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Molecular characterization of factors which can influence the reproductive success of cattle

(SSD VET/01)

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
MARIE CURIE

Imagination is more important than knowledge.
ALBERT EINSTEIN

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ABSTRACT

My PhD project addressed the paternal and maternal contribution associated with correct early embryonic development, focusing on microRNAs (miRNAs, short non-coding RNAs regulating gene expression at post-transcriptional level) involved in controlling reproductive function in cattle. To this end, next generation sequencing (NGS) methods and bioinformatic analysis pipelines were set up and validated using low quantities of starting materials, *e.g.* blastocysts and oocytes. The role of miRNAs carried by spermatozoa during early embryogenesis was investigated by assessing *in vitro* produced blastocysts from semen of bulls with high and low blastocyst rates. Moreover, follicular fluid and oocyte miRNAs were analyzed in order to unravel molecular mechanisms involved in poor fertility cows. In particular, a model based on low and high antral follicle count ovaries was used to study the influence of ovarian function on oocyte developmental competence. The results indicated that: 1) sperm miRNAs may impact embryo developmental competence affecting the expression of gene networks involved in several cellular processes including cell adhesion, communication and metabolism of the blastocyst; 2) the different oocyte quality is associated with a different miRNA blueprint in both follicular fluids and oocytes through the mis-regulation of biological processes critical for oocyte competence. In conclusion, these studies contributed to improve our knowledge on the function of miRNAs in the sperm, oocytes and pre-implantation embryos. Several miRNAs were identified as potential biomarkers of blastocyst and oocyte competences, which will be valuable to optimize Reproductive Biotechnologies.

Chapter 1

General Introduction

For several decades, selection for production traits has been the main goal for cattle breeding. However, many functional traits, such us milk production, have negative correlation with genetic merit for health and fitness [1]. Indeed, milk production of the modern dairy cows has increased, but the fertility of these cows has steadily declined [2]. The reasons for this are difficult to understand because fertility is a complex trait affected by the environment, genetics and management. It is estimated that the calving rate in high producing cows is less than 40% and foetal loss is closer to 60%. Moreover, 70-80% of these losses occur during the pre-implantation period of embryonic growth, between days 8 and 16 [3, 4].

The PhD project was focused on the study of early stages of reproduction to unravel the molecular mechanisms associated with poor cow and bull fertility.

1.1. Advances in cattle reproductive biotechnologies: state of the art

The problem of bovine reproduction is closely linked to the livestock industry as breeding techniques have usually been motivated by curiosity and consolidated by breeder's needs and interests. Indeed, artificial insemination (AI) was initially developed as a hygiene measure to prevent disease transmission but is now widely used for genomic selection. The advent of embryo genotyping, in combination with Assisted Reproduction Techniques (ARTs) [5, 6], allows the reproduction of the desired genotype from selected parents. The close similarities between humans and livestock species, such as cattle, sheep and horses, have allowed the improvement of knowledge in ARTs, to be translated to humans. ARTs are dependent on two factors: the easy availability of both the gametes and pre-implantation embryos and the ethical requirements [7]. In the last 25 years new ARTs, which were

developed for cattle, have been transferred and adapted for other species, such as human, buffalo and horses. The most important of these are: (a) ovum pick up (OPU), which followed the establishment of reproducible techniques for *in vitro* maturation [8], fertilization [9,10] and culture of sheep [11] and cattle embryos [12], in OPU oocytes are collected from living donors of known genetic value; (b) intracytoplasmic sperm injection (ICSI), which is useful in cases of sperm-related male infertility but its efficiency in cattle is still not comparable to that in humans so it is usually only used for research purposes [13]; (c) nuclear transfer and somatic cell nuclear transfer (SCNT) widely used in cattle for cloning animals of great commercial interest [14]. In livestock species, the main objective for these reproductive biotechnologies is to give rise to *in vitro* produced (IVP) embryos, which are then transferred to recipients. According to Embryo Transfer Society statistics (Fig. 1), the number of embryo transfers (ET) has increased more than 10 times [15] in recent years and is now approaching the number of embryos produced *in vivo*. In conclusion, ARTs in cattle are becoming a consolidated reproductive biotechnology and, in particular, OPU is increasingly preferred for ET over conventional superovulation.

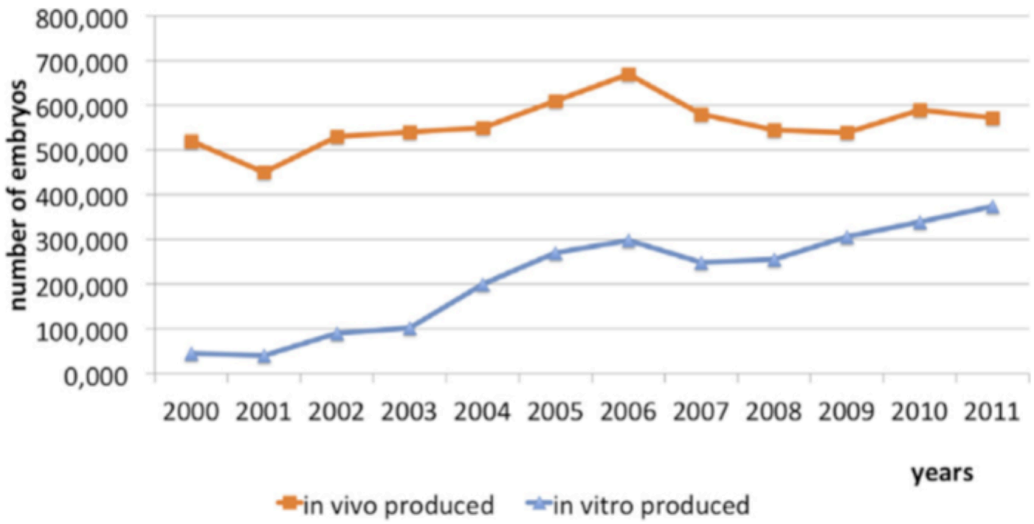

Figure 1. Summary of the International Embryo Transfer Society statistical data collected by the data retrieval committee. *In vitro* production of embryos is steadily increasing with the majority of them now being produced by ovum pick up (OPU) [16].

1.2. Overview of physiology of reproduction and pathways of differentiation of the gametes

The gonads are the primary reproductive organs responsable for producing both gametes and steroid hormones which control reproductive function. Normal gametogenesis in the gonads is a prerequisite for mammalian reproduction. This involves complex molecular mechanisms for cellular functions as well as the interaction of different gonadal cell types. Germ cells develop into oogonia (primitive eggs) or spermatogonia (stem cell precursors of sperm). Germ cells develop also in the supporting somatic cells, which are either the Sertoli cells and Leydig cells in the male or the granulosa cells and the theca cells in the female. A remarkable aspect of gametogenesis in the testis or ovary is that germ cells exhibit strictly regulated spatiotemporal gene expression for functional development of sperm or oocytes [17, 18]. Moreover, a large proportion of mRNAs are partially stored and translationally inactive in mammalian haploid germ cells [19, 20].

Spermatogenesis is initiated in the male testis with the beginning of puberty, where male primary germ cells undergo mitotic division and produce spermatogonia, from which the primary spermatocytes are derived. Primary spermatocytes undergo successive meiotic division producing four spermatids, and a metamorphic change (spermiogenesis) occurrs in the spermatids to produce spermatozoa. The molecular mechanisms, which regulate primary germ cells development, differentiation and exit from the mitotic cell cycle, are associated with a timed expression of meiotic genes. A number of mRNAs located in the nucleous are transcribed and stored for some days in spermatocytes without detectable protein expression [21]. The expression of meiotic genes which is required for initiation of meiotic processes during spermatogenesis and the expression of a multitude of testicular genes are known to be under control of post-transcriptional regulatory mechanisms [22-24].

Female reproduction is characterized by a dynamically regulated sequential recruitment, selection and growth of the follicles, atresia, ovulation, and luteolysis, which occur in the ovary and are dependent on precise expression and interaction of several intraovarian gene products in both an autocrine and paracrine manner. These mechanisms of follicular growth can be classified

into three phases: i) the gonadotropin-independent phase that involves follicular growth through primordial, primary, and secondary stages; ii) the gonadotropin-responsive phase that involves transition of preantral follicles to early antral stage, and iii) the gonadotropin-dependent phase that involves continual growth beyond the early antral stage and includes follicle recruitment, selection, and ovulation [25]. The differentiation of the primordial follicles into the diverse follicle developmental stages are initiated by primordial germ cells. These cells enter meiosis at birth and develop into mature oocytes capable of fertilization. They are nurtured, retained and subsequently developed in the ovary through a complex follicular developmental process on a cyclic basis [26, 27]. The growth and development of mammalian oocytes is critically dependent on a bidirectional communication between the oocyte and its companion somatic cells [28]. The viability of primordial and primary follicles is determined mainly by survival factors derived from the oocyte, whereas the relative expression level of tumor suppressors, apoptotic proteins, and survival factors in granulosa cells determines whether an ovarian follicle will grow or undergo atresia in the late pre-antral stage [29].

The development of the sexual organs and gametes, i.e. oocyte and spermatozoa, is characterized by a substantial reorganization of their transcriptome. Understanding the regulation of gene expression at the level of ribonucleic acids (RNAs) in gametes and early embryos has improved as the technology available has advanced. In particular, the discovery of the microRNAs (miRNAs) in 1993 by Ambros and colleagues [30] demonstrated that small non-coding RNAs have regulatory roles and impact on cellular phenotype and function. Several studies have accumulated evidence on the expression of miRNAs and their biogenesis pathway genes in germ cells and gonadal somatic cells [31-35], which suggest the potential involvement of miRNAs in translational control during reproductive function. The improvement of our knowledge of, in particular miRNAs, is revealing numerous pathways and mechanisms which may impact on fertility.

1.3. Small RNAs: classes and biogenesis in mammalian

Small non-coding RNAs are post-transcriptional regulators of gene expression, which range in size from 18 to 32 nucleotides (nt). They are generally divided into three functional classes: microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and Piwi-interacting RNAs (piRNAs) [37-41]. These classes differ in their biogenesis, *i.e.* their maturation from the primary transcription product to the active form (Fig. 1).

MiRNAs can be divided into two sub-classes: canonical and non-canonical groups. Canonical miRNAs are initially transcribed as long RNAs containing self-complementary hairpins, also called pri-miRNAs (Fig. 1A). During maturation the 60-75 nucleotide hairpin structures are bound by the RNA-binding protein Dgcr8 (*Di George syndrome critical region 8*), which directs the RNase III enzyme Drosha to cleave the base of the hairpin [42-47]. Following cleavage mediated by Drosha-Dgcr8 complex, also called the microprocessor, the released hairpin (pre-miRNAs) is transported to the cytoplasm, where Dicer, another RNase III enzyme, then cleaves it into a single short 18-25 nt dsRNA [48-51]. Non-canonical miRNAs bypass processing of the microprocessor or Dicer [37, 52] using other endonucleases or directing transcription of a short hairpin. For example, mirtrons, which are miRNAs matured from introns, come from mRNA splicing [53-55]. After splicing from host mRNAs, the lariat is de-branched and refolds into a short stem–loop structure that resembles as a pre-miRNA. Some mirtrons have also extra sequences at the 5'- or 3'- end and are trimmed by exonucleases [56]. In other case, endogenous short hairpin RNAs are generated directly through transcription [37, 52] and, in particular, although these endogenous short hairpin RNAs were initially thought to be transcribed by RNA Polymerase III, it has been recently shown that some of them, for example mir-320, are transcribed by RNA Polymerase II [57]. Another important example is that of miR-145, which is produced in a dicer-indipendent manner. This miRNA requires the catalytic activity of AGO2 [58-60]. Pri-miR-145 cleavage is first mediated by Drosha, which gives rise to a 18 bp hairpin. This hairpin is too short to be bound and processed by Dicer, thus, AGO2 slices pri-miR-145 in the middle of the 3' strand. Then ribonuclease PARN trims

down the 3'- end and produces the mature miRNA [61]. The existence of alternative pathways reflects the evolutionary flexibility of miRNA biogenesis. However, it is notable that the vast majority of functional miRNAs follow the canonical pathways for their biogenesis, and that only about 1% of conserved miRNAs, for example, miR-320 and miR-451, are produced independently of Dicer or Drosha in vertebrates. Most other non-canonical miRNAs are low in abundance and poorly conserved. Thus, the functional relevance of non-canonical miRNAs should be interpreted with caution. Indeed, many miRNAs are conserved among different species while some are species-specific [62-66].

In contrast, siRNAs, which are also 18-25 nt in length and derive from long dsRNAs (Fig. 1B) in the form of either sense or antisense RNA pairs or as long hairpins, are directly processed by Dicer to produce multiple functional siRNAs from the same primary transcript [67-72]. In summary, canonical, non-canonical miRNAs and endo-siRNAs involve generally Dicer processing and in the mature form are 18-25 nucleotides in length.

The piRNAs have a different biogenesis: they are processed by a different mechanism, which is not mediated by Dicer [73, 74]. Indeed, how piRNAs are produced and their modes of action are not fully understood [75]. The piRNAs are 25-32 nt in length and are expressed abundantly in the mammalian gametes [76-78]. These kind of small RNAs interact with a distinct family of Argonaute proteins, which are the Piwi proteins and include Miwi, Miwi2 and Mili, which in mouse are also known as Piwil1, Piwil4 and Piwil2, respectively. Although the mechanism of piRNA biogenesis has not been fully resolved, they are generated from long single-stranded RNA precursors that are often encoded by complex and repetitive intergenic sequences. One proposed model for their biogenesis is the '*ping-pong mechanism*' (Fig. 1C) [79-96]. In this model, the Argonaute protein Mili cleaves the primary piRNA to define the 5'-end of piRNAs, which is subsequently bound by Miwi2. Miwi2 cleaves the other strand of the precursor, thereby generating a 5'-end of the piRNA that can bind to Mili, thus forming a positive amplification loop. Many of the details of this model remain to be uncovered. Furthermore, the ping-pong model is likely to explain the biogenesis of only a subset of mammalian piRNAs, those that are derived from repetitive sequences, such as transposons, and those

that are associated with Miwi2 and Mili. These piRNAs are involved in the early stages of spermatogenesis. The mechanism of biogenesis of piRNAs derived from complex intergenic sequences, associated with Miwi2 and Mili, which function in the later stages of spermatogenesis, is not known.

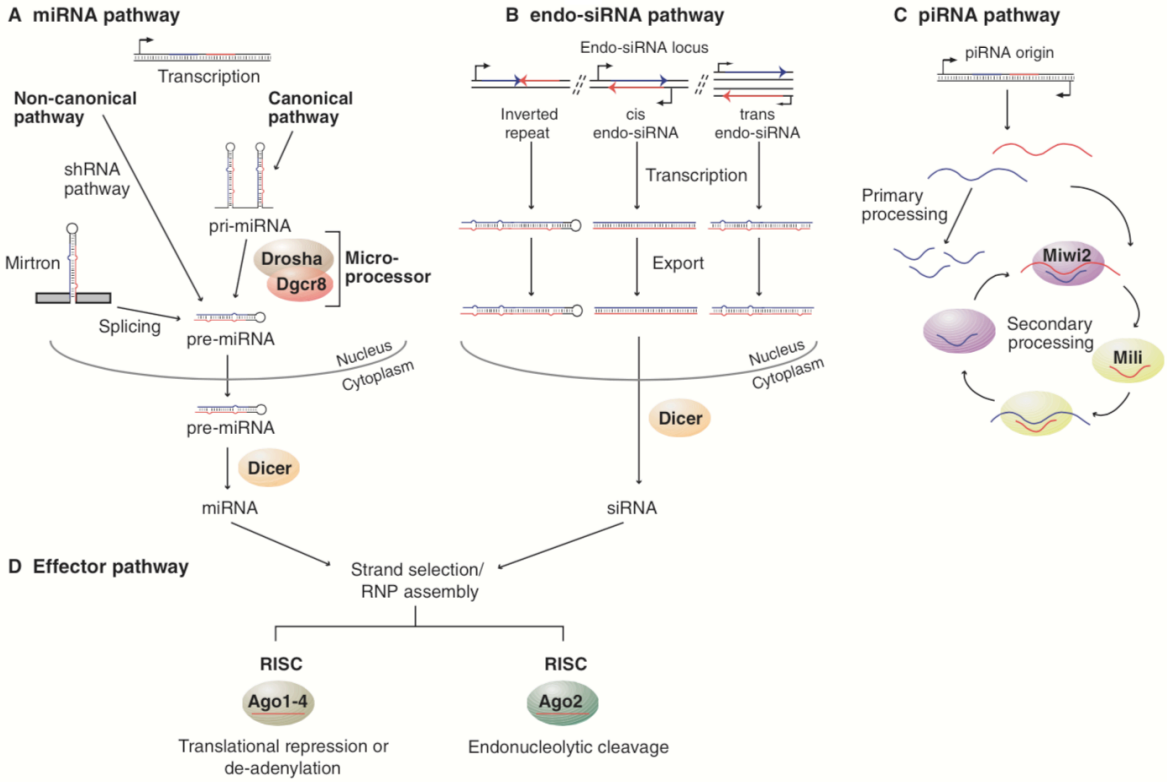

Figure 1. Mechanisms of biogenesis of the different small RNA classes [82].

1.4. Mechanism of action of small RNAs

1.4.1. MiRNAs and endo-siRNAs act in the cytoplasm

Mature miRNAs and endo-siRNAs interact with Argonaute proteins (AGO 1–4 and AGO2 in mammals; also known as EIF2C1-4) forming the RNA-induced silencing complex, RISC (Fig. 1D) [83]. RISC interacts with target mRNA using the complementarity between the miRNA or endo-siRNA sequence and the 3'-UTR of the mRNA target. If complete complementarity exists, cleavage of the target mRNA can occur through AGO2, which has an endonucleolitic activity. If there is incomplete complementarity, the primary mechanism of post-transcriptional gene regulation (PTGR) occurs blocking translation

through AGO 1-4, which have translational repression and de-adenylation activities [84-86]. However, PTGR can act via mRNA degradation even if the complementarity is not complete between the small RNA and 3'-UTR of the target mRNA [87].

The cellular location, where miRNAs and endo-siRNAs act, could be the Processing bodies (P-bodies). P-bodies are discrete cytoplasmic foci that contain proteins involved in mRNA degradation. They are involved in several post-transcriptional processes: mRNA decay, translational repression, non-sense-mediated mRNA decay and RNAi-mediated repression. All four Ago proteins [88-90], GW182 [88] and two RNA helicases RCK/p54 [91] and MOV10 [92] have been found in P-bodies, suggesting that miRNA suppression is localized to the P-body. However, it has also been suggested that P-body formation is a consequence rather than the cause of miRNA-mediated gene silencing, because when siRNA or miRNA silencing pathways are blocked, P-bodies are not formed [93].

1.4.2. PiRNA function is in the nuclear and/or perinuclear region

PiRNAs can act only after their association with PIWI proteins. These small RNAs and PIWI proteins are located in the nucleus or perinuclear region [80, 94, 95], where they repress genetic elements, mainly transposons [61]. PiRNAs link the N-terminal PAZ (Piwi/Argonaute/Zwille) and MID (middle) domains of the Piwi protiens, which together recognize and bind the 3'- end of piRNAs [97]. The C-terminal domain of PIWI proteins has RNase H activity, which is capable of recognizing the 5'- end of piRNAs and facilitates the cleavage of the target sequence [95, 98]. In gametes, the PIWI-piRNA complex acts on transposons before or after their transcription. When they act at transcription level, the mechanism of action can be through chromatin silencing of transposable elements via histone modification or altering the DNA epigenetic status [99, 100]. While post-trascription repression is achieved by cleaving trasposons through the PIWI-piRNA complexes, thereby producing secondary piRNA through the '*ping-pong model*' described above [80] (see 1.1.). This mechanism of action is supported by the observation that transposons are expressed at a higher level in the presence of PIWI protein

mutations [101]. In Drosophila, piRNAs are involved in telomere function, including telomere protection complex assembly, thereby maintaining chromosome integrity [102]. Expression of PIWI proteins in human somatic stem cells [103] and neoplastic cells [104-107] suggests that piRNA may also be involved in regulating stem cell function and carcinogenesis. However, the range of mechanisms and pathways regulated by the Piwi-piRNA complex remains unclear.

1.5. Small RNAs in reproductive function

MiRNAs are involved in both female and male reproduction. They are regulated by a paracrine or autocrine signalling, and are produced by a wide array of cells including oocytes, embryos, endometrial and granulosa cells [108, 109]. Moreover, they are found in biofluids, such as plasma and serum or follicular ovarian fluid as freely circulating stable molecules [110] or enclosed in exosomes [111, 112]. The extracellular miRNAs may be taken up by specific cells of endometrium, placenta or ovarian follicles, where they bind to their target mRNAs, repressing their translation and modulating cellular events and functions [110, 199, 200].

1.5.1. Small RNAs and ovarian function

1.5.1.1. Folliculogenesis

Follicle development is a highly orchestrated cyclic process that depends on gonadotropin action. Folliculogenesis starts with the activation of resting follicles and gradually leads to the growth and development of a pre-ovulatory follicle. The follicle development is accompanied by the sequential differentiation of oocytes and their surrounding somatic cells, which form the granulosa and theca layers [113].

In cattle, recruitment of follicles (primordial and primary follicles, <2 mm in diameter), selection and growth of leading follicles (small and mid-antral follicles, 2-8 mm in diameter), ovulation of the pre-ovulatory dominant follicle (large antral follicle, >8 mm in diameter) and degeneration of an-ovulatory subordinate follicles (Follicular atresia) takes place in a wave-like progression, with typically 2 or 3 follicular waves per oestrous cycle [114, 115] (Figure 2).

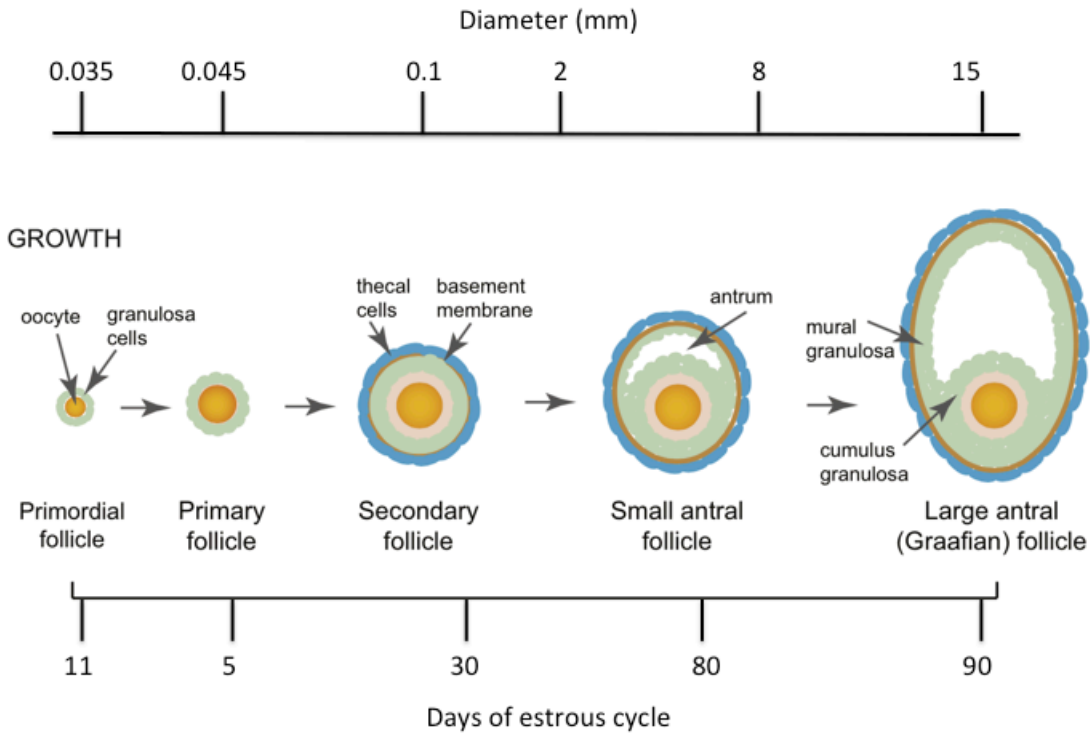

Figure 2. Folliculogenesis in cattle [116].

In several species folliculogenesis has been shown to be post-transcriptional regulated by small RNAs [117-119] and a particular interest is focused on the role of miRNAs. Cellular differentiation processes, which occur during follicular development, seem to be regulated by the expression and interaction of many miRNAs in a spatio-temporal manner in different follicle compartments: granulosa cells [120, 121], theca cells [122], follicular fluid and oocyte [123].

Folliculogenesis may be coordinated by individual miRNAs, which regulate ovarian steroid hormones, by targeting hormone receptors as well as affecting hormone biosynthesis and release. There are several findings on Estradiol (E2), which is important in inducing ovarian follicle development and its production is correlated to aromatase. In particular, miR-378 is positively linked to aromatase expression and E2 synthesis in granulosa cells [124]. Moreover, miR-133b stimulates ovarian oestradiol synthesis by targeting Foxl2, which mediates the transcriptional repression of StAR and CYP19A1 to promote E2 biosynthesis [125]. Also miR-383 promotes E2 byosintesis in ovarian granulosa cells. It inhibits RNA binding motif single-stranded interacting protein 1 (RBMS1) altering its mRNA stability and leading to the inactivation of c-Myc, which induces steroidogenesis in these cells [126].

Finally, miR-423-5p and miR-378 regulate E2 synthesis by targeting CYP19A1 mRNA and repressing CYP19A1 protein content and enzyme activity in newborn piglets [127].

In addition to the expression of individual miRNAs, there are subsets of miRNAs, such as miR-183 cluster and miR-132 cluster, which are organized in genomic groups and are differentially expressed during different phases of folliculogenesis by specific cells of follicle. This indicates they have a functional role during follicular development in granulosa cells [1118]. However, only a small set of miRNAs among a large number of expressed miRNAs is found to be specific for follicle stages, while majority of the miRNAs (>80%) are expressed at all stages [1118]. This indicates that commonly expressed miRNAs may play a role in maintaining normal physiological ovarian function during all the follicular phases of the oestrus cycle. Information on the stage specific miRNAs may help to decipher the molecular mechanism of follicular development, ovulation and atresia.

Finally, follicular fluid miRNAs have been recently identified in cattle. In particular, their profiles can be different between developing and mature oocytes. Moreover, differences can be associated also with several miRNAs, which are present as freely and exosome-vehiculated forms [110]. Those miRNAs present at the different stages may be associated with the growth status of oocyte and may act as regulators of oocyte developmental competence by facilitating cell-to-cell communication in the follicular environment [110].

1.5.1.2. Oocyte development and maturation: the role of small RNAs

Mammals are born with a finite oocyte number that originates from the primordial germ cells during embryonic development [128] (table 1). In particular, in mammals the developmental process begins during oogenesis when both maternal RNAs and proteins accumulate as the oocyte grows and matures [129]. Oogenesis is reliant on a dynamic gene regulatory network that includes oocyte-specific transcriptional regulators [130]. A hallmark of the oocyte is its high level of transcription, which is driven on maternal mRNAs, and proteins that are crucial for the early development of the newly fertilised zygote [131]. The early stages of embryogenesis are, therefore, regulated by

maternally inherited components stored within the oocyte. As development proceeds the process of early embryogenesis then becomes dependent on the expression of genetic information from the embryo [129].

Table 1. Maximum number of female germ cells reached in foetal ovaries at the time of birth or late gestation in different species [201].

Species	**Maximum number of germ cells (Day of pregnacy)**	**Number of germ cells close after birth (Day after birth)**
Bovine	2,700,000 (110)	68,000(13 day after birth)
Pig	1,100,000 (50)	500,000 (at birth)
Buffalo	23,540 (210)	20,000 (at birth)
Rat	75,000 (18)	27,000 (2 days after birth)
Human	6,800,000 (150)	2,000,000 (at birth)

Bovine oocyte maturation involves the resumption and completion of the first meiotic division from germinal vesicle (GV) stage to metaphase II (MII) with corresponding cytoplasmic maturation. Storage of mRNA during oocyte maturation and its timely availability during early embryo development are essential for oocyte quality and developmental competence. There is differential expression of transcripts in *in vitro* matured and immature bovine oocytes, which underlines the tight temporal control of protein synthesis required for oocyte maturation in preparation for subsequent fertilisation and early embryo development [132]. As with mRNA, miRNA expression in mouse shows a dynamic change during oogenesis, where a large proportion of maternal genes directly or indirectly under control of miRNAs [133].

MiRNA, siRNA and piRNA are expressed in oocytes of many species at different stages of development [76, 124, 134-138]. However, only siRNAs seem to have a critical role in oocyte maturation. This has been inferred by comparing the knockout phenotypes of Dicer and Dgcr8 mutant mice. Dicer knockout in the oocyte resulted in meiotic arrest with severe spindle and chromosomal segregation defects and loss of both miRNAs and endo-

siRNAs, as they are usually processed by Dicer [139-141]. In contrast, Dgcr8 knockout has not observable phenotype, and mRNA levels remain unchanged even though the oocytes are characterized by the loss of miRNAs [140]. These findings suggest that endo-siRNAs, and not miRNAs, underlie the meiotic defect of Dicer knockout oocytes. Reporter assays using Dgcr8 knock out mice oocytes have shown siRNA activity in mature oocytes, but little to no miRNA function [142]. Finally, miRNA function is suppressed in fully-grown oocytes although miRNA biogenesis is unaffected and miRNA targets are present (Figure 3). The mechanism of suppression is unknown. P-bodies seem to be correlated with miRNA destabilization. This hypothesis is supported by the loss of P-bodies in maturing oocytes followed by their resumption at the blastocyst stage [143-146]. PiRNAs are also expressed in mouse oocytes [137], but deletion of the Piwi proteins does not produce an observable oocyte phenotype [93, 146, 147]. Therefore, it is unclear whether they play a role in oogenesis.

1.5.1.3. Implications for female fertility

Increasing evidences on the role of miRNAs during female reproduction have allowed the association of these molecules to several diseases [149-150], which generally affect oocyte developmental competence. However, the underlying mechanism of developmental competence has not been yet identified and a crucial part of this process is certainly the latter phase of oocyte growth, in which developmental potential is gained. This is known in mice [151], humans [152] and cattle [153]. While the building process is now better understood, we are still far from a clear blueprint of what composes a competent oocyte.

Polycystic ovarian syndrome (PCOS)

Polycystic ovarian syndrome (PCOS) is an anovulatory disorder, which is characterized by the development of ovarian cysts and affects women of all reproductive age [154]. The underlying mechanisms resulting in PCOS are not understood, however, the expression of several miRNAs are characteristic of PCOS patients [155]. Using microarray and quantitative PCR, five (let-7i-3p, miR-5706, miR-4463, miR-3665, miR-638) miRNAs were found to be

overexpressed in blood of women with PCOS compared to healthy controls, while four (miR-124-3p, miR-128, miR- 29a-3p, let-7c) were underexpressed [156]. When deep sequencing was used to profile exosomes from the follicular fluid of women with PCOS, miR-132 and miR-320 were found to be less abundant in the follicular fluid compared to controls [157]. These authors also demonstrate that miR-132 and miR-320 stimulated production of estradiol (E2) in a human granulosa-like tumor cell line, while inhibition of these miRNAs supressed E2 production.

In addition to variations in serum and follicular miRNA populations associated with PCOS, differences are also observed miRNA in the developing embryos in these women, which could have an impact on oocyte developmental competence. Blastocysts derived from oocytes obtained from PCOS patients have an under-regulated subset of miRNAs, like let-7a, miR-19a, miR-19b, miR-24, miR-92, and miR-93 [158], which can compromise embryo development and, thus, fertility.

Premature ovarian failure (POF)

Premature ovarian failure (POF) is a disorder with multifactorial origin, which affects ovarian function in women under 40 years of age. The condition is characterized by early ovarian senescence and diminished antral follicle count (AFC) [159]. Several studies have identified alterations in miRNA levels of women with POF. Interestingly, these studies focused on circulating miRNAs in plasma and serum. MiR-22 plasma levels were reduced in women with POF compared with control women. Under-regulation of miR-22 was also correlated with a lower AFC [160]. Moreover, the miRNAs circulating in plasma, which are associated with POF, have important roles in regulating many signalling pathways. MiR-23a, which was abundant in the plasma of POF patients inhibits XIAP and caspase-3 expression, resulting in apoptosis in human granulosa cells [161]. These results indicate that circulating miRNAs can be potentially used as non-invasive biomarkers of POF.

Interestingly, Single-nucleotide polymorphisms (SNPs) are found in genomic DNA coding for miRNAs, which have been associated with disease susceptibility. A study of miRNA polymorphisms identified an association between combined genotypes in the genome coding for miR-146aC>G, miR-

196a2T>C, and miR-499A>G and POF in women. Therefore transcriptional changes in miR-146a and miR-196a2 induced by miRNA SNPs may be involved in POF development [162].

The study of miRNAs in the follicular fluid and oocyte could improve the missing knowledge on the role of miRNAs in the acquisition of oocyte developmental competence.

1.5.2. Male reproduction and small RNAs

Spermatogenesis is a process where spermatogonia differentiate into motile spermatozoa within the seminiferous epithelium of the testis. During the course of differentiation numerous mRNAs are regulated post-transcriptionally [104]. A repertoire of small RNAs is present in differentiating male gametes throughout spermatogenesis [163-166]. Among these, the miRNAs are abundant in male gametes at times of active gene transcription in meiotic stages, specifically pachytene, as well as in early spermatids prior to nuclear silencing. They are proposed to play roles in post-transcriptional silencing of genes during process of spermatogenesis (Figure 3) [167, 168]. The functional role of both miRNAs and endo-siRNA is demonstrated but the deletion of Dicer results in a loss of mature sperm [163, 169]. This could be attributed to the loss of either miRNAs or endo-siRNAs. However, the deletion of Ago2, a protein that is essential for cleavage of mRNA targets by endo-siRNAs, has not obvious testis phenotype, suggesting that the regulation of spermatogenesis is miRNA based [163]. Furthermore, RNA-binding protein Dead end 1 (DND1), which is implicated in preventing miRNA access to cell cycle-related target mRNAs, has been shown to be essential for male gamete development during embryogenesis [170, 171]. The roles of individual miRNAs during spermatogenesis have also been investigated, for example, miR-122a regulates the expression of TNP2, which is a testis-specific gene involved in chromatin remodelling during spermatogenesis [164].

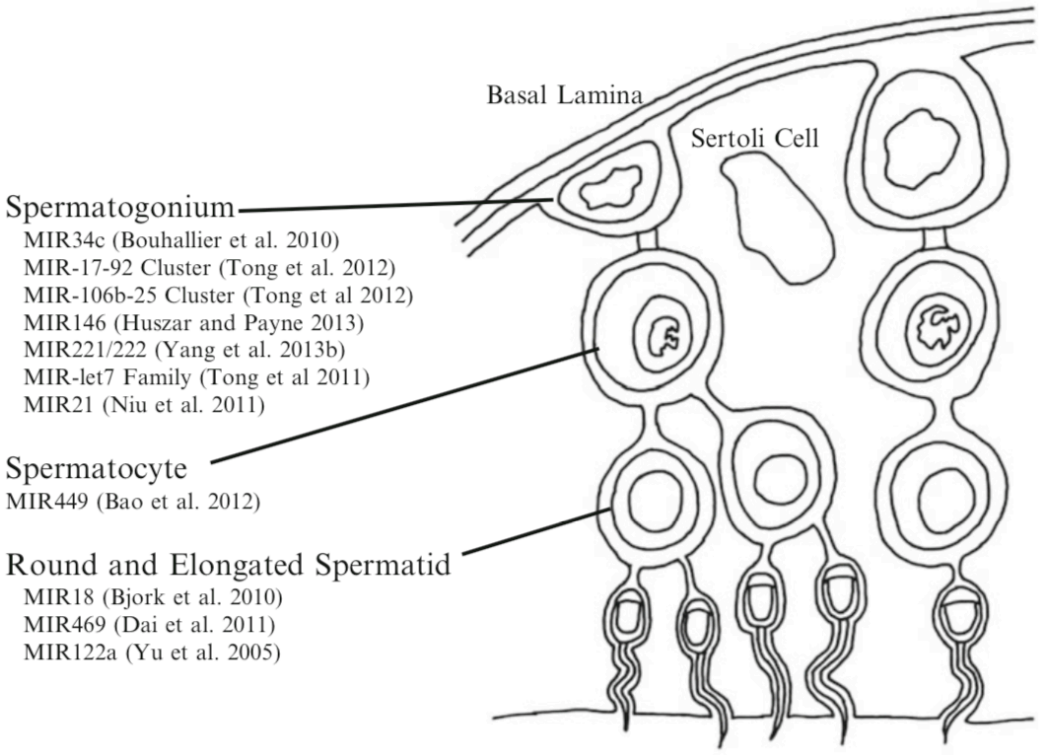

Figure 3. MicroRNA associated with different stages of spermatogenesis [125, 164, 172-174].

