UNIVERSITEIT GENT

FACULTEIT DIERGENEESKUNDE
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TRANSCRIPTOMIC AND EPIGENETIC PATTERNS IN BOVINE AND EQUINE PREIMPLANTATION EMBRYOS

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences, Faculty of Veterinary Medicine, Ghent University, 2016.

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This research was funded by the agency for Innovation by Science and Technology (agentschap voor Innovatie door Wetenschap en Technologie, IWT) – Flanders. Project number 111438 - Epigenetic modifications induced by *in vitro* culture in bovine embryos: is there a benefit in dynamic versus static culture conditions?

Printed by: University press, Zelzate

Only if we stop and think about the little things,
we will come to understand the great ones.
José Saramago

Sólo si nos detenemos a pensar en las pequeñas cosas,
llegaremos a comprender las grandes
José Saramago

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LIST OF ABBREVIATIONS

|FC|	Absolute fold change
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
6-DMAP	6-Dimethylaminopurine
AI	Artificial insemination
ANOVA	Analysis of variance
ART	Assisted reproductive technology
AS	Angelman syndrome
BER	Base excision repair machinery
BP	Band pass
bp	Base pair
BSA	Bovine serum albumin
BSA-ITS	Serum-free supplementation
BWS	Beckwith-Wiedemann syndrome
CC	Cumulus cell
cDNA	Complementary DNA
CG	Cortical granule
CGI	CpG islands
COCs	Cumulus oocyte complexes
DAPI	4',6-diamidino-2-phenylindole
DE	Differentially expressed
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic acid

Dnmts	DNA methyltransferase enzymes
DPSS	Diode-pumped solid-state
DTT	DT-Dithiothreitol
EGF	Epidermal growth factor
EthD-2	Ethidium homodimer 2
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery ratio
FITC	Fluorescein isothiocyanate-conjugated
FSH	Follicle-stimulating hormone
gDNA	Genomic DNA
GO	Gene ontology
GV	Germinal vesicle
H2AK119ub1	Histone 2A lysine 119 ubiquitination
H2BK120ub1	Histone 2B lysine 120 ubiquitination
H3K27me2-3	Histone 3 lysine 27 di-tri-methylation
H3K36me3	Histone 3 lysine 36 tri-methylation
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K79me3	Histone 3 lysine 79 tri-methylation
H3K9me3	Histone 3 lysine 9 tri-methylation
HCl	Hydrogen chloride
HELP	*Hpa*II tiny fragment enrichment by ligation-mediated PCR
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hpi	Hours post insemination
ICM	Inner cell mass
ICR	Imprinting control region
ICSI	Intracytoplasmic sperm injection
IETS	International Embryo Transfer Society

IgG	Immunoglobulin G
ITS	Insulin-transferrin-selenium
IVC	*In vitro* embryo culture
IVF	*In vitro* fertilization
IVM	*In vitro* oocyte maturation
IVP	*In vitro* embryo production
lncRNAs	Long non-coding RNAs
LOS	Large offspring syndrome
LP	Long pass
MeDIP	Methylated DNA immunoprecipitation
MII	Metaphase of the second meiotic division
MIQE	Minimum information for publication of quantitative real-time PCR experiments
miRNA	Micro RNA
MOET	Multiple ovulation and embryo transfer
mPN	Maternal pronucleus
mRNA	Messenger RNA
MSCC	Methylation sensitive cut counting
ncRNA	Non-coding RNA
NGS	Next generation sequencing
OPU	Ovum pick up
oxBS-seq	Oxidative bisulfite sequencing
pb	Polar body
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCGs	Primordial germ cells
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide

PN	Pronucleus
pPN	Paternal pronucleus
qPCR	Quantitative real-time polymerase chain reaction
R^2	Correlation coefficient
redBS-seq	Reduced bisulfite sequencing
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
ROSI	Round spermatid injection
RPKM	Reads per kilobase per million
RRBS	Reduced representation bisulfite sequencing
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
SOF	Synthetic oviduct fluid
Spz	Spermatozoon
ssDNA	Single-stranded DNA
TALP	Tyrode's albumin lactate pyruvate
TCM-199	Tissue culture medium 199
TCN	Total cell number
TDG	Thymine-DNA glycosylase
TE	Trophectoderm
TETs	Ten-eleven translocation enzymes
TMM	Trimmed mean of M-values
tsRNA	Sperm transfer RNA-derived small RNAs

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTJ	Utero-tubal junction
UV	Ultraviolet
WOW	Well-of-the-well
ZP	Zona pellucida

CHAPTER 1

GENERAL INTRODUCTION

1.1 ONCE UPON A TIME...LIFE

Preimplantation embryo development is a very complex period in which two highly specialized cells, called gametes, will combine and generate a new organism. During the first week of mammalian embryo development many important events take place, including the first cleavage, embryonic genome activation, compaction of the morula and differentiation with blastocyst formation (Lonergan *et al*. 2006). Despite its complexity, this process happens quite autonomously in mammals, and can be supported *in vitro* by assisted reproductive technologies (ARTs). The use of ARTs is of major importance for the treatment of human infertility and for livestock production, but it implies the exposure of gametes and embryos to suboptimal conditions during this crucial period. As consequence, the use of ARTs has led to increased incidence of particular syndromes, many of them related to epigenetic alterations.

In the following pages, the events that are taking place during preimplantation development, and the different ARTs will be described, with special focus on their use for bovine and horse embryo production. Additionally, an overview will be provided of the different techniques used for embryo quality determination, and the reported effects of ARTs.

