (Yan *et al.*, 2011) and in tiger frog virus (TFV) (Yuan *et al.*, 2016), both members of the *Iridoviridae* family, and also, it has been identified virus encoded miRNAs by ascovirus infecting mosquitoes (Hussain *et al.*, 2008). In this last case, a viral miRNA reduces DNA polymerase I levels by transcriptional degradation and modulates the ascovirus replication. All these viruses belong, as the asfivirus, to the considered monophyletic superfamily of nucleocytoplasmic large DNA virus (NCLDV). Taking all the above into account ASFV was a good candidate to become a virus capable of encoding miRNAs.

There is no clear knowledge about when viral miRNAs are expressed at highest levels after infection according to Cullen (Cullen, 2013), who showed the variability of the viral miRNAs expression with time among different viruses. Therefore, we have analyzed samples from infected animals at different times in addition to different viruses with different virulence and animals infected with an attenuated strain and re- inoculated with an heterologous virulent strain in order to increase the spectrum.

Highthroughput sequencing revealed nine reads that matched the viral genome with 100% identity. When <100% identity was allowed, to take into account the sequence variability among miRNAs (IsomiRs) (Neilsen *et al.*, 2012), thirty one reads

matched the viral genome. From the total of forty, twenty six candidates as potential viral miRNAs were obtained. These candidates were blasted against the Vmir hairpins prediction from E75 and Ba71 genomes. Also, Ba71V, a Vero highly adapted strain, has been included in the analysis in order to avoid the loss of candidates because of the possibility of genome variability in cell culture passages (Yanez *et al.*, 1995). However, it was observed that none of the candidates matched any of the predicted hairpins and had appropriate secondary structure and minimum free energy, showing that the candidates did not have the necessary characteristics to be identified as a viral miRNAs.

Therefore, we can conclude that, ASFV, in the experimental conditions that have been performed, does not encode viral miRNAs and can be added to the group of viruses that do not express miRNAs as Cowpox virus or PCV2 (Skalsky and Cullen, 2010, Nunez-Hernandez *et al.*, 2015). However, studies with higher number of reads, together with analysis of the presence of viral miRNAs in cell culture and other *in vivo* conditions should be necessary to discard if ASFV codifies miRNAs.

6

CONCLUSIONS

I. Porcine circovirus type 2 infection in pigs produces differences in porcine miRNA expression in mediastinal lymph node. The miRNAome in tonsil is less affected by the PCV2 infection.

II. From the eight miRNAs differentially expressed with the highest numbers of reads, miR-126-5p, miR-126-3p, let-7d-3p, miR-129a and let-7b-3p were up-regulated while miR-193a-5p, miR-574-5p and miR-34a were down-regulated. The target genes from these miRNAs were determined and their involvement in pathways related to immune response and pathogenesis of PCV2 were assessed.

III. PCV2 does not encode viral miRNAs in an in vivo experimental infection.

IV. A porcine miRNA, miR-29a-5p, shows similarity with PCV2 genome, which could be involved in virus- host interactions.

V. miR-451, miR-145-5p, miR-92a, miR-23a and miR-122 were differentially expressed in spleen and miR-126-3p and miR-126-5p in submandibular lymph node at different times in animals infected with a virulent ASFV.

VI. miR-125b, miR-92a and miR- 451 in spleen and miR-15a and miR-30e-5p in submandibular lymph node were differentially expressed in animals infected with two ASFV differing in virulence, E75 and E75CV1.

VII. Porcine miRNAs differentially expressed in ASFV infected animals in both conditions interact with genes involved in pathways related to immune system and to pathways related to pathogenesis of ASFV, showing their relevance in the course of the ASFV infection. miR-451 and miR-145-5p

interact with the viral gene EP1242L, the RNA polymerase subunit 2, and can regulate the viral replication. A179L, the viral gene homologous to BcL2, is not regulated by the differentially expressed miRNAs that interact with BcL2, participating in the apoptosis induced by ASFV.

VIII. ASFV does not express miRNAs in an in vivo experimental infection.

7

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