PiRNAs are found at relatively high levels in spermatocytes. Their expression begins at the pachytene stage of meiosis and persists until after the spermatid stage, when they gradually disappear during spermatid differentiation [79, 175]. In mouse, piRNAs have been separated into two classes based on the timing of their expression, their repetitive versus non-repetitive nature, and the Piwi proteins with which they are associated [76-78, 175,176]. The first class is highly repetitive and is expressed before meiotic pachytene. This class of piRNAs interacts with Mili and Miwi2 protiens [79, 177]. The second class of piRNAs is non-repetitive, becomes abundant during the pachytene stage and is also associated with Mili and Miwi proteins [79, 175, 177]. Deletion of Mili and Miwi2 results in early arrest of meiosis in meiosis I (at the primary spermatocyte stage), whereas deletion of Miwi results in arrest following meiosis II (the round spermatid stage) [94, 99, 146]. The functional role of both repetitive and non-repetitive piRNAs has to be clarified.

1.5.2.1. MiRNAs and motile spermatozoa: a repertoire for early embryonic development

While a large number of miRNAs are found in mature spermatozoa in testes [178], most of the RNAs with computationally predicted 3′UTR targets are absent [179]. This suggests that the role of the miRNAs present in the spermatozoa is in the oocyte at or following fertilization. They can act as signals for early embryonic histone replacement [180] or affect epigenetic modifications, or may regulate gene expression during early embryonic development, either via epigenetic modifications or at the level of post-transcriptional control. The role of miRNAs in histone modifications is supported by finding that more than 10% of all small RNAs are located to histone-enriched transcription start sites (TSS) and promoter regions [163].

How miRNAs carried by spermatozoa impact embryogenesis can also be investigated at the level of individual miRNAs. For example, one of the most abundant miRNA carried by spermatozoa is human miR-34c [179, 181], which has also been identified in sperm of horses and mice [65, 66]. MiR-34c has been shown to be essential for the first cleavage division in mouse zygotes [34]. The mechanism of action and functional role in spermatogenesis and fertility of miR-34c remain to be fully clarified [63, 64, 66, 183]. In mouse testes, miR-34c expression is p53 independent [165], whereas miR-34c targets p53 in cancer cells [184]. This suggests an influence on growth [185, 186]. Furthermore, miR-34c has been found in bovine spermatozoa, oocyte and early embryos, suggesting a paternal contribution of this miRNA [187].

Spermatozoa also contain several intact miRNA precursors (pri-miRNAs, 100 –150 nucleotides). Since the zygote has the capacity to process immature miRNAs [182], the potential role of the pri-miRNAs requires consideration, *e.g.* pri-miRNA-181c is the most abundant immature miRNA in human spermatozoa.

1.5.2.2. MiRNAs as biomarkers of male fertility

Infertility is estimated to affect 15% of the couples worldwide and male infertility is expected to be responsible for 50% of this [188]. Generally, male infertility is accompanied by qualitative and quantitative abnormalities in spermatozoa [189, 190]. When the problem in fertility is associated to alteration in morphology of spermatozoa, such us normozoospermia, asthenozoospermia and oligoasthenozoospermia, it is possible to identify different miRNA profiles between fertile and infertile men [191]. Regulation of function can be exerted by low levels of specific miRNAs, such as miR-34c, which is very abundant in spermatozoa and is under-regulated in oligoasthenozoospermia compared to normozoospermia. Moreover, alterations in miRNA profiles can be demonstrated by a subset of miRNAs. A panel of five miRNAs, miR-34b*, miR-34b, miR-34c, miR-429, and miR-122, has been proposed as potential biomarkers for the diagnosis and assessment of male subfertility. The power to discriminate between affected and normal men was assessed using these five miRNAs with the support vector machine classification (SVM) analysis, which is method performing classification tasks by constructing hyperplanes in a multidimensional space. In this case, the panel of miRNAs was capable of discriminating between individual with subfertility from normal subjects with reliability of 98.7 %, specificity of 98.8 %, and sensitivity of 98.4 % [192].

Bovine spermatozoa are also rich in miRNAs, which have different expression levels in bulls with pronounced differences in Non-Return Rates (NRR), implicating these miRNAs in differences in fertility. Seven miRNAs were identified as differentially expressed in spermatozoa from high and moderate fertility bulls, which suggests that miRNAs may play important roles in mammalian gametogenesis and early development [64]. Information derived from miRNAs in spermatozoa could be considered as biomarker for male fertility [193].

MiRNAs in spermatozoa may influence gene expression in the embryo post fertilization and impact on development. Thus, it is important to focus on how the differences in the miRNA blueprint are associated to high and low fertility and the way this may affect developmental potential of early embryo.

1.5.3. New insights in gametes small RNAs: from fertilization to early embryogenesis

A considerable number of small RNAs are present in oocytes, many of which can be associated to dynamic expression profiles during oogenesis and folliculogenesis. Indeed, expression of miRNAs generally increases during oocyte maturation and embryo development. Conversely, there are miRNAs, which are abundant in the immature oocyte and diminish throughout maturation, while others remain relatively stable [136]. The expression profiles of small RNAs, particularly miRNAs, throughout gamete maturation and early embryogenesis strongly implicate them in the timely regulation of embryonic gene expression. There is an inverse correlation between piRNAs and miRNAs, suggesting a different role of these small RNAs in the reproductive function (Figure 4).

MiRNAs in spermatozoa could influence gene expression in the embryo post fertilization. Paternal contributions to the zygote and its developmental competence are increasingly recognized as important element of successful fertilization. Sperm are known to deliver proteins, RNAs and small RNAs, which are critical for embryo development [194]. Moreover, some RNAs and small RNAs present in spermatozoa and early embryos are absent from non-fertilized mature oocytes (MII oocytes), suggesting a unique role for these RNAs in post-fertilization mechanisms [195].

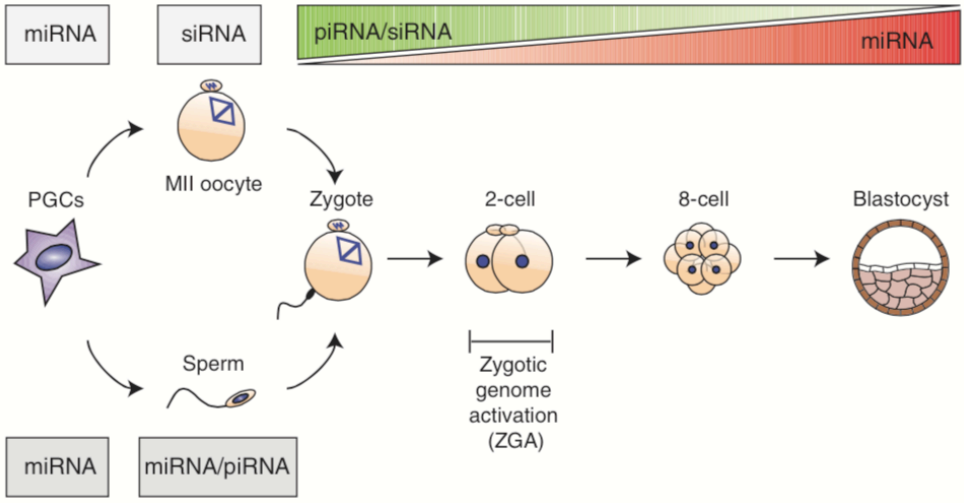

Figure 4. Piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs) are essential in the developing male germline, whereas endogenous small interfering RNAs (endo-siRNAs) play their most crucial role in oocyte maturation. There is a transition from

endo-siRNAs or piRNAs to miRNAs during pre-implantation development. PGCs indicates primordial germ cells [82].

The small RNAs may be responsible for the transmission of information across generations. Small non-coding RNAs that, are produced in gametes and which may be transmitted to progeny, may be influenced by, for example, environment or diet [116, 196]. Exposure of adult males and females to poor environments may result in adverse effects in their progeny. Such effects have been linked to epigenetic variations in spermatozoa and oocytes [197, 198]. This indicates that small RNAs, which are present in both spermatozoa and oocytes at the time of fertilization, have a critical role, not only on reproductive function, but also on the phenotype of the offspring [116, 196].
To unravel the mechanisms underlying the transmission of epigenetic information detailed studies of small RNAs will need to be carried out in developing and mature gametes of individuals exposed to different environments, as well as early embryos and somatic tissues in the next generation.

In conclusion, our knowledge of miRNAs with respect to reproduction and fertility is increasing rapidly. Further studies of miRNAs will contribute to understanding the molecular mechanisms important in regulating sperm and oocyte maturation and may identify potential biomarkers of quality for gametes and pre-implantation embryos.

1.6. References

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AIMS OF THE THESIS

The following aims were addressed using a deep sequencing protocol which was set up during the PhD program. The protocol, which is based on high-throughput approach, facilitated the study of both the known and putative novel bovine sequences in a sample. These data were used to look at the whole gene networks regulated by miRNAs and provide insights into cellular metabolism and complex biological systems involved in gamete and embryo development. The main objectives of the PhD were:

1) to use the small RNA sequencing methods and bioinformatic analysis pipelines exploring *in vitro* produced blastocysts. This increased knowledge of the bovine miRNAs *repertoire* and improved our understanding of the role of miRNAs in pre-implantation embryos.

2) to study the influence of miRNAs derived from spermatozoa on embryo developmental potential. In particular, to study *in vitro* produced embryos fertilized with sperm associated to high and low blastocyst rates.

3) to examine molecular mechanisms of poor cow fertility using oocytes from ovaries with high and low function.

CHAPTER 2

Profiling of microRNAs in bovine blastocysts using deep sequencing

2.1. Introduction

Knowledge of the key factors regulating pluripotency and differentiation during pre-implantation embryo development is essential to optimize *in vitro* embryo production (IVP) protocols and evaluate the developmental competence of the IVP embryos. Producing good quality blastocysts is essential for Artificial Reproductive Rechniques (ARTs) in humans [1] and economically important animals including ruminants [2, 3]. The number of bovine embryos produced *in vitro* and transferred into cow recipients has increased more than 10 fold in the last dozen years [4], which indicates that IVP is considered a reliable and cost-effective technique, and is increasingly being used in cattle breeding to accelerate the rate of genetic gain.

MicroRNAs (miRNA) are short non-coding RNAs of 20-25 nucleotides, that have been shown to be important in control of, amongst others, reproductive functions, such as oocyte maturation [5, 6], implantation [7] and early embryonic development [8, 9]. They are regulated by a paracrine or autocrine signaling and are produced by cells, *e.g.* of the embryo, endometrium, and granulosa cells and oocytes [10, 11]. MiRNAs are found in biofluids such as plasma or follicular ovarian fluid as circulating stable forms [12] or in exosomes [13, 14]. Extracellular miRNAs may be taken up by specific cells in tissues like endometria, placenta or ovarian follicles, where they bind to their target mRNAs, repressing their translation and modulating cellular events and and functions [12, 72, 73]. MiRNAs of presumed placental origin have been detected in the plasma of pregnant females in several mammal species [15, 16]. However, miRNAs are present in all biofluids and this characteristic has prompted their study as potential non-invasive biomarkers for physiological and pathological processes [16, 17]. In some pathologies associated with

reproduction, such as polycystic ovary syndrome or male factor infertility, miRNA profiles are altered [7, 18]. Several study have focused on the analysis of miRNAs in bovine blastocyst but their role during the early stage of embryo development is not well understood [19-21]. Recently, the presence of miRNAs secreted into culture media by embryos during IVP has been associated with embryo quality or developmental competence [22, 23]. Biomarkers that have been developed to assess embryo developmental competence are generally based on proteins and/or variations in the expression of mRNA [24, 25].

Advances in technology for deep sequencing has made possible to analyze all the miRNAs present in the sample. However, the very low quantities of RNA present in 100-150 cells, which compose a blastocyst, make miRNA profiling of early embryos technically challenging. This work describes the use of 3 procedures, which aim to optimize a low input deep sequencing approach for the analysis of all miRNAs present in bovine blastocysts and investigates the molecular mechanisms, which characterize the early stage of embryo development that are regulated by miRNAs.

2.2. Materials and methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (Milan, Italy).

2.2.1. Oocyte collection and *in vitro* maturation

Bovine ovaries were collected post slaughter and transported to the laboratory in PBS at physiological temperature (about 37°C). Cumulus oocyte complexes (COCs) were isolated from mid-antral follicles (2-6 mm) within 2 hr of collection, washed 4 times in modified PBS (supplemented with 36 µg/L pyruvate, gentamycin (50ug/mL), and 0.5 mg BSA/mL, sigma, fraction V, A-9647) and once in maturation medium (TCM199 + 10 µg/mL epidermal growth factor (EGF) + 10 % (v/v) fetal calf serum (FCS)). COCs were matured in group of 50 per well for 24 hours (h) at 38.8° C with an atmosphere of 5% CO_2 in air with maximum humidity.

2.2.2. *In vitro* fertilization (IVF) and culture (IVC)

Following 24 h maturation, COCs were washed 4 times in PBS and once in the fertilization medium (TALP: 10 µg/mL heparin salt – 184 units/mg, Calbiochem, San Diego, CA) before being transferred individually into fertilization plates in 250 µl of fertilization medium. Mature COCs were fertilised using cryopreserved semen of the same bull, which was rountinely used for IVF.

Motile spermatozoa were obtained by centrifugation of thawed spermatozoa through a Percoll (Pharmacia, Upsalla, Sweden) discontinuous density gradient (3.0 mL of 45% Percoll layered over 2.5 mL of 90%) for 10 min at 700 g, at room temperature. Viable spermatozoa were collected at the bottom of the 90% Percoll fraction. The pellet was suspended in TALP and then centrifuged at 100 g for 5 min at room temperature, following which the supernatant was removed. The concentration of Spermatozoa was estimated using a haemocytometer, and were then diluted in the appropriate volume of TALP to give a concentration of 2 X 10^6 spermatozoa/mL; 250 µL of this suspension was added to each fertilization drop to obtain a final concentration of 1 X 10^6 spermatozoa/mL. Fertilization dishes were then incubated for 24 h in an atmosphere of 5% CO_2 at 38.8°C in air with maximum humidity. At 18-20 h post fertilization, cumulus cells were removed from presumptive zygotes by moderate mixing using a vortex mixer in 1-2 mL PBS for 3 min. The presumptive zygotes were then washed twice in modified PBS and once in culture media (synthetic oviduct fluid supplemented with 10% FCS) before being transferred into 25 µl drops of culture media under mineral oil (Sigma, Italy) and cultured at maximum humidity in an atmosphere of 5% CO_2 and 5% O_2 at 38.8°C [71]. The resulting blastocysts were collected at the day 7 of *in vitro* embryo development (day 0 = day of fertilization) and snap frozen in pools of 5, in liquid nitrogen, and stored at -80°C.

2.2.3 RNA extraction procedures

Total RNA extraction was carried out using three different protocols using pools of 30 embryos for each sample independently of the RNA extraction procedure. RNA quality and quantity of each sample was assessed using RNA Pico Chip and 2100 Agilent Bioanalyzer.

2.2.3.1. Procedure 1 : Classical Trizol – M1

Briefly, pools of 30 embryos were lysed in 1 mL Trizol reagent (Life Technologies, CA, USA). Two hundred microliters of chloroform was added to the sample and mixed for 15 seconds using a vortex mixer, and then incubated at room temperature for 5 minutes. The samples were then centrifuged at 12000 g for 10 minutes in order to separate the aqueous phase from organic phase. The aqueous phase was carefully remove and 1 volume of isopropanol added and mixed by inversion, then centrifuged at 12000 g for 15 minutes. The supernatant was discarded without disturbing the RNA pellet. The pellet was washed using 1 volume of 75 % ethanol and was centrifuged at 7500 g for 5 min. The ethanol was discarded and the pellet was dried for 5-10 minutes at room temperature. The RNA was dissolved using RNAse free-water and stored at -80°C.

2.2.3.2. Procedure 2: Classical Trizol plus RNA clean and concentrator-5 – M2

The procedure was as described above until the final step of precipitation from the aqueous phase isolation. At this point, the aqueous phase was concentrated to 5 µl using the "*RNA Clean and Concentrator*" spin column procedure as described by the supplier (Zymo research corp, USA). The RNA was eluted using RNAse free water and stored at -80°C.

2.2.3.3. Procedure 3: Allprep DNA/RNA/miRNA and RNA clean and concentrator-5 – M3

Embryos were mixed in 1 mL of lysis buffer as supplied with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, USA) and lysed by repeated pipetting with P1000 Gilson pipette for about one minute. RNA extraction was carried out according to the manufacturers' recommended procedure (Qiagen, UK). RNA was eluted into 50 µl of RNAse free water and was concentrated into 5 µl following the recommended RNA clean and concentrator procedure (Zymo research corp, USA). RNA samples were stored at -80°C.

2.2.4. Library preparation and deep sequencing

A small RNA library was prepared from 5 µl of the total RNA from each sample using the Truseq Small RNA kit (Illumina Inc., USA, Figure 1) with some modifications, as follows: to minimize primer dimer formation, total RNA was mixed with half of the TruSeq Small RNA sample reagents, followed by 15 cycles of PCR to amplify the library. Five µl of each unique indexed library were pooled in 6-plex and resolved on a Pippin gel cassette 3% Agarose Dye free (BluPippin, Sage Science, MA, USA). Library RNA fragments in the 140-160 base pairs size range (the length of miRNA inserts plus the 3′ and 5′ adaptors) size ranges were recovered in 40 µL of Pippin elution buffer and then purified by Qiagen MinElute PCR Purification kit (Qiagen, CA, USA). The indexed libraries were quantified in triplicate on a ABI9700 qPCR instrument using the KAPA Library Quantification Kit, according to the manufacture's protocol (Kapa Biosystems, Woburn, MA, USA). Then, ten µL of the pooled libraries at a final concentration of 2 nM were used for sequencing on a Illumina HiSeq2000 using a 50bp Single-Read sequencing protocol. Finally, eight libraries (3 for M1, 2 for M2 and 3 for M3) were sequenced into a lane of Illumina Hiseq2000 flow cell.

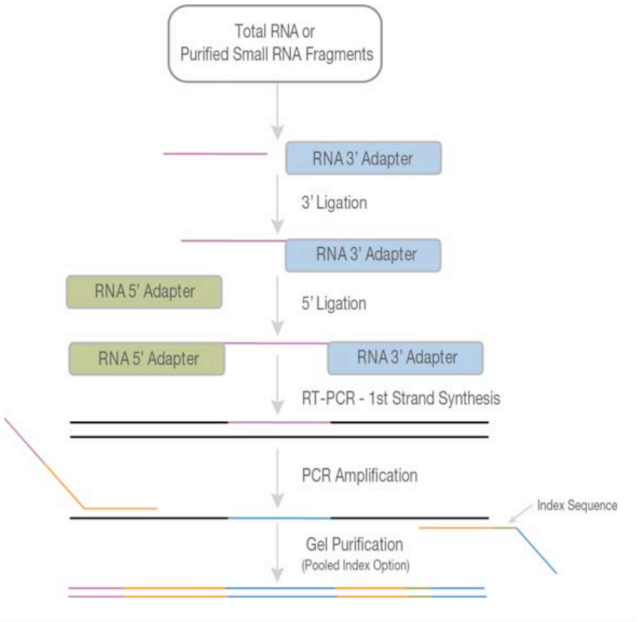


Figure 1. The principal phases of library preparation procedure with Truseq Truseq Small RNA kit (Illumina Inc., USA) are: adapter ligation, reverse transcription, PCR amplification, and pooled gel purification to generate a library. The protocol takes advantage of the natural structure common to the most known miRNAs, which have a 5'-phosphate and a 3'-hydroxyl group as a result of the cellular pathway used to create them. Because of this, the Illumina adapters in the kit are directly, and specifically, ligated to miRNAs.

2.2.5. Bioinformatic pipeline: setting up the methods for the analysis of sequencing data

Raw sequences (*e.g.* raw reads) were obtained from Illumina Hiseq25000 as FASTQ files after preliminary sequence quality control procedures using FastQC v0.11.2. Then, raw reads were trimmed using '*Trimmomatic software*' to remove adapter sequences of library [26]. The Quality Control threshold was set as a minimum base quality of 15 over a 4 bases sliding window and only sequences with length above 15 nucleotide were retained. Trimmed miRNA sequences (*i.e.* filtered or clean reads passing the threshold), which pass the threshold, were annotated using miRDeep2 software [27] This software was used for detection of novel and know miRNAs using bovine and homologous human sequences. In particular, the FASTA files of all matured bovine miRNAs, precursor bovine miRNAs and miRNAs of human were

downloaded from miRBase database (release 21: June 2014) (http://mirbase.org/ftp.shtml).

Known bovine and human miRNAs (mature and precursors) were used as a reference in the discovery process. Sequence reads were mapped to bovine reference genome and aligned sequence reads were blasted against both mature and precursor miRNAs of bovine and human. All known bovine and human miRNAs were used to quantify the frequency of miRNA sequences in each sample and produce a list of miRNA IDs and the relative abundance of mapped reads expressed as counts.

Novel miRNAs and their respective read counts were inferred using the same software (Figure 2). MiRDeep2 predicts the probability of un-annotated sequence being novel miRNA based on the genomic context, which surrounds the sequence and the capability of the sequence to fold into hairpin structure with low free energy [27]. Secondary structure of miRNA precursor was predicted using RNAfold [28] and minimum free energy algorithm [29].

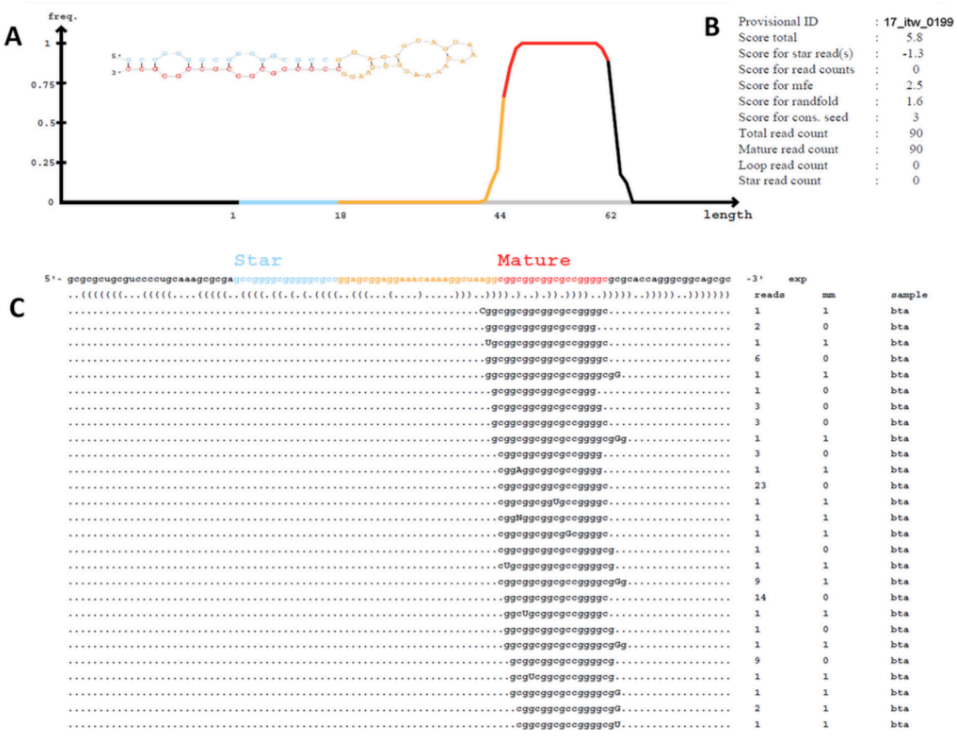


Figure 2. Graphic illustration of a representative predicted novel miRNA by miRDeep2. The primary miRNA hairpin with both mature and star miRNAs highlighted with red and blue colors, respectively (A). MiRDeep2 scores and

provisional ID are shown (B). The consensus matured miRNA sequence and other isomiRs and their corresponding read counts are indicated. Mismatched nucleotides of isomiRs with the miRNAs hairpin are written in capital letter (C) [28].

2.2.6. MiRNA target prediction and functional analysis

Gene target prediction for the miRNAs was carried out using DIANA miRPath v2.0 with homologous human miRNA and gene union options. DIANA miRPath can use predicted miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-CDS algorithm or experimentally validated miRNA interactions derived from DIANA-TarBase v6.0 [69]. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was obtained using DIANA miRPath in order to identify significantly targetted canonical pathways (P<0.0001) and predict the target genes putatively regulated by miRNAs. The list of target genes was used for Gene ontology (GO) analysis to infer gene function and molecular mechanisms enriched by the predicted miRNA target genes. To this end, the list of target genes were imported to DAVID Bioinformatics systems (http://david.abcc.ncifcrf.gov/) to visualize the non-redundant biological terms for large clusters of genes in a functionally grouped clusters [30].

2.3. Results

2.3.1. Sample quality and quantity

At the day 7 after fertilization, blastocysts were examined under the stereomicrosocpe and only those embroys, which were classified as grade 1 quality according to IETS standards, were collected for small RNAseq analysis. RNA was extracted from 8 pools of 30 blastocysts using the three different procedures (3 for M1, 2 for M2 and 3 for M3, Table 1). RNA integrity number (RIN) was between 8.9 and 9.7 for all the samples. However, the quantity of the total RNA, assayed using Agilent Bioanalyzer 2100 varied from 3.5 to 18.0 ng, indicating that this difference was not dependent on the procedures of RNA extraction (table 1).

Table 1. RNA quantity and quality in nanograms (ng) and RNA integrity (RIN), respectively, for each sample (sample ID) and procedure (procedure ID).

Procedure ID	Sample ID	Total RNA quantity (ng)	RIN
Procedure 1	M1A	10.5	9.4
	M1B	3.9	9.7
	M1C	18.0	8.9
Procedure 2	M2A	8.3	9.7
	M2B	11.6	9.7
Procedure 3	M3A	3.5	9.6
	M3B	10.5	9.7
	M3C	7.8	9.7

2.3.2. Overview of sequencing data

Sequencing produced 26M, 5M and 15M of reads for M1, M2 and M3, respectively. A mean of 81.2% (M1), 81.9% (M2) and 86.6% (M3) of reads were maintained after filtering of low quality reads. Between 5 and 12% of filtered reads could be mapped to known mature miRNAs in the human and bovine miRBase (table 2).

Comparing the three procedures, a similar number of miRNAs was identified in M1 and M3, for which the number of filtered reads was higher than M2. The procedure was characterized by a proportionately fewer miRNAs and in addition the number of novel miRNAs that were not present in miRBase differed among the procedures of RNA extraction: 62% (M1), 37% (M2) and 40% (M3) (table 3).

Table 2. Sequencing data reported by procedure and sample showing the number (n) of: raw reads (raw sequences or data obtained from the sequencer), trimmed reads (filtered sequences or data obtained by removing adapters and low quality sequence), percentage (%) of trimmed reads, mapped reads (n) and percentage (%) obtained from MiRDeep2.

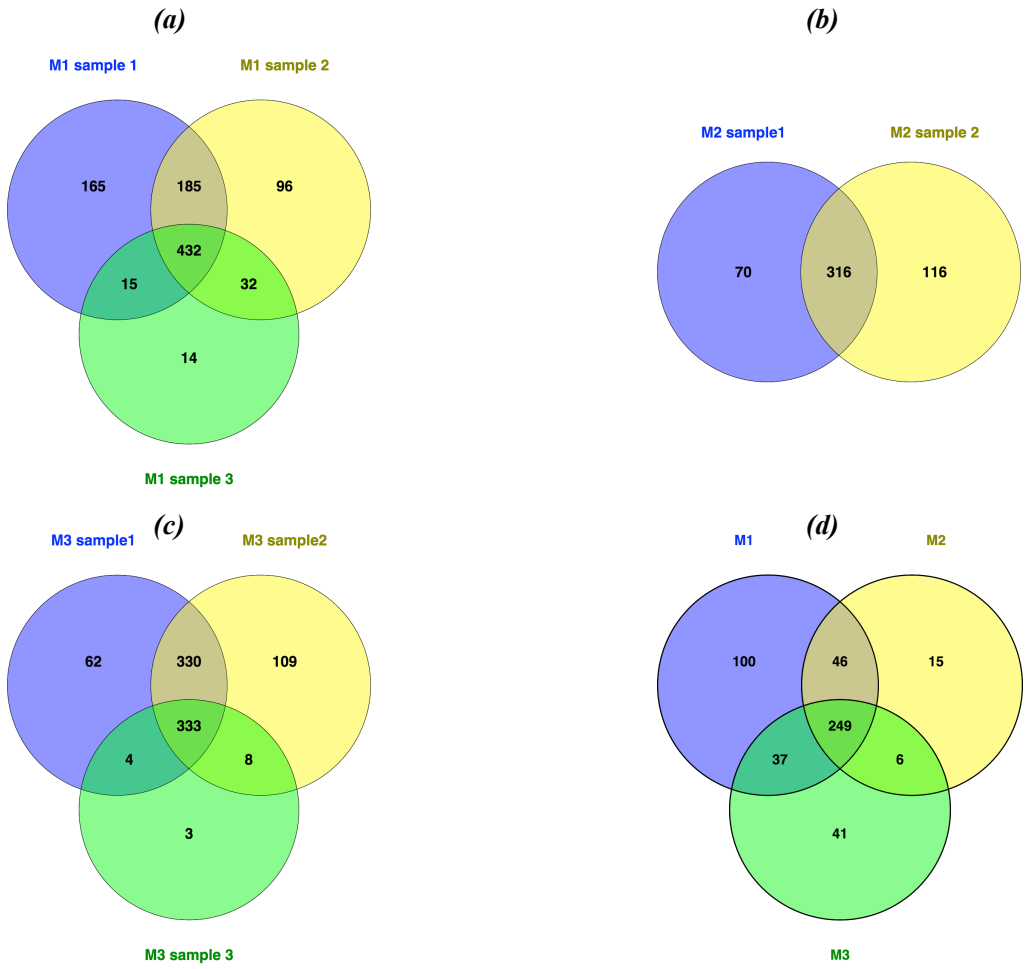
Procedure ID	Sample ID	Filtered reads (n)	Mapped read s(n)	Known MiRNA mapped reads(n)	%Known miRNA mapped reads
Procedure 1	M1A	37420115	25483222	1359181	5.3
	M1B	19740535	4933294	1194270	24.2
	M1C	6576100	4345780	228544	5.3
Procedure 2	M2A	3188883	2436344	98444	4.0
	M2B	5762574	4573463	146986	3.2
Procedure 3	M3A	14760122	13061986	1305092	10.0
	M3B	22348265	19376332	2476582	12.8
	M3C	1936178	1594601	105116	6.6

Table 3. The annotation and discovery of miRNAs obtained by MiRDeep2 analysis are reported as total miRNAs, novel miRNAs, known miRNAs and percentage (%) of novel miRNA.

Procedure ID	Sample ID	Total miRNAs	Novel miRNA	Known miRNA	% novel miRNA
Procedure 1	M1A	797	420	377	52.7
	M1B	745	328	417	44.0
	M1C	494	181	313	36.6
Procedure 2	M2A	386	144	242	37.3
	M2B	432	155	277	35.9
Procedure 3	M3A	729	307	422	42.1
	M3B	780	325	455	41.7
	M3C	348	117	231	33.6

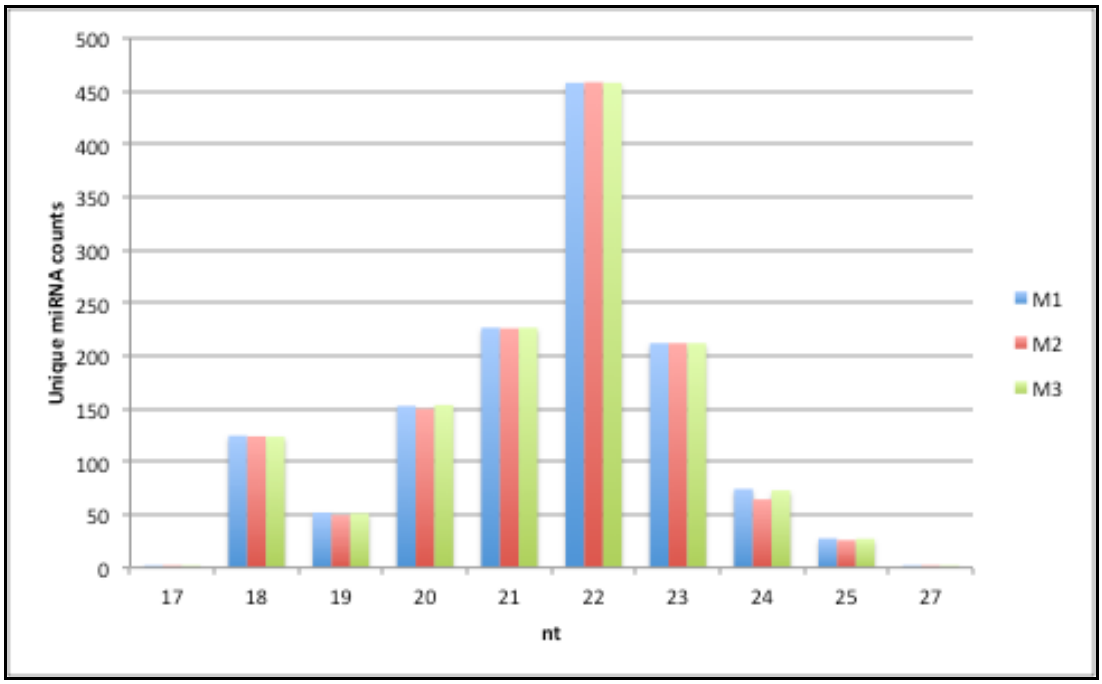
There were miRNAs which differed among the technical replicates of the procedures: only 432 miRNAs overlapped for the samples of M1, 316 overlapped between M2 samples and 333 between M3 samples (Graph 1A, 1B and 1C). Only 249 miRNAs were in common among all the RNA extraction

procedures (Graph 1D). This difference may be explained by the very low abundance of some of the miRNAs. Of the known miRNAs, 562 (M1), 1045 (M2) and 699 (M3) miRNAs had between 0 to 10 counts; 443 (M1), 201 (M2) and 316 (M3) had between 100 and 1000 counts and 222 (M1), 82 (M2) and 205 (M3) had more less than 1000 counts; only 135 (M1), 35 (M2) and 143 (M3) had over 1000 (data not shown).

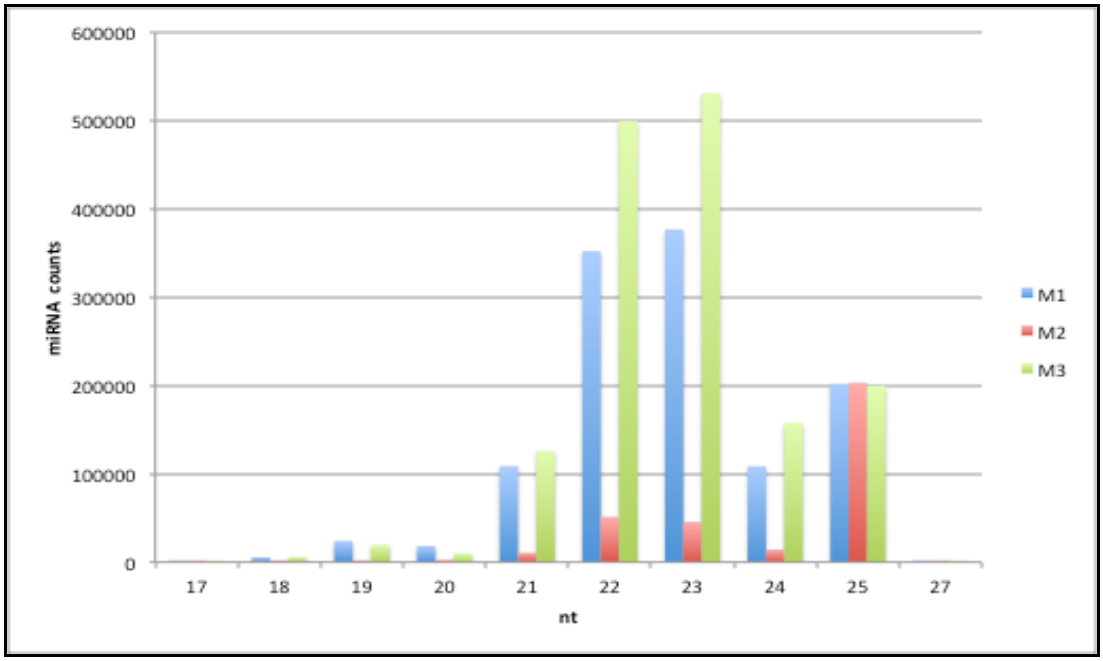


Graph 1. Venn diagrams per sample for each RNA extraction procedure, indicating the number of overlapping miRNAs among and within samples and proceedures M1, M2, M3 (1A, 1B, 1C respectively). The number of overlapping miRNAs between the procedures is also indicated, identifying those, which were common between samples (1D).