1.1.1 Preimplantation Embryo Development *In vivo*

In mammals, preimplantation embryo development is the series of events that takes place from the time of fertilization in the oviduct until the implantation in the uterus. For fertilization to be successful, a single capacitated spermatozoon needs to fuse with a mature oocyte. During ovulation, one or more mature oocytes arrested in metaphase of the second meiotic division (MII), are released in the oviduct. At that time they are surrounded by a glycoprotein matrix, called zona pellucida (ZP), and a few layers of cumulus cells. The number of oocytes released per ovulatory cycle is species-specific, with monovulatory species releasing typically one, and sometimes two oocytes, such as human (Fauser and Van Heusden 1997), cattle (Adams 1999) and horse (Bergfelt and Adams 2000), and polyovulatory species releasing multiple oocytes, typically 10 or more, such as pig (Bazer *et al*. 1969), rat (Blandau 1952) and rabbit (Boving 1956). At mating, more than 10^9 actively motile spermatozoa are delivered in the female genital tract, either in the vagina, cervix (cattle) or uterus (horse), depending on the species. However, this ejaculated sperm is not able to fertilize yet, even if placed in direct contact with the oocyte. Before they are able to fertilize, they need to undergo

a series of physiological changes called capacitation (Yanagimachi 1981). Only a small fraction of the ejaculated sperm will reach the utero-tubal junction (UTJ), where a sperm reservoir will be formed. Near the time of ovulation, a small population of the sperm within the reservoir will become capacitated, with a special type of vigorous motility, called hyperactivation (Yanagimachi 1981). These capacitated spermatozoa will be released from the reservoir and move towards the ampulla (Hunter 1993), which ensures that only a limited number of spermatozoa will reach the oocyte at the ampullary-isthmic junction. Here, capacitated sperm will meet the mature oocyte and bind to the ZP or to the cumulus-oocyte complex (Inoue *et al*. 2011b; Jin *et al*. 2011), where it will acrosome react, releasing hydrolytic enzymes, and enter the perivitelline space. Once in the perivitelline space, the sperm cell will fuse with the oolemma, and get incorporated in the oocyte (Figure 1.1).

The entrance of the fertilizing spermatozoon induces the cortical reaction, in which the content of the cortical granules (secretory granules just underlying the oolemma) is secreted in order to modify the properties of the zona ZP and the oolemma, thereby preventing the penetration of new spermatozoa and therefore, polyspermy (Austin 1956). The penetration of the spermatozoon also activates the oocyte, which finishes the second meiotic division, extruding the second polar body (pb). This leaves a haploid (n) maternal nucleus, forming a one-cell embryo called zygote. Subsequently, both the maternal metaphase plate and the paternal sperm head will decondense, and form the maternal and paternal pronucleus (PN) (Hyttel *et al*. 1988). This maternal and paternal pronuclei will increase their size and migrate towards each other (Figure 1.2). During this migration, recondensation of chromatin takes place, indicating that the prophase of the first mitotic division has begun. Therefore, at this stage, DNA replication has taken place. Soon after pronuclear encounter, the first mitosis will be finished, and a series of mitotic divisions will start (Hyttel *et al*. 1989).