The most abundant length of all sequences classified as miRNA was 22 bp, which corresponds to the mature miRNAs length for all samples of each extraction procedures (Graph 2). Considering only the mature miRNA sequences, the enriched lengths were 22, 23 and 25 bp (Graph 3).



Graph 2. Mapped reads are reported as unique mature miRNAs and length of distribution (length is expressed as nucleotide = nt).



Graph 3. Mapped reads are reported as sum of all mature miRNA sequences and length of distribution (length is expressed as nucleotide = nt).

In summary: a total of 1363 unique miRNAs were identified, following discovery and annotation using miRdeep software. These miRNAs differed quantitatively and qualitatively among the extraction procedures: M1 identified 679 miRNA (ranging from 494 to 797) of which a mean of 420 were novel, M3 identified 619 (±33) miRNAs of which a mean of 250 were novel and M2 allowed to identify 409 (±236) of which 150 were novel (table 3).

Of the most abundant miRNAs, bta-miR-148a, bta-miR-92a, bta-miR-192, bta-miR-378 and bta-miR-10b were highly represented in all the procedures of RNA extraction (table 4).

Table 4. The top 20 most abundant miRNAs reported as miRNA ID and avarage read counts for samples of each procedure.

Procedure 1		Procedure 2		Procedure 3	
miRNA ID	Avarage read counts	miRNA ID	Avarage read counts	miRNA ID	Avarage read counts
bta-miR-10b	191422	bta-miR-10b	24128	bta-miR-10b	299902
bta-miR-378	84726	bta-miR-92a	13797	bta-miR-378	163837
bta-miR-371	37424	bta-miR-378	11791	bta-miR-192	78539
bta-miR-22-3p	56652	bta-miR-148a	6893	bta-miR-22-3p	63588
bta-miR-92a	58504	bta-miR-371	5721	bta-miR-148a	68241
bta-miR-26a	39459	bta-miR-192	4235	bta-miR-92a	71244
bta-miR-192	47495	bta-miR-423-5p	3870	bta-miR-30e-5p	49358
bta-miR-148a	49916	bta-miR-22-3p	5564	bta-miR-371	46660
bta-miR-30e-5p	38521	bta-miR-21-5p	4281	bta-miR-26a	45982
bta-miR-6119-5p	25061	bta-miR-30e-5p	3884	bta-miR-6119-5p	32787
Novel:hsa-miR-4706	17008	bta-miR-26a	3683	bta-miR-21-5p	39731
bta-miR-423-5p	19791	bta-miR-6119-5p	2321	bta-miR-30d	33302
bta-miR-191	18576	bta-miR-30d	2357	bta-miR-191	18569
bta-miR-21-5p	21224	bta-miR-191	1748	bta-miR-6526	16201
bta-miR-30d	19946	bta-miR-25	1585	bta-miR-423-5p	19765
bta-miR-186	12851	bta-miR-1468	1234	bta-miR-27b	16496
bta-miR-6526	9988	bta-miR-2478	973	bta-miR-186	13654
bta-miR-151-5p	9670	bta-miR-1246	1361	Novel:hsa-miR-4706	10275
bta-miR-16b	9737	bta-miR-27b	1311	bta-miR-25	13997

2.3.3. Target prediction and functional analysis

The 20 most highly expressed miRNAs were compared between procedures (table 4). A total of 14 miRNAs were common to all 3 procedures The most abundant miRNAs were bta-miR-148a, bta-miR-92a, bta-miR-192, bta-miR-378 and bta-miR-10b (Table 4). Bta-miR-6119-5p was exclusively annotated in miRbase for cattle. Thus, functional analysis was carried out using the 13 miRNAs which had human homologues (hsa-miR-10b-5p, hsa-miR-378a-3p, hsa-miR-192-5p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-30e-5p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-191-5p and hsa-miR-423-5p). KEGG pathway analysis identified many canonical signaling pathways which were enriched with genes targeted by these miRNAs (P<0.0001, table S1 – supplemental material). The more significant pathways were related to cancer *(e.g.* Pathways in cancer (hsa05200), Prostate cancer (hsa05215), Colorectal cancer (hsa05210), Bladder cancer (hsa05219) Transcriptional misregulation in cancer (hsa05202), Chronic myeloid leukemia (hsa05220), Endometrial cancer (hsa05213), Pancreatic

cancer (hsa05212)) and diseases (e.g. Prion diseases (hsa05020), HTLV-I infection (hsa05166), Hepatitis B (hsa05161), Fanconi anemia pathway (hsa03460)). Other significant canonical pathways were related to control cell growth and death (e.g. Cell cycle (hsa04110), p53 signaling pathway (hsa04115)) and signal transduction (e.g. Wnt signaling pathway (hsa04310), TGF-beta signaling pathway (hsa04350), ErbB signaling pathway (hsa04012)).

Considering the most significant KEGG pathways, a total of 179 genes were targeted by abundantly expressed miRNAs in blastocysts. Of these genes, 5 genes (STAT3, TGFBR1, VEGFA, HLA-G, HBEGF and TGFBR1) were identified as blastocyst specific in the NCBI gene database. DAVID cluster functional analysis grouped the genes into 124 GO clusters. The first 10 clusters (P< 0.0001) code for proteins involved in biological processes, molecular function and cellular components (Table S2 – supplementary material). The top ten gene clusters included: (1) regulation of phosphorus metabolic process; (2) cell cycle; (3) components of intracellular organelles and nucleoplasm; (4) immune system development; (5) regulation of transferase activity; (6) regulation of kinase activity; (7) protein phosphorylation mechanisms (8) phosphatase activity; (9) regulation of cellular component movement; (10) regulation of macromolecule biosynthetic process.

2.4. Discussion

The blastocyst consists of about 100-150 cells and preparing sufficient total RNA to carry out the profiling of miRNAs using a deep sequencing protocol is difficult. Studies to date that have profiled miRNAs in low input samples, such as blastocysts or gametes, have used targetted approaches to detect the espression of known miRNAs like miRNAs expression in mouse blastocysts performed with an in-house designed microarray with 743 mature miRNAs on [31] and the analysis of 366 mature miRNAs assessing pools of 10 bovine blastocysts with a Taqman assay [32]. Other studies have used commercial miRNA chips. For example, a study of bovine gametes, immature and mature oocytes (MII) used a mammalian miRNA chip with 3272 miRNAs including 94 bovine miRNAs [21]. The selected miRNAs on such chips are represented by

known and predicted miRNAs from diverse species, like human, mouse or cattle. The protocols described invariably use an amplification step before the analysis, which may distort apparent relative expression levels. This study analyzed all the miRNAs present in the bovine blastocyst and was carried out using three procedures for total RNA extraction and a high-throughput sequencing approach, adapted for low input samples, in order to reduce bias. Specifically amplification steps for the miRNAs before library preparation were avoided. The quantity of RNA obtained varied considerable among samples even for the same procedure. These variations in input of RNA quantity impacted the number of reads per sample, which was independent on the procedure. Libraries were prepared successfully from all the samples with a minimum of 10 ng as total input RNA input (data not shown).

In total, across all three approaches, 1363 miRNAs were identified in bovine blastocysts among which 41.5% were novel bovine miRNAs. The annotation and discovery processes carried out using miRDeep was able to attribute 218 of the novel bovine sequences to a human homologous miRNA. However, 60% of novel bovine miRNA sequences did not have a matching human miRNA. This suggests that these miRNAs expressed in bovine blastocysts may have a restricted pattern of expression, or are expressed at very low levels and therefore have not been described in other studies. The number of novel bovine miRNAs reported here is in line with the proprtion of unknown bovine small RNAs identified in other studies, for example, 50-60% novel miRNAs were recently described in studies of bovine spermatozoa [33] and granulosa cells of preovulatory dominant and subordinate follicles [34] using a small RNAseq. Tipically, studies of human tissues like human embryonic stem cells [35] have a lower proportion of unknowmn miRNAs.

The most abundant mature miRNAs identified in the present study were characterized with respect to their target genes and the pathways in which these genes were represented. This analysis showed an enrichment of target genes, which are mostly oncogenic factors and, thus, were associated with biogenesis of several cancers. Many studies have correlated the gene expression patterns in tumor cells with expression in gametes, embryos or during foetal development [36-38], suggesting that early embryonic development and tumor transformation have similar metabolic programs.

Indeed, miR-21, which was one of the most abundant miRNAs identified in the blastocysts is widefully described as have oncogenic activity and is up-regulated in several cancers, including breast, colon, pancreas, lung and liver cancers, and leukemia [39-43]. MiRNA-21 blocks diffentation and apoptosis by regulating several genes involved in cell cycle, including PTEN, PDCD4, MYC, BCL-2, E2F2, TP53, VEGFA, TGFβ and TPM1. Some of these genes, particularly E2F, BCL2, MYC, VEGFA and TP53 have been shown to be involved in oncogenic pathways [44-46]. TP53, which has a direct inhibition of expression of the tumor suppressor gene LATS2 [47], is also targeted by miR-30d, which was expressed in blastocysts in the present analysis. The target genes of miR-21 are also targeted by several of the other abundant miRNAs such as miR-26a, miR-92a, miR-378a and miR-192. In particular, miR-192 has been shown to inhibite apoptosis of cancer cells by targeting BCL2 and BAX [48]. MiR-10b is involved in cancer biogenesis and has been found to be associated with breast cancer, in particular, through the negative regulation of BRCA1, which is a tumor suppressor gene [49]. MiRNA-26a has also been implicated in metastasis through epithelial-to-mesenchymal transition of cancer stem cells, where it acts in cooperation with miR-21 [50]. In the present study, miR-10b was predicted to target NR4A3, which is also known as NOR1 and is involved in trascriptional regulation [51]. MiR-191, another found to be abundant in blastocystsis, was overexpressed in acute myloid leukemia [41] and solid cancers like that of breast and lung [42]. It may also be involved in metastasis through regulation of the transforming growth factor beta (TGFβ) signaling pathway inducing the expression of genes, which promote cell migration [53]. MiR-92a is part of the miR-17~92a cluster, which includes miR-17, miR-18a, miR-19a and miR-92a-1, and which has oncogenic activity through the regulation of TGFBR2, SMAD2, and BMP family genes [54]. However, other miRNAs, which belong to this genomic cluster, were not found to be abundant in bovine blastocysts. Nevertheless, predicted target genes, including SMAD4 and TGF-beta-RII, which are important for cell proliferation, were among the targets of abundant miRNAs, specifically miR-21, miR-26a, miR-371a and miR-423. These miRNAs have been suggested as pontential biomarkers of liver cancer in the circulation [55] although their mechanism of action is not fully undestood. Some miRNAs seem to have a

carcinogenic effect when they are expressed at low levels, like miR-22 which can have an opposing effects depending on the type of cancer. Indeed, this miRNA was found to be down-regulated in solid cancer, such as liver [56] or prostate [57], and hence it may have an anti-tumor action. In other cancers, however, such as chronic lymphocytic leukemia (CLL), which is characterized by accumulation of clonal B cells arrested in G0/G1 stages, it was up-regulated and induced phosphatase and tensin homolog down-regulation and the activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway [58]. This miRNA also targets genes involved in the cell cycle, like E2F and CDK6, which are targeted also by miR-21. The down-regulation of miR-148a is associated with cancer metastasis [59]. In the present study this miRNA was predicted to target HSP90B1 and CYCS, which have an apoptotic action [60] and are also regulated by cell cycle genes, such as BCL-2, MYC, which are involved by the way in the MAPK and TGFβ/SMAD signaling pathways. Finally, miR-30e down-regulation induces the development of cancer through the activation of TGF-β and NF-κB signaling pathways [61].

In summary, there is simple evidence that the most abundant miRNAs, which are shown in the present study to be important for bovine blastocyst development, are also associated with the regulation of oncogenic pathways and have been described for several cancers. However, it is not clear what makes the difference between tumour cells, which are generally characterized by loss of cell cycle regulation, and embryonic cells, which are rapidly dividing and pluripotent, but are delicately regulated to avoid uncontrolled cell proliferation. Some studies focused on early embryos cell proliferation have recently evidenced that contact inhibition may make the difference between embryonic and tumor cells [62]. However, the mechanisms underlying contact inhibition are still unclear.

Some of the most abundant miRNAs expressed in bovine blastocysts identified in this study have been described in other reproductive functions: bta-miR-10b has been shown to be important in ovarian function and has been implicated in early stage embryo development because it is highly abundant in oocytes during maturation although its role in early stage embryos is not known [63, 64]. Bta-miR-378 and bta-miR-26a were associated to ovarian follicle growth [34, 64] and have been identified in

embryonic tissues [70]. In particular, miR-378 is expressed in porcine cumulus cells and lack of its expression is associated with impaired oocyte maturation [65]. It reduces progesteron receptor expression and that of other genes such as ADAMTS1, CTSL1, and PPARG, which are important for follicular maturation and remodeling [66]. This miRNA is also involved in regulation of genes involved in neuronal developmental in human embryonic stem cells [67]. Bta-miR-21 is highly expressed during the early stages of embryo development, including blastocyst, and may be involved in maternal-embryo communication [21]. As described above, this miRNA cooperates with other abundant miRNAs in bovine blastocyst. Homologous human miR-191 and miR-192 are associated with oocytes quality and, in particular, are more highly expressed in blastocysts from oocytes recoved from young women compared with those from older ones [68]. Our analysis is consistent with this, in so far that miR-191 has a higher expression in blastocyst of good quality. However, miRNA-192 has recently been reported to be present in culture media of degenerated bovine embryos [22].

MiRNAs are likley to play a active part in regulating blastocyst development, where they control and coordinate the expression of a range of genes. Various miRNAs, which have a higher level of expression, are associated with good quality embryos as all the blastocysts analyzed in this study were classified according to IETS standards as good grade of quality. Understanding the roles of these miRNAs and manipulating their expression may help to investigate molecular mechanisms underlying correct embryo development.

In conclusion, this study focused on the miRNAs present in the bovine blastocyst and identified several novel miRNAs. The study also identified genes and pathways that are potentially regulated by miRNAs in early embryo development. Many of these genes are involved in the cell cycle and have also been implicated in tumor cells. Indeed, pathway analysis inferred the enrichment of genes targetted by the miRNAs and being involved in cancer related pathways. Similarities have been in the miRNA regulation of genes targeted by the abundant miRNAs in bovine blastocyst with changes in gene regulation associated with cancer transformation. Further studies will be necessary to understand the true function of miRNAs in the developing cells,

especially to define genes, which are actually regulated, and the implications for the pathways in which they play a role. Moreover, the analysis of miRNA expression in parallel with transcritiome studies will better elucidate their function. Because miRNAs are released by the embryo and can be detected in culture media, the miRNAs identified in this study are potential biomarkers of blastocyst quality and developmental compentece, which may be used to improve the success rate for reproductive biotechnologies.

2.6. References

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CHAPTER 3

Differences in microRNAs after *in vitro* fertilization using sperm with high and low blastocyst rates

3.1. Introduction

Early embryogenesis is affected by oocyte and sperm quality, which is correlated with specific biomolecules, including proteins and RNAs, which are delivered to the zygote at fertilization [1-4]. Among these biomolecules, the repertoire of RNAs is important in directing the embryo during the first steps of division until the embryo genome is activated [5]. In cattle this occurs later than other mammalian species, at the 8-16 cell-stage embryo [6]. The parentally derived RNAs influence zygote developmental potential to the blastocyst stage. Generally 30-40% of the fertilized oocytes [7] reach this stage, therefore understanding the factors that regulate early development is an important step for improving reproductive efficiency.

It is well known that the maternal contribution has a large impact on early embryo development. Maternally derived mRNAs present in the oocyte decrease rapidly following fertilisation [8]. The lack of some maternal mRNAs has been indeed associated with a low oocyte competence and quality [9]. However, the role of the sperm contribution during embryogenesis is unclear. For a long time, sperm have been considered to carry only the paternal genetic component, but now, accumulating evidence suggests that various sperm biomolecules actively participate in early development [10-13]. Indeed, during fertilization, the sperm transmits not only nuclear DNA but also other biomolecules, such as oocyte activation factors (OAF), which are critical for fertilization [14], centrosomes critical for cell division [14] and a population of messenger RNA (mRNA) that are critical for early development [10, 15]. Moreover, studies investigating epigenetic modifications in the developing

spermatozoa have provided new insights into paternal contribution during embryogenesis. Sperm epigenetic programming can have a significant influence on both the developing spermatozoa and embryo. In humans, alterations of the epigenetic programming have been related to fertilization potential and the early development of the embryo [16]. Epigenetic programming includes microRNAs (miRNA), which are regulators of gene expression at the mRNA level. Recently, the study of miRNAs in the oocyte and early embryo showed that a small set of miRNAs can influence the developing embryo, and mis-programming may result in early embryonic death [17, 18]. Oocyte miRNAs may be important during oocyte-to-zygote transition [19]. Spermatozoa are enriched in miRNAs, which have been investigated in mature bovine spermatozoa by comparing high and low fertility bulls [20]. However, few miRNAs were associated with differences in bull fertility and there is currently no information on their impact on pre-implantation embryo. Understanding the influence of sperm miRNAs during early embryogenesis will broaden our knowledge on the paternal contribution of mature spermatozoa. Morevoer, miRNAs are also used as biomarkers for diseases, and are therefore potentially new biomarkers for gamete and embryo quality.

This study investigated the paternal influence on the early stages of bovine embryo development through miRNA profiling. The experiments were carried out in two parts. First, I investigated whether a correlation could be establish between field fertility data and *in vitro* embryo production (IVP), considering two aspects: (i) the existency of genetic factors that determine semen quality and (ii) the effect of season on sperm quality (part 1). The results of the first part indicated that differences in blastocyst rates obtained after IVP depended on the individual bull and not on the field estimates of fertility or the season. Thus, in the second part of this study, miRNA profiling was carried out comparing high and low blastocyst rate bulls identified in part I (part II).

Part I. Effect of sire fertility and season on *in vitro* embryo development using Estimated Relative Conception Rate in high index dairy bulls

3.2. Materials and Methods

3.2.1. Bull samples and experimental design

Semen from 10 holstein-fresian bulls was collected and cryopreserved by an Artificial Insemination (AI) center located in Northern Italy (Inseme Spa, Lodi, Italy) and was commercially available for AI. In particular, the bulls of this study were located in the tails of the Gaussian distribution of Estimated Relative Conception Rate (ERCR) in Italy: 5 high (H) and 5 low (L) fertility bulls (Table 1). ERCR is a measure of conception rate used worldwide as field fertility index. It is based on non-return rate at 56 days corrected for herd-year-month of insemination, energy-corrected milk production adjusted to 3.5% fat and 3.2% protein content, days open at first mating and parity and is expressed as the conception rate of a sire relative to that of other sires. ERCR is mainly determined from many services in a large number of herds and is indeed routinely used as fertility index in Italy by the Italian Breeders Association (AIA). The estimation of conception rate in ERCR is corrected for the main factors affecting fertility, therefore the biological factors controlling differences in index value for individual bulls are not well defined.

Reliability for all considered bulls was higher than 85% (Reliability = 100 × [1 – (1 – (Number of inseminations/ (Number of inseminations + 200))½) × 2.3]). Semen was collected from all bulls during the summer and winter (indicated as S and W, see table 1) in order to look at the heat effects on spermatogenesis, which spans approximately 61 days in cattle [21]. In Northern Italy, the warmest and coolest months are July/August and January/February, respectively.

Table 1. The table reports the Estimated Relative Conception Rate (ERCR) for the 10 bulls used in the study, the sampling season (W = winter or cool month; S = summer or warm month) and Reliability for the high and low fertility bulls.

ID Bull	ERCR (value)	Artificial inseminations (n)	Reliability (%)	SAMPLING MONTH OF SEMEN	
				W (Winter)	S (Summer)
A	2.4	4202	99	March	September
B	2.0	3209	98	February	September
C	4.1	2371	98	February	September
D	2.0	4074	99	February	September
E	2.8	16767	99	February	September
F	-2.9	1427	96	March	September
G	-1.7	1892	97	March	September
H	-2.4	346	85	March	September
I	-1.8	2929	98	February	September
J	-1.8	360	86	February	late August

IVF outcomes are influenced by several variables, including the origin and handling of oocytes, the number of inseminations carried out at the same time, the ambient temperature, etc. Each replicate (*i.e.* “set up” or day of ovary collection, number of oocyte retrieved and *in vitro* fertilization) was designed to ensure that a high fertility versus a low fertility bull sampled in the S and W months was compared (Figure 1).

Figure 1. Matrix of comparison of bulls during each replicate (e.g. experiment day or "set up"). Each replicate allowed the comparison of a high ERCR bull versus a low ERCR bull using semen collected in the warm (s) and cool (w) month of the year. Each bull was repeated at least three times.

Experimental replicate	Fertility Status (ERCR)	
	High	Low
SET UP 1	A, W and S	F, W and S
SET UP 2	B, W and S	G, W and S
SET UP 3	C, W and S	H, W and S
SET UP 4	D, W and S	I, W and S
SET UP 5	E, W and S	J, W and S
SET UP 6	A, W and S	G, W and S
SET UP 7	B, W and S	H, W and S
SET UP 8	C, W and S	I, W and S
SET UP 9	D, W and S	J, W and S
SET UP 10	E, W and S	F, W and S
SET UP 11	A, W and S	H, W and S
SET UP 12	B, W and S	I, W and S
SET UP 13	C, W and S	J, W and S
SET UP 14	D, W and S	F, W and S
SET UP 15	E, W and S	G, W and S

3.2.2. *In vitro* embryo production

IVP was carried out using the procedures described in chapter 2 (par. 2.2.1. and 2.2.2.). The resulting blastocysts were collected at the day 7 of *in vitro* embryo development and snap frozen in pools of 5-10, in liquid nitrogen and stored at -80°C.

3.2.3 Data collection and statistical analysis

Assessment of *in vitro* embryo development was carried out by recording embryo cleavage (48 hour post insemination, hpi) and blastocyst rates at days 6, 7, 8 and 9 following fertilization. The mean percentage and standard error (SEM) were calculated using at least three replicates for the same bull and the same season of semen collection.

Statistical analysis was carried out using R software using a generalized linear mixed model (logit algorithm for a binomial distribution of data) to test significant differences between H and L groups in the S and W months of semen collection. The model was fitted using the function “glmer” of the R package “lme4”. This model incorporates both fixed-effects and random effects in a linear predictor, using maximum likelihood [51].

One-way ANOVA was carried out in order to compare each bull within the two seasons using the cleavage and Day 7 embryo development rates which are the parameters routinely used to assess in vitro embryo development [7].

3.3. Results

A total of 5987 COC were fertilized *in vitro*, 20% of which (n=1170) developed to the blastocyst stage by Day 7 post fertilization. The H group bulls differed only for blastocyst rate for S and for both the cleavage and blastocyst rates for W (P<0.05). Bulls A and E had a lower value than the other bulls in S and W and did not differ for the cleavage rate between seasons (Table 2). The cleavage rate of the H group bulls was normally distributed, ranging from 65.3% to 81.1% for S and from 63.0% to 79.1% for W. The blastocyst rate for the same bulls ranged from 13.5% to 27.0% for S and from 13.1% to 29.6% for W (Table 2).

The cleavage rate for the L group bulls ranged from 35.9% to 78.8% for S and from 22.8% to 75.6% for W. Blastocyst rates of L bulls were 5.2 % to 35.9% for S and 1.5% to 25.5 % for W. In this case, differences were seen in cleavage and blastocyst rates among bulls of the L group between the seasons in which the semen was collected (P<0.05). Bull F, in particular, had a very low rate of *in vitro* embryo development (*i.e.* cleavage and blastocyst

rates) compared with the other low fertility bulls both in S and W. This bull produced only 3 blastocysts from 202 COCs when S semen was used and 8 blastocysts from 153 COCs for W versus a mean of 16.9% for S and 20.1% for W for the group as a whole.

H group did not differ for the cleavage and blastocyst rates when using semen collected in S and W (*P*>0.05, Table3). Similar results were obtained for the L group, which did not differ for both the same parameters when comparing the seasons (P>0.05, Table 3). Interestingly, cleavage rate was higher in the H group than the L group (Figure 1) for W (P<0.001). Blastocysts rates at 6, 7, 8 and 9 days post insemination were similar for H and L groups and for both S and W (Table 3).

Table 2. Cleavage and D7 blastocyst rates following *in vitro* fertilization of oocytes with sperm by bull within high and low ERCR groups. Cleavage and blastocyst rates were calculated as mean value ± Standard Error of Mean (SEM). The rate is represented as a % of cleaved oocytes.

Fertility status	Bull ID	Warm month (S)			Cool month (W)		
		N	cleavage n (%)	D7 blastocyst rate n (%)	N	cleavage n (%)	D7 blastocyst rate n (%)
High ERCR	A	283	194 (68.6±4.8)	42 (14.8±3.2^{a})	284	179 (63.1±4.9^{a})	49 (17.3±3.3^{a})
	B	190	154 (81.1±2.5)	63 (33.2±4.6^{b})	196	155 (79.1±1.6^{b})	58 (29.6±5.2^{b})
	C	318	238 (74.8±3.2)	86 (27.1±3.7^{ab})	313	221 (70.6±3.8^{ab})	53 (16.9±2.1^{a})
	D	349	257 (73.6±4.3)	70 (20.1±3.9^{ab})	338	259 (76.6±2.9^{b})	79 (23.4±1.8^{ab})
	E	297	194 (65.3±3.6)	40 (13.5±3.3^{a})	366	243 (66.4±3.9^{ab})	48 (13.1±3.1^{a})
Low ERCR	F	153	55 (35.9±8.3^{c})	8 (5.2±2.2^{b})	202	46 (22.8±3.4^{b})	3 (1.5±0.9^{b})
	G	379	238 (62.8±2.8^{b})	51 (13.5±2.8^{b})	397	233 (58.7±5.8^{a})	67 (16.9±4.1^{b})
	H	295	224 (75.9±3.4^{a})	106 (35.9±3.7^{ac})	291	220 (75.6±1.2^{a})	66 (22.7±4.1^{ab})
	I	251	192 (76.5±1.5^{a})	67 (26.7±2.5^{a})	243	170 (69.9±4.1^{a})	62 (25.5±3.5^{ab})
	J	481	379 (78.8±3.2^{a})	86 (17.9±4.9^{ab})	361	244 (67.6±7.4^{a})	66 (18.3±6.1^{b})

^{A,b,c}Values with different superscripts differ significantly (*P*<0.0001).

Table 3. Cleavage and blastocyst rates (from day 6 to day 9 of *in vitro* embryo development, day = D) following *in vitro* fertilization of oocytes with sperm from high and low ERCR bulls. Cleavage and blastocyst rates were calculated as mean value ± Standard Error of Mean (SEM). The rate is represented as a % of cleaved oocytes.

Season temperature of semen collection	Fertillity status	Cleavage rate	Blastocyst rate			
			D6	D7	D8	D9
Cool	High ERCR bulls	71.2±3.1[a]	9.9±1.6	19.8±2.9	23.3±2.4	23.6±2.7
	Low ERCR bulls	58.9±9.4[b]	9.1±3.1	16.9±4.2	19.9±4.7	20.2±4.7
Warm	High ERCR bulls	72.7±2.71	8.8±1.5	21.7±3.7	26.2±3.8	26.8±3.9
	Low ERCR bulls	65.9±8.1	8.9±0.8	20.1±0.5	23.8±0.5	24.3±0.6

[a,b]Values with different superscripts differ significantly (P<0.0001).

3.4. Discussion

Field fertility is normally assessed using 56-90 day non-return rates (NNR) [22-27], or Estimated Relative Conception Rate estimated (ERCR), the latter is corrected using factors influencing bull fertility [28-31]. This study examined the relationship between the estimated field fertility, measured as ERCR of the sire, and *in vitro* embryo production (IVP), and also tested the effect of heat stress.

The cleavage rate following IVF has been usually associated with NRR [22,23, 31-33] but a weak correlation has been found between blastocyst rate and NRR [24, 35-37]. In the present study, large differences between bulls were observed for *in vitro* embryo development, following IVF success, however, this was not dependent on ERCR. There were bulls, which were outliers with respect to the cleavage and blastocyst rates in both high and low fertility groups, *i.e.* high ERCR bulls with a low cleavage and blastocyst rates and low ERCR bulls with a high cleavage and blastocyst rates. These values influenced the means of both the H and L group, but, nevertheless, differences between the H and L groups were observed for cleavage rates for semen collected in the cool month. However, no differences between high and low fertility bulls were seen for *in vitro* blastocyst rate at 6 to 9 days of *in vitro* embryo development. These results are similar to those obtained for

NRR [22-24, 31-37]. This suggested that using a fertility index based such as ERCR, which estimates overall fertility and includes traits such as the milk production, may be not appropriate for predicting *in vitro* fertilization efficiency and early embryo development. Low fertility bulls may be associated with lower pregnancy rate for several reasons, *e.g.* through early embryonic loss, which is not routinely detected by breeders. Embryonic loss mostly occurs during the pre-implantation period of embryonic growth, between days 8 and 16 and is considered to be the main factor influencing the reproductive success in cattle [39, 40]. However, the reasons of embryo mortality are not fully understood, and may be associated with errors in molecular programming, which can affect viability of developing embryos [41, 42]. The association between bull field fertility, which was assessed in a way similar to ERCR, and IVF success was recently associated DNA fragmentation in spermatozoa [43]: low fertility bulls have a relatively high number of DNA-nicks. In these cases fertility can be addressed by increasing sperm concentration during IVF. In the present study, sperm was diluted as normally practiced for IVF. Using a large number of *in vitro* matured oocytes, differences were observed between H and L groups for cleavage rate following IVF. Bull fertility, estimated as ERCR, is poorley correlated with IVF success. Fertility may be affected by other factors, which were not considered in the present study and should be further investigated.

Male fertility is affected by ambient temperature [44, 45], therefore the IVF rate of semen from the same bulls collected following the warmest and coolest months in Italy was compared to test for an effect of temperature stress on *in vitro* embryo development. Most species show seasonal variations in fertility, including ovulation frequency, spermatogenic activity, gamete quality, and also sexual behaviour [46]. Although highly selected breeds of cattle do not show significant reproduction seasonality [47, 48], they can be affected by heat stress, which may impact negatively on spermatogenesis [49, 50]. Summer temperatures may reach ~40°C and fall to 0°C in winter in the Mediterranean area. The results presented here, however, indicate that *in vitro* fertilization and developmental competence of embryos is not affected by season of semen collection, and cleavage and blastocyst rates were similar for both high and low ERCR bulls in S and W.

In conclusion, the results indicate that semen of low fertility bulls, measured as ERCR, is as capable of fertilizing oocytes *in vitro* and to produce pre-implantation blastocysts with the same quality and quantity as high fertility bulls. Indeed, the *in vitro* produced blastocysts were morphologically of good quality for both high and low fertility bulls (data not reported). Therefore, an alternative approach was explored to assess high vs low fertility.

Part II. Profiling of blastocyst microRNAs from high and low blastocyst rate bulls using deep sequencing

3.5. Material and Methods

As the correlation between field fertility and *in vitro* embryo development was not found, the experimental design was changed and bulls were classified based on number of blastocysts produced *in vitro* using semen collected during the same period, in order to exclude the effect of season, to give the "Blastocyst rate".

3.5.1. RNA extraction

Total RNA extraction was carried out using pool of 30 blastocysts for each sample using Allprep DNA/RNA/miRNA and RNA clean and concentrator-5 procedures (described in chapter 2 par. 2.2.3.3. – procedure 3). Then, the RNA quality and quantity of each sample was assessed using RNA Pico Chip and a 2100 Agilent Bioanalyzer.

3.5.2. Small RNA sequencing

Library preparation and deep sequencing were carried out using the procedure set up in Chapter 2 (par 2.2.4.).

3.5.3. Bioinformatic and functional analysis

Filtering of raw sequences, annotation and discovery of miRNAs were carried out according to the pipeline described in Chapter 2 (par 2.2.5.).

Gene expression analysis was perfomed using the R package 'EdgeR' (http://bioconductor.org/packages/release/bioc/html/edgeR.html) in order to identify differentially expressed miRNAs. MiRNAs with log2 fold change differences ≥ 1, *P-value* ≤ 0.05 and False Discovery Rate (FDR) ≤ 0.1 were considered as significantly differentially expressed. The interaction between differentially expressed miRNAs and their target mRNAs was predicted using miRWalk 2.0; a widely used web-based database to predict animal miRNA-target mRNA interactions [52]. A total of 6 prediction programs were combined into a pipeline for the analysis as follows: miRanda-rel2010 [53], PicTar2 [54], PITA [55], RNA22v2 [56], RNAhybrid2.1 [57] and Targetscan6.2 [58]. The lists of predicted target genes of individual miRNAs were imported to DAVID Bioinformatics systems; a freely available bioinformatic tool (http://david.abcc.ncifcrf.gov/). Gene ontology (GO) analysis was carried out using this tool to identify the most important enriched biological and cellular processes. The DAVID web-tool facilitated also the identification of the canonical signaling pathways significantly enriched with the predicted target genes of each miRNAs, which were then analyzed using Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [59]. To improve the functional meaning of the results, KEGG pathway analysis was also performed using DIANA miRPath v2.0 with homologous human miRNA and gene union options. DIANA miRPath uses predicted miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-CDS algorithm, or experimentally validated miRNA interactions derived from DIANA-TarBase v6.0 [60].

3.6. Results

The distribution of bulls was based on their Day 7 blastocyst rate (graph 4). Two main categories were created depending on the blastocyst rate: high (≥

25.1 %) and low (≤15.1 %) groups (figure 1). The high group (H group) differed significantly from low group (L group) (32.1 ± 4.5 vs 10.7 ± 4.8, *P*<0.01). The 3 extreme bulls of each category were chosen for small RNA sequencing and are indicated in the rest of the thesis as follows: bull 1 (H), bull 2 (B), bull 3 (C), as high blastocyst rate and bull 4 (A), bull 5 (E) and bull 6 (G) as Low.

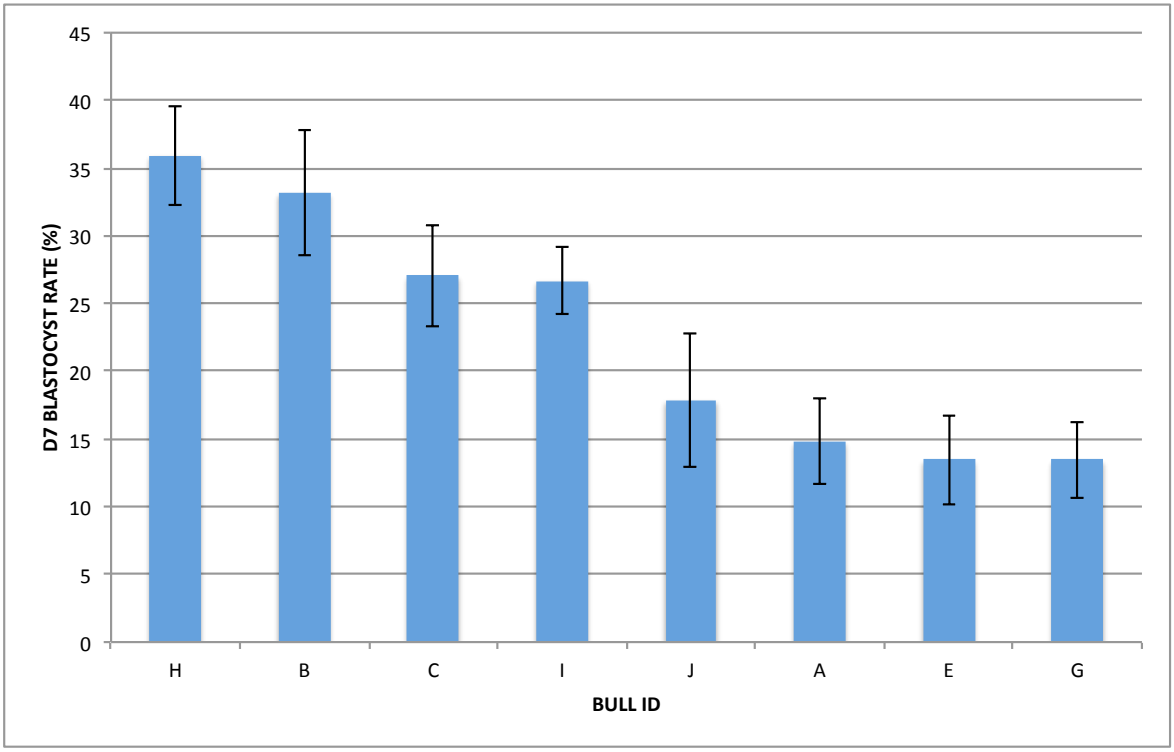


Figure 2. Distribution was carried out using Day 7 blastocyst rate of each bull. Mean value and standard error of mean (SEM, error bars) are depicted.