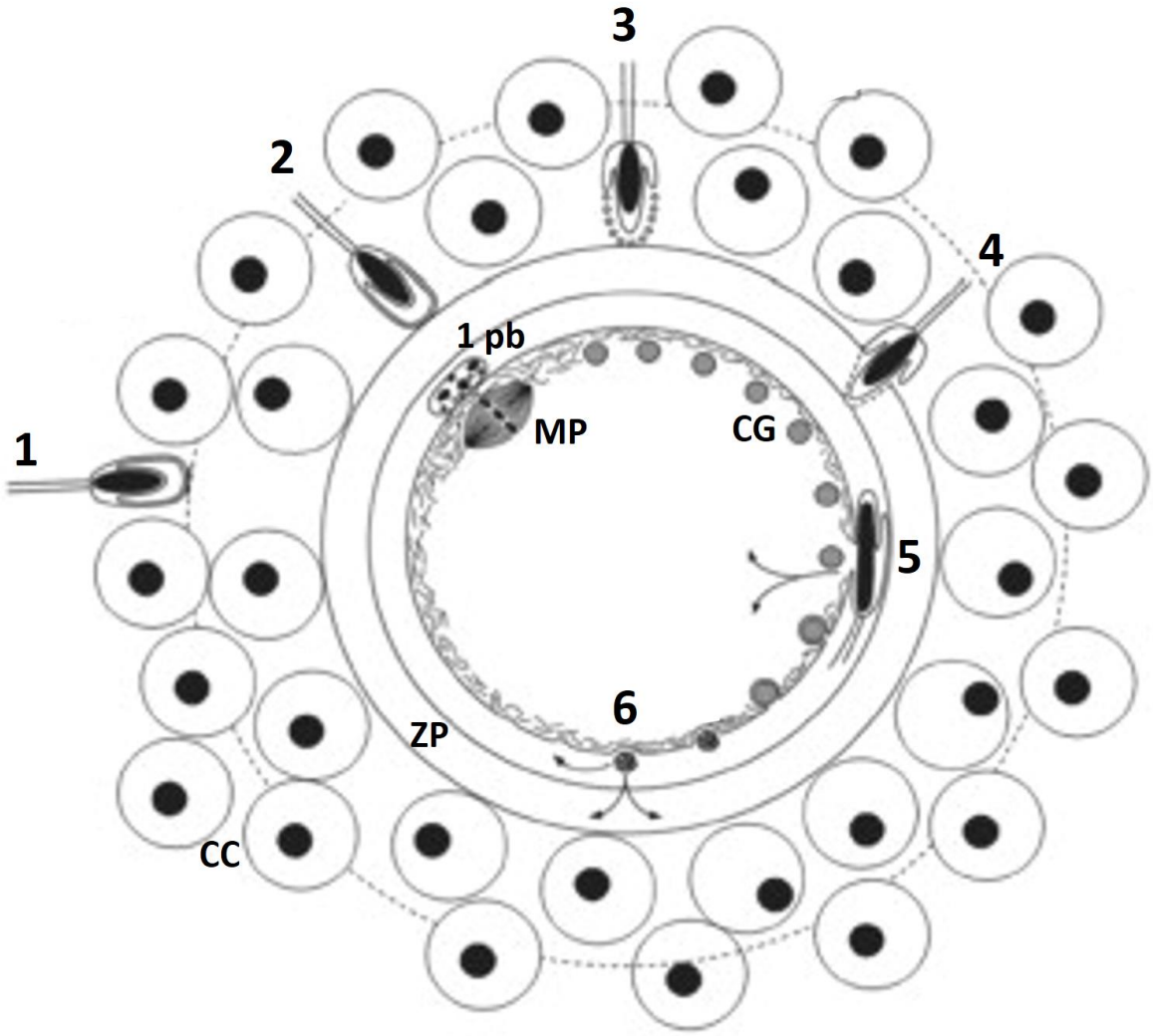

Figure 1.1. *Schematic representation of the fertilization of the mammalian oocyte. The capacitated spermatozoon binds either at the level of the cumulus layer (1) or at the zona pellucida (ZP) (2), which initiates the acrosome reaction (3). The exposure of the acrosomal content, which is capable to lyse extracellular matrices of the cumulus and/or the zona pellucida, and the hyperactivated motility are required for allowing a few spermatozoa to reach the perivitelline space (space between the ZP and the oolemma) (4). Subsequently, the spermatozoon adheres to the oolemma, fuses with it and thus fertilizes the oocyte (5). The fertilized oocyte immediately allows the cortical reaction, which avoids another spermatozoa to fuse and thus prevents polyspermic fertilization (6). The fertilizing spermatozoon brings the signal for oocyte activation and thus also causes polyspermy blockage. Adapted from Gadella and Luna 2014.*

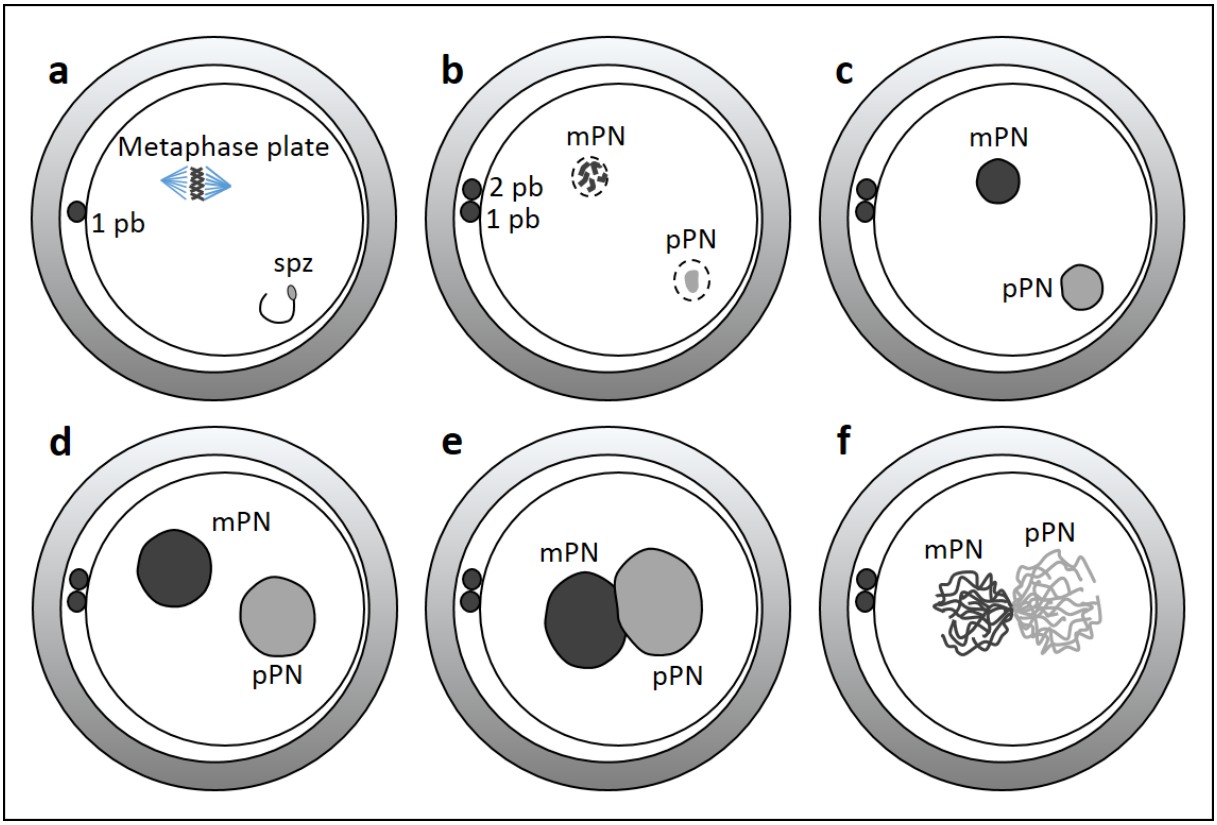

Figure 1.2. *Schematic representation of pronuclear development. (a) The spermatozoon just entered the mature oocyte arrested in metaphase II, which induces the restart of meiosis II. (b) PN0, the sperm head is decondensing. Meiosis II is finished, with the extrusion of the second polar body (2 pb), and the chromosomes start to decondense. The nuclear envelope is starting to form. (c) PN1, The nuclear envelope is completed. There are two small round pronuclei one of maternal (mPN) and one of paternal (pPN) origin. (d) PN2, the PN have increased in size and are starting to migrate towards the center. (e) PN3, the PN have reached their maximum size and are in apposition. (f) PN4, there is a nuclear membrane break down. The S phase takes place between PN2 and PN3. ZP: zona pellucida.*