3.6.1. Characterization of miRNA deep sequencing data

To investigate the involvement of miRNAs in bovine sperm associated with a diverse blastocyst rate, 3 miRNA libraries of H and L blastocyst groups, respectively, were generated using total RNA from Day 7 IVP blastocysts. These were sequenced using the Illumina HiSeq2500 small RNA deep sequencing technology with 50 bases long sequence reads, *i.e.* raw reads, generated for bull1, bull2, bull3 (H group) and bull5, bull6 (L group). Bull 4 sequencing failed and was excluded from data analysis. Accordingly, 24.6 and 12.9 million reads, *i.e.* clean reads, were obtained after filtering of low

quality reads from libraries of the H and L group, respectively (table 4). Quality filtered sequence reads were used to identify known annotated and prediction of novel miRNAs. From all reads which passed the quality control criteria, 18.2M reads in H group and 8.7M in L group were mapped to the bovine reference genome, comprising 74.1 % and 66.8 % of the total quality reads obtained, respectively. Furthermore, 549,090 reads in H group and 218,537 in L group were found to be similar to known bovine and human miRNAs reported in miRBase release 21.

Table 4. Summary of sequence reads alignment to bovine reference genome and known miRNAs annotated in miRBase.

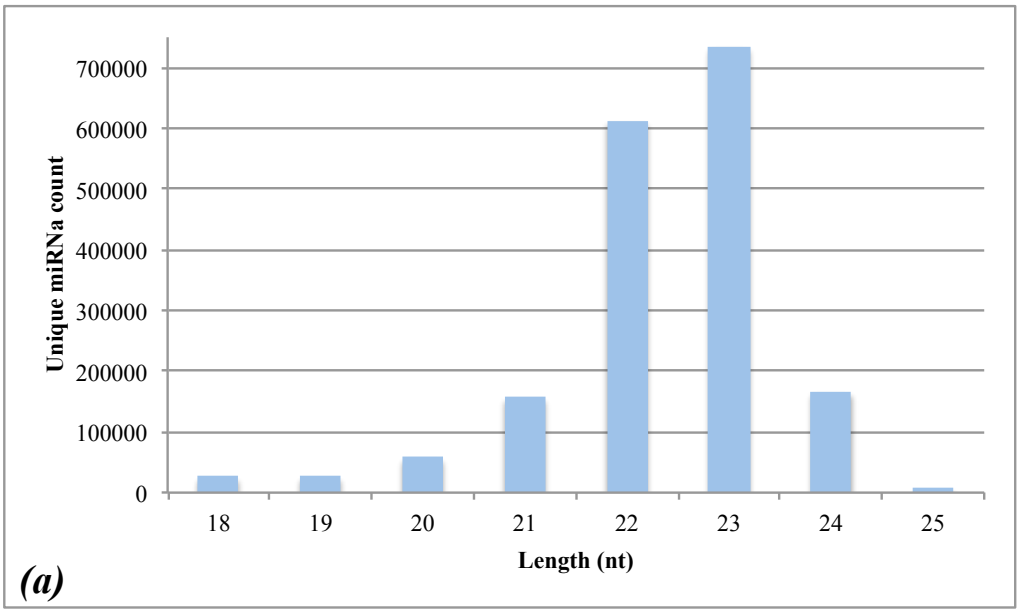
Bull group	Sample ID*	Filtered reads (n)	Mapped reads (n)#	% Mapped reads	Known MiRNA mapped reads (n)§	%know miRNA mapped reads
High blastocyst rate	Bull 1	38843847	29298950	75.4	913546	3.1
	Bull 2	16085421	11992592	74.6	352711	2.9
	Bull 3	18961284	13412562	70.7	381012	2.8
Low blastocyst rate	Bull 5	14440423	9506437	65.8	268079	2.8
	Bull 6	11468277	7850467	68.5	168995	2.2

*: Bull1, Bull2, Bull3 denote for biological triplicates of high blastocyst rate bulls and Bull5, Bull6 denote for biological triplicates of low blastocyst rate bulls.
#: Number of quality filtered reads aligned to bovine reference genome (assembly UMD3.1).
§ Proportion of mapped sequence reads aligned to known annotated miRNAs in miRBase release 21

3.6.2. MiRNAs expressed in Day 7 embryos of H and L blastocyst rate bulls

MiRNAs with at least 1 read count in at least two of the three biological replicates were considered as detected. Among the short RNAs that could be mapped to known bovine and human miRNA precursors, the most abundant

length was 22 nucleotides (nt) for both the H and L group, which corresponds with the expected miRNA size (Figure 3).



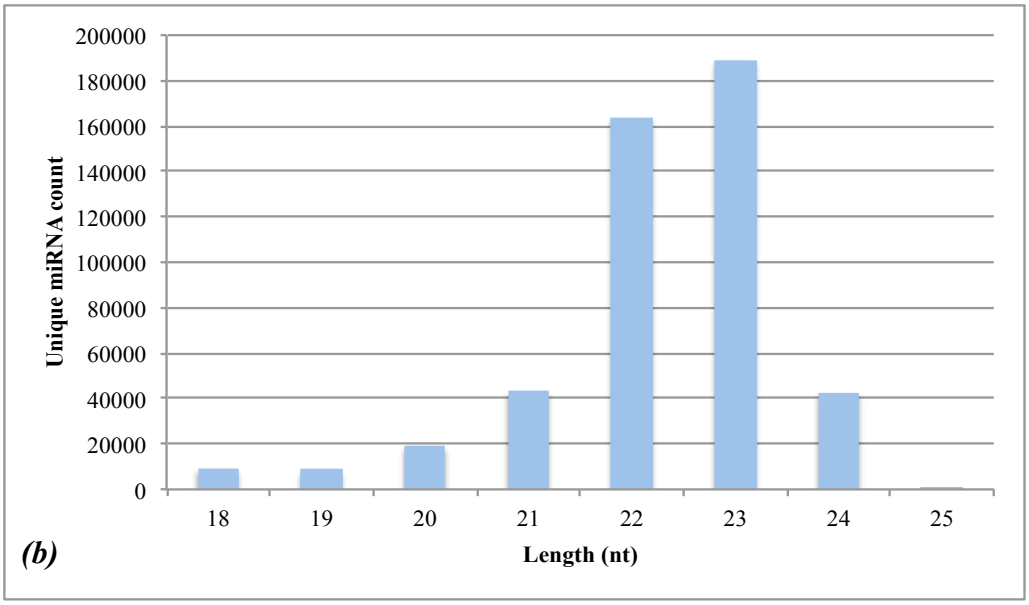
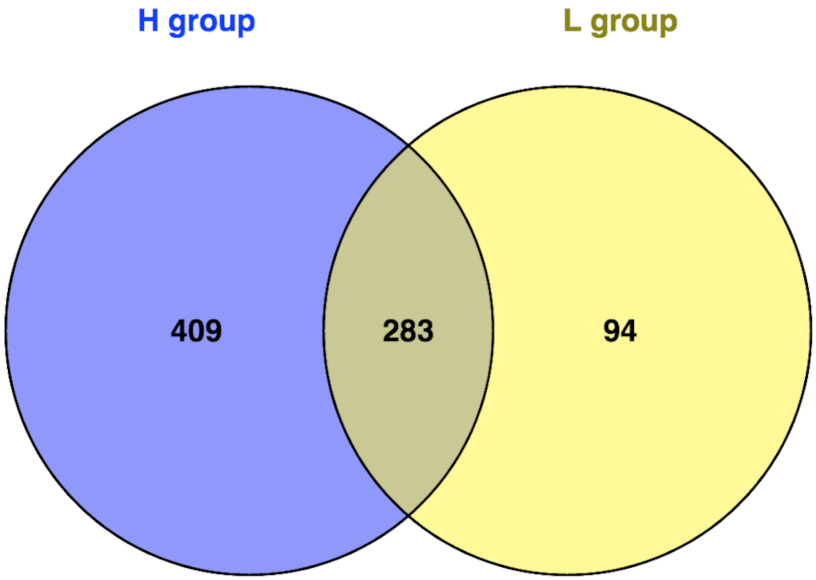


Figure 3. Length distribution (nucleotide = nt) of miRNA populations in low (a) and high (b) blastocyst rate bulls.

A total of 692 and 377 known bovine and homologous human miRNAs were detected in H and L group, respectively, of which 283 miRNAs were in common between groups. However, 409 miRNAs including homologous

human hsa-miR-4755-5p and has-miR-548-3p were found to be specific to H group. While, 94 miRNAs were unique to L group (Graph 1).

Many of the detected miRNAs were found in both the H and L groups. Among the top 10 abundantly expressed miRNAs in each group, 8 miRNAs (bta-miR-10b, bta-miR-423, bta-miR-92a, bta-miR-191, bta-miR-378a, bta-miR-148a, bta-miR-192, bta-miR-22) were expressed in common in both H and L groups (table 5). Among these, bta-miR-10b and bta-miR-423 were the two most abundantly expressed miRNAs with a read count of 129,517 and 36,460 in H group and 47,775 and 21,813 in L group and accounting for 48.3 and 15.9% of the sequence reads aligned to known miRNAs, respectively. Nevertheless, the overwhelming majority of the detected miRNAs in both libraries had fewer than 50 read counts (data not shown).



Graph 1. Comparison of known miRNAs, which were expressed in the blastocysts of H and L groups and were annotated in MiRBASE for human and cattle (Venn diagram; H group = high blastocyst rate bulls, B group = low blastocyst rate bulls). The Venn diagram was built using mean average read count of biological replicates.

Table 5. List of top 10 highly abundantly expressed miRNAs in high and low blastocyst rate bulls.

High blastocyst rate bulls		Low blastocyst rate bulls	
miRNA ID	**Avarage read counts***	**miRNA ID**	**Avarage read counts***
bta-miR-10b	129517	bta-miR-10b	47775
bta-miR-423-5p	36460	bta-miR-423-5p	21813
bta-miR-92a	51670	bta-miR-92a	25310
bta-miR-191	29438	bta-miR-191	11804
bta-miR-378	47152	bta-miR-378	16875
bta-miR-148a	23568	bta-mR-148a	7253
bta-miR-192	18989	bta-miR-22	5686
bta-miR-22	18735	bta-miR-192	5600
bta-miR-30d	11657	bta-miR-16b	4844
bta-miR-30e	11029	bta-miR-92b	4680

3.6.3. MiRNAs gene expression and functional analysis

Differential expression analysis revealed that 3 miRNAs were significantly differentially expressed between H and L blastocyst rate bulls. The expression level of these miRNAs, which were annotated for human in miRBase release 21, was significantly reduced in the L group with respect to H group, *e.g.* Log_2 Fold Change (table 3). Moreover, 2 of these miRNAs (hsa-miR-4755-5p and hsa-miR-548d-3p) were expressed only in the blastocysts of H group. While, hsa-miR-1225-3p was expressed in the blastocysts of both groups.

Table 6. List of miRNAs down-regulated in Day 7 blastocysts of L group (miRNA ID, Fold change = Log_2 Fold Change, P-value and FDR = False Discovery Rate).

miRNA ID	Fold Change	p-value	FDR
Novel:hsa-miR-4755-5p	-10,5	<0.0001	0.0045
Novel:hsa-miR-548d-3p	-10,1	0.0002	0.0372
Novel:hsa-miR-1225-3p	-6,0	0.0002	0.0372

To understand the functional involvement of these miRNAs in bovine early embryo development, target genes of each differentially expressed miRNAs were predicted and used to determine the most significantly enriched GO biological functions and KEGG signaling pathways using DAVID. In addition, KEGG pathway analysis was also performed using miRPath v 2.0.

First, gene ontology (GO) analysis on predicted target genes revealed that biological processes associated with transcription regulation, cellular biosynthetic processes, including nucleic acid metabolism, and cell and embryonic morphogenesis, were among the highly enriched GO terms (Table S3 – supplementary material). KEGG pathway analysis, which was performed using DAVID web-tool, identified 56 enriched canonical signaling pathways (Table S4 – supplementary material**).** These canonical pathways were grouped into classes based on functions and biological processes using the KEGG pathway database (http://www.genome.jp/kegg/pathway.html). Many of the pathways were associated with **oncogenesis**. The most enriched pathway was indeed that of **cancer** but there were tumor-specific enriched pathways including colorectal, melanoma and chronic myeloid leukemia, and general cancers including reproductive tissues and prostate cancers. The other enriched classes were linked to: 1) cellular processes, where the most

enriched pathways were those associated with cell adhesion (**Regulation of actin cytoskeleton, Tight Junction and cell adhesion molecules (CAMs)**) cell proliferation (**MAPK signaling pathway)** and cell survival and growth, (**TGF-beta, apoptosis and neurotrophin pathways)**; 2) Cell communication (**Endocytosis** and **calcium pathways**); 3) cell metabolism (**Insulin** and **phosphatidylinositol pathways**). Finally, DIANA MirPath analysis was carried out using hsa-miR-4755-5p and hsa-miR-548d-3p simultaneously (table 7) and hsa-miR-1225-5p alone (table 8).

Table 7. Kegg pathways from DIANA miRPath v 2.0, which were enriched by target genes of hsa-miR-4755-5p and hsa-miR-548d-3p.

KEGG pathway	p-value	Target genes	MiRNAs
ErbB signaling pathway (hsa04012)	0.0001389508	CAMK2D, ERBB2, SHC1, BTC	hsa-miR-4755-5p, hsa-miR-548d-3p
Transcriptional misregulation in cancer (hsa05202)	0.001395331	HMGA2, CCND2, NCOR1, DDX5, PPARG, MEIS1	hsa-miR-4755-5p
African trypanosomiasis (hsa05143)	0.002354351	SELE, APOL1	hsa-miR-4755-5p
Ubiquitin mediated proteolysis (hsa04120)	0.005949636	RFWD2, UBA6, PARK2, BIRC2	hsa-miR-4755-5p
Apoptosis (hsa04210)	0.009663038	CSF2RB, IRAK1, BIRBC2	hsa-miR-4755-5p
Huntington's disease (hsa05016)	0.01833565	CREB5, AP2A2, PPARG, CNAQ, PPARGC1A, SDHD	hsa-miR-4755-5p
Ubiquinone and other terpenoid-quinone biosynthesis (hsa00130)	0.04608754	NQO1	hsa-miR-4755-5p

Table 8. Kegg pathways from DIANA miRPath v 2.0, which were enriched by target genes of hsa-miR-1225-5p.

KEGG pathway	p-value	Target genes	MiRNAs
Protein processing in endoplasmic reticulum(hsa04141)	0.003416487	SSR1, DERL1, ERO1LB	hsa-miR-1225-3p
Proteasome (hsa03050)	0.007827826	PSME4, IFNG	hsa-miR-1225-3p
Amphetamine addiction (hsa05031)	0.009343598	CAMK4, GRIA4	hsa-miR-1225-3p
Biosynthesis of unsaturated fatty acids (hsa01040)	0.0284081	ELOVL2	hsa-miR-1225-3p
Type I diabetes mellitus (hsa04940)	0.0284081	IFNG	hsa-miR-1225-3p
Protein digestion and absorption (hsa04974)	0.0284081	ATP1A4, DPP4	hsa-miR-1225-3p
Lysine degradation (hsa00310)	0.03504665	KMY2C	hsa-miR-1225-3p
Graft-versus-host disease (hsa05332)	0.04021567	IFNG	hsa-miR-1225-3p

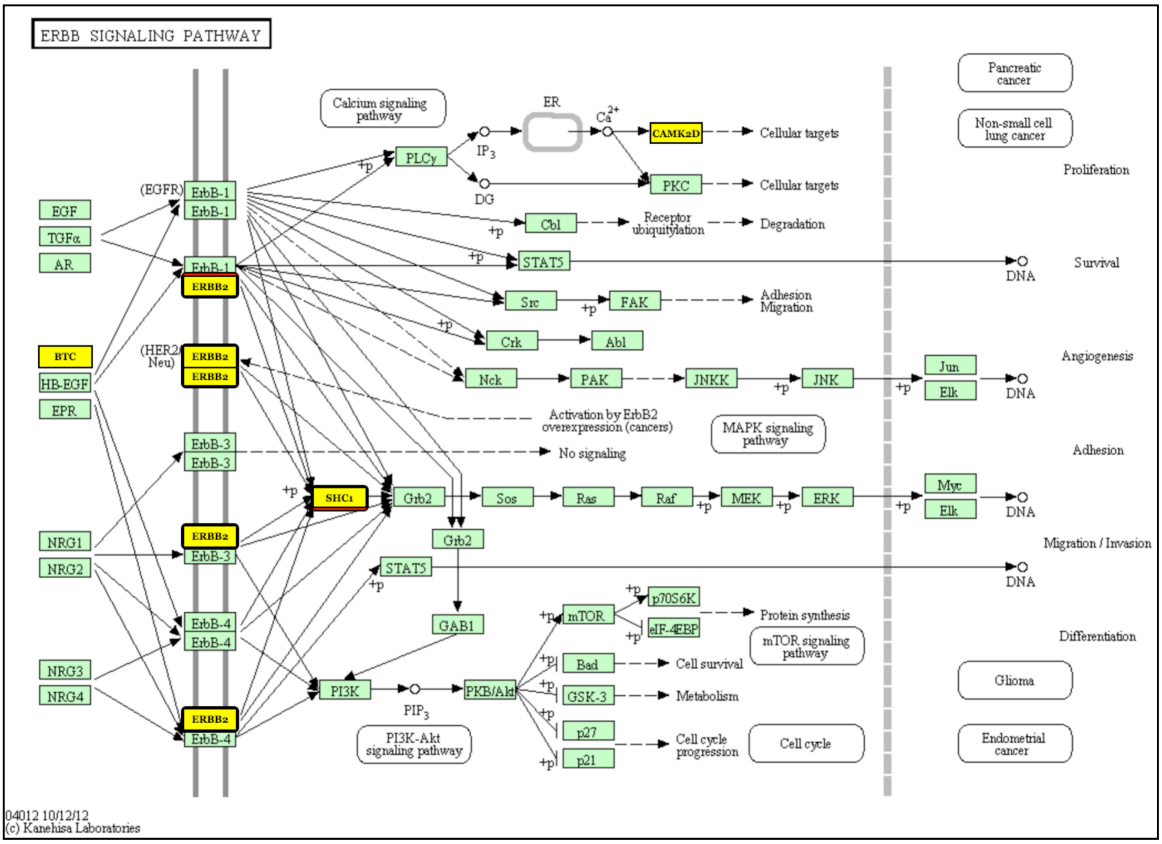


Figure 4. ErbBB signaling pathway and predicted target genes of miR-4755-5p and miR-548d-3p. Target genes of miRNAs are indicated in yellow.

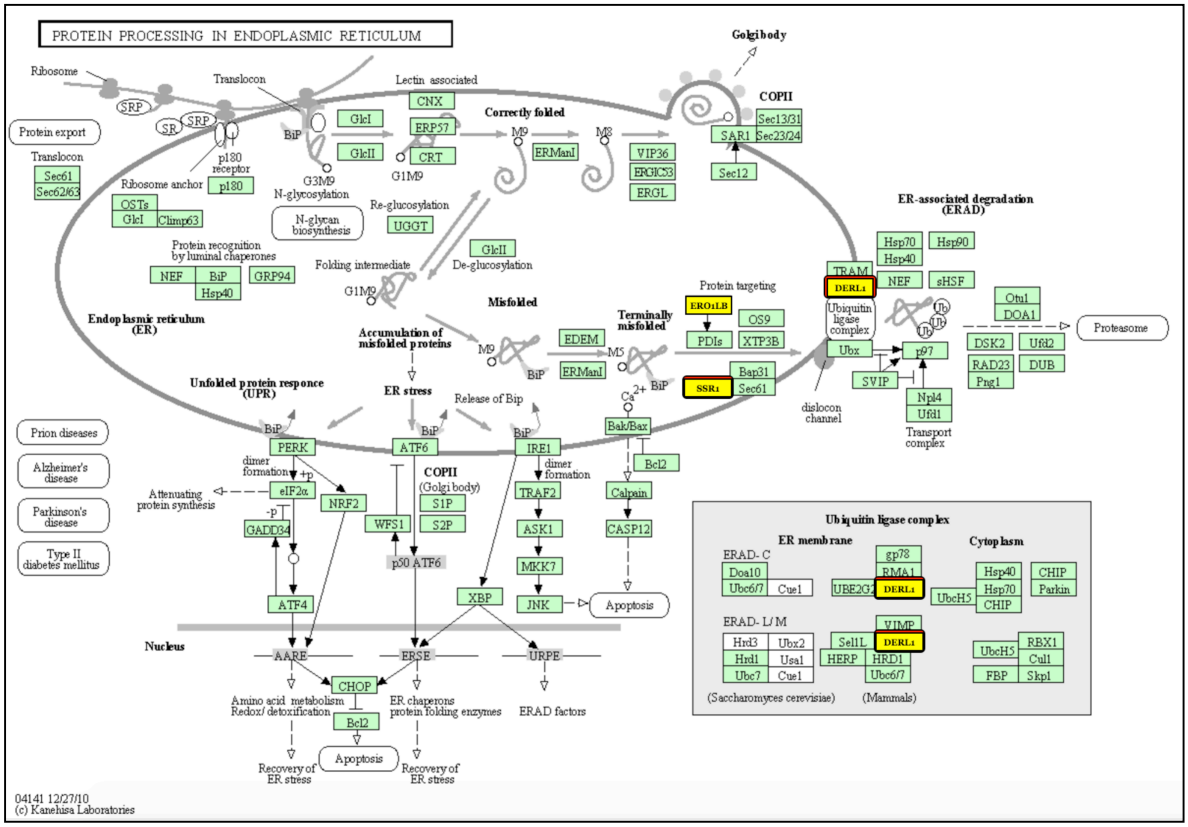


Figure 5. Protein processing in endoplasmatic reticulum pathway and predicted target genes of miR-1225-3p. Target genes of miRNAs are indicated in yellow.

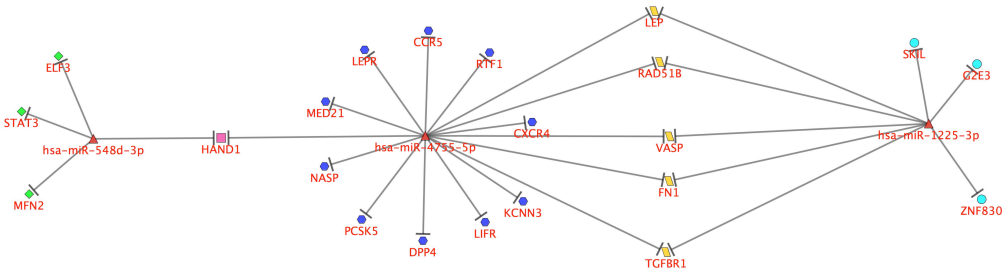


Figure 6. Gene network representation of interaction between the three differentially expressed miRNAs and their predicted target genes, which are important in blastocyst formation and differentiation processes. Three different hubs can be seen: 5 genes (VASP, TGFBR1, FN1, LEP, RAD51B) are common target of miR-4755-5p and miR-1225-3p, while one gene (HAND1) is a common target between miR-4755-5p and miR-548d-3p.

3.7. Discussion

In total, the three miRNAs which were the most significantly reduced in the group of bulls with low competent sperm associated with a lower *in vitro* blastocyst rate, have not been annotated in cattle and are novel homologous human. Moreover, they have not been previously described in regard to early embryogenesis. The results presented here suggest their involvement in bovine blastocyst development. Using miRPath v2.0 the most significant KEGG pathways associated with miR-4755-5p and miR-548d-3p (-10.1 and -10.1 $\log_2$ Fold Change lower expression in low compared with high group), which were identified only in the blastocysts of high competent sperm, were predicted to regulate, the expression of genes involved in the ErbBB signaling pathway. This result is in agreement with findings in mice embryos where overxpression of ErBB signaling genes is associated to placental abnormalities and embryonic lethality [61]. An important gene involved in this molecular mechanism is ErBB1, expression of which is increased when placenta hyperplasia occurs [62]. Expression of this gene is strictly correlated to ErBB2 (figure 4), which is a predicted target of miR-548d. Furthermore, SHC1 activity, which is dependent on ErBB2, is a predicted target of miR-4755-5p. The expression of this gene is used to assess blastocyst quality in cattle [63, 64]. As these miRNAs have higher expression in the high blastocyst competent sperm embryos, those of low competent sperm may have an higher expression of this gene, which is not what would be expected. Blastocysts obtained after fertilization using low competent sperm may have compromised expression of ErBB signalling, which may be associated to altered fetal developmental programming and overal survival.

Expression of miR-1225-5p is reduced in blastocysts obtained from low competent sperm. This miRNA may reduce the expression of genes involved in protein degradation processes in the endoplasmic reticulum (ER, figure 5). Protein degradation, which is also reflected by an enrichment of byosinthetic processes in GO terms involving genes targeted by this miRNA, may be higher in embryos produced using low competent sperm. Alterations in ER protein processing have been recently associated with a higher sensitivity of early embryo to exogenous factors, which impact on epigenetic modifications,

including miRNAs levels and embryo survival [65]. Thus, the lower sperm competence may be associated with increased sensitivity of the embry to environmental factors.

To improve the functional interpretation of the results, target prediction analysis was performed using miRWalk, which allows the combination of several gene prediction tools. This approach identified many other canonical pathways, which are known to be important in early embryo development, and which may be affected as a result of differences in miRNAs levels in the blastocysts. These pathways included: regulation of actin cytoskeleton, tight Junction and cell adhesion molecules (CAMs), MAPK, TGF-beta, apoptosis, neurotrophin, endocytosis, calcium, GnRH, insulin and phosphatidylinositol signaling pathway. All these are intracellular gene networks and are linked to cellular processes, which may affect embryonic development and differentiation. The Actin cytoskeleton pathway is reported to be essential for regulation of actin dynamics, which occur during processes of maturation and development in early embryogenesis [66]. Tight junction and cell adhesion molecules (CAMs) signaling pathways control cell interactions and are involved in blastocyst compaction, which is important for blastocele formation and consequent implantation [67, 68]. MAPK signals are involved in embryo development and reported to be important in pluripotency [69]. The inhibition of MAPK genes during early bovine embryogenesis is associated with incresed expression of NANOG, SOX2 and POU5F1 [70]. TGF-beta, apoptosis and neurotrophin signaling pathways are also important in controlling cell proliferation. Among these, TGF-beta signals have been reported to be critical for bovine embryo development. Increased expression of these genes is associated with degeneration of embryos [71]. In the results presented here blastocyts associated to a lower sperm competence may have overexpression of apoptotic signals. Endocytosis signals are reported to be important in pre-attachment development of bovine embryos. Indeed, induction of autophagic activity during early embryogenesis increases the blastocyst developmental rate [72]. Furthermore, autophagy is a negative regulator of ER stress in early embryos. This is in agreement with the results obtained here, where ER signals, which are associated to protein degradation, may be overexpressed in blastocysts obtained from low

competent sperm. Hence the embryos may be more sensible to stress fators. Calcium signals are important in several reproductive processes *e.g.* oocyte activation during fertilization in mouse [73], and interacts with other canonical pathways, including MAPK and apopotosis pathways, which are important as indicators of embryo quality. Calcium signalling may have a role during embryo development and blastocyst competence. Insulin and phosphatidylinositol signaling pathways are both important in cell metabolism. Loss of insulin repectors, such as IGFR1, which is a predicted target of miR-548d, may have affect imprinted genes [74]. Finally, phosphatidylinositol signaling pathway, which has diverse effects on cell processes, may have a potential role in growth and survival of bovine blastocysts [75, 76].

Among all the predicted target genes of the differentially expressed miRNAs, there were 22 genes, which are known to be involved in blastocyst formation and developmental processes. Among these, 5 genes (VASP, TGFBR1, FN1, LEP, RAD51B) are predicted targets of miR-1225-3p and miR-4755-5p. While HAND1 is targetted by miR-4755-5p and miR-458d-3p (Figure 6). VASP is involved in conceptus elongation [77] and trophoblast invasion during implantation [78]. TGFBR1 may be important during gastrulation as is abundant in human epiblastic cells [79]. Fibronectin 1 (FN1) is also a candidate for gastrulation and implantation in bovine embryo [80]. LEP is an important autocrine signal for trophoblastic growth [81]. While, RAD51B plays a role in cell proliferation and early embryonic development [82]. Finally, HAND1 is associated with trophoblastic cell differentation also in cattle [83]. All these genes are implicated in embryo development and implantion processes, and some play a role in placenta development. Thus, changing the timing or levels of their expression may affect tardiver processes including implantation and placenta formation in the embryos.

In conclusion, the results presented here identified miRNAs that are likely to be involved in the regulation of bovine embryo development through various gene targets and signaling pathways. Three of all the expressed miRNAs were differentially expressed between blastocysts produced using high and low competent sperms. These results suggest a paternal influence during early embryogenesis, which may impact on the developmental potential of the early embryos. Further studies will be necessary to confirm these data. A

focused study of the expression of miRNAs and together with their predicted target genes in early stages up to blastocyst from spermatozoa of different developmental potential will reveal the role of these small RNAs at fertilization through to zygote and morula.

3.8. References

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CHAPTER 4

Identification of microRNAs as biomarkers of competent oocytes

4.1. Introduction

Cows with a low antral follicle count (AFC) have reduced fertility, a poor response to superovulation, low circulating concentrations of progesterone [1] and poor pregnancy rate [2]. Indeed, the number of mid-antral follicles of 2-6 mm in diameter, which is used to assess AFC, is negatively correlated with several ovarian traits [3]. Low AFC ovaries are small, have poor vascularization [4] and few healthy follicles. However, follicular fluids from low AFC ovaries have high levels of ovarian hormones, including Growth Hormone (GH) and Progesterone (P4) [5]. These factors result in low ovarian function and poor quality oocytes, which have low developmental [6,7].

Previous studies indicate that reduced oocyte developmental competence may be the consequence of vascular defects and reduced nitric oxide (NO) availability. NO elicits a wide spectrum of intracellular effects and can be stimulatory or inhibitory during oocyte maturation depending on the concentration [4]. The poor quality oocytes from low AFC ovaries have a high frequency of chormosomal mutations, which have been associated with alterations in the localization of Progesterone Receptor Membrane Component 1 and Aurora Kinase B [8]. The molecular mechanisms affecting the developmental competence oocytes are unknown, however, in humans [9], mice [10] and cattle [11], it has been shown that it is the final period of oocyte growth where developmental potential is gained [30]. MicroRNAs (miRNAs), which are involved in ovarian function, are found in the diverse compartments of ovarian follicles, including granulosa cells [12, 13], theca cells [14], follicular fluid and oocyte itself [15]. The role of miRNAs during follicle development has been studied in humans [16-18], mice [19, 20], cattle [21-23], pigs [24, 25] and horses [26]. These studies suggest that the miRNAs may regulate cellular differentiation processes, which occur during follicular

development in a defined spatio-temporal manner. In cattle, follicular fluid miRNAs were found to change during folliculogenesis [27]. Moreover, they were found both free and associated with exosomes, the latter may facilitate transport of specific miRNAs into follicular cells [21].

Dynamic changes in miRNA expression have been described during oocyte maturation, where a large proportion of maternal genes are directly or indirectly under the control of miRNAs [28]. However, in normal condition, miRNA activity seems to be reduced in fully grown oocytes, although their biogenesis is unaffected and their mRNA targets are present [29]. Therefore, reducing miRNA activity may be associated with acquisition of developmental competence, and miRNAs may not be required until the zygotic genome activation is completed and the pluripotency program, which also controls miRNA expression [67], is established.

The goal of the present study was to unravel some of the molecular pathways regulated by miRNAs, which are associated with oocyte developmental competence.

4.2. Materials and methods

4.2.1. Follicular fluid and oocyte collection

Ovaries were obtained from a local abattoir and were transported to the laboratory in warmed (27-30°C) Dulbecco Phospate Buffered Saline (PBS). Ovaries with more than 10 follicles of 2-6 mm in diameter and a dominat follicles (>8 mm) were assigned to high Antral Follicle Count group (H group), while those with less than 10 follicles of 2-6 mm were classified as low AFC group (L group). The classification did not take into account corpus luteum as suggested in previous studies [3, 6].

Follicles were aspirated using a 19-gauge needle and both follicular fluids and cumulus-oocyte complexes (COC) were pooled according to the classification into 50 mL Falcon tubes, which were warmed at 38.8°C. Blood cells and debris were removed from the follicular fluids by centrifugation at 10 minutes at 1500 g and were then stored at -80° C.

Cumulus-oocyte complexes (COCs), which had sedimented to the bottom of tube, were identified using a stereomicroscope and COCs that were medium brown in color with five or more complete layers of cumulus cells were collected [31]. Oocyte denuding was carried out by incubating COCs in modified PBS (supplemented with 36 µg/L pyruvate and 50 µg/mL gentamycin), and 0.5 mg/mL BSA (Sigma Aldrich, fraction V, A-9647, USA) with 100 UI/mL hyaluronidase (Sigma Aldrich, H3757, USA) for 3 minutes and then mechanically pipetting. Pools of 10 denuded oocytes were stored at -80° C.

4.2.2. Progesterone (P4) quantification

Progesterone (P4) in follicular fluids was determined in undiluted samples using a competitive enzyme immunoassay. An in-house produced anti-P4 monoclonal antibody was used as capture antibody and progesterone-11-HS-HRP (Fitzgerald Industries International, Concord, MA) as labeled hormone. The procedure was carried out as described by Borromeo and colleagues [32] and was determined in 5 biological replicates of folicular fluid from good and poor AFC sampled on different slaughter days, concomitantly with follicular fluid and oocyte collection. Statistical analysis was performed by *T-student test*. *P-values* less than 0.05 were considered to be statistically significant.

4.2.3. Determination of oocyte mithocondrial activity

Mitochondrial activity was determined by staining oocytes with two mitotracker probes (Invitrogen by Life Technologies, Carlsbad, CA, USA): MitoTracker FM Green (MTG), which allows the analysis of relative mitochondrial activity because it stains all mitochondria without distinguishing them according to the membrane potential (Δψ), and MitoTracker Orange CMTMRos (MTO), which stains only active mitochondria.

Briefly, denuded oocytes, which were obtained as indicated above (par. 4.2.1.), were incubated in 0.4 % PBS/BSA (Sigma Aldrich, fraction V, A-9647, USA) to which 280 nM MTG, 200 nM MTO and 5 µg/µl Hoechst 33452 were

added and they were then incubated for 30 minutes at 38.8°C with 5% CO_2 in air and maximum humidity. The oocytes were then washed three times in PBS/polyvinylpyrrolidone (PVP), mounted on slides and were observed using an epifluorence microscope (Axio scope A1, ZEISS) at specific wavelengths for MTG (488 nm), MTO (546 nm) and Hoechst (380 nm) dyes.
Mitochondrial activity was estimated as the MTO/MTG Ratio using digital images of both mitotracker probes obtained at the same exposure time. Quantification of fluorescence was carried out using ImageJ. All experiments were repeated 5 times on different slaughter days. Statistical differences were analyzed using a *T-student test*. *P-values* less than 0.05 were considered to be statistically significant.

4.2.4. Small RNA extraction from follicular fluids

MiRNAs were extracted from three pools of 300µl of follicular fluid from H and L groups using Nucleospin MiRNA plasma (MACHEREY-NAGEL GmbH & Co. KG, Germany), which is specific for biofluid small RNA extraction, following the manufacturer's instructions. The high AFC group samples are indicated in the rest of the chapter as: FF1, FF2 and FF3 and the low group samples as FF4, FF5 and FF6.