The timing of the cleavage divisions varies depending on the species, with the first cleavage division taking place around 20h after the estimated time of fertilization (Betteridge 1995) (Figure 1.3). Within the first mitotic divisions the nucleus/cytoplasm ratio increases, as the size of the embryo remains constant, producing with each cleavage division smaller blastomeres. After a species specific number of cleavage divisions, the embryo reaches the morula stage. Soon after, the compaction of the morula starts. In this process, the cells flatten and the contact between blastomeres increases by the establishment of adherent junctions, until the embryo appears as a uniform mass. This generates a cell contact-induced cell

polarization, which constitutes the first differentiation event. These differentiating cells are the precursors of the trophectoderm (TE) and the inner cell mass (ICM) (Watson and Barcroft 2001). The time of compaction varies greatly among species (Betteridge 1995; Watson and Barcroft 2001). These events are essential for blastocyst formation, and by the late morula stage, the embryo acquires the remaining macromolecules necessary for cavitation.

The first step of blastocyst formation is the differentiation of the TE. It acquires the characteristics of an epithelium and it is involved in the transport of ions and water, forming one or several cavities that will fuse forming the so called blastocoel (Watson and Barcroft 2001; Marikawa and Alarcon 2009). On the other hand, the ICM aggregates as a single mass attached to the basal surface of the TE. The cells from the ICM will produce all embryonic tissues and a part of the extra-embryonic membranes. The TE combined with the ICM-derived extra embryonic membranes, will form the fetal part of the placenta.

It is at the blastocyst stage that the size of the embryo increases, due to the expansion of the blastocoel, which can occupy up to 90% of the volume of the embryo. This expansion thins the ZP until the embryo frees itself by contraction and expansion cycles, in a process called hatching. In human and rodents, the embryo implants soon after hatching. In cattle, pig and horse, the implantation in the uterus is delayed. In ruminants, the embryo changes from a spherical to an ovoid structure and further to tubular and filamentous morphology, filling the uterine horn completely while finally attaching, which in cattle starts around day 18-21 post insemination (Betteridge and Fléchon 1988). On the contrary, the equine blastocyst is surrounded by a glycoprotein capsule even after hatching, which prevents its expansion. Instead, the embryo moves from one horn to another until, around day 16, it is fixed at the base of one of the horns (Klein 2015).

Species	Pronuclear formation	2-cell	4-cell	8-cell	Compact morula	Early blastocyst	Normal blastocyst	Expanded blastocyst	Hatching	Reference
Human	-	24-26h	42h	66h	16 cells	100h	114h	120h	144h	(Veeck 2003)
Mouse	4-9h	21-23h	38-50h	50-60h	8-16 cells	66-82h	-	-	96-110h	(Betteridge 1995)
Cow	20h	24-32h	36-50h	50-64h	32 cells	120-168h	-	185h	192-216h	(Betteridge 1995; Holm *et al*. 2002; Catteeuw *et al*. 2015)
Horse	15h	24h	24-48h	72h	8-16 cells	120h	-	-	168h	(Betteridge 1995)

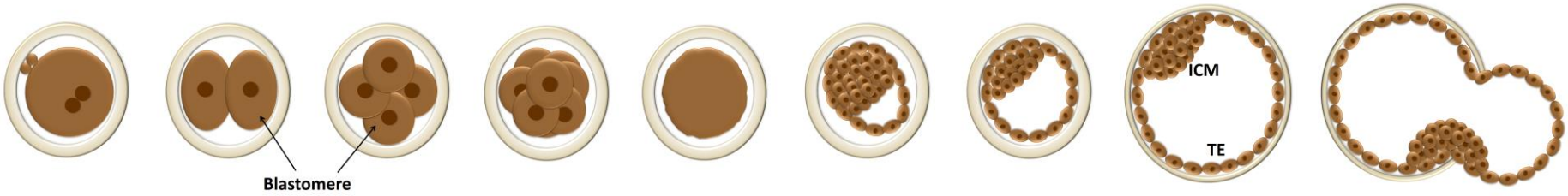

Figure 1.3. *Schematic representation of the in vitro preimplantation development, and its timing in human, mouse, cattle and horse. The times are given in hours after insemination. ICM: inner cell mass; TE: trophectoderm.*

1.1.2 Assisted Reproductive Technology (ART)

Assisted reproductive technologies are a miscellaneous group of techniques that can be combined or used individually and aim to produce embryos and/or support their development until transfer. Since the development of ART, these techniques have been applied in many mammalian species for clinical, commercial and research purposes. These techniques have been developed progressively, becoming more sophisticated as the knowledge of gametes and embryo development has increased. They include four generations of technologies: 1) artificial insemination (AI), 2) multiple ovulation and embryo transfer (MOET), 3) *in vitro* embryo production (IVP) and 4) cloning and transgenesis (Thibier 2005).

1.1.2.1 Artificial insemination (AI)

Artificial insemination consists in the deliberate introduction of sperm into a female´s uterus or cervix for the purpose of achieving a pregnancy, through *in vivo* fertilization by other means than sexual intercourse.

AI was developed as a hygienic measure to prevent disease transmission. Spallanzani is considered to be the first one to describe AI, since he reported in 1780 the successful use of AI in dogs. It was not until 1931, in Russia, that the first mass breeding of cows via AI was reported. Nowadays, it is vastly used in many species, especially in livestock, for practical and economic reasons.

Before AI can be performed, semen has to be collected, processed and stored, and then it can be introduced in the female reproductive tract (Webb 1992), which is normally combined with the detection of oestrus in females. For this reason, AI can maximize the use of semen of genetically valuable males, since from one ejaculate, many insemination doses can be obtained, e. g. with one ejaculate of a bull between 300 and 1,000 cows can be inseminated. When the semen is frozen, it can easily be transported worldwide.

1.1.2.2 Multiple ovulation and embryo transfer (MOET)

Multiple ovulation and embryo transfer has been used in farm animals since the early eighties (Thibier 2005). It consists of the use of multiple hormone injections to induce the release of multiple oocytes from the ovaries (so called superovulation), followed by AI,

flushing of the embryos from the uterus and transfer of the resulting embryos into synchronized recipients. Therefore, this technique maximizes offspring from genetically valuable females, while reducing also the time between generations. The major disadvantage of MOET is the unpredictable response of the donor female to exogenous superovulation treatment. Horses, for instance, do not respond to the hormone treatment, and embryo transfer, without hormone treatment is used instead of the MOET.

1.1.2.3 *In vitro* embryo production (IVP)

In vitro embryo production entails the combination of three steps that need to be performed following a strict timing. It covers all steps from the maturation and fertilization of the oocyte to the embryo development. The oocytes can be derived both from a living (by transvaginal ovum pick-up) or dead animal (slaughterhouse derived ovaries). It is also combined with embryo transfer in many species.

- **Ovum pick up (OPU)**

Ovum pick up was first developed for human assisted reproduction. In the early eighties it was already a regularly performed procedure used in combination with hormone stimulation (Lopata *et al*. 1974; Lauritsen 1983). In 1987, bovine oocytes were successfully collected using transvaginal ultrasonography, for the first time in animals (Pieterse *et al*. 1988). The technique has been refined over the years, but the basis remains the same. It consists in the retrieval of oocytes from living donors using ultrasound guided transvaginal aspiration of the contents of ovarian follicles (Bols 2005). It can be used in combination with gonadotrophin treatments to increase the size of smaller follicles.

Nowadays, it is commonly used in farm animals, including cattle and horses. However, the results of OPU are very variable depending on the donor, the moment and frequency of follicular aspiration with respect to the estrous cycle, the use of hormone stimulation, and the operator´s experience.

- ***In vitro* oocyte maturation (IVM)**

Immature follicles can be obtained by OPU, as explained above, or from slaughtered animals. In both cases, cumulus oocytes complexes (COCs) are aspirated from the follicles and incubated in maturation medium for a period of time that is species specific, e. g. ˜22h for cattle and ˜26-30h for horses. This time is necessary to acquire two types of maturation, nuclear and cytoplasmic. For the nuclear maturation, oocytes need to resume their meiotic division up to metaphase II (MII) and extrude the first polar body. The cytoplasmic maturation is more difficult to evaluate, but it involves accumulation of mRNA, proteins, substrates and nutrients (Watson 2007). To correctly achieve both types of maturation, the embryos are incubated in maturation medium, which is designed according to the specific requirements of the species, but it is based on a complex medium, such as tissue culture medium (TCM)-199, commonly supplemented with energy substrates (pyruvate), hormones (gonadotrophin and/or steroid hormones), growth factors (EGF, IGF, FGF), and complex mixtures such as fetal bovine serum (FBS) or serum replacement. Importantly, a tendency to reduce the use of FBS, and move towards the use of completely defined maturation medium is apparent in animal IVP, following the example of the practices in human.

- ***In vitro* fertilization (IVF)**

The first species in which the birth of offspring was reported after *in vitro* fertilization were rabbits (Chang 1959), mice (Whittingham 1968), rats (Toyoda and Chang 1974) and human (Steptoe and Edwards 1978), with the birth of Louise Brown in 1978. IVF consists in the co-incubation of the mature oocytes with capacitated sperm, in a medium suitable for fertilization, for a number of hours that allow sperm penetration. It can be performed with fresh or frozen semen, which has been purified previously. The most commonly used purification method is the separation of motile sperm from dead cells over a discontinuous density gradient. Recently, the use of sex-sorted semen, that allows the production of embryos of a desired sex, has become more popular in farm animals. The separation is based on the differences in DNA content between the X and the Y chromosome, and it is performed by flow cytometry. It was implemented in 1989, and it has an accuracy of 90% (Seidel 2009). However, it has been reported to reduce the fertilization rates (Maxwell *et al*. 2004).