4.2.5. Total RNA extraction from oocytes

Total RNA extraction was performed from three pools of 30 oocytes from H and L groups and processing procedure 2 (see Chapter 2, par. 2.2.3.1.). RNA quality and quantity of each sample was assessed using a RNA Pico Chip and a 2100 Agilent Bioanalyzer. The samples are indicated as: Ovo1, Ovo2 and Ovo3 for the H group and Ovo4, Ovo5 and Ovo6 for the L group.

4.2.6. Small RNA sequencing

Library preparation and deep sequencing were carried out for both follicular fluid and oocyte RNA samples using the procedure described in Chapter 2 (par 2.2.4.). Libraries from follicular fluids and oocytes were sequenced into

two lanes of an Illumina HiSeq2500 using a 50 bases long sequence read module.

4.3.7. Bioinformatic and functional analysis

Filtering of raw sequences, annotation and discovery of miRNAs were carried out according to the pipeline described in Chapter 2 (Chapter 2, par. 2.2.5.).
Gene expression analysis was perfomed using the R package '*EdgeR*' (http://bioconductor.org/packages/release/bioc/html/edgeR.html) to identify differentially expressed miRNAs. MiRNAs with log2 fold change differences ≥ 1, *P-value* ≤ 0.05 and False Discovery Rate (FDR) ≤ 0.1 were considered as statistically differentially expressed. The interaction between differentially expressed miRNAs and their target mRNAs was predicted using two procedures:

1) miRWalk 2.0; a widely used web-based database to predict animal miRNA-target mRNA interactions [33]. In total 6 prediction programs were combined into a pipeline for the analysis as follows: miRanda-rel2010 [34], PicTar2 [35], PITA [36], RNA22v2 [37], RNAhybrid2.1 [38] and Targetscan6.2 [39]. The lists of predicted target genes of individual miRNAs were imported to DAVID Bioinformatics systems; a freely available bioinformatic tool (http://david.abcc.ncifcrf.gov/). Gene ontology (GO) analysis was carried out using this tool to identify most important enriched biological and cellular processes. The DAVID web-tool facilitated the identification of the canonical signaling pathways significantly enriched with the predicted target genes of each miRNAs, which were then analyzed using Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [40].

2) DIANA miRPath v2.0 using homologous human miRNA and gene union options. DIANA miRPath predicts miRNA targets (in CDS or 3'-UTR regions) provided by the DIANA-microT-CDS algorithm, or experimentally validated miRNA interactions derived from DIANA-TarBase v6.0 [41]. This information is used by DIANA miRPath for KEGG pathway analysis. Only the canonical pathways with *P-value* <0.01 were chosen and the predicted target genes of miRNAs from these pathways were taken into account for GO analysis and

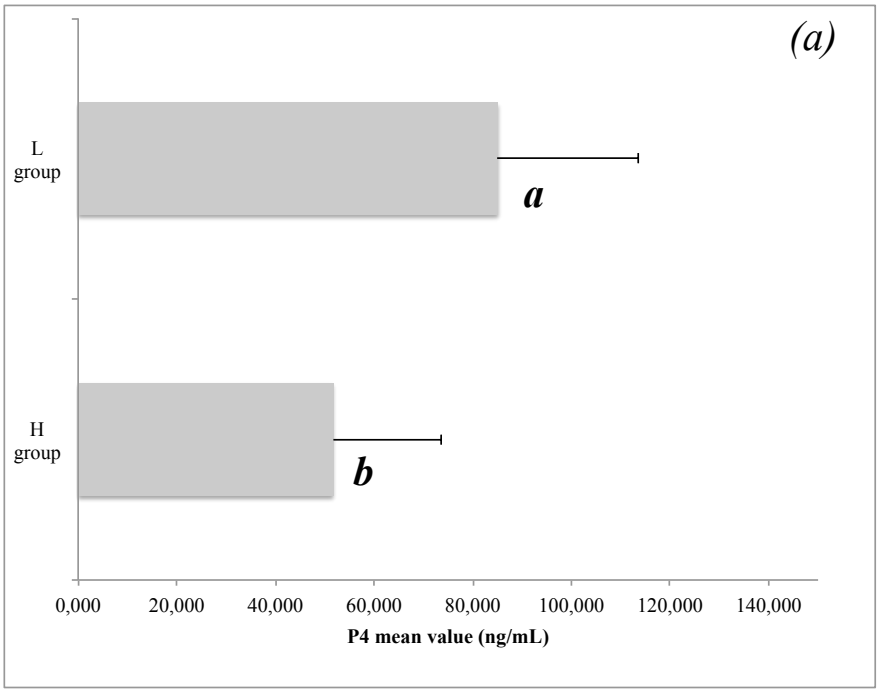
gene interactions, which were performed using ClueGo plugin into Cytoscape V 3.2.1 [42].

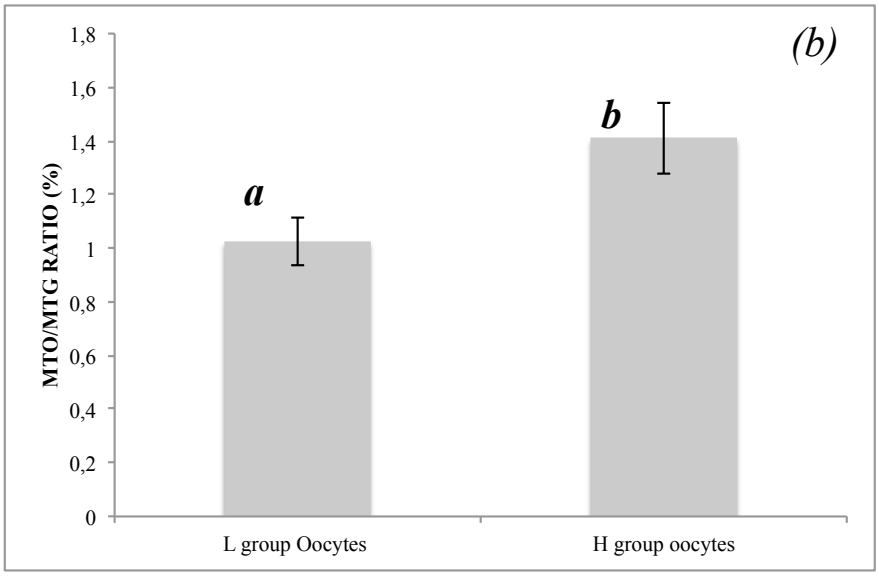
4.3. Results

4.3.1. Mithocondrial activity and P4 quantification

To assess the overall mitochondria activity, 26 Germinal vesicle (GV) oocytes of L group and 29 of H group were immunostained and the MTO/MTG Ratio was calculated in order to evaluate overall activity of mithocondria (Table 1b and figure 2). In particular, all the GV oocytes were similar in their developmental stage as they had a GV2-GV3 chromatin configuration of nucleus (Figure 2) [43, 44]. But, the GV oocytes of L group seem to have a lower activity of mithocondria. Indeed, these oocytes had a lower MTO/MTG ratio than those of H group (Table 1(*b)*: 1.0 ± 0.1 versus 1.4 ± 0.1, respectively, $P<0.05$). Furthermore, Follicular fluids, which were collected from L group, had higher P4 concentration (ng/mL) than those of H group (Table 1(*a)*: 85.1 ± 28.5 *versus* 51.7 ± 21.9, respectively, $P<0.05$).

Table 1. (*a*) P4 quantification of follicular fluids from L and H Antral Follicle Count ovaries. Data are represented as mean value ± Standard Error of Mean (SEM); (*b*) MTO/MTG ratio between H and L group oocytes. Data are represented as mean value ± Standard Error of Mean (SEM).





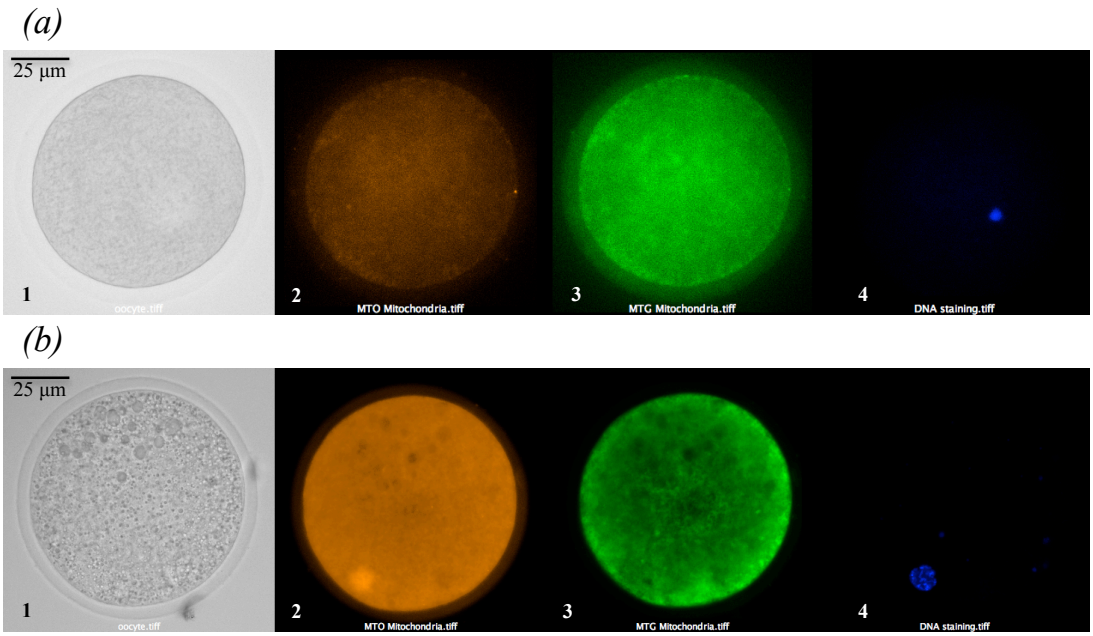


Figure 2. Images of an L (a) and H (b) group oocyte after MTO, MTG and HOECHST 33452 staining. **1)** real image of the oocyte **2)** mitotracker Orange image which, was acquired at 546 nm length ; 3) mitotracker green image, which was acquired at 488 nm length; 4) Hoechst 33452 image, which was acquired at 380 nm. MTO and MTG probes bind to the mitochondria and allow the evaluation of mitochondria activity. Hoechst 33452 binds DNA and allows the determination of developmental stage of the oocyte associated with the nuclear chromatin configuration [43, 44].

4.3.2. Characterization of miRNA deep sequencing data

To investigate the miRNAs associated with oocyte developmental competence, 3 miRNA libraries of L and H groups were generated using RNA from follicular fluids and oocytes. After filtering of low quality reads 13.7M (95.8 %) and 15.2M (95.7 %) reads were mapped to the bovine reference genome from follicular fluid libraries of the L and H groups, respectively (Table 2*a*) and 71.3M (94.8 %) and 47.1M (91.9 %) reads from oocyte libraries of L and H group, respectively (Table 2*b*). Quality filtered sequence reads were used to identify annotated human and bovine miRNAs present in miRBase release 21. For all reads which passed the quality control criteria from the follicular fluid libraries, 6.0M and 3.9M from L and H groups, respectively,

were found to be similar to known bovine or human miRNAs, comprising 45.8% and 25.5% of the total quality reads obtained. For oocyte libraries 2.3M (3.2%) and 1.1M (2.4%) of L and H groups, respectively, were mapped to known bovine or human miRNAs.

Table 2. Summary of sequence reads aligned to bovine reference genome and known miRNAs annotated in miRBase for follicular fluid (*a*) and oocyte (*b*) samples.

(a)

Antral Follicle count group	Sample ID*	Filtered reads (n)	Mapped reads (n)#	% Mapped reads	Known MiRNA mapped reads (n)§	%know miRNA mapped reads
High group	FF1	10456471	9968593	95.3	2932009	28.0
	FF2	18668263	17896115	95.9	3593507	19.2
	FF3	18668263	19745922	95.9	515234	27.6
Low group	FF4	28014477	26861734	95.9	13280007	47.4
	FF5	3500296	3337883	95.4	1100184	31.4
	FF6	3500296	9078429	95.6	3600075	37.9

(b)

Antral Follicle count group	Sample ID*	Filtered reads (n)	Mapped reads (n)#	% Mapped reads	Known MiRNA mapped reads (n)§	%know miRNA mapped reads
High group	OVO1	40099509	36547022	91.1	729050	1.8
	OVO2	55537666	50589909	91.1	1024431	1.8
	OVO3	58196435	54283950	93.3	1673109	2.9
Low group	OVO4	89670665	84988590	94.8	2681559	3.0
	OVO5	51264984	48621998	94.8	1914506	3.7
	OVO6	84609787	80227468	94.8	2321895	2.7

*: FF1, FF2, FF3, OVO1, OVO2 and OVO3 denote for biological triplicates of Follicular fluid and oocyte from high group and FF4, FF5, FF6, OVO4, OVO5, OVO6 denote for biological triplicates of follicular fluid and oocyte from low group.
#: Number of quality filtered reads aligned to bovine reference genome (assembly UMD3.1).
§ Proportion of mapped sequence reads aligned to known annotated miRNAs in miRBase release 21

4.3.3. MiRNAs expressed in follicular fluids and oocytes

MiRNAs with as least 1 read in at least two of the three biological replicates were considered as detected. Among the short RNAs that could be mapped to known bovine and human miRNA precursors, the most abundant length was 23 nucleotides (nt) for both follicular fluid (a) and oocyte miRNAs (b) of L and H group, which corresponds with mature miRNA size (Figure 3).

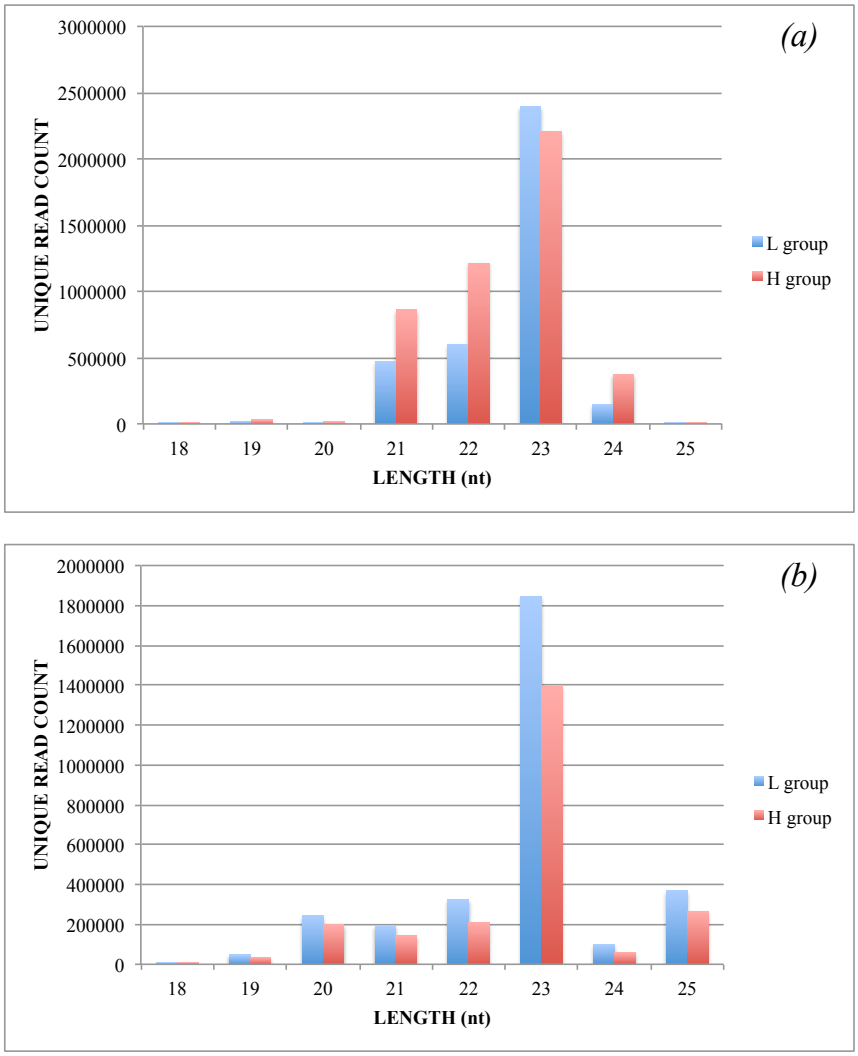


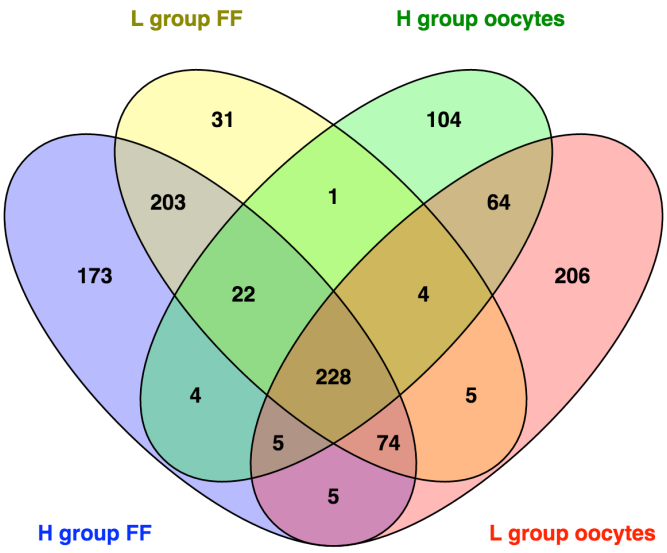
Figure 3. Length distribution (nucleotide = nt) of miRNA populations in follicular fluid (a) and oocyte (b) miRNAs from L and H groups, respectively.

Annotation of miRNA sequences with miRDeep identified 1236 known miRNAs, across follicular fluid and oocyte samples (Graph 3). The mean number of follicular fluid miRNAs was similar between L and H groups, which were on average 497 ± 85 in L and 520 ± 22 in H (P>0.05), of which 203 were

in common. The mean number of miRNA expressed in oocytes was higher in the L group with respect to the H group: 373 ± 12 vs 285 ± 36 (P<0.05), respectively, of these 301 were in common between the groups. Moreover, follicular fluids and oocytes of H group (P<0.0001) had a different mean number of expressed miRNAs, which was not different between follicular fluids and oocytes of L group (P>0.05).

The miRNAs bta-miR-10b, bta-miR-27b, bta-miR-143 and bta-miR-22 were the abundantly expressed in follicular fluids of both L and H groups; of these miRNA bta-miR-10b had the highest level of expression with a read count of 3.0M in L and 1.5M in H groups; accounting for 50.1% and 39.2% of the sequence reads aligned to known miRNAs, respectively. The miRNAs bta-miR-10b, bta-miR-92a and a miRNA with the human homologue hsa-miR-6509-3p had the highest level of expression in the oocytes of L and H groups. Bta-miR-10b was the most abundant with a read count of 1.7 M in L and 758,461 in H group, which represented the 32.3% and 66.4% of the mapped reads to known miRNAs, respectively. Moreover, hsa-miR-6509-3p and hsa-miR-513c-5p, which were only annotated for human, were abundantly expressed in the oocytes of both H and L groups. In particular, the reads of hsa-miR-6509-3p accounted the 8.5% and 9.7% in L and H groups, respectively, while those of hsa-miR-513c-5p were represented by 1.0 % in L and 1.2 % in H group.

Apart from these highly expressed miRNAs, tthe majority of annotated miRNAs had fewer than 50 reads (data not shown). Among the top 10 abundantly expressed miRNAs in each group, 4 miRNAs (bta-miR-10b, bta-miR-423, bta-miR-22 and bta-miR-148) were expressed in both follicular fluids and oocytes of L and H groups (Table 3 and 4).



Graph 3. Venn diagram. Comparison of common known and homologous human miRNAs between High (H) group and Low (L) group of both follicular fluid and oocyte samples.

Table 3. List of Top 10 highly abundantly expressed miRNAs in follicular fluids of L and H groups.

Follicular fluid miRNAs in L group		**Follicular fluid miRNAs in H group**	
miRNA ID	**Avarage read counts***	**miRNA ID**	**Avarage read counts***
bta-miR-10b	3007349	bta-miR-10b	1519637
bta-miR-27b	519897	bta-miR-27b	399262
bta-miR-143	389404	bta-miR-22	248940
bta-miR-22	324146	bta-miR-143	223850
bta-miR-423	124454	bta-miR-30a	101416
bta-miR-148a	118611	bta-miR-25	96767
bta-miR-21	97675	bta-miR-30e	83203
bta-miR-30a	79320	bta-miR-186	70867
bta-miR-25	71122	bta-miR-148a	66214
bta-miR-378	62790	bta-miR-423	53478

Table 4. List of Top 10 highly abundantly expressed miRNAs in oocytes of L and H groups.

Oocyte miRNAs in L group		Oocyte miRNAs in H group	
miRNA ID	**Avarage read counts***	**miRNA ID**	**Avarage read counts***
bta-miR-92a	1693243	bta-miR-10b	758461
bta-miR-10b	1497538	Novel:hsa-miR-6509-3p	110264
Novel:hsa-miR-6509-3p	195705	bta-miR-92a	44550
bta-miR-26a	106697	bta-miR-423	20994
bta-miR-22	57394	bta-miR-148a	17476
bta-miR-423	49303	bta-miR-22	17175
bta-miR-148a	45677	Novel:hsa-miR-513c-5p	14042
bta-miR-27b	23421	bta-miR-16b	10557
Novel:hsa-miR-513c-5p	23310	bta-miR-92b	9352
bta-miR-30d	14527	Novel:hsa-miR-4446-5p	9315

4.3.4. Differential expression of follicular fluid miRNAs in L and H group

Comparison of the expression level of miRNAs between L and H groups in the follicular fluids identified 66 differentially expressed miRNAs. The majority of the annotated miRNAs were expressed in follicular fluids of both L and H groups (data not shown; $P \leq 0.05$ and FDR≤ 0.1). Among the differentially expressed miRNAs, there were 50 with a reduced and 16 with increased level of expression in the group of low antral follicle count ovaries with respect to those with an high antral follicle count (Table S5 – supplementary material). Some of these miRNAs were clusters located in the same chomosomal region and may be co-regulated; these included: miR-450a and miR-450b, miR-99a and let-7c, miR-24-3p and miR-195, miR30c and miR-30a, miR-18a and miR-92a. The Log2 fold change values in L group ranged from -2.71 (bta-miR-885) up to -4.11 (bta-miR-150).

4.3.5. Differential expression of oocyte miRNAs in L and H group

Analysis of H and L group oocytes identified 6 miRNAs which were differentially expressed ($P \leq 0.05$ and FDR ≤ 0.1). Among these miRNAs, five had higher expression in the L group (bta-mir-145, bta-mir-150, bta-mir-342, bta-mir-450b and bta-mir-380) and one (bta-miR-10a) had lower expression with respect to H group. Interestingly, three of differentially expressed miRNAs (bta-miR-10a, bta-miR-150 and bta-miR-450b) in the oocytes were also differentially expressed between follicular fluids of H and L groups, but with inverse expression, *i.e.* the miRNA, with increased expression in the oocyte of L group, was reduced in the L group follicular fluid and viceversa. The Log_2 fold change values in L group ranged from -1.62 (bta-miR-10a) up to 9.51 (bta-miR-145).

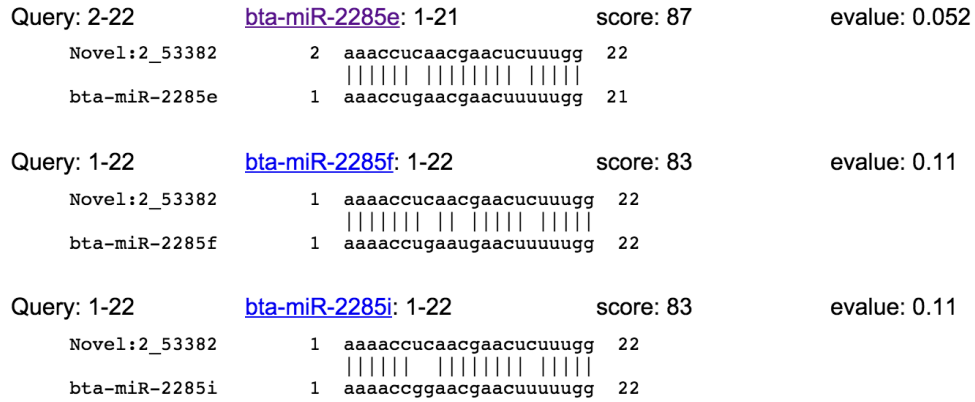
4.3.6. Prediction of novel bovine miRNAs in follicular fluids and oocytes

The small RNA sequences were used to identify known annotated miRNAs present in the oocytes and follicular fluids and also to identify novel miRNA sequences. Potential novel miRNAs were identified using miRDeep2 software and, only when the sequences were identified into two of the three biological replicates of L and H groups of both follicular fluids and oocytes with at least 1 read count, they were considered to be a putative novel miRNAs. There were 708 putative novel miRNAs identified. These sequences were searched against miRBase release 21 to identify homologous known annotated matured miRNAs, and BLASTN tool (www.ensembl.org) was used to identify the genomic location. Only one sequence (novel_2_53382) had some similarity to the bta-miR-2285 family, even though this sequence was not annotated in miRBase release 21 (Figure 4), in addition it aligned with a different chromosomal region from mir-2285 family (Table 5). Thus, indicating that it may be a novel miRNA which may belong to that miRNA family. Genomic context analysis of these predicted novel miRNAs revealed that 5 novel miRNAs were transcribed from intergenic region, 3 from intronic region of transcripts (BCAT2, COPG2 and GART) and one other from exonic region of the ZNF358 gene (Table 5). Of the novel miRNAs identified, 7 were differentially expressed in follicular fluids and 2 in oocytes.

Table 5. Putative novel miRNAs expressed in follicular fluids and oocytes of L and H antral follicle count ovaries.

Sample type	Provisional Matured miRNA miRNA ID Sequence	Mature miRNA sequence	genomic coordinates and strand of miRNA precursors	Average read count in L group	Average read count in H group	Genomic region of novel miRNAs and overlapping transcript
Follicular fluid	Novel:19_46128	UUCCCGCCGGCGUAUGCUGCUGU	19:43830014-43830039[Forward]	3.7	28	intergenic
	Novel:18_44358	UUCUCAGCCCCAGGGGUUCCU	18: 55872705-55872727[Reverse]	1.3	15.8	intronic, BCAT2
	Novel:4_87488	ACUUUUGCCCCUAGUAACGGACU	4:95112096-95112121[Forward]	46.7	132	intronic, COPG2
	Novel:X_110269	UGAGCACACCUGCCUGAGCAGA	X:30280443-30280467[Forward]	2.7	24.3	intergenic
	Novel:2_53382	AAAACCUCAACGAACUCUUUGG	2:115554887-115554911[Reverse]	21.7	4.3	intergenic
	Novel:7_99116	AUGAAGAUCUGGAGCCUGUCUCC	7:17610270-17610295[Forward]	5	13	exonic, ZNF358
	Novel:1_108989	UCCUAAAGGAGAUCAGUCCUGGGUG	1:1268082-1291083[Forward]	194	87.3	intronic, GART
Oocyte	Novel:20_55299	UCCUUUCUGAGCCACCAGGGA	20:19697855-19697877[Reverse]	123.3	691.3	intergenic
	Novel:1_3066	UUCCUGCAAACUUAUCCUAUG	1:89855043-89855065[Reverse]	3154	540	intergenic

Figure 4. Alignment of the novel_2_53382 sequence using miRBase release 21.



4.3.7. Target gene prediction, Gene ontology and pathways enriched by differentially expressed miRNAs in follicular fluids

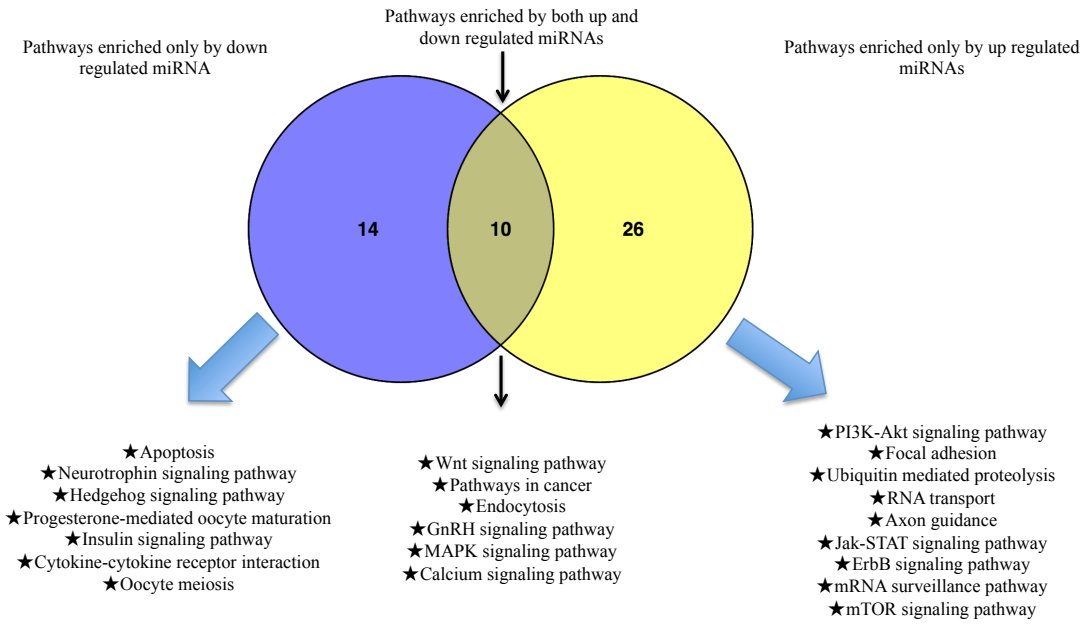
Analysis of miRNA expression in follicular fluids identified 66 miRNAs that were differentially expressed between L and H groups with significance of *P<0.005*, increasing the stringency, using a threshold of *P*<0.001, 25 remained significant and these differentially expressed miRNAs were used in the functional analysis (Table S5 – supplementary material). DIANA miRPath identified 53 KEGG pathways which were enriched by these miRNAs (Table S6 – supplementary material). Among the most enriched KEGG pathways, there were several canonical pathways which were associated with cellular processes, in particular: cell adhesion (Focal adhesion signaling pathway), cell proliferation (Cell cycle, MAPK, RNA transport, Wnt, mTOR, PI3K-Akt and ErbB signaling pathways), cell surival and growth (Neurotrophin signaling pathway, Oocyte meiosis, TGF-beta signaling pathway) and cell metabolism (Insulin signaling pathway).

Among the 10 most significant KEGG pathways, there were 161 predicted target genes of miRNAs. Of these, only five (BCL2, FOXO3, KIT, TP53, VEGFA) are known to be involved in ovarian follicle development. GO analysis showed that these genes are predominantly involved in biological processes including cell cycle regulation, cell proliferation, apoptosis, post-

traslational modifications, macromolecule biosynthesis and cell migration (Table S7 – supplementary material).

4.3.8. Target gene prediction, Gene ontology and pathways enriched by differentially expressed miRNAs in oocytes

Functional analysis of predicted target genes of miRNAs identified the biological functions that were putatively differentially regulated between the L and H group oocytes. The target genes for the abundant expressed miRNAs and, thus, were likely to have lower expression in L group, influenced GO terms such us cell proliferation, RNA transport and localization, catabolic modification of macromolecules and response to hormone stimulus (Table S8 – supplementary material). While, GO terms enriched with genes targeted by miRNAs with lower expression in the L vs H groups were mainly associated with mechanisms involving RNA transcription (Table S9 – supplementary material). KEGG pathway analysis indicated that 36 canonical pathways were enriched with target genes of miRNAs with higher expression in the L vs H group oocytes and 18 were associated with target genes of reduced miRNAs. Moreover, 10 of the KEGG pathways were found to be commonly enriched by both abundant and reduced miRNAs in the L vs H group oocytes (Graph 4). The miRNAs highly expressed in the L group oocytes targeted genes involved in signaling pathways that are relevant for oocyte quality including PI3K-Akt, Jak-STAT, mTOR and ErbB. Other signaling pathways identified are important in controlling oocyte maturation, including meiosis, progesterone-mediated oocyte maturation and apopotosis; these had higher expression of miRNAs in L group with respect to H group.



Graph 4. Venn diagram comparing the up- and down-regulated canonical signaling pathways.

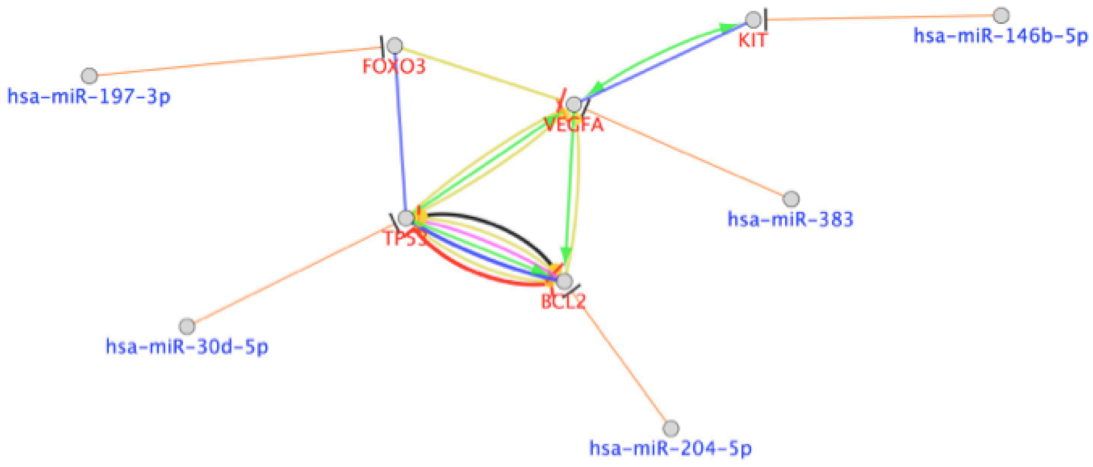


Figure 5. Representation of gene interactions between FOXO3, TP53, BCL2, VEGFA and KIT and their miRNA regulation.

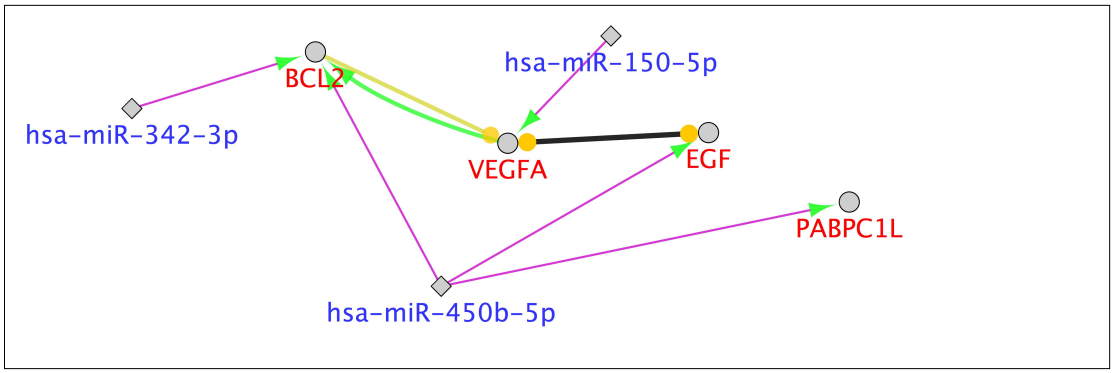


Figure 6. Representation of gene interactions between BCL2, VEGFA, EGF and PABPC1L and their miRNA regulation.

4.4. Discussion

This study used a model based on low and high antral follicle count ovaries, which is known to be associated with oocyte developmental potential [6]. Follicular fluids and immature oocytes from mid-antral follicles of 2-6 mm in diameter were collected and studied. The number of these follicles is representative of the ovarian reserve and is a practical way to measure ovarian function, which has been used in several species including humans [45] and cattle [5].