- **Intracytoplasmic sperm injection (ICSI)**

Conventional ICSI was initially developed in Belgium for the treatment of male factor infertility in humans in 1992 (Palermo *et al*. 1992), and it is an alternative to *in vitro* fertilization when sperm fails to penetrate when it is co-incubated with the oocyte. This technique consists of the injection of a single spermatozoon into the cytoplasm of a mature oocyte by a sharp injection pipette. Soon after its development, several upgrades were implemented to facilitate the perforation of the zona pellucida (ZP). The most commonly used upgrade is the piezo drill. This is a device that creates minute vibrations of a blunt-ended injection pipette. The piezo drill was applied in mice in 1995, resulting in the first ICSI mice offspring (Kimura and Yanagimachi 1995). It is commonly used now in many species, including the horse (Choi *et al*. 2002) (Figure 1.4). Another alternative is the laser-assisted ICSI. By using a laser, a hole is made in the zona pellucida prior to sperm injection. This causes less oocyte disturbance than conventional ICSI, and, in human, has been shown to be beneficial for patients with fragile oolemma or with high rates of oocyte degeneration after conventional ICSI (Abdelmassih *et al*. 2002; Rienzi *et al*. 2004). ICSI has become the preferred method of *in vitro* fertilization in human and has been successfully used in many species, such as horse, mouse, cat (Pope *et al*. 1998), pig (Nakai *et al*. 2011), rhesus macaques (Wolf 2004), cattle and sheep (reviewed by Garcia-Rosello *et al*. 2009).

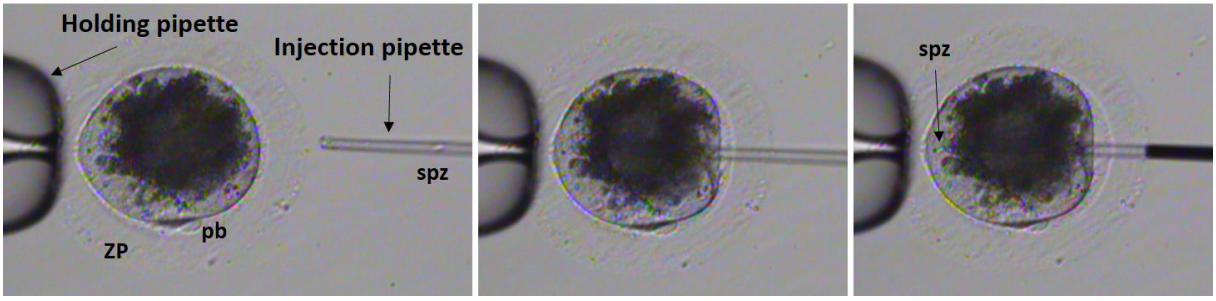

Figure 1.4. *Piezo drill assisted ICSI. The injection pipette captures a spermatozoon (spz). The mature oocyte is immobilized with the polar body (pb) at the 6 or 12 o'clock position by aspiration with a holding pipette at 9 o'clock. The injection pipette drills the zona pellucida (ZP) at 3 o'clock, penetrates the oolemma, and deposits the immobilized spermatozoon in the cytoplasm of the oocyte.*

- ***In vitro* embryo culture (IVC)**

The first successful culture of mammalian embryos *in vitro* was achieved in the fifties (Whitten 1956; Whitten 1957). IVC is intended to support embryo development until transfer. In farm animals, embryos are mostly transferred at the blastocyst stage. However, in humans, embryos can also be transferred earlier to the uterus, at the cleavage stages, to limit the time of exposure of the embryo to *in vitro* conditions. After fertilization, the remaining cumulus cells and sperm are removed from the presumptive zygotes, which are subsequently placed in culture medium for a variable time, depending on the species and the subsequent use of the embryos.

Three conditions are fundamental for IVC to be successful: 1) air and temperature, 2) composition of the culture medium, and 3) embryo/medium ratio. All these conditions are aimed to mimic as much as possible the *in vivo* situation.

Regarding air and temperature, an atmosphere with 5% O_2, 5% CO_2 is preferred for embryo culture (Smits *et al*. 2012; Li *et al*. 2014; Appeltant *et al*. 2015). It has been proven, that the reduction of the proportion of O_2 from 20% to 5%, which is more similar to the oxygen tension in the oviduct, reduces the formation of reactive oxygen species (ROS) (Guerin *et al*. 2001). In addition, most culture media are buffered to reach their optimum pH when the volume of CO_2 is 5%, whereas the pH rises rapidly at the 0.04% CO_2 in air. A 100% humidity is also necessary to avoid evaporation of the drops of culture media. The temperature of the incubator is also crucial for the correct embryo development, an increase of 1 degree above the optimal temperature can have a detrimental effect on the developmental competence (Hansen 2007). This optimal temperature is species specific, with 38.5˚C being the optimal temperature for bovine embryos and 38.2˚C for equine embryos.

The composition of the culture medium has also a major influence on embryo development. Since the development of IVP, many different medium formulations have been tried in order to best support embryo development and produce embryos that resemble their *in vivo* counterparts.

Historically, surrogate sheep oviducts were commonly applied for embryo culture of several species, as such improving embryo quality to near *in vivo* standards (reviewed by

Lazzari *et al*. 2010). However, in recent years it was abandoned for ethical and sanitary reasons. Subsequently, the co-culture of embryos with oviduct, cumulus and granulosa cells and BRL and Vero cell lines, as source of paracrine factors became popular (reviewed by Gordon 2003 and Wydooghe *et al*. 2015). Co-culture was later substituted by cell-free culture systems. Many different culture media are currently used in different species, such as KSOM, SOF, DMEM-F12, CR1 and Whitten, and many different media supplementations are used to support embryo development. Traditionally, the most commonly used supplement was fetal bovine serum (FBS). However, its use has declined in ruminants because it can induce large offspring syndrome (LOS). In horse, FBS is still frequently used since no cases of LOS have occurred until now. Human embryo culture has moved to completely defined culture conditions already for a long time, and this evolution has promoted the change towards semi-defined culture conditions based on bovine serum albumin (BSA) or fatty acid-free BSA supplementation, in many species.