The results identified several biological processes, which are critical for oocyte developmental potential and may be compromised in the follicles and oocytes of low quality ovaries because of a miRNA mis-regulation. Several miRNAs identified have been described in previous studies of normal ovarian physiology focusing on follicular fluid and cells which form the ovarian follicles, *i.e.* oocytes, theca and granulosa cells during correct follicle development of cattle [21-23, 27, 46-48] and human [16-18, 49, 50]. Among these, miR-769, miR-1343, miR-450a, miR-204, miR-100, miR-99a, miR-1271 and miR-451 were abundantly expressed in granulosa cells during development of follicles from subordinate to dominant preovulatory [48]. In the present study, these miRNAs had lower levels of expression in low quality ovaries. Similarly, miR-190b, which has been found to increase in theca cells of dominant with respect to subordinate follicles [27], was reduced

in follicular fluids of the poor quality ovaries. The small RNAs let-7c and miR-451 have been shown to be present at a higher level in exomes in the follicular fluid of follicles from growing oocytes compared with fully-grown oocytes [21]. In the present study exomes were not separated from circulating miRNAs, however, these miRNAs were reduced in follicular fluids of mid-antral follicles in low quality ovaries.

All these miRNAs in follicular fluid are likely to affect granulosa and theca cells, however, there are miRNAs such as miR-320, which was reduced in follicular fluids of low quality ovaries, and which may have an effect directly on the oocyte. Indeed, this miRNA is reduced in follicular fluids of women with polycystic ovarian syndrome and the reduction of this miRNA impacts on oocyte quality in mouse during early embryogenesis affecting embryo developmental potential [49].

The present study identified 1236 miRNAs, some of which were differentially expressed in follicular fluids and oocytes from high and low AFC ovaries. To assess the gene networks, which may be involved in the alteration of biological processes during follicle development in low quality ovaries, KEGG pathway analysis was performed. Many canonical signaling pathways in the follicles of low quality ovaries were targeted by miRNAs with reduced levels of expression. As miRNAs usually repress gene expression, target pathways of these miRNAs may have an increased expression. Among the canonical pathways, the most enriched for differentially expressed miRNA gene targets was the PI3K-Akt signaling pathway. This pathway is known to control follicle growth, differentiation and survival [51, 52], and includes the genes BCL2, FOXO3, TP53, VEGFA and KIT, which were predicted targets of miR-204-5p, miR-197-3p, miR-146b-5p, miR-30d-5p and miR-383. The reduction of these miRNAs in follicular fluids of low quality ovaries may be associated with abnormal expression of cell proliferation signals, which may in turn affect the development of follicles. It has been reported that inhibition of VEGFA increases apoptosis in granulosa cells through an imbalance among the BCL2 family members in mouse [53]. This study showed that FOXO3 expression may be reduced via miR-197 resulting in the repression of VEFGA and consequent misregulation of BCL2 (figure 5). These molecular

processes may increase apoptosis and lead to premature atresia of follicles and corresponding oocytes in the low AFC ovaries.

Among the predicted target genes of the differentaly expressed miRNAs, four genes PABPC1L [64], VEGFA [65], BCL2 [66] and EGF [68], are known to be important in oocyte biological processes and interact with each other (Figure 6). Among these genes, BCL2, as discussed above, has been shown to have an aberrant level of expression in poor quality porcine oocytes, and is correlated with a low mitochondrial activity [66]. These results indicate that the overall lower mitochondrial activity of the low quality oocytes may result from inhibition of BCL2 expression mediated by miR-342 and miR-150.

The negative impact of miRNAs in the follicles of low quality ovaries may also be exercised through other signaling pathways such as Neurotrophin, Oocyte meiosis and TGF-beta signaling. Neurotrophins are known to regulate follicle formation and development [54]. TGF-Beta signaling plays an important role in controlling the production of peptide hormones by the ovary, including Antimüllerian hormone (AMH) [55], and is associated with the differential expression of TGF-β/BMP genes in human ovary from the primordial to the late secondary stage follicles [56]. Several of the differentially expressed miRNAs predicted to target genes involved in this pathway such as SMAD4, SMAD7, TGFBR1 and TGFBR2. These genes, if not correctly regulated, may induce an earlier atresia of follicles. Pertubation of TGF-beta signals may explain the higher concentration of Progesterone found in follicular fluids of low quality ovaries. Previous studies have reported that the lower concentration of AMH is associated to the higher concentration of progesterone [3]. It is known that high concentration of Progesterone impact negatively on the oocyte quality in cattle [57] and humans [58].

The poor follicular environment is likely to impact on the oocyte maturation and quality. The immature oocytes collected from follicles of high and low quality ovaries, had 8 miRNAs, which were differentially expressed. KEGG pathway and Gene Ontology analysis indicated that several molecular mechanisms and biological processes may have been affected in these oocytes. Among the differentially expressed miRNAs, miR-150 is described to be abundant in immature bovine oocytes and diminishes at the time of

embryo genomic activation [59]. In the present study miR-150 was more abundant in low than high quality oocytes, although this miRNA was among the most expressed miRNA in oocyte of both groups. The level of expression of miR-150 is likely to be important and hence the elevated expression in low quality oocyte may have an adverse effect on the target pathways.

Among the other differentially expressed miRNAs between high an low AFC ovaries, miR-145 affects the TGF-beta signaling pathway and is important in the development of primordial follicle in mouse: inhibition decreases the proportion of primordial follicle and increases that of growing follicles [60]. MiR-150 is abundant in low quality oocytes demonstrating that the oocyte maturation may be affected by TGF-beta signalling. In particular, initiation of primordial follicle development and primordial follicle quiescence may be seriously compromised in low quality ovaries.

Functional analysis of differentially expressed miRNAs in the oocytes identified that the principal biological processes, which are targetted in oocytes from low AFC ovaries, are RNA synthesis and translation. These molecular processes are required for the accumulation of maternal RNAs and to build proteins in the oocyte, which is associated with acquiring developmental competence [30]. The expression of oocyte miRNAs may follow maternal RNA accumulation [59, 61, 62] and, thus, oocyte miRNAs may be silent during oocyte maturation. It is likely that there are miRNAs, which are actively involved in coordinating maternal RNA accumulation during oocyte maturation. Indeed, KEGG pathway analysis showed that the target genes of the abundant miRNAs expressed in the oocyte include RNA transport, ubiquitin mediated proteolysis and mRNA surveillance signaling pathways. These results indicate that there are gene networks in the low quality oocytes which may regulate the accumulation of maternal RNA that are misregulated through variations in miRNA levels. Other canonical pathways may be compromised. Canonical pathways in which target genes of the miRNAs with increased expression in the L group include: Focal Adhesion, Jack-stat, ERBB and mTOR signaling patways. Among these, focal adhesion pathway regulates the communication between oocyte and its cumulus cell [63] and may be down-regulated. The canonical pathways,

which may have an increased expression, are: apoptosis, progesterone-mediated oocyte maturation and oocyte meiosis. Increased expression of apoptosis signals is likely to be harmful for oocyte quality. Oocyte meiosis signals are mediated in part by progesterone, which is important in stimulating the resumption of the two meiotic division cycles required in the maturation of the oocyte, and if excessive may be associated with low quality oocytes leading to earlier atresia.

Interestingly, miR-450b, miR-150 and miR-10a were found to be differentially expressed in both follicular fluids and oocytes. MiR-450b and miR-150 were abundant in low quality oocytes but were reduced in the follicular fluid, while miR-10a, which was reduced in the oocytes, was abundant in the follicular fluid. These differences may be due to the secretion of these miRNAs by the cells of the follicle, such us theca or granulosa cells, and stored in follicular fluid, then contribute to the development of the different cell types during the follicle maturation process. Indeed, among these miRNAs, miR450b was found to be abundant in granulosa cells of dominant follicles in cows [48].

In conclusion, the results of the present study indicate that miRNAs may be important for oocyte maturation and the acquisition of developmental competence, which may be influenced by several biological processes regulated by miRNAs and important for follicle health. Moreover, the oocyte quality may be associated with the pattern of miRNAs present in both follicular fluids and oocytes. Thus, follicular fluid derived miRNAs may be real-time markers of oocyte quality, which could be used to improve Reproductive Biotechnologies. Further studies will be necessary to determine if the miRNAs shown to be differentially expressed between high and low AFC ovaries in this study regulate the predicted target genes. The study should then be extended to explore the regulation of gene expression as the oocyte matures and gains competence to be ready for the fertilization. This knowledge of the biological processes and gene networks involved during folliculogenesis and oogenesis is important for improving fertility in livestock and humans.

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CONCLUSIONS

The PhD program created new knowledge on the paternal and maternal contribution on early embryo development to help decipher early reproductive physiology. All the experiments were focused on profiling miRNA expression, which was carried out using high-throughput sequencing. These methodologies potentially identify all the miRNAs present in a sample and can provide a global vision of the gene networks and molecular mechanisms relevant, in this case, to blastocysts, oocytes and follicular fluids. The data produced has improved knowledge of miRNAs in bovine oocytes and embryos. Both known and novel bovine miRNAs, were used for prediction, including the identification of bovine miRNAs which were homologous to human ones. The data confirmed the evolutionary conservation of miRNAs among species.

The study of paternal influences on early embryogenesis identified the sperm contribution of a small set of miRNAs to blastocyst developmental competence. These results indicate that miRNAs are actively involved in regulating embryogenesis through the control of important gene networks relevant for embryo development. Further study will be necessary to confirm these data and, in particular, it may be useful to look at the expression of both differentially expressed miRNAs and predicted target genes in spermatozoa and early embryo stages, from zygote, through 2-16 cell stage embryos to morula. This will enable the assessment of the role of parental mRNA during the process from fertilization to the pre-implantation embryo. These extended studies may identify miRNAs that can be used as biomarkers of blastocyst quality.

The miRNA data for follicular fluids and oocytes from ovaries of different functionality identified a miRNA expression blueprints associated with the high and low quality of oocytes, which may alter the expression of a number of genes relevant for both folliculogenesis and oogenesis. These results

contribute to our understanding of molecular mechanisms involved in oocyte maturation and acquisition of competence.

Further experiments should be performed to confirm these findings both *in vitro* and *in vivo*. Gene expression analysis of both miRNAs and relevant target genes associated to oocyte competence should be further investigated to confirm the regulatory targets for the miRNAs. In addition, miRNA and mRNA expression should be examined in immature and *in vitro* matured oocytes. RNA interference experiments could also be carried out to examine the effects of miRNAs on other cells which constitute to the ovarian follicle, specifically theca and granulosa cells. Moreover, *in vivo* studies will be necessary to determine whether follicular fluid miRNAs can act outside the ovary and if their level in the bloodstream can be detected thereby providing non-invasive, real-time markers to determine oocyte quality in living animals.

In conclusion, knowledge derived from this 3-year PhD study has provided background information for new tools to improve in vitro embryo production (IVP) for advanced reproductive biotechnologies.

SUPPLEMENTARY MATERIAL

CHAPTER 2

PROFILING OF microRNAs IN BOVINE BLASTOCYSTS USING DEEP SEQUENCING

Table S1. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways obtained using DIANA miRPath v2.0 are reported up to *P-value* <0.0001. The target genes and homologous human miRNAs are reported for each KEGG pathways.

KEGG pathways	*P-value*	TARGET GENES	miRNAs
Cell cycle (hsa04110)	$7.59e^{-11}$	ESPL1, CDC6, GSK3B, RBL2, E2F1, SMC1A, E2F2, CDC14A, CDC25,MCM6, CCND2, ORC1, CDKN1B, STAG2, CDKN2A, CDK6, TP53, ANAPC10, SMAD, CCNE2, E2F5, SKP2, MYC, TTK, RB1, CDC7, CDC20, BUB1B, MAD2L1, TGFB2, CCNE1, CDKN1A, CDKN2D, RAD21, MCM3, CDC25A	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
Prion diseases (hsa05020)	$2.98e^{-08}$	EGR1, PRKACA, PRNP, IL1A	hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-191-5p, hsa-miR-423-5p, hsa-miR-192-5p

Pathways in cancer (hsa05200)	$2.01e^{-06}$	FZD7, FOS, GSK3B, STAT3, E2F1, TGFBR1, ERBB2, E2F2, BID, TCF4, APC, CRK, WNT1, RAD51, WNT5A, BCL2, CDKN1B, PLD1, WNT3, BRCA2, IGF1R ,EGFR, CDKN2A, APPL1, RET, CDK6, PML, TP53, PTK2, ITGAV, FZD4, MMP2, MAPK9, SMAD4, MSH6, CCNE2, SKP2, MYC, MMP9, MSH2, PIK3R1, RB1, SOS1, HSP90B1, FGF2, FZD1, FAS, TGFB2, PPARG, NKX3-1, CCNE1, CDKN1A, CYCS, SUFU, TCF7, VEGFA, PTEN, TGFB R2, TFG	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
Prostate cancer (hsa05215)	$5.86e^{-05}$	GSK3B, E2F1, ERBB2, E2F2, TCF4, BCL2, CDKN1B, INSRR, IGF1R, EGFR, TP53, C CNE2, PIK3R1, RB1, SOS1, HSP90B1, NK X3- 1, CREB3L2, CCNE1, PDGFD, CDKN1A, T CF7, PTEN	hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p,hsa-miR-21-5p hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
Colorectal cancer (hsa05210)	$1.19e^{-04}$	FOS, GSK3B, TGFBR1, TCF4, APC, BCL2, APPL1, TP53, MAPK9, SMAD4, MSH6, M YC, MSH2, PIK3R1, TGFB2, CYCS, TCF7, TGFBR2	hsa-miR-378a-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
Bladder cancer (hsa05219)	$1.47e^{-04}$	E2F1, ERBB2, E2F2, THBS1, EGFR, CDK N2A, RPS6KA5, TP53, MMP2, MYC, MMP9 , RB1, CDKN1A, VEGFA	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
HTLV-I infection (hsa05166)	$1.47e^{-04}$	FZD7, EGR1, FOS, GSK3B, E2F1, TGFBR 1, MYB, RANBP1, IL1R1, E2F2, APC, WNT 1, WNT5A, CCND2, ZFP36, HLA- C, WNT3, MRAS, MAP3K1, XBP1, HLA- DOA, CDKN2A, TP53, NFATC4, RANBP3, I L15, CRTC2, FZD4, NFAT5, MAPK9, ANAPC10, SMAD4, MYC, HLA-	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p,hsa-miR-192-5p

		DPA1, PRKACA, PIK3R1, RB1, ATF1, CDC 20, BUB1B, MAD2L1, FZD1, HLA-G, KAT2B, TGFB2, CDKN1A, XPO1, TGFB R2	
Hepatitis B (hsa05161)	$7.61e^{-04}$	FOS, STAT3, E2F1, TGFBR1, E2F2, IFNB1 , BCL2, CDKN1B, MAP3K1, TLR4, CDK6,D DX3X, TP53, NFATC4, APAF1, LAMTOR5, NFAT5, MAPK9, SMAD4, CCNE2, MYC, M MP9, PIK3R1, RB1, FAS, TGFB2, CREB3L 2, CCNE1, CDKN1A, CYCS, PTEN	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p,hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
Wnt signaling pathway (hsa04310)	$1.75e^{-03}$	FZD7, CTNNBIP1, GSK3B, TBL1X, TCF4, APC, VANGL1, WNT1, WNT5A, CCND2, R OCK2, WNT3, TP53, NFATC4, FRAT2, PRI CKLE1, NLK, PLCB1, FZD4, NFAT5, MAPK9, GPC4, SM AD4, CSNK1A1, MYC, PRKACA, FZD1, PR ICKLE2,TCF7, DAAM1, TBL1XR1	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
p53 signaling pathway (hsa04115)	$2.75e^{-03}$	CCNG1, ZMAT3, BID, THBS1, CCND2, PE RP, CDKN2A, CDK6, TP53, APAF1, CCNE 2 , SESN1, MDM4, FAS, SERPINB5, CCNE1, CDKN1A, CYCS, PTEN	hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-92a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
Transcriptional misregulation in cancer (hsa05202)	$4.79e^{-02}$	CCNT2, NFKBIZ, ID2, HMGA2, CCND2, H OXA9, PBX1, CDKN1B, IGF1R, RUNX2, P ML, SUPT3H, TP53, SS18, PTK2, HIST1H3 I, MYC, NR4A3, MMP9, BMP2K, CDK14, T F1, SP1, PPARG, WHSC1, H3F3B, CDKN1 A, SSX2B, SIX4, PLAU, SMAD1, TGFBR2, NGFR, MEIS1	hsa-miR-10b-5p, hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p

Chronic myeloid leukemia (hsa05220)	$6.39e^{-02}$	E2F1, TGFBR1, E2F2, CRK, CDKN1B, CDKN2A, CDK6, TP53, SMAD4, MYC, PIK3R1, RB1, SOS1, TGFB2, GAB2, CDKN1A, TGFBR2	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p, hsa-miR-192-5p
TGF-beta signaling pathway (hsa04350)	$1.35e^{-01}$	TGFBR1, ID2, THBS1, ROCK2, ID4, ACVR2B, ZFYVE16, SMAD4, E2F5, MYC, ID1, CVR2A, SP1, TGFB2, BMP7, SMAD1, TGFBR2, BMPR2, RPS6KB1	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-192-5p
Pancreatic cancer (hsa05212)	$1.71e^{-01}$	STAT3, E2F1, TGFBR1, ERBB2, E2F2, RAD51, PLD1, BRCA2, EGFR, CDKN2A, CDK6, TP53, MAPK9, SMAD4, PIK3R1, RB1, TGFB2, VEGFA, TGFBR2	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-92a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-192-5p
Fanconi anemia pathway (hsa03460)	$7.26e^{+00}$	BLM, RMI1, RAD51, USP1, REV1, EME1, BRCA2, BRCA1, FANCM, ERCC4, REV3L, POLQ, FANCI	hsa-miR-21-5p, hsa-miR-192-5p
Small cell lung cancer (hsa05222)	$4.48e^{+01}$	E2F1, E2F2, BCL2, CDKN1B, CDK6, TP53, APAF1, PTK2, ITGAV, CCNE2, SKP2, MYC, PIK3R1, RB1, CCNE1, CYCS, PTEN	hsa-miR-378a-3p, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-192-5p
ErbB signaling pathway (hsa04012)	$6.15e^{+01}$	GSK3B, HBEGF, ERBB2, NRG4, CRK, CDKN1B, EGFR, PTK2, MAPK9,MYC, PIK3R1, SOS1, PAK6, BTC, CDKN1A, RPS6KB	hsa-miR-378a-3p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-423-5p, hsa-miR-192-5p
Endometrial cancer (hsa05213)	$6.15e^{+01}$	GSK3B, ERBB2, TCF4, APC, EGFR, TP53, MYC, PIK3R1, SOS1, TCF7	hsa-miR-378a-3p, hsa-miR-192-5p, hsa-miR-371a-3p, hsa-miR-26a-5p, hsa-miR-21-5p, hsa-miR-30d-5p, hsa-miR-423-5p

Table S2. Annotation cluster analysis carried out using DAVID web-tool is reported for the first 10 gene clusters identified. Each cluster is described by enrichment score, which is based on an EASE scores (an alternative name of Fisher Exact Statistics in DAVID system, referring to one-tail Fisher Exact Probability Value used for gene-enrichment analysis) of each term members; the higher value represents the more enriched cluster. Moreover, GO category and term, genes associated in the cluster according to their function are reported. *P-value* and False Discovery Rate (FDR) were less than 0.0001 (data not shown).

Annotation Cluster	Enrichment Score	GO Term	Genes
1	15.76	GO:0042325~regulation of phosphorylation	CCNT2, BLM, ERBB2, BMPR2, TTK, TLR4, CCNG1, PTEN, TGFB2, CCNE2, CDKN2A, CDKN2D, MAP3K1, BCL2, ILK, PRKACA, PDGFD, THBS1, FGF2, APC, EGFR, CDC6, RBL2, TGFBR1, TGFBR2, SMAD4, RB1, CDC25A, CDC25B, ACVR2A, ACVR2B, CDKN1A, CDKN1B, CCND2, BMP7
		GO:0051174~regulation of phosphorus metabolic process	CCNT2, BLM, ERBB2, BMPR2, TTK, TLR4, CCNG1, PTEN, TGFB2, CCNE2, CDKN2A, CDKN2D, MAP3K1, BCL2, ILK, PRKACA, PDGFD, THBS1, FGF2, APC, EGFR, CDC6, RBL2, TGFBR1, TGFBR2, SMAD4, RB1, CDC25A, CDC25B, ACVR2A, ACVR2B, CDKN1A, CDKN1B, CCND2, BMP7
		GO:0019220~regulation of phosphate metabolic process	CCNT2, BLM, ERBB2, BMPR2, TTK, TLR4, CCNG1, PTEN, TGFB2, CCNE2, CDKN2A, CDKN2D, MAP3K1, BCL2, ILK, PRKACA, PDGFD, THBS1, FGF2, APC, EGFR, CDC6, RBL2, TGFBR1, TGFBR2, SMAD4, RB1, CDC25A, CDC25B, ACVR2A, ACVR2B, CDKN1A, CDKN1B, CCND2, BMP7
2	14.31	GO:0043067~regulation of programmed cell death	BID, MMP9, ZMAT3, ERBB2, BTC, PML, TLR4, PTEN, TGFB2, IGF1R, CDKN2A, CDKN2D, SOS1, MAP3K1, BCL2, ILK, FAS, THBS1, MYC, FGF2, IL1A, APC, EGFR, MSH6, MSH2, TGFBR1, CYCS, SKP2, TP53, BRCA2, BRCA1, HSP90B1, CDKN1A, CDKN1B, IFNB1, GSK3B, VEGFA, MAPK9, APAF1, NGFR, PRNP, PERP, BMP7
		GO:0010941~regulation of cell death	BID, MMP9, ZMAT3, ERBB2, BTC, PML, TLR4, PTEN, TGFB2, IGF1R, CDKN2A, CDKN2D, SOS1, MAP3K1, BCL2, ILK, FAS, THBS1, MYC, FGF2, IL1A, APC, EGFR, MSH6, MSH2, TGFBR1, CYCS, SKP2, TP53, BRCA2, BRCA1, HSP90B1, CDKN1A, CDKN1B, IFNB1, GSK3B, VEGFA, MAPK9, APAF1, NGFR, PRNP, PERP, BMP7

		GO:0042981~regulation of apoptosis	BID, MMP9, ZMAT3, ERBB2, BTC, PML, TLR4, PTEN, TGFB2, IGF1R, CDKN2A, CDKN2D, SOS1, MAP3K1, BCL2, ILK, FAS, THBS1, MYC, IL1A, APC, EGFR, MSH6, MSH2, TGFBR1, CYCS, SKP2, TP53, BRCA2, BRCA1, HSP90B1, CDKN1A, CDKN1B, IFNB1, GSK3B, VEGFA, MAPK9, APAF1, NGFR, PRNP, PERP, BMP7
3	14.08	GO:0005654~nucleoplasm	CCNT2, E2F1, E2F2, SUPT3H, XPO1, BLM, E2F5, PML, ANAPC10, ATF1, CCNE2, CCNE1, FOS, CDKN2A, DDX3X, FANCI, HOXA9, TCF4, POLQ, RUNX2, MYC, ERCC4, CDC7, TBL1XR1, CDC6, KAT2B, RBL2, USP1, TP53, SMAD4, BRCA2, CDC20, RB1, SMAD1, APPL1, MCM3, BRCA1, CDC25A, CDC25B, RAD51, MCM6, RPS6KA5, CDKN1A, PBX1, NGFR, TBL1X
		GO:0031981~nuclear lumen	E2F1, CCNT2, E2F2, XPO1, E2F5, ZMAT3, CCNE2, FOS, CCNE1, CDKN2A, FANCI, MYB, MYC, CDC7, CDC6, TBL1XR1, RBL2, USP1, TP53, RB1, MCM3, RAD51, MCM6, NGFR, SUPT3H, BLM, PML, ANAPC10, SUFU, ATF1, DDX3X, HOXA9, POLQ, TCF4, RUNX2, ERCC4, KAT2B, NLK, EME1, SMAD4, BRCA2, CDC20, SMAD1, APPL1, BRCA1, CDC25A, STAT3, CDC25B, RPS6KA5, CDKN1A, SP1, PBX1, TBL1X
		GO:0043233~organelle lumen	E2F1, CCNT2, E2F2, XPO1, E2F5, ZMAT3, TGFB2, CCNE2, FOS, CCNE1, CDKN2A, FANCI, MYB, MYC, CDC7, CDC6, TBL1XR1, RBL2, USP1, CYCS, TP53, RB1, MCM3, RAD51, MCM6, VEGFA, NGFR, SUPT3H, BLM, PML, ANAPC10, SUFU, ATF1, DDX3X, HOXA9, POLQ, TCF4, THBS1, RUNX2, ERCC4, KAT2B, NLK, EME1, SMAD4, BRCA2, CDC20, SMAD1, APPL1, STAT3, CDC25A, BRCA1, CDC25B, RPS6KA5, HSP90B1, CDKN1A, SP1, PBX1, TBL1X
		GO:0031974~membrane-enclosed lumen	E2F1, CCNT2, E2F2, XPO1, E2F5, ZMAT3, TGFB2, CCNE2, FOS, CCNE1, CDKN2A, FANCI, MYB, MYC, CDC7, CDC6, TBL1XR1, RBL2, USP1, CYCS, TP53, RB1, MCM3, RAD51, MCM6, VEGFA, NGFR, SUPT3H, BLM, PML, ANAPC10, SUFU, ATF1, DDX3X, HOXA9, POLQ, TCF4, THBS1, RUNX2, ERCC4, KAT2B, NLK, EME1, SMAD4, BRCA2, CDC20, SMAD1, APPL1, STAT3, CDC25A, BRCA1, CDC25B, RPS6KA5, HSP90B1, CDKN1A, SP1, PBX1, TBL1X
		GO:0070013~intracellular organelle lumen	E2F1, CCNT2, E2F2, XPO1, E2F5, ZMAT3, CCNE2, FOS, CCNE1, CDKN2A, FANCI, MYB, MYC, CDC7, CDC6, TBL1XR1, RBL2, USP1, CYCS, TP53, RB1, MCM3, RAD51, MCM6, NGFR, SUPT3H, BLM, PML, ANAPC10, SUFU, ATF1, DDX3X, HOXA9, POLQ, TCF4, RUNX2, ERCC4, KAT2B, NLK, EME1, SMAD4, BRCA2, CDC20, SMAD1, APPL1, STAT3, BRCA1, CDC25A, CDC25B, RPS6KA5, HSP90B1,

			CDKN1A, SP1, PBX1, TBL1X
4	12.40	GO:0002520~immune system development	BLM, MMP9, PPARG, PML, IL15, TGFB2, BCL2, HOXA9, FAS, PIK3R1, APC, EGR1, MSH6, MSH2, TGFBR1, TGFBR2, TP53, BRCA2, CDK6, RB1, SIX4, ID2, SP1, VEGFA, PBX1
		GO:0048534~hemopoietic or lymphoid organ development	EGR1, BLM, MSH2, MMP9, TGFBR1, PPARG, TGFBR2, TP53, PML, BRCA2, CDK6, RB1, IL15, SIX4, TGFB2, SP1, ID2, BCL2, VEGFA, HOXA9, PBX1, FAS, PIK3R1, APC
		GO:0030097~hemopoiesis	EGR1, BLM, MSH2, MMP9, PPARG, TGFBR2, TP53, PML, BRCA2, CDK6, RB1, IL15, TGFB2, ID2, SP1, BCL2, VEGFA, HOXA9, PBX1, FAS, PIK3R1, APC
5	12.21	GO:0051338~regulation of transferase activity	CCNT2, BLM, ERBB2, PPARG, CCNG1, PTEN, TGFB2, CCNE2, CDKN2A, MAP3K1, CDKN2D, ILK, PRKACA, THBS1, FGF2, APC, EGFR, CDC6, RBL2, TGFBR1, TGFBR2, RB1, CDC25A, CDC25B, ACVR2B, CDKN1A, CDKN1B, CCND2
		GO:0043549~regulation of kinase activity	CCNT2, BLM, ERBB2, CCNG1, PTEN, TGFB2, CCNE2, CDKN2A, MAP3K1, CDKN2D, ILK, PRKACA, THBS1, FGF2, APC, EGFR, CDC6, RBL2, TGFBR1, TGFBR2, RB1, CDC25A, CDC25B, ACVR2B, CDKN1A, CDKN1B, CCND2
		GO:0045859~regulation of protein kinase activity	CCNT2, BLM, ERBB2, CCNG1, PTEN, TGFB2, CCNE2, CDKN2A, MAP3K1, CDKN2D, ILK, PRKACA, THBS1, FGF2, APC, EGFR, CDC6, TGFBR1, TGFBR2, RB1, CDC25A, CDC25B, ACVR2B, CDKN1A, CDKN1B, CCND2
6	11.19	kinase	ERBB2, BMPR2, TFG, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, CDKN2A, MAP3K1, ILK, PRKACA, CDK14, CDC7, EGFR, CSNK1A1, RET, ROCK2, NLK, TGFBR1, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, CDKN1A, CDKN1B, GSK3B, BMP2K, BUB1B, MAPK9, PLAU
		binding site:ATP	ERBB2, BMPR2, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, EGFR, CSNK1A1, CDC7, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, HSP90B1, GSK3B, BMP2K, BUB1B, MAPK9
		domain:Protein kinase	ERBB2, BMPR2, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, EGFR, CSNK1A1, CDC7, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, ACVR2A, ACVR2B, GSK3B, BMP2K, BUB1B, MAPK9
		IPR017441:Protein kinase, ATP binding site	ERBB2, BMPR2, TFG, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, EGFR, CSNK1A1, CDC7, RET, ROCK2, TGFBR1, NLK, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, BUB1B, MAPK9

		IPR000719:Protein kinase, core	ERBB2, BMPR2, TFG, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, EGFR, CSNK1A1, CDC7, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, MAPK9
		GO:0004672~protein kinase activity	ERBB2, BMPR2, TFG, TTK, RPS6KB1, TGFB2, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, CDC7, EGFR, CSNK1A1, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, BUB1B, MAPK9
		active site:Proton acceptor	ERBB2, BMPR2, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, PRKACA, CDK14, EGFR, CSNK1A1, CDC7, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, BUB1B, MAPK9
		transferase	ERBB2, BMPR2, TFG, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, CDC7, EGFR, CSNK1A1, RET, KAT2B, REV1, ROCK2, NLK, TGFBR1, TGFBR2, CDK6, WHSC1, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, BUB1B, MAPK9, REV3L
7	10.47	IPR000719:Protein kinase, core	ERBB2, BMPR2, TFG, TTK, RPS6KB1, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, EGFR, CSNK1A1, CDC7, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, MAPK9
		GO:0006468~protein amino acid phosphorylation	ERBB2, BMPR2, PML, TFG, TTK, RPS6KB1, INSRR, TGFB2, PAK6, IGF1R, PTK2, MAP3K1, BCL2, ILK, PRKACA, THBS1, FGF2, CDK14, CDC7, CSNK1A1, EGFR, RET, ROCK2, NLK, TGFBR1, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, MAPK9, BMP7
		GO:0006796~phosphate metabolic process	CDC14A, ERBB2, PML, BMPR2, TTK, TFG, RPS6KB1, PTEN, INSRR, TGFB2, PAK6, IGF1R, PTK2, MAP3K1, BCL2, ILK, PRKACA, THBS1, FGF2, CDK14, PIK3R1, CDC7, CSNK1A1, EGFR, RET, ROCK2, MSH2, NLK, TGFBR1, TGFBR2, CDK6, CDC25A, CDC25B, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, MAPK9, BMP7
		GO:0006793~phosphorus metabolic process	CDC14A, ERBB2, PML, BMPR2, TTK, TFG, RPS6KB1, PTEN, INSRR, TGFB2, PAK6, IGF1R, PTK2, MAP3K1, BCL2, ILK, PRKACA, THBS1, FGF2, CDK14, PIK3R1, CDC7, CSNK1A1, EGFR, RET, ROCK2, MSH2, NLK, TGFBR1, TGFBR2, CDK6, CDC25A, CDC25B, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, MAPK9, BMP7

		GO:0016310~phosphorylation	ERBB2, BMPR2, PML, TTK, TFG, RPS6KB1, INSRR, TGFB2, PAK6, IGF1R, PTK2, MAP3K1, BCL2, ILK, PRKACA, THBS1, FGF2, CDK14, PIK3R1, CDC7, CSNK1A1, EGFR, RET, MSH2, ROCK2, NLK, TGFBR1, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, MAPK9, BMP7
		GO:0004672~protein kinase activity	ERBB2, BMPR2, TFG, TTK, RPS6KB1, TGFB2, INSRR, PAK6, IGF1R, PTK2, MAP3K1, ILK, PRKACA, CDK14, CDC7, EGFR, CSNK1A1, RET, ROCK2, TGFBR1, NLK, TGFBR2, CDK6, RPS6KA5, ACVR2A, ACVR2B, GSK3B, BMP2K, BUB1B, MAPK9
8	10.35	GO:0043065~positive regulation of apoptosis	BID, MMP9, ZMAT3, PML, TLR4, PTEN, TGFB2, CDKN2A, MAP3K1, BCL2, SOS1, FAS, MYC, APC, MSH6, TGFBR1, TP53, SKP2, BRCA2, BRCA1, CDKN1A, CDKN1B, IFNB1, MAPK9, NGFR, BMP7, PERP
		GO:0043068~positive regulation of programmed cell death	BID, MMP9, ZMAT3, PML, TLR4, PTEN, TGFB2, CDKN2A, MAP3K1, BCL2, SOS1, FAS, MYC, APC, MSH6, TGFBR1, TP53, SKP2, BRCA2, BRCA1, CDKN1A, CDKN1B, IFNB1, MAPK9, NGFR, BMP7, PERP
		GO:0010942~positive regulation of cell death	BID, MMP9, ZMAT3, PML, TLR4, PTEN, TGFB2, CDKN2A, MAP3K1, BCL2, SOS1, FAS, MYC, APC, MSH6, TGFBR1, TP53, SKP2, BRCA2, BRCA1, CDKN1A, CDKN1B, IFNB1, MAPK9, NGFR, BMP7, PERP
9	9.40	GO:0051272~positive regulation of cell motion	EGFR, PLD1, MMP9, TGFBR1, RPS6KB1, TGFB2, IGF1R, BCL2, ILK, VEGFA, HBEGF, THBS1, FGF2, PIK3R1, APC
		GO:0030335~positive regulation of cell migration	EGFR, PLD1, MMP9, RPS6KB1, TGFB2, IGF1R, BCL2, VEGFA, ILK, HBEGF, THBS1, FGF2, PIK3R1, APC
		GO:0040017~positive regulation of locomotion	EGFR, PLD1, MMP9, RPS6KB1, TGFB2, IGF1R, BCL2, VEGFA, ILK, HBEGF, THBS1, FGF2, PIK3R1, APC
		GO:0051270~regulation of cell motion	EGFR, PLD1, MMP9, TGFBR1, RPS6KB1, PTEN, TGFB2, IGF1R, CDKN1B, BCL2, MAP3K1, ILK, VEGFA, HBEGF, THBS1, FGF2, PIK3R1, APC

		GO:0040012~regulation of locomotion	EGFR, PLD1, MMP9, RPS6KB1, PTEN, TGFB2, IGF1R, IFNB1, BCL2, MAP3K1, ILK, VEGFA, HBEGF, THBS1, FGF2, PIK3R1, APC
		GO:0030334~regulation of cell migration	EGFR, PLD1, MMP9, RPS6KB1, PTEN, TGFB2, IGF1R, BCL2, MAP3K1, ILK, VEGFA, HBEGF, THBS1, FGF2, PIK3R1, APC
10	9.13	GO:0010557~positive regulation of macromolecule biosynthetic process	E2F1, BLM, PPARG, TLR4, TGFB2, CCNE1, FOS, WNT1, IGF1R, MAP3K1, NFAT5, TCF4, THBS1, FGF2, MYC, RUNX2, IL1A, EGR1, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, BRCA1, STAT3, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0009891~positive regulation of biosynthetic process	E2F1, BLM, PPARG, TLR4, TGFB2, CCNE1, FOS, WNT1, IGF1R, MAP3K1, NFAT5, TCF4, THBS1, FGF2, MYC, RUNX2, IL1A, EGR1, EGFR, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, BRCA1, STAT3, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0031328~positive regulation of cellular biosynthetic process	E2F1, BLM, PPARG, TLR4, CCNE1, FOS, WNT1, IGF1R, MAP3K1, NFAT5, TCF4, THBS1, FGF2, MYC, RUNX2, IL1A, EGFR, EGR1, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, BRCA1, STAT3, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0051173~positive regulation of nitrogen compound metabolic process	E2F1, BLM, PPARG, CCNE1, FOS, WNT1, IGF1R, MAP3K1, NFAT5, TCF4, FGF2, MYC, RUNX2, EGFR, EGR1, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, BRCA1, STAT3, RAD51, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0010628~positive regulation of gene expression	E2F1, BLM, PPARG, CCNE1, FOS, WNT1, MAP3K1, NFAT5, TCF4, FGF2, MYC, RUNX2, EGR1, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, STAT3, BRCA1, SP1, VEGFA, MAPK9, PBX1, BMP7, TBL1X
		GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	E2F1, BLM, PPARG, CCNE1, FOS, WNT1, IGF1R, MAP3K1, NFAT5, TCF4, FGF2, MYC, RUNX2, EGR1, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, BRCA1, STAT3, RAD51, SP1, VEGFA, PBX1, BMP7, TBL1X

		GO:0045893~positive regulation of transcription, DNA-dependent	E2F1, PPARG, FOS, WNT1, CCNE1, MAP3K1, NFAT5, TCF4, FGF2, MYC, RUNX2, EGR1, TBL1XR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, STAT3, BRCA1, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0051254~positive regulation of RNA metabolic process	E2F1, PPARG, FOS, WNT1, CCNE1, MAP3K1, NFAT5, TCF4, FGF2, MYC, RUNX2, EGR1, TBL1XR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, STAT3, BRCA1, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0045941~positive regulation of transcription	E2F1, BLM, PPARG, FOS, WNT1, CCNE1, MAP3K1, NFAT5, TCF4, FGF2, MYC, RUNX2, EGR1, TBL1XR1, TGFBR1, SMAD4, TP53, RB1, SMAD1, NR4A3, SIX4, STAT3, BRCA1, SP1, VEGFA, PBX1, BMP7, TBL1X
		GO:0045944~positive regulation of transcription from RNA polymerase II promoter	E2F1, EGR1, TBL1XR1, PPARG, TP53, SMAD4, RB1, NR4A3, SMAD1, SIX4, STAT3, FOS, SP1, MAP3K1, VEGFA, NFAT5, PBX1, BMP7, TBL1X, FGF2, RUNX2, MYC

CHAPTER 3

Differences in miRNAs after *in vitro* fertilization using sperm with high and low blastocyst developmental potential

Table S3. Gene Ontology (GO) analysis was performed using the list of predicted target genes of differentially expressed miRNAs. GO terms for biological function, gene count, which indicates how much the GO term is enriched of predicted genes, *P-value* and False Discovery Rate (FDR) are reported.