Embryo culture in group has been shown to improve developmental rates and embryo quality in many mammalian species, including mice, cattle, pigs, cats and humans (reviewed by Wydooghe *et al*. 2015). It is believed that it is due to the embryotrophic autocrine factors that are secreted by the embryos, and can act on the embryo itself and on its neighbors. This effect was observed for the first time when an increase in the embryo density of murine and bovine embryos led to an increased blastocyst development (Paria and Dey 1990; Odoherty *et al*. 1997). An embryo density of 1 embryo per 2 µL of media was found to be optimal in these two species. Unfortunately, besides in laboratory experiments, it is not common to reach the optimal number of embryos for culture. Fortunately, the development of the well-of-the-well (WOW) system allows to culture individual embryos in very reduced amounts of culture medium, preventing the dilution of the autocrine factors (Vajta *et al*. 2000). This method has proven to improve embryo development in bovine, mouse, porcine, buffalo and human embryos (reviewed by Wydooghe *et al*. 2015).

- **Cloning by somatic cell nuclear transfer (SCNT)**

The first time that a mammal was generated by nuclear transfer was in 1984, with the birth of three sheep (Willadsen 1986). However, the nuclear donors were early embryonic cells. The first time that a mammal was originated by SCNT was in 1996, producing the famous

sheep, Dolly. To this end, the nucleus of a mammary gland cell from an adult sheep was transferred to an enucleated oocyte (Wilmut *et al*. 1997). Nowadays, nuclear transfer in general, and SCNT in particular, has become routine practice in many laboratories worldwide for lab and farm animals. However, it is a complicated technique that requires dedication to master it. The protocols used today are still based on the one developed by Willadsen. First, mature oocytes need to be enucleated, then, the donor cell must be fused or injected in the enucleated oocyte. Finally, the reconstructed embryo needs to be activated, developed *in vitro*, and transferred into a surrogate mother (Galli *et al*. 2014). Many cell types have been used for SCNT with different degrees of success, and there is not a cell type that consistently performs better than others (Colleoni *et al*. 2005).

1.1.2.4 ART in cattle

The use of ART in cattle is mainly motivated by the need of generating large numbers of embryos, preferably of predetermined sex and known genotype (with focus on milk or meat production). It has contributed to the improvement of the genetics of the animals, maximizing the use of highly valuable bulls by AI, and of highly valuable cows by the use of MOET. Bovine embryos are also used in research, to improve ART in cattle, but also as a model for human, to test the feasibility and safety of new techniques and culture media.

Embryo transfer in cattle began to grow in the late seventies following the introduction of non-surgical embryo recovery techniques. In the early years, embryos were transferred into recipients via flank surgical approach. However, by the mid-eighties, most of the embryos were transferred by non-surgical trans-cervical approach (Hasler 2006).

The birth of the first *in vitro*-produced calf was reported in 1981 (Brackett *et al*. 1982). It was born after *in vivo* maturation followed by *in vitro* fertilization. It was not until 1990 that the first completely *in vitro* produced calves were born (Fukuda *et al*. 1990). OPU was introduced for cattle in the Netherlands at the end of the eighties, by the adaptation of an existing technique in human assisted reproduction (Pieterse *et al*. 1988). It was first applied to cows that did not respond to superovulation treatments, but later it was applied on a wider scale, including pregnant cows and heifers, including prepubertal heifers (reviewed by Galli *et al*. 2014).

In the early years, most of the embryos transferred were from *in vivo* origin. However, the use of IVP in cattle has increased more than 10 times in the last 15 years. Considering the data provided by IETS, in 2014, 44% of all the embryos transferred worldwide were produced *in vitro* and most of them were originated by OPU combined with IVP (Table 1.1). Still, these tendencies largely vary depending on the continent: Asia is an important producer of IVP embryos mainly originating from slaughterhouse donors. On the contrary, in Europe, the large majority of embryos transferred in 2014 were derived *in vivo* (Table 1.2). The extension of the use of AI in cattle, also largely varies depending on the continent. In 2005, in Europe, approximately 60% of the cows were fertilized by AI, while it was only used in 2% of the cows in Africa, and 1% in South America (Thibier 2005).

The MOET programs in cattle have been used commercially for more than 30 years, with an average of 5 embryos per cow per flush (van Wagtendonk-de Leeuw 2006). Using OPU, between 5 and 10 oocytes per donor cow (*Bos taurus*) can be collected per session, a number that can be increased by the use of hormonal stimulation (Bols 2005). Without the use of hormone stimulation, an average of two OPU sessions per week can be performed.

As mentioned above, the use of IVP for commercial purposes has greatly increased in the last years. Additionally, IVP is the main technique used for research purposes. Approximately 90% of the immature oocytes undergo nuclear maturation and about 80% of them will be properly fertilized (Lonergan *et al*. 2003a). Despite this fact, the rate of blastocyst development from an immature oocyte is limited to 30-40%. Therefore, many efforts have been made in order to improve the number and quality of the embryos obtained. Historically, bovine embryos were co-cultured with cumulus, oviduct or uterine cells, or BRL and Vero cell lines (Massip *et al*. 1996; Gordon 2003). With some exceptions (Goovaerts *et al*. 2009), the co-culture was replaced by cell-free culture systems. Several culture media were tested for bovine embryo culture throughout the years, with synthetic oviduct fluid (SOF) being the one most commonly used in Europe. The supplements added to this medium to support embryo development vary depending on the laboratory. Traditionally, the supplementation with FBS for the culture of bovine embryos was the most popular, because it is increasing blastocyst rates, and because it is producing more consistent results in the laboratory (Gordon 2003). However, the increased risk of LOS (Farin *et al*. 2010) has stimulated the transition to semi-defined culture media, based on the addition of BSA as protein source, for bovine embryo

culture. The combination of BSA with insulin, transferrin and selenium (ITS) is frequently used, leading to excellent blastocyst rates (George *et al*. 2008; Wydooghe *et al*. 2014) and improving embryo quality compared to BSA alone (Wydooghe *et al*. 2014). The use of BSA-ITS supplementation reduced the lipid content of the embryos, while increasing their freezability (George *et al*. 2008). However, FBS is still used in some laboratories for research, but not when embryos are being used for embryo transfer.