GO Term	Gene count	*P-Value*	FDR
GO:0006350~transcription	623	$9.41e^{+13}$	$1.79e^{+06}$
GO:0045449~regulation of transcription	750	$1.02e^{+13}$	$1.94e^{+06}$
GO:0006357~regulation of transcription from RNA polymerase II promoter	251	$1.37e^{+14}$	$2.61e^{+07}$
GO:0010557~positive regulation of macromolecule biosynthetic process	227	$1.02e^{+14}$	$1.93e^{+07}$
GO:0009891~positive regulation of biosynthetic process	238	$1.43e^{+14}$	$2.72e^{+07}$
GO:0031328~positive regulation of cellular biosynthetic process	235	$1.58e^{+14}$	$3.00e^{+07}$
GO:0010604~positive regulation of macromolecule metabolic process	282	2.42e+13	$4.61e^{+07}$
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	217	$2.49e^{+14}$	$4.74e^{+08}$
GO:0051173~positive regulation of nitrogen compound metabolic process	222	$3.73e^{+14}$	$7.10e^{+07}$
GO:0010628~positive regulation of gene expression	203	$7.40e^{+14}$	$1.41e^{+08}$
GO:0045941~positive regulation of transcription	198	$8.10e^{+13}$	$1.54e^{+09}$
GO:0031175~neuron projection development	105	$2.91e^{+14}$	$5.54e^{+08}$
GO:0045893~positive regulation of transcription, DNA-dependent	171	$2.92e^{+14}$	$5.56e^{+08}$
GO:0051254~positive regulation of RNA metabolic process	172	$3.22e^{+14}$	$6.13e^{+08}$

GO:0007167~enzyme linked receptor protein signaling pathway	131	$3.64e^{+13}$	$6.94e^{+08}$
GO:0048666~neuron development	130	$3.92e^{+14}$	$7.47e^{+08}$
GO:0051252~regulation of RNA metabolic process	526	$6.40e^{+14}$	$1.22e^{+09}$
GO:0007242~intracellular signaling cascade	380	$1.42e^{+14}$	$2.71e^{+10}$
GO:0006355~regulation of transcription, DNA-dependent	512	$2.42e^{+14}$	$4.61e^{+09}$
GO:0030030~cell projection organization	136	$2.53e^{+14}$	$4.82e^{+10}$
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	92	$3.74e^{+14}$	$7.13e^{+09}$
GO:0006468~protein amino acid phosphorylation	220	$3.92e^{+14}$	$7.46e^{+09}$
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	136	$4.60e^{+14}$	$8.76e^{+09}$
GO:0048812~neuron projection morphogenesis	87	$1.41e^{+14}$	$2.68e^{+11}$
GO:0030182~neuron differentiation	153	$2.01e^{+14}$	$3.84e^{+11}$
GO:0006793~phosphorus metabolic process	297	$4.62e^{+13}$	$8.81e^{+09}$
GO:0006796~phosphate metabolic process	297	$4.62e^{+13}$	$8.81e^{+09}$
GO:0048858~cell projection morphogenesis	95	$5.92e^{+14}$	$1.13e^{+12}$
GO:0048667~cell morphogenesis involved in neuron differentiation	84	$6.02e^{+14}$	$1.15e^{+12}$
GO:0007409~axonogenesis	79	$6.24e^{+14}$	$1.19e^{+12}$
GO:0007267~cell-cell signaling	196	$6.79e^{+14}$	$1.29e^{+11}$
GO:0032990~cell part morphogenesis	98	$7.45e^{+14}$	$1.42e^{+12}$
GO:0000902~cell morphogenesis	126	$1.76e^{+14}$	$3.35e^{+11}$
GO:0016192~vesicle-mediated transport	186	$3.71e^{+13}$	$7.07e^{+10}$
GO:0048598~embryonic morphogenesis	110	$5.75e^{+13}$	0.001
GO:0032989~cellular component morphogenesis	135	$8.21e^{+14}$	0.002
GO:0000904~cell morphogenesis involved in differentiation	91	$8.62e^{+14}$	0.002
GO:0033674~positive regulation of kinase activity	86	$1.93e^{+14}$	0.004
GO:0045184~establishment of protein localization	233	$2.87e^{+14}$	0.005

GO:0045860~positive regulation of protein kinase activity	83	$3.00e^{+14}$	0.006
GO:0015031~protein transport	231	$3.03e^{+14}$	0.006
GO:0016310~phosphorylation	238	$9.43e^{+14}$	0.018
GO:0008104~protein localization	259	$1.06e^{+14}$	0.020
GO:0051347~positive regulation of transferase activity	86	$1.09e^{+14}$	0.021
GO:0019220~regulation of phosphate metabolic process	153	$1.80e^{+14}$	0.034
GO:0051174~regulation of phosphorus metabolic process	153	$1.80e^{+14}$	0.034
GO:0010629~negative regulation of gene expression	158	$1.90e^{+14}$	0.036
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	92	$2.58e^{+14}$	0.049
GO:0016481~negative regulation of transcription	145	$2.81e^{+14}$	0.053
GO:0048729~tissue morphogenesis	67	$2.96e^{+14}$	0.056
GO:0044057~regulation of system process	103	$4.59e^{+14}$	0.087
GO:0046578~regulation of Ras protein signal transduction	75	$4.77e^{+14}$	0.091
GO:0046907~intracellular transport	196	$5.25e^{+14}$	0.100

Table S4. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis. which was performed using DAVID web-tool and predicted target genes of differentially expressed miRNAs. KEGG pathways, gene count, which indicates how much the KEGG pathway is enriched of predicted genes, and *P-value* are reported.

KEGG pathway	Gene count	*P-Value*
hsa04310:Wnt signaling pathway	69	$1.57e^{+07}$
hsa04010:MAPK signaling pathway	92	$2.11e^{+11}$
hsa04020:Calcium signaling pathway	65	$4.11e^{+11}$
hsa04012:ErbB signaling pathway	37	$1.08e^{+12}$
hsa04920:Adipocytokine signaling pathway	30	$1.96e^{+12}$
hsa04370:VEGF signaling pathway	30	0.002
hsa04720:Long-term potentiation	27	0.004
hsa04514:Cell adhesion molecules (CAMs)	45	0.005
hsa04340:Hedgehog signaling pathway	23	0.005
hsa04960:Aldosterone-regulated sodium reabsorption	17	0.017
hsa04350:TGF-beta signaling pathway	30	0.019
hsa04540:Gap junction	30	0.026
hsa04730:Long-term depression	24	0.035
hsa04630:Jak-STAT signaling pathway	47	0.035
hsa05210:Colorectal cancer	45	$4.57e^{+05}$
hsa05200:Pathways in cancer	113	$2.02e^{+10}$
hsa05214:Glioma	32	$5.08e^{+09}$
hsa05213:Endometrial cancer	28	$5.70e^{+09}$
hsa05215:Prostate cancer	39	$3.04e^{+11}$
hsa05223:Non-small cell lung cancer	27	$4.74e^{+10}$

hsa05220:Chronic myeloid leukemia	34	$4.86e^{+11}$
hsa05217:Basal cell carcinoma	26	$2.12e^{+11}$
hsa05212:Pancreatic cancer	31	$3.44e^{+11}$
hsa04912:GnRH signaling pathway	39	$3.49e^{+11}$
hsa04270:Vascular smooth muscle contraction	43	$4.05e^{+12}$
hsa04150:mTOR signaling pathway	24	$6.10e^{+11}$
hsa05218:Melanoma	30	$6.39e^{+11}$
hsa05211:Renal cell carcinoma	27	0.006
hsa05014:Amyotrophic lateral sclerosis (ALS)	21	0.011
hsa05221:Acute myeloid leukemia	22	0.017
hsa05222:Small cell lung cancer	29	0.021
hsa05216:Thyroid cancer	13	0.022
hsa04520:Adherens junction	39	$3.98e^{+08}$
hsa04144:Endocytosis	65	$1.87e^{+11}$
hsa04510:Focal adhesion	68	$5.33e^{+11}$
hsa04210:Apoptosis	35	$5.96e^{+11}$
hsa04914:Progesterone-mediated oocyte maturation	33	0.002
hsa04810:Regulation of actin cytoskeleton	69	0.002
hsa04530:Tight junction	46	0.004
hsa04114:Oocyte meiosis	38	0.008
hsa04910:Insulin signaling pathway	58	$5.11e^{+08}$
hsa04916:Melanogenesis	43	$1.42e^{+11}$
hsa04722:Neurotrophin signaling pathway	50	$2.85e^{+11}$
hsa04360:Axon guidance	50	$9.44e^{+10}$
hsa04660:T cell receptor signaling pathway	41	$7.37e^{+11}$
hsa04062:Chemokine signaling pathway	62	0.002

hsa04666:Fc gamma R-mediated phagocytosis	36	0.002
hsa04664:Fc epsilon RI signaling pathway	30	0.004
hsa04670:Leukocyte transendothelial migration	41	0.005
hsa04662:B cell receptor signaling pathway	26	0.028
hsa00310:Lysine degradation	20	0.003
hsa04070:Phosphatidylinositol signaling system	27	0.013
hsa05414:Dilated cardiomyopathy	32	0.013
hsa04930:Type II diabetes mellitus	19	0.014
hsa04130:SNARE interactions in vesicular transport	16	0.018
hsa04120:Ubiquitin mediated proteolysis	42	0.039

CHAPTER 4

Differences in miRNAs after *in vitro* fertilization using sperm with high and low blastocyst developmental potential

Table S5. List of differentially expressed miRNAs between follicular fluids of high and low antral follicle count ovaries. MiRNA ID, Log Fold Change (LogFC), *P-value* and False Discovery Rate (FDR) are reported.

MiRNA ID	LogFC	*P-Value*	FDR
bta-miR-769	-2.524	7.92E-09	4.22E-06
bta-miR-1343	-2.924	2.24E-08	5.96E-06
bta-miR-450a	-2.915	6.06E-08	8.79E-06
bta-miR-30d	-2.170	9.62E-08	1.03E-05
bta-miR-204	-4.025	1.34E-07	1.19E-05
bta-miR-100	1.897	3.94E-07	3.00E-05
bta-miR-99a	1.748	2.70E-06	0.000
Novel:hsa-miR-320a	2.071	2.53E-05	0.001
bta-miR-197	-2.884	3.38E-05	0.002
bta-miR-151	-1.551	3.69E-05	0.002
bta-miR-146b	-1.660	3.70E-05	0.002
bta-miR-24-3p	1.434	5.45E-05	0.002
bta-miR-374b	-2.647	0.000	0.004
bta-miR-1271	-2.656	0.000	0.004
bta-miR-98	-2.087	0.000	0.005
Novel:hsa-miR-4640-5p	-2.857	0.000	0.006
bta-miR-10a	1.522	0.000	0.006

bta-miR-190b	-1.901	0.000	0.006
bta-let-7c	-1.748	0.000	0.006
bta-miR-451	-2.578	0.000	0.006
bta-miR-31	-1.927	0.000	0.007
bta-miR-4286	-2.308	0.001	0.011
bta-miR-3613	-3.772	0.001	0.013
bta-miR-30c	-1.500	0.001	0.013
Novel:hsa-miR-4707-5p	-3.653	0.001	0.013
bta-miR-450b	-1.308	0.001	0.017
Novel:hsa-miR-6878-5p	-2.058	0.001	0.017
bta-miR-449a	-1.723	0.001	0.020
bta-miR-301a	-1.524	0.001	0.020
bta-miR-150	-4.112	0.002	0.022
bta-miR-383	-1.957	0.002	0.024
bta-miR-18a	-2.447	0.002	0.027
bta-miR-149	-1.422	0.002	0.027
bta-miR-16b	-1.276	0.002	0.028
bta-miR-92a	-1.084	0.002	0.028
bta-miR-362	1.351	0.002	0.030
bta-miR-30f	-1.474	0.003	0.033
bta-miR-324	-1.856	0.004	0.042
bta-miR-93	-1.143	0.004	0.046
bta-miR-148b	-1.359	0.004	0.046
bta-miR-885	2.715	0.004	0.046
bta-miR-16a	-1.472	0.005	0.054
bta-miR-628	-1.798	0.006	0.058

bta-miR-33b	-1.674	0.006	0.059
bta-miR-874	1.043	0.006	0.059
bta-miR-504	1.526	0.006	0.061
bta-miR-320a	1.051	0.006	0.061
bta-miR-127	0.968	0.007	0.063
bta-miR-2399	2.549	0.007	0.064
bta-let-7b	-1.053	0.007	0.064
bta-miR-374a	-1.609	0.008	0.071
bta-miR-29d	1.441	0.009	0.073
Novel:hsa-miR-4722-5p	2.073	0.010	0.077
bta-miR-27a	0.919	0.010	0.081
bta-miR-195	-1.419	0.010	0.081
bta-miR-1247	1.475	0.011	0.083
Novel:hsa-miR-665	1.380	0.012	0.087
bta-miR-223	-2.890	0.012	0.087
bta-miR-328	-1.240	0.012	0.090
bta-miR-30e	-1.033	0.012	0.090
bta-miR-30a	-0.841	0.013	0.093
Novel:hsa-miR-3960	-1.286	0.013	0.093
bta-miR-2313	-1.581	0.013	0.093
bta-miR-194	-1.229	0.014	0.093
bta-miR-3431	-0.945	0.014	0.095
bta-miR-365-3p	-1.470	0.015	0.099

Table S6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, *P-value*, count of predicted target genes (#genes) and differentially expressed miRNAs (#miRNAs), which were obtained using DIANA miRPath v2.0, are reported.

KEGG pathway	*P-value*	#genes	#miRNAs
Pathways in cancer	$1.054e^{-11}$	62	17
Chronic myeloid leukemia	$6.655e^{-11}$	21	10
Focal adhesion	$8.588e^{-11}$	43	13
Viral carcinogenesis	$1.576e^{-10}$	45	14
Bladder cancer	$3.949e^{-10}$	15	10
PI3K-Akt signaling pathway	$1.907e^{-08}$	59	18
ErbB signaling pathway	$3.958e^{-08}$	21	8
Neurotrophin signaling pathway	$4.724e^{-08}$	27	13
TGF-beta signaling pathway	$1.588e^{-07}$	21	11
Prostate cancer	$2.544e^{-07}$	20	13
Hepatitis B	$1.185e^{-06}$	29	14
Colorectal cancer	$2.149e^{-06}$	15	11
HIF-1 signaling pathway	$1.855e^{-05}$	23	8
MAPK signaling pathway	$5.036e^{-05}$	43	14
Non-small cell lung cancer	0.000	13	8
Small cell lung cancer	0.000	17	8
Pancreatic cancer	0.000	17	12
NF-kappa B signaling pathway	0.000	18	8
Endometrial cancer	0.000	12	9
Insulin signaling pathway	0.001	24	10
Transcriptional misregulation in cancer	0.001	31	10

Fc gamma R-mediated phagocytosis	0.001	18	7
HTLV-I infection	0.001	40	14
Amoebiasis	0.001	19	6
mTOR signaling pathway	0.001	13	8
Acute myeloid leukemia	0.001	12	7
Melanoma	0.001	13	8
Glioma	0.001	15	9
NOD-like receptor signaling pathway	0.002	13	4
Endocytosis	0.002	32	12
Long-term depression	0.003	15	5
African trypanosomiasis	0.003	8	4
GnRH signaling pathway	0.003	16	6
Alcoholism	0.003	30	8
Vascular smooth muscle contraction	0.004	20	7
Wnt signaling pathway	0.005	25	11
Osteoclast differentiation	0.005	22	8
Long-term potentiation	0.005	13	6
Cell cycle	0.006	24	11
Chemokine signaling pathway	0.010	28	10
Epstein-Barr virus infection	0.011	31	10
Thyroid cancer	0.014	7	7
Melanogenesis	0.019	16	8
Basal cell carcinoma	0.024	10	7
Lysine degradation	0.028	10	7
Regulation of actin cytoskeleton	0.029	32	11
Measles	0.030	22	10

Chagas disease (American trypanosomiasis)	0.036	16	6
Shigellosis	0.044	11	7
p53 signaling pathway	0.044	12	7
Oocyte meiosis	0.048	19	9
RNA transport	0.048	22	6
Cytokine-cytokine receptor interaction	0.049	34	9

Table S7. Gene Ontology (GO) analysis was performed using the list of predicted target genes of differentially expressed miRNAs between follicular fluids of high and low antral follicle count ovaries. GO terms for biological function, predicted targe genes of differentially expressed miRNAs, *P-value* and False Discovery Rate (FDR) are reported. Data are reported only for FDR less than 0.01.

GO Term	Genes	*P-Value*	FDR
GO:0006468~protein amino acid phosphorylation	FGFR3, ERBB3, STK11, ERBB2, STK36, RPS6KB2, RPS6KB1, KIT, IGF1R, PTK2, BCL2, MAP3K1, CAMK2D, PRKAA2, THBS1, FRS2, PDK1, IRAK1, CTBP1, RET, FLT1, ROCK1, SMAD7, MYLK3, TGFBR1, TGFBR2, MYLK4, RAF1, MYLK2, CDK6, CDK4, PRKCB, DAPK1, NTRK3, MAPK1, ACVR2A, RPS6KA3, CCND1, CAMK4, GSK3B, RIPK2, RELN, MAPK8, MTOR, ABL2, MYLK, ACVR1, F2R	$4.70e^{-11}$	$8.13e^{-07}$
GO:0042127~regulation of cell proliferation	FGFR3, OSMR, ERBB3, STK11, ERBB2, MITF, EGLN3, FOXO1, FGF10, RPS6KB1, KIT, GLI2, IGF1R, CUL2, CDKN2B, BCL2, CAMK2D, THBS1, MYC, CCNA2, APC, CEBPA, CTBP1, IL6, IL2RA, FLT1, TGFBR1, NODAL, TGFBR2,	$6.90e^{-09}$	$1.19e^{-03}$

	TP53, SMAD4, CDK6, IL6R, CDK4, BRCA1, MAPK1, NRAS, CDKN1A, CCND1, CDKN1B, HDAC1, ID2, ETS1, VEGFA, HBEGF, RIPK2, MTOR, LAMC1, F2R		
GO:0043067~regulation of programmed cell death	TRAF1, MCL1, ERBB3, BCAR1, ERBB2, MITF, FOXO1, NFKB1, PMAIP1, FOXO3, KIT, CUL2, IGF1R, COMP, MAP3K1, BCL2, RHOA, FAS, THBS1, TRAF6, MYC, ARHGDIA, APC, IRAK1, IL6, IL2RA, VAV3, ROCK1, TGFBR1, TP53, BIRC5, IL6R, BIRC3, YWHAE, BRCA1, TP73, DAPK1, MAPK1, NRAS, CDKN1A, CDKN1B, HDAC1, ETS1, GSK3B, VEGFA, RIPK2, MAPK8, ACVR1, F2R	$2.63e^{-06}$	$4.55e^{-04}$
GO:0010941~regulation of cell death	TRAF1, MCL1, ERBB3, BCAR1, ERBB2, MITF, FOXO1, NFKB1, PMAIP1, FOXO3, KIT, CUL2, IGF1R, COMP, MAP3K1, BCL2, RHOA, FAS, THBS1, TRAF6, MYC, ARHGDIA, APC, IRAK1, IL6, IL2RA, VAV3, ROCK1, TGFBR1, TP53, BIRC5, IL6R, BIRC3, YWHAE, BRCA1, TP73, DAPK1, MAPK1, NRAS, CDKN1A, CDKN1B, HDAC1, ETS1, GSK3B, VEGFA, RIPK2, MAPK8, ACVR1, F2R	$3.08e^{-08}$	$5.33e^{-04}$
GO:0043069~negative regulation of programmed cell death	MCL1, ERBB3, ERBB2, MITF, FOXO1, NFKB1, KIT, IGF1R, BCL2, COMP, RHOA, FAS, THBS1, TRAF6, MYC, ARHGDIA, APC, IRAK1, IL6, ROCK1, TGFBR1, TP53, BIRC5, BIRC3, TP73, DAPK1, NRAS, CDKN1A, HDAC1, GSK3B, VEGFA, RIPK2, MAPK8, ACVR1, F2R	$4.43e^{-07}$	$7.67e^{-04}$
GO:0060548~negative regulation of cell death	MCL1, ERBB3, ERBB2, MITF, FOXO1, NFKB1, KIT, IGF1R, BCL2, COMP, RHOA, FAS, THBS1, TRAF6, MYC, ARHGDIA, APC, IRAK1, IL6, ROCK1, TGFBR1, TP53, BIRC5, BIRC3, TP73, DAPK1, NRAS, CDKN1A, HDAC1, GSK3B,	$4.85e^{-07}$	$8.39e^{-04}$

	VEGFA, RIPK2, MAPK8, ACVR1, F2R		
GO:0016310~phosphorylation	FGFR3, ERBB3, STK11, ERBB2, STK36, RPS6KB2, RPS6KB1, KIT, IGF1R, PTK2, BCL2, MAP3K1, CAMK2D, PRKAA2, THBS1, FRS2, PDK1, IRAK1, CTBP1, RET, FLT1, ROCK1, SMAD7, MYLK3, TGFBR1, TGFBR2, MYLK4, RAF1, MYLK2, CDK6, CDK4, PRKCB, DAPK1, NTRK3, MAPK1, ACVR2A, RPS6KA3, CCND1, CAMK4, GSK3B, RIPK2, RELN, MAPK8, MTOR, ABL2, MYLK, ACVR1, F2R	$1.05e^{-06}$	$1.81e^{-02}$
GO:0042981~regulation of apoptosis	TRAF1, MCL1, ERBB3, ERBB2, BCAR1, MITF, FOXO1, NFKB1, PMAIP1, FOXO3, IGF1R, CUL2, BCL2, MAP3K1, COMP, RHOA, FAS, TRAF6, THBS1, MYC, ARHGDIA, APC, IRAK1, IL6, IL2RA, VAV3, ROCK1, TGFBR1, TP53, BIRC5, IL6R, BIRC3, YWHAE, BRCA1, TP73, DAPK1, MAPK1, NRAS, CDKN1A, CDKN1B, HDAC1, ETS1, GSK3B, VEGFA, RIPK2, MAPK8, ACVR1, F2R	$1.29e^{-06}$	$2.23e^{-02}$
GO:0043066~negative regulation of apoptosis	MCL1, ERBB3, ERBB2, MITF, FOXO1, NFKB1, IGF1R, BCL2, COMP, RHOA, FAS, THBS1, TRAF6, MYC, ARHGDIA, APC, IRAK1, IL6, ROCK1, TGFBR1, TP53, BIRC5, BIRC3, TP73, DAPK1, NRAS, CDKN1A, HDAC1, GSK3B, VEGFA, RIPK2, MAPK8, ACVR1, F2R	$3.21e^{-05}$	$5.56e^{-04}$
GO:0006796~phosphate metabolic process	FGFR3, STK11, ERBB3, ERBB2, STK36, RPS6KB2, RPS6KB1, KIT, IGF1R, PTK2, MAP3K1, BCL2, CAMK2D, PRKAA2, THBS1, FRS2, PDK1, IRAK1, RET, CTBP1, FLT1, ROCK1, SMAD7, MYLK3, TGFBR1, MYLK4, TGFBR2, RAF1, MYLK2, CDK6, CDK4, DAPK1, PRKCB, NTRK3, MAPK1, ACVR2A, RPS6KA3, CCND1, PPP1CA, CAMK4, GSK3B, RIPK2, MAPK8, RELN, MTOR, ABL2, MYLK,	$8.16e^{-05}$	$1.41e^{-01}$

	PPP2R2A, ACVR1, F2R		
GO:0006793~phosphorus metabolic process	FGFR3, STK11, ERBB3, ERBB2, STK36, RPS6KB2, RPS6KB1, KIT, IGF1R, PTK2, MAP3K1, BCL2, CAMK2D, PRKAA2, THBS1, FRS2, PDK1, IRAK1, RET, CTBP1, FLT1, ROCK1, SMAD7, MYLK3, TGFBR1, MYLK4, TGFBR2, RAF1, MYLK2, CDK6, CDK4, DAPK1, PRKCB, NTRK3, MAPK1, ACVR2A, RPS6KA3, CCND1, PPP1CA, CAMK4, GSK3B, RIPK2, MAPK8, RELN, MTOR, ABL2, MYLK, PPP2R2A, ACVR1, F2R	$8.16e^{-05}$	$1.41e^{-01}$
GO:0008284~positive regulation of cell proliferation	FGFR3, OSMR, ERBB2, FGF10, RPS6KB1, KIT, GLI2, IGF1R, BCL2, CAMK2D, MYC, CCNA2, IL6, IL2RA, FLT1, TGFBR1, NODAL, TGFBR2, CDK6, IL6R, CDK4, MAPK1, NRAS, CDKN1A, CCND1, CDKN1B, ID2, HDAC1, VEGFA, RIPK2, HBEGF, MTOR, LAMC1, F2R	$4.07e^{-03}$	$7.04e^{-01}$
GO:0010604~positive regulation of macromolecule metabolic process	E2F1, MITF, FOXO1, NFKB1, FOXO3, KIT, GLI2, SRF, IGF1R, REL, MAP3K1, BCL2, TCF4, THBS1, TRAF6, MYC, CCNA2, APC, CEBPA, IRAK1, IL6, AR, EGR2, SMAD7, TGFBR1, NODAL, TP53, SMAD4, CREB5, IL6R, CDK4, BRCA1, TP73, RAD51, MAPK1, ACVR2A, CCND1, HDAC1, ETS1, VEGFA, MTOR, ACVR1, F2R	$1.82e^{-01}$	$3.89e^{+02}$
GO:0051272~positive regulation of cell motion	PLD1, IL6, FLT1, BCAR1, TGFBR1, MYLK2, RPS6KB1, IL6R, KIT, MAPK1, IGF1R, ETS1, BCL2, VEGFA, HBEGF, THBS1, F2R, APC	$7.31e^{-01}$	$1.34e^{+04}$
GO:0012501~programmed cell death	TRAF1, PHLPP1, E2F1, E2F2, MCL1, ERBB3, EGLN3, NFKB1, PMAIP1, FOXO3, KIT, GSN, MAP3K1, BCL2, FAS, THBS1, TRAF6, MYC, IL6, IL2RA, LTBR, VAV3, ROCK1, TP53, RAF1, BIRC5, BIRC3, YWHAE, BRCA1, TP73, DAPK1, NRAS,	$1.05e^{+01}$	$1.73e^{+04}$

	CDKN1B, RIPK2, MAPK8, F2R		
GO:0051270~regulation of cell motion	PLD1, IL6, FLT1, SMAD7, BCAR1, TGFBR1, MYLK2, RPS6KB1, IL6R, KIT, MAPK1, IGF1R, CDKN1B, ETS1, BCL2, MAP3K1, VEGFA, HBEGF, THBS1, CHRD, F2R, APC	$4.53e^{+00}$	$7.87e^{+02}$
GO:0007167~enzyme linked receptor protein signaling pathway	IRAK1, RET, FGFR3, FLT1, ERBB3, SMAD7, TGFBR1, BCAR1, ERBB2, TGFBR2, SMAD4, RAF1, FOXO1, FGF10, KIT, FLNA, NTRK3, IGF1R, ACVR2A, PTK2, ID1, MAP3K1, VEGFA, HBEGF, CHRD, FRS2, ACVR1	$1.23e^{+02}$	$2.13e^{+05}$
GO:0007242~intracellular signaling cascade	FGFR3, ERBB3, ERBB2, RPS6KB2, RPS6KB1, KIT, HIST2H4A, HIST2H4B, IGF1R, GSN, MAP3K1, RHOA, TRAF6, THBS1, GNG5, FRS2, CCNA2, ARHGDIA, SHC4, HIST1H4H, PDK1, IRAK1, AR, RET, PLD1, FLT1, VAV3, ROCK1, SMAD7, TGFBR1, TP53, RAF1, YWHAE, BRCA1, FLNA, TP73, PRKCB, IFNAR1, DAPK1, MAPK1, NRAS, IFNAR2, CCND1, RPS6KA3, CRKL, GSK3B, MAPK8, RAP1B, MTOR, CRK, F2R	$1.56e^{+01}$	$2.71e^{+04}$
GO:0008219~cell death	TRAF1, PHLPP1, E2F1, E2F2, MCL1, ERBB3, EGLN3, NFKB1, PMAIP1, FOXO3, KIT, GSN, MAP3K1, BCL2, FAS, THBS1, TRAF6, MYC, IL6, AR, IL2RA, LTBR, VAV3, ROCK1, TP53, RAF1, BIRC5, BIRC3, YWHAE, BRCA1, TP73, DAPK1, NRAS, CDKN1B, RIPK2, MAPK8, F2R	$2.55e^{+02}$	$4.42e^{+04}$
GO:0010033~response to organic substance	MCL1, OSMR, ERBB3, ERBB2, BCAR1, FOXO1, RPS6KB1, PMAIP1, SRF, IGF1R, CDKN2B, GSN, BCL2, ATF6B, FAS, THBS1, MYC, CCNA2, GNG5, IRAK1, IL6, AR, PLD1, EGR2, TGFBR1, TGFBR2, IL6R, BRCA1, MAPK1, CDKN1A, CCND1, ID2, ID1, RIPK2, MTOR, PPP2R2A, F2R	$2.78e^{+02}$	$4.80e^{+04}$

GO:0006915~apoptosis	TRAF1, PHLPP1, E2F1, E2F2, MCL1, ERBB3, EGLN3, NFKB1, PMAIP1, FOXO3, GSN, MAP3K1, BCL2, FAS, THBS1, TRAF6, MYC, IL6, IL2RA, LTBR, VAV3, ROCK1, TP53, RAF1, BIRC5, BIRC3, YWHAE, BRCA1, TP73, DAPK1, NRAS, RIPK2, MAPK8, F2R	$2.94e^{+02}$	$5.09e^{+04}$
GO:0016265~death	TRAF1, PHLPP1, E2F1, E2F2, MCL1, ERBB3, EGLN3, NFKB1, PMAIP1, FOXO3, KIT, GSN, MAP3K1, BCL2, FAS, THBS1, TRAF6, MYC, IL6, AR, IL2RA, LTBR, VAV3, ROCK1, TP53, RAF1, BIRC5, BIRC3, YWHAE, BRCA1, TP73, DAPK1, NRAS, CDKN1B, RIPK2, MAPK8, F2R	$3.15e^{+02}$	$5.46e^{+04}$
GO:0010557~positive regulation of macromolecule biosynthetic process	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, IGF1R, REL, MAP3K1, TCF4, THBS1, TRAF6, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, TGFBR1, NODAL, SMAD4, TP53, CREB5, CDK4, BRCA1, TP73, MAPK1, HDAC1, ETS1, VEGFA, MTOR, ACVR1, F2R	$5.22e^{+01}$	$9.03e^{+04}$
GO:0031328~positive regulation of cellular biosynthetic process	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, IGF1R, REL, MAP3K1, TCF4, THBS1, TRAF6, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, TGFBR1, NODAL, SMAD4, TP53, CREB5, CDK4, BRCA1, TP73, MAPK1, HDAC1, ETS1, VEGFA, MTOR, ACVR1, F2R	$1.98e^{+03}$	$3.43e^{+06}$
GO:0009967~positive regulation of signal transduction	IL6, LTBR, FLT1, ERBB3, ERBB2, TGFBR1, SMAD4, FGF10, IL6R, KIT, FLNA, NRAS, ACVR2A, CDKN2B, REL, ITGA8, VEGFA, RHOA, RIPK2, RELN, MTOR, TRAF6, THBS1, F2R	$2.83e^{+03}$	$4.89e^{+05}$
GO:0040017~positive regulation of locomotion	IL6, PLD1, FLT1, BCAR1, FGF10, RPS6KB1, IL6R, KIT, IGF1R, MAPK1, BCL2, VEGFA, HBEGF, THBS1, F2R, APC	$2.84e^{+03}$	$4.92e^{+05}$

GO:0009891~positive regulation of biosynthetic process	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, IGF1R, REL, MAP3K1, TCF4, THBS1, TRAF6, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, TGFBR1, NODAL, SMAD4, TP53, CREB5, CDK4, BRCA1, TP73, MAPK1, HDAC1, ETS1, VEGFA, MTOR, ACVR1, F2R	$3.00e^{+02}$	$5.18e^{+05}$
GO:0010647~positive regulation of cell communication	COL4A4, IL6, LTBR, FLT1, ERBB3, TGFBR1, ERBB2, SMAD4, FGF10, IL6R, KIT, FLNA, NRAS, ACVR2A, CDKN2B, REL, ITGA8, VEGFA, RHOA, RIPK2, RELN, MTOR, TRAF6, THBS1, F2R	$3.51e^{+02}$	$6.07e^{+05}$
GO:0006928~cell motion	ERBB2, BCAR1, RPS6KB1, KIT, GLI2, SRF, PTK2, THBS1, ARHGDIA, APC, RET, IL6, FLT1, VAV3, EGR2, ROCK1, NODAL, TGFBR1, IL6R, YWHAE, COL5A1, ID1, ITGA5, ETS1, HBEGF, RELN, MAPK8, LAMC1, ACVR1	$6.05e^{+02}$	$1.05e^{+07}$
GO:0016477~cell migration	RET, IL6, VAV3, FLT1, ROCK1, NODAL, BCAR1, TGFBR1, RPS6KB1, IL6R, KIT, SRF, YWHAE, COL5A1, PTK2, ID1, ITGA5, HBEGF, RELN, LAMC1, THBS1, ACVR1, APC	$6.23e^{+02}$	$1.08e^{+07}$
GO:0051674~localization of cell	RET, IL6, VAV3, FLT1, ROCK1, NODAL, BCAR1, TGFBR1, RPS6KB1, IL6R, KIT, SRF, YWHAE, COL5A1, PTK2, ID1, ITGA5, ETS1, HBEGF, RELN, LAMC1, THBS1, ACVR1, APC	$6.54e^{+00}$	$1.13e^{+07}$
GO:0048870~cell motility	RET, IL6, VAV3, FLT1, ROCK1, NODAL, BCAR1, TGFBR1, RPS6KB1, IL6R, KIT, SRF, YWHAE, COL5A1, PTK2, ID1, ITGA5, ETS1, HBEGF, RELN, LAMC1, THBS1, ACVR1, APC	$6.54e^{+00}$	$1.13e^{+07}$
GO:0051094~positive regulation of developmental process	IL6, IL2RA, TGFBR2, SMAD4, RPS6KB1, NFKB1, IL6R, KIT, FOXO3, GLI2, SRF, NTRK3, ACVR2A, CDKN2B, ID2, ETS1, IL4R, BCL2, RHOA, THBS1, ARHGDIA,	$7.21e^{+02}$	$1.25e^{+07}$

	ACVR1, APC		
GO:0030335~positive regulation of cell migration	IL6, PLD1, FLT1, BCAR1, RPS6KB1, IL6R, KIT, IGF1R, MAPK1, BCL2, VEGFA, HBEGF, THBS1, F2R, APC	$1.24e^{+04}$	$2.14e^{+06}$
GO:0045597~positive regulation of cell differentiation	IL6, IL2RA, TGFBR2, SMAD4, NFKB1, IL6R, KIT, FOXO3, GLI2, SRF, NTRK3, ACVR2A, CDKN2B, ID2, ETS1, IL4R, BCL2, RHOA, ARHGDIA, APC, ACVR1	$1.40e^{+03}$	$2.42e^{+07}$
GO:0006916~anti-apoptosis	IRAK1, MCL1, TGFBR1, FOXO1, BIRC5, NFKB1, BIRC3, DAPK1, IGF1R, HDAC1, GSK3B, COMP, BCL2, VEGFA, RIPK2, FAS, TRAF6, THBS1, MYC, ARHGDIA	$2.00e^{+04}$	$3.46e^{+07}$
GO:0010628~positive regulation of gene expression	E2F1, MITF, FOXO1, NFKB1, FOXO3, KIT, GLI2, SRF, REL, MAP3K1, TCF4, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, NODAL, TGFBR1, SMAD4, TP53, CREB5, BRCA1, TP73, MAPK1, HDAC1, ETS1, VEGFA, ACVR1, F2R	$2.43e^{+04}$	$4.21e^{+06}$
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, IGF1R, REL, MAP3K1, TCF4, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, TGFBR1, NODAL, SMAD4, TP53, CREB5, BRCA1, TP73, RAD51, MAPK1, HDAC1, ETS1, VEGFA, ACVR1, F2R	$2.70e^{+04}$	$4.68e^{+06}$
GO:0040012~regulation of locomotion	PLD1, IL6, FLT1, SMAD7, BCAR1, FGF10, RPS6KB1, IL6R, KIT, MAPK1, IGF1R, BCL2, MAP3K1, VEGFA, HBEGF, THBS1, CHRD, F2R, APC	$6.01e^{+03}$	$1.04e^{+08}$
GO:0051173~positive regulation of nitrogen compound metabolic process	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, IGF1R, REL, MAP3K1, TCF4, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, TGFBR1, NODAL, SMAD4, TP53, CREB5, BRCA1, TP73, RAD51, MAPK1, HDAC1, ETS1, VEGFA, ACVR1, F2R	$6.13e^{+03}$	$1.06e^{+08}$
GO:0045941~positive regulation of transcription	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, REL, MAP3K1, TCF4, CCNA2, MYC, CEBPA, IRAK1, AR, IL6, EGR2, NODAL, TGFBR1, SMAD4, TP53, CREB5,	$6.63e^{+04}$	$1.15e^{+08}$

	BRCA1, TP73, MAPK1, HDAC1, ETS1, VEGFA, F2R, ACVR1		
GO:0030334~regulation of cell migration	PLD1, IL6, FLT1, SMAD7, BCAR1, RPS6KB1, IL6R, KIT, MAPK1, IGF1R, BCL2, MAP3K1, VEGFA, HBEGF, THBS1, CHRD, F2R, APC	$7.72e^{+03}$	$1.34e^{+08}$
GO:0009725~response to hormone stimulus	PLD1, AR, IL6, EGR2, ERBB3, BCAR1, ERBB2, TGFBR1, TGFBR2, FOXO1, RPS6KB1, IL6R, SRF, BRCA1, MAPK1, IGF1R, CCND1, CDKN1A, BCL2, MTOR, FAS, THBS1, CCNA2, GNG5	$2.59e^{+04}$	$4.47e^{+07}$
GO:0051174~regulation of phosphorus metabolic process	IRAK1, IL6, VAV3, FLT1, SMAD7, TGFBR1, ERBB2, TGFBR2, SMAD4, IL6R, KIT, YWHAE, TP73, ACVR2A, CCND1, CDKN1A, CDKN1B, CDKN2B, BCL2, MAP3K1, RELN, MTOR, TRAF6, THBS1, FRS2, APC, F2R	$3.78e^{+04}$	$6.53e^{+07}$
GO:0019220~regulation of phosphate metabolic process	IRAK1, IL6, VAV3, FLT1, SMAD7, TGFBR1, ERBB2, TGFBR2, SMAD4, IL6R, KIT, YWHAE, TP73, ACVR2A, CCND1, CDKN1A, CDKN1B, CDKN2B, BCL2, MAP3K1, RELN, MTOR, TRAF6, THBS1, FRS2, APC, F2R	$3.78e^{+04}$	$6.53e^{+07}$
GO:0042325~regulation of phosphorylation	IRAK1, IL6, VAV3, FLT1, SMAD7, TGFBR1, ERBB2, TGFBR2, SMAD4, IL6R, KIT, TP73, ACVR2A, CCND1, CDKN1A, CDKN1B, CDKN2B, BCL2, MAP3K1, RELN, MTOR, TRAF6, THBS1, FRS2, APC, F2R	$9.26e^{+04}$	$1.60e^{+09}$
GO:0009719~response to endogenous stimulus	PLD1, AR, IL6, EGR2, ERBB3, BCAR1, ERBB2, TGFBR1, TGFBR2, FOXO1, RPS6KB1, IL6R, SRF, BRCA1, MAPK1, IGF1R, CCND1, CDKN1A, BCL2, MTOR, FAS, THBS1, CCNA2, GNG5	$1.86e^{+05}$	$3.21e^{+09}$
GO:0007243~protein kinase cascade	IRAK1, RET, FGFR3, FLT1, ERBB3, ERBB2, TGFBR1, RPS6KB2, RPS6KB1, KIT, DAPK1, IFNAR1, MAPK1, IFNAR2, IGF1R, RPS6KA3, CRKL, MAP3K1, MAPK8, TRAF6, THBS1, FRS2, F2R	$2.01e^{+05}$	$3.47e^{+08}$

GO:0045596~negative regulation of cell differentiation	SMAD7, NODAL, TGFBR1, TP53, FGF10, CDK6, KIT, GLI2, HIST2H4A, HIST2H4B, MAPK1, PTK2, CCND1, HDAC1, IL4R, RHOA, CHRD, ARHGDIA, HIST1H4H, APC	$3.82e^{+05}$	$6.61e^{+08}$
GO:0045893~positive regulation of transcription, DNA-dependent	E2F1, CEBPA, AR, IL6, EGR2, NODAL, MITF, TP53, SMAD4, FOXO1, CREB5, NFKB1, FOXO3, GLI2, SRF, BRCA1, TP73, REL, HDAC1, ETS1, MAP3K1, VEGFA, TCF4, MYC, F2R	$8.42e^{+05}$	$1.46e^{+10}$
GO:0051254~positive regulation of RNA metabolic process	E2F1, CEBPA, AR, IL6, EGR2, NODAL, MITF, TP53, SMAD4, FOXO1, CREB5, NFKB1, FOXO3, GLI2, SRF, BRCA1, TP73, REL, HDAC1, ETS1, MAP3K1, VEGFA, TCF4, MYC, F2R	$9.95e^{+05}$	$1.72e^{+10}$
GO:0051726~regulation of cell cycle	E2F1, E2F2, TP53, MYLK2, CDK6, BIRC5, CDK4, BRCA1, CCND1, CDKN1A, CDKN1B, CDKN2B, HDAC1, PRDM4, ID2, ETS1, BCL2, CAMK2D, CCNA2, MYC, APC	$1.09e^{+07}$	$1.88e^{+10}$
GO:0007166~cell surface receptor linked signal transduction	OSMR, BCAR1, MITF, FOXO1, FGF10, GLI2, HHIP, FRS2, GNG5, IRAK1, WNT10B, RET, FLNA, IFNAR1, MAPK1, IFNAR2, ACVR2A, CCND1, VEGFA, RIPK2, ACVR1, FGFR3, ERBB3, C3, ERBB2, ITGA10, KIT, IGF1R, PTK2, MAP3K1, TRAF6, APC, FZD8, FLT1, VAV3, IL2RA, SMAD7, TGFBR1, TGFBR2, SMAD4, RAF1, BIRC3, NTRK3, FZD10, ITGA5, ID1, GSK3B, ITGA8, HBEGF, CHRD, F2R	$1.78e^{+07}$	$3.08e^{+09}$
GO:0006357~regulation of transcription from RNA polymerase II promoter	E2F1, MITF, FOXO1, NFKB1, FOXO3, GLI2, SRF, MAP3K1, RHOA, TCF4, MYC, CHD4, CEBPA, IL6, CTBP1, AR, EGR2, SMAD7, NODAL, SMAD4, TP53, BRCA1, IFNAR2, ID2, HDAC1, ID1, ETS1, VEGFA, CRK, HDAC8	$2.69e^{+07}$	$4.66e^{+10}$

GO:0043065~positive regulation of apoptosis	IL2RA, VAV3, TGFBR1, TP53, PMAIP1, FOXO3, YWHAE, BRCA1, TP73, DAPK1, MAPK1, CUL2, CDKN1A, CDKN1B, ETS1, MAP3K1, BCL2, RIPK2, MAPK8, FAS, TRAF6, MYC, APC	$3.37e^{+06}$	$5.84e^{+09}$
GO:0043068~positive regulation of programmed cell death	IL2RA, VAV3, TGFBR1, TP53, PMAIP1, FOXO3, YWHAE, BRCA1, TP73, DAPK1, MAPK1, CUL2, CDKN1A, CDKN1B, ETS1, MAP3K1, BCL2, RIPK2, MAPK8, FAS, TRAF6, MYC, APC	$3.84e^{+06}$	$6.64e^{+09}$
GO:0010942~positive regulation of cell death	IL2RA, VAV3, TGFBR1, TP53, PMAIP1, FOXO3, YWHAE, BRCA1, TP73, DAPK1, MAPK1, CUL2, CDKN1A, CDKN1B, ETS1, MAP3K1, BCL2, RIPK2, MAPK8, FAS, TRAF6, MYC, APC	$4.17e^{+06}$	$7.22e^{+10}$
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	RET, FGFR3, FLT1, ERBB3, BCAR1, ERBB2, FGF10, FOXO1, RAF1, KIT, FLNA, NTRK3, IGF1R, PTK2, VEGFA, HBEGF, FRS2	$5.29e^{+06}$	$9.16e^{+08}$
GO:0007389~pattern specification process	EGR2, FLT1, NODAL, TGFBR1, TGFBR2, SMAD4, FGF10, GLI2, ACVR2A, CRKL, VEGFA, RELN, HHIP, FRS2, CHRD, PITX2, ACVR1, APC	$9.71e^{+06}$	$1.68e^{+10}$
GO:0035239~tube morphogenesis	RET, FLT1, NODAL, TGFBR2, SMAD4, FGF10, GLI2, SRF, PPP1CA, BCL2, VEGFA, TRAF6, ACVR1	$2.15e^{+07}$	$3.72e^{+10}$
GO:0043549~regulation of kinase activity	IRAK1, VAV3, FLT1, ERBB2, TGFBR1, TGFBR2, KIT, TP73, CCND1, CDKN1A, CDKN1B, CDKN2B, MAP3K1, RELN, MTOR, TRAF6, THBS1, FRS2, F2R, APC	$2.32e^{+07}$	$4.01e^{+10}$
GO:0051329~interphase of mitotic cell cycle	E2F1, CUL2, CDKN1A, CCND1, CDKN1B, CDKN2B, BCL2, CAMK2D, CDK6, BIRC5, CDK4, ACVR1	$2.36e^{+08}$	$4.08e^{+10}$
GO:0010627~regulation of protein kinase cascade	IL6, LTBR, ERBB3, ERBB2, TGFBR1, MYLK2, IL6R, TP73, FLNA, REL, MAP3K1, RHOA, RIPK2, MTOR, TRAF6, F2R, APC	$2.39e^{+08}$	$4.13e^{+11}$

GO:0008285~negative regulation of cell proliferation	CEBPA, IL6, CTBP1, IL2RA, STK11, ERBB2, TGFBR2, TP53, SMAD4, FGF10, CDK6, CUL2, CDKN1A, CDKN1B, CDKN2B, ETS1, BCL2, THBS1, F2R, APC	$2.77e^{+08}$	$4.80e^{+10}$
GO:0035295~tube development	CEBPA, RET, FLT1, NODAL, TGFBR1, TGFBR2, SMAD4, FGF10, GLI2, SRF, PPP1CA, BCL2, VEGFA, HHIP, TRAF6, ACVR1	$3.05e^{+07}$	$5.28e^{+11}$
GO:0051325~interphase	E2F1, CUL2, CDKN1A, CCND1, CDKN1B, CDKN2B, BCL2, CAMK2D, CDK6, BIRC5, CDK4, ACVR1	$3.19e^{+08}$	$5.52e^{+10}$
GO:0051338~regulation of transferase activity	IRAK1, VAV3, FLT1, ERBB2, TGFBR1, TGFBR2, KIT, TP73, CCND1, CDKN1A, CDKN1B, CDKN2B, MAP3K1, RELN, MTOR, TRAF6, THBS1, FRS2, F2R, APC	$4.47e^{+06}$	$7.74e^{+10}$
GO:0044087~regulation of cellular component biogenesis	COL4A4, TGFBR1, KIT, SRF, PTK2, CDKN1B, GSN, MAP3K1, GSK3B, RHOA, MTOR, THBS1, APC	$7.51e^{+07}$	$1.30e^{+12}$
GO:0045859~regulation of protein kinase activity	IRAK1, FLT1, ERBB2, TGFBR1, TGFBR2, KIT, TP73, CCND1, CDKN1A, CDKN1B, CDKN2B, MAP3K1, RELN, MTOR, TRAF6, THBS1, FRS2, F2R, APC	$7.55e^{+07}$	$1.31e^{+11}$
GO:0032535~regulation of cellular component size	HPN, AR, FGFR3, TGFBR1, TP53, SMAD4, CDK4, TP73, NTRK3, CDKN1A, CDKN1B, PRDM4, GSN, BCL2, MAP3K1, HBEGF, MTOR	$7.78e^{+06}$	$1.35e^{+11}$
GO:0008361~regulation of cell size	HPN, AR, FGFR3, TGFBR1, TP53, SMAD4, CDK4, TP73, NTRK3, CDKN1A, CDKN1B, PRDM4, BCL2, HBEGF, MTOR	$9.38e^{+07}$	$1.62e^{+11}$
GO:0008283~cell proliferation	E2F1, AR, IL2RA, OSMR, BCAR1, ERBB2, TP53, RAF1, FGF10, KIT, GLI2, IFNAR2, ACVR2A, PRDM4, BCL2, VEGFA, RIPK2, RAP1B, HHIP, FRS2, MYC	$1.13e^{+09}$	$1.95e^{+12}$
GO:0065003~macromolecular complex assembly	TRAF1, E2F2, HIST2H4A, HIST2H4B, HIST1H2BO, IGF1R, PTK2, HIST1H2BN, HIST1H2BK, HIST1H2BL, GSN, MAP3K1, HIST1H2BI, FAS, PRKAA2, MYC, APC, HIST1H4H, IRAK1, HIST1H2BC, HIST1H2BD, HIST1H2BF, HIST1H2BG,	$1.24e^{+09}$	$2.14e^{+12}$

	HIST1H2BH, SMAD4, TP53, BIRC3, FLNA, RAD51, HIST2H2BF, LAMC1		
GO:0006917~induction of apoptosis	VAV3, TGFBR1, TP53, FOXO3, PMAIP1, YWHAE, BRCA1, TP73, DAPK1, MAPK1, CUL2, CDKN1A, CDKN1B, ETS1, MAPK8, FAS, TRAF6, MYC	$1.38e^{+09}$	$2.38e^{+12}$
GO:0012502~induction of programmed cell death	VAV3, TGFBR1, TP53, FOXO3, PMAIP1, YWHAE, BRCA1, TP73, DAPK1, MAPK1, CUL2, CDKN1A, CDKN1B, ETS1, MAPK8, FAS, TRAF6, MYC	$1.44e^{+08}$	$2.49e^{+12}$
GO:0051130~positive regulation of cellular component organization	C3, TGFBR1, SMAD4, FGF10, KIT, SRF, NTRK3, CDKN1B, MAP3K1, GSK3B, RHOA, MTOR, ARHGDIA, APC	$1.45e^{+09}$	$2.50e^{+12}$
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	E2F1, CEBPA, AR, IL6, EGR2, NODAL, MITF, TP53, SMAD4, FOXO1, NFKB1, FOXO3, GLI2, SRF, HDAC1, ETS1, MAP3K1, VEGFA, MYC	$2.24e^{+09}$	$3.88e^{+11}$
GO:0048545~response to steroid hormone stimulus	IL6, ERBB2, TGFBR1, TGFBR2, RPS6KB1, IL6R, BRCA1, MAPK1, CCND1, CDKN1A, BCL2, FAS, THBS1, CCNA2	$2.88e^{+08}$	$4.98e^{+11}$
GO:0043933~macromolecular complex subunit organization	TRAF1, E2F2, HIST2H4A, HIST2H4B, HIST1H2BO, IGF1R, PTK2, HIST1H2BN, HIST1H2BK, HIST1H2BL, GSN, MAP3K1, HIST1H2BI, FAS, PRKAA2, MYC, APC, HIST1H4H, IRAK1, HIST1H2BC, HIST1H2BD, HIST1H2BF, HIST1H2BG, HIST1H2BH, SMAD4, TP53, BIRC3, FLNA, RAD51, HIST2H2BF, LAMC1	$4.26e^{+08}$	$7.36e^{+11}$
GO:0010740~positive regulation of protein kinase cascade	IL6, LTBR, ERBB3, TGFBR1, ERBB2, IL6R, FLNA, REL, RHOA, RIPK2, MTOR, TRAF6, F2R	$4.43e^{+08}$	$7.66e^{+11}$
GO:0046777~protein amino acid autophosphorylation	NTRK3, IGF1R, IRAK1, PTK2, MAP3K1, ERBB2, TGFBR1, CAMK2D, MTOR, KIT	$5.00e^{+08}$	$8.66e^{+10}$
GO:0009611~response to wounding	IL6, IL2RA, ERBB3, C3, ERBB2, TGFBR2, FGF10, RPS6KB1, NFKB1, IL6R, SRF,	$6.06e^{+08}$	0.001

	COL5A1, TP73, ITGA5, GSN, BCL2, MAP3K1, RIPK2, HBEGF, THBS1, F2R, ACVR1		
GO:0048754~branching morphogenesis of a tube	PPP1CA, FLT1, BCL2, VEGFA, TGFBR2, SMAD4, FGF10, GLI2, ACVR1	$6.85e^{+08}$	0.001
GO:0001944~vasculature development	FLT1, SMAD7, NODAL, TGFBR1, TGFBR2, FOXO1, FGF10, SRF, COL5A1, PTK2, CRKL, ID1, VEGFA, THBS1, ACVR1	$1.04e^{+10}$	0.002
GO:0007507~heart development	CRKL, ID2, ID1, ERBB3, SMAD7, ERBB2, NODAL, TGFBR1, TGFBR2, MYLK2, GLI2, SRF, COL5A1, ACVR1	$1.05e^{+10}$	0.002
GO:0048661~positive regulation of smooth muscle cell proliferation	IL6, FLT1, VEGFA, TGFBR2, HBEGF, RPS6KB1, IL6R	$1.15e^{+10}$	0.002
GO:0045637~regulation of myeloid cell differentiation	ACVR2A, ID2, ETS1, MITF, CDK6, FAS, FOXO3, HIST2H4A, APC, HIST1H4H, HIST2H4B	$1.22e^{+09}$	0.002
GO:0043434~response to peptide hormone stimulus	IGF1R, PLD1, AR, EGR2, ERBB3, BCL2, BCAR1, FOXO1, RPS6KB1, IL6R, MTOR, CCNA2	$1.45e^{+10}$	0.003
GO:0048534~hemopoietic or lymphoid organ development	CEBPA, CRKL, ID2, TGFBR1, BCL2, VEGFA, TGFBR2, TP53, FGF10, CDK6, KIT, FAS, NFKB2, TRAF6, APC	$1.59e^{+09}$	0.003
GO:0022402~cell cycle process	E2F1, STK11, TP53, CDK6, BIRC5, CDK4, BRCA1, TP73, RAD51, CUL2, CCND1, CDKN1A, CDKN1B, CDKN2B, GSK3B, BCL2, CAMK2D, THBS1, CCNA2, MYC, APC, ACVR1	$1.70e^{+09}$	0.003
GO:0006333~chromatin assembly or disassembly	HIST1H2BC, HIST1H2BD, HIST1H2BF, HIST1H2BG, HIST1H2BH, HIST2H4A, HIST2H4B, HIST1H2BO, HIST1H2BN, HIST1H2BK, HIST1H2BL, HIST1H2BI,	$1.82e^{+10}$	0.003

	HIST2H2BF, HDAC8, CHD4, HIST1H4H		
GO:0001763~morphogenesis of a branching structure	PPP1CA, FLT1, BCL2, VEGFA, TGFBR2, SMAD4, FGF10, GLI2, ACVR1	$1.88e^{+10}$	0.003
GO:0032101~regulation of response to external stimulus	NTRK3, IL6, IL2RA, C3, OSMR, VEGFA, TGFBR2, FGF10, IL6R, MTOR, THBS1, F2R	$1.98e^{+10}$	0.003
GO:0007423~sensory organ development	ERBB3, ERBB2, MITF, FGF10, COL5A2, COL5A1, CDKN1B, BCL2, ITGA8, MAP3K1, VEGFA, FRS2, MYC, APC	$2.12e^{+10}$	0.004
GO:0033674~positive regulation of kinase activity	IRAK1, VAV3, FLT1, ERBB2, TGFBR1, TGFBR2, KIT, CCND1, MAP3K1, RELN, TRAF6, THBS1, FRS2, F2R	$2.34e^{+09}$	0.004
GO:0007178~transmembrane receptor protein serine/threonine kinase signaling pathway	ACVR2A, IRAK1, ID1, SMAD7, MAP3K1, TGFBR1, TGFBR2, SMAD4, CHRD, ACVR1	$2.56e^{+10}$	0.004
GO:0007050~cell cycle arrest	CUL2, CDKN1A, CDKN1B, CDKN2B, STK11, TP53, THBS1, MYC, TP73, APC	$2.56e^{+10}$	0.004
GO:0030278~regulation of ossification	ACVR2A, IL6, EGR2, BCL2, CDK6, IL6R, CHRD, ACVR1, APC	$2.81e^{+09}$	0.005
GO:0048584~positive regulation of response to stimulus	IRAK1, IL6, C3, OSMR, BCAR1, FGF10, IL6R, BRCA1, NTRK3, MAPK1, VEGFA, RIPK2, TRAF6, THBS1	$2.96e^{+10}$	0.005
GO:0000082~G1/S transition of mitotic cell cycle	CUL2, CDKN1A, CCND1, CDKN1B, BCL2, CAMK2D, CDK4, ACVR1	$3.14e^{+10}$	0.005
GO:0001702~gastrulation with mouth forming second	ACVR2A, NODAL, SMAD4, FRS2, CHRD, ACVR1	$3.15e^{+08}$	0.005
GO:0002520~immune system development	CEBPA, CRKL, ID2, TGFBR1, BCL2, VEGFA, TGFBR2, TP53, FGF10, CDK6,	$3.20e^{+10}$	0.006

	KIT, FAS, NFKB2, TRAF6, APC		
GO:0002684~positive regulation of immune system process	IRAK1, IL6, IL2RA, C3, BCAR1, TGFBR2, IL6R, MAPK1, CDKN1A, IL4R, VEGFA, RIPK2, TRAF6, THBS1	$3.25e^{+10}$	0.006
GO:0051347~positive regulation of transferase activity	IRAK1, VAV3, FLT1, ERBB2, TGFBR1, TGFBR2, KIT, CCND1, MAP3K1, RELN, TRAF6, THBS1, FRS2, F2R	$3.56e^{+09}$	0.006
GO:0030155~regulation of cell adhesion	VAV3, ERBB3, GSN, SMAD7, BCL2, ERBB2, CDK6, THBS1, CHRD, ARHGDIA, APC	$3.63e^{+10}$	0.006
GO:0045786~negative regulation of cell cycle	CDKN1A, CDKN1B, PRDM4, HDAC1, ETS1, BCL2, TP53, CDK6, APC	$3.74e^{+09}$	0.006
GO:0051247~positive regulation of protein metabolic process	IL6, SMAD7, TGFBR1, SMAD4, IL6R, CDK4, BRCA1, ACVR2A, MAPK1, CCND1, BCL2, MTOR, THBS1, APC	$4.08e^{+09}$	0.007
GO:0001568~blood vessel development	FLT1, SMAD7, TGFBR1, TGFBR2, FOXO1, FGF10, SRF, COL5A1, PTK2, CRKL, ID1, VEGFA, THBS1, ACVR1	$4.47e^{+09}$	0.008
GO:0006334~nucleosome assembly	HIST1H2BC, HIST1H2BD, HIST1H2BF, HIST1H2BG, HIST1H2BH, HIST2H4A, HIST2H4B, HIST1H2BO, HIST1H2BN, HIST1H2BK, HIST1H2BL, HIST1H2BI, HIST2H2BF, HIST1H4H	$4.93e^{+09}$	0.009
GO:0032844~regulation of homeostatic process	ACVR2A, IL2RA, ETS1, SMAD7, BCL2, VEGFA, CDK6, FOXO3, MYC, F2R	$5.95e^{+09}$	0.010

Table S8. Gene Ontology (GO) analysis was performed using the list of predicted target genes of differentially miRNAs, which were significantly abundant in the immature oocytes of low antral follicle count ovaries. GO terms for biological function and count of the genes. which indicates how much the GO term is enriched of predicted genes, *P-value* and False Discovery Rate (FDR) are reported. Data are reported only for FDR less than 0.1.

GO Term	Gene count	*P-Value*	FDR
GO:0042127~regulation of cell proliferation	32	$2.77e^{+06}$	$4.63e^{+08}$
GO:0008284~positive regulation of cell proliferation	23	$6.76e^{+05}$	$1.13e^{+10}$
GO:0051236~establishment of RNA localization	11	$9.47e^{+07}$	$1.58e^{+11}$
GO:0050658~RNA transport	11	$9.47e^{+07}$	$1.58e^{+11}$
GO:0050657~nucleic acid transport	11	$9.47e^{+07}$	$1.58e^{+11}$
GO:0006403~RNA localization	11	$1.27e^{+08}$	$2.12e^{+11}$
GO:0006913~nucleocytoplasmic transport	13	$1.27e^{+09}$	$2.12e^{+12}$
GO:0051169~nuclear transport	13	$1.45e^{+09}$	$2.43e^{+12}$
GO:0043632~modification-dependent macromolecule catabolic process	23	$2.45e^{+09}$	$4.10e^{+12}$
GO:0019941~modification-dependent protein catabolic process	23	$2.45e^{+09}$	$4.10e^{+12}$
GO:0044265~cellular macromolecule catabolic process	26	$2.52e^{+09}$	$4.22e^{+11}$
GO:0009719~response to endogenous stimulus	19	$4.03e^{+08}$	$6.74e^{+11}$
GO:0015931~nucleobase, nucleoside, nucleotide and nucleic acid transport	11	$4.03e^{+08}$	$6.75e^{+11}$
GO:0051028~mRNA transport	10	$4.12e^{+08}$	$6.90e^{+11}$
GO:0009725~response to hormone stimulus	18	$4.88e^{+08}$	$8.16e^{+11}$

GO:0051603~proteolysis involved in cellular protein catabolic process	23	$5.21e^{+08}$	$8.71e^{+11}$
GO:0044257~cellular protein catabolic process	23	$5.66e^{+08}$	$9.48e^{+11}$
GO:0030163~protein catabolic process	23	$9.53e^{+08}$	0.002
GO:0009057~macromolecule catabolic process	26	$1.00e^{+10}$	0.002
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	23	$1.01e^{+09}$	0.002
GO:0043066~negative regulation of apoptosis	17	$1.49e^{+09}$	0.002
GO:0010604~positive regulation of macromolecule metabolic process	27	$1.62e^{+09}$	0.003
GO:0051173~positive regulation of nitrogen compound metabolic process	23	$1.70e^{+10}$	0.003
GO:0051329~interphase of mitotic cell cycle	10	$1.74e^{+10}$	0.003
GO:0043069~negative regulation of programmed cell death	17	$1.79e^{+10}$	0.003
GO:0060548~negative regulation of cell death	17	$1.86e^{+10}$	0.003
GO:0010557~positive regulation of macromolecule biosynthetic process	23	$2.18e^{+10}$	0.004
GO:0051325~interphase	10	$2.22e^{+09}$	0.004
GO:0010033~response to organic substance	24	$3.06e^{+10}$	0.005
GO:0007049~cell cycle	25	$3.11e^{+09}$	0.005
GO:0051726~regulation of cell cycle	16	$3.14e^{+10}$	0.005
GO:0051168~nuclear export	8	$3.70e^{+09}$	0.006
GO:0031328~positive regulation of cellular biosynthetic process	23	$4.63e^{+09}$	0.008
GO:0009891~positive regulation of biosynthetic process	23	$5.83e^{+09}$	0.010
GO:0051272~positive regulation of cell motion	9	$1.11e^{+11}$	0.020

GO:0042325~regulation of phosphorylation	18	$1.23e^{+11}$	0.021
GO:0006916~anti-apoptosis	12	$1.52e^{+11}$	0.025
GO:0045893~positive regulation of transcription, DNA-dependent	18	$1.66e^{+11}$	0.028
GO:0010628~positive regulation of gene expression	20	$1.72e^{+10}$	0.029
GO:0042981~regulation of apoptosis	24	$1.82e^{+10}$	0.030
GO:0051254~positive regulation of RNA metabolic process	18	$1.84e^{+11}$	0.031
GO:0019220~regulation of phosphate metabolic process	18	$2.05e^{+10}$	0.034
GO:0051174~regulation of phosphorus metabolic process	18	$2.05e^{+10}$	0.034
GO:0043067~regulation of programmed cell death	24	$2.13e^{+10}$	0.036
GO:0010941~regulation of cell death	24	$2.26e^{+11}$	0.038
GO:0045859~regulation of protein kinase activity	15	$2.36e^{+11}$	0.039
GO:0000082~G1/S transition of mitotic cell cycle	7	$3.08e^{+11}$	0.051
GO:0043549~regulation of kinase activity	15	$3.43e^{+10}$	0.057
GO:0045941~positive regulation of transcription	19	$4.01e^{+11}$	0.067
GO:0001501~skeletal system development	14	$4.47e^{+10}$	0.075
GO:0051338~regulation of transferase activity	15	$5.37e^{+10}$	0.090

Table S9. Gene Ontology (GO) analysis was performed using the list of predicted target genes of bta-miR-10a, which was significantly reduced in the immature oocytes of low antral follicle count ovaries. GO terms for biological function and count of the genes. which indicates how much the GO term is enriched of predicted genes, P-value and false discovery rate are reported. Data are reported only for FDR less than 0.1.

GO Term	Gene count	P- Value	FDR
GO:0045449~regulation of transcription	217	$1.04e^{+09}$	$1.88e^{+11}$
GO:0006357~regulation of transcription from RNA polymerase II promoter	80	$1.82e^{+09}$	$3.29e^{+11}$
GO:0006350~transcription	181	$2.22e^{+09}$	$4.01e^{+11}$
GO:0051252~regulation of RNA metabolic process	157	$1.39e^{+10}$	0.003
GO:0006355~regulation of transcription, DNA-dependent	152	$4.02e^{+09}$	0.007
GO:0030182~neuron differentiation	50	$2.00e^{+09}$	0.036
GO:0010557~positive regulation of macromolecule biosynthetic process	66	$4.24e^{+10}$	0.077

APPENDIX A

PUBLICATIONS - ABSTRACTS AND FULL PAPERS

1. STROZZI F, P**ASQUARIELLO R**, RAMELLI P, LAZZARI G, GALLI C, WILLIAMS LJ, IAMARTINO D. Analysis of the transcriptome of bovine embryos obtained from sex sorted and non-sorted semen by RNA-sequencing. P0104, XXI Plant and Animal Genome Congress, San Diego - California, January 11-16 2013.

2. Daniela IAMARTINO, John L. WILLIAMS, Tad SONSTEGARD, James REECY, Curtis Van TASSELL, Ezequiel L. NICOLAZZI, Stefano BIFFANI, Filippo BISCARINI, Steven SCHROEDER, Denise A.A. de OLIVEIRA, Angelo COLETTA, Jose Fernando GARCIA, Ahmad ALI, Luigi RAMUNNO, **Rolando PASQUARIELLO**, Marcela G. DRUMMONDd, Eduardo BASTIANETTO, Eric FRITZ, James KNOLTES and the INTERNATIONAL BUFFALO CONSORTIUM. The buffalo genome and the application of genomics in animal management and improvement. Buffalo Bulletin 2013 Vol.32 (Special Issue 1): 151-158

3. Daniela Iamartino, Francesco Strozzi, Rossana Capoferri, Paola Ramelli, **Rolando Pasquariello**, Alessandra Stella, John L. Williams. Genotyping By Sequencing (GBS) protocol in Water Buffalo Species. P-034, 20th ASPA congress. Italian Journal of Animal Science 2013; volume 12: supplement 1.

4. Daniela Iamartino, John L. Williams, Luigi Ramunno, Paolo Ajmone-Marsan, Alessio Valentini, Bianca Moioli, Giovanni Chillemi, Francesco Strozzi, Fabrizio Ferrè, Tommaso Biagini, Giordano Mancini, **Rolando Pasquariello**, Gianfranco Cosenza, Angelo Coletta, Bianca Castiglioni,

Luigi Zicarelli, Leopoldo Iannuzzi, Nicolò Macciota, Donato Matassino, Fabio Pilla, Mariasilvia D'Andrea, Federica Corrado, Vincenzo Peretti, The International Buffalo Genome Consortium. The Sequencing of the Water Buffalo genome: results and perspectives. C-051, 20th ASPA Congress. Italian Journal of Animal Science 2013; volume 12: supplement 1.

5. **Rolando Pasquariello**, Riccardo Giannico, Andrea Viglino, Raffaele Mazza, Beatriz Fernandez, Francesco Strozzi, Chiara Ferrandi, Fulvio Gandolfi, Patrick Lonergan, John L. Williams. Analysis of small RNAs in bovine blastocysts using deep sequencing. P-118, vol 14, N. 1s (2015): Proceedings of the 21st ASPA Congress, Milano, June 9-12, 2015.

6. Giustino Gaspa, Daniela Iamartino, Ezequiel Nicolazzi, Stefano Biffani, Paola Rammelli, **Rolando Pasquariello**, Massimo Cellesi, John Williams, Nicolò P.P. Macciotta, the International Buffalo Genome Consortium. A preliminary survey on distribution of Runs of Homozygosity in Italian Water buffalo. P-054, vol 14, N. 1s (2015): Proceedings of the 21st ASPA Congress, Milano, June 9-12, 2015.

7. **Pasquariello R**, Fiandanese N, Viglino A, Pocar P, Williams JL, Gandolfi F. Profiling of follicular fluid microRNAs in high and low Antral Follicle Count ovaries in cattle. Abstract, Veterinary and Animal Science Days, Milan, Italy, July 15-17, 2015.

8. **Pasquariello R**, Fiandanese N, Viglino A, Pocar P, Williams JL, Gandolfi F. Follicular fluid microRNAs as biomarkers of competent oocytes in cattle. The International Embryo Transfer Society 42nd Annual Conference 2016 (IETS 2016), Louisville, Kentucky, USA, January 23-26, 2016. *In course of pubblication* on Reproduction, Fertility and Development.

9. **Pasquariello R,** Fernandez-Fuertes B, Lonergan P, Gandolfi F, Strozzi F, Pizzi F, Mazza R, Williams JL. Profiling of microRNAs in bovine

blastocysts using deep sequencing. *Submitted to the Journal 'Reproduction, Fertility and Development'.*

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