Table 1.1. *Number of bovine embryos produced worldwide in 2014 (Source: IETS, 2015).*

		***In vivo* (n) (Flushing)**	***In vitro* (n) (OPU)**	***In vitro* (n) (slaughtered donor)**
Collections		94,666	129,098	1,462
Collected oocytes		-	1,808,878	39,843
Transferable embryos		614,464	590,359	2,091
Transferred embryos	**Fresh**	201,960	296,666	863
	Frozen	262,622	68,061	35

Table 1.2. *Number of bovine embryos produced in Europe in 2014 (Source: AETE, 2015).*

		***In vivo* (n) (Flushing)**	***In vitro* (n) (OPU)**	***In vitro* (n) (slaughtered donor)**
Collections		22,490	9,710	1,335
Collected oocytes		-	83,785	37,414
Produced embryos		200,939	-	-
Transferable embryos		138,418	15,693	1,369
Transferred embryos	**Fresh**	59,546	11,430	
	Frozen	63,834	2,992	

1.1.2.5 ART in the horse

Artificial insemination is routinely used to increase the offspring of genetically valuable stallions. Additionally, embryo flushing from highly valuable mares combined with transfer to recipient mares is a common procedure to increase offspring from genetically valuable mares (Table 1.3 and 1.4). Instead, the use of IVP in the horse is mainly motivated to overcome fertility problems of mares and stallions of high economic value. Research in horse is limited by the low amount of oocytes that can be obtained from slaughtered mares.

In 1990, the birth of a foal produced after *in vitro* fertilization of an *in vivo* matured oocyte was reported (Palmer *et al*. 1991). In that case the sperm was treated with Ionophore A23187. Despite the fact that one more foal was born using this technique (Bezard *et al*. 1992), and that multiple attempts were tried to produce foals using different sperm treatments, no protocol producing reproducible results for equine *in vitro* fertilization is established yet (Choi *et al*. 1994; Dell'Aquila *et al*. 1997a; Dell'Aquila *et al*. 1997b; Alm *et al*. 2001; Hinrichs *et al*. 2002). In addition, OPU combined with ICSI is gaining more acceptance in horse reproduction, with a growing number of groups performing it worldwide. The first pregnancy derived from an *in vitro* matured oocyte fertilized by ICSI was reported in 1996 (Squires *et al*. 1996). Since then, the results obtained with ICSI were inconsistent due to a heterogeneous and thick zona difficult to penetrate by conventional pipettes, especially of *in vitro* matured oocytes. The introduction of the piezo drill eliminated the inconsistency of the technique (Galli *et al*. 2014). To date, there are many laboratories that have reported the birth of foals from *in vivo* and *in vitro* matured equine oocytes (reviewed by Galli *et al*. 2014). Many different culture conditions have been tested for ICSI fertilized oocytes. Several defined media have been tested, including G1.2 (Choi *et al*. 2002), Dulbecco´s Modified Eagle Medium/Nutrient mixture F-12 (DMEM/F12) and CZB (Choi *et al*. 2004), and modified synthetic oviduct fluid (Ritchie 2006). Furthermore, co-culture with Vero cells (Dell'Aquila *et al*. 1997b), oviduct epithelial cells (Battut *et al*. 1991), cumulus cells (Li *et al*. 2001), granulosa cells (Rosati *et al*. 2002), or culture in conditioned media (Choi *et al*. 2001) were also used. However, in most of these systems, only between 4 and 16% of blastocyst rates were achieved. Interestingly, when the early cleaved ICSI produced embryos were transferred to a mare´s oviduct or temporarily to the oviduct of a surrogate sheep, the blastocyst development increased to 36% (reviewed by Galli *et al*. 2014). Additionally, when the ICSI produced zygotes were cultured in a medium based

on DMEM/F12 combined with a mixed gas atmosphere, between 27 and 38% blastocysts rate was reached. Still, the cell number of the *in vitro* produced blastocysts was lower than that of their *in vivo* counterparts (reviewed by Galli *et al*. 2014).

The birth of the first mules cloned by somatic cell nuclear transfer (SCNT) was reported in 2003 (Woods *et al*. 2003). The same year, a foal was born after transfer of an adult somatic cell into an *in vitro*-matured oocyte (Galli *et al*. 2003). Since then, the use of this technique for cloning horses of high sporting value have become more popular. However, there are no scientific reports on the results of commercial laboratories.

Table 1.3. *Number of equine embryos produced and transferred in Europe in 2014. In vivo data from France, Portugal, Poland and Switzerland, and in vitro data from Italy (Source: AETE, 2015).*

		***In vivo* (n) (Flushing)**	***In vitro* (n) (OPU-ICSI)**
Collections		654	195
Collected oocytes		-	2,050
Produced embryos		380	141
Transferred embryos	**Fresh**	376	8
	Frozen	2	75

Table 1.4. *Number of equine embryos produced and transferred in worldwide in 2014. In vivo data from Argentina, Canada, France, Mexico, Poland, Portugal, South Africa, Switzerland and United States. In vitro data from Italy (Source: IETS, 2015).*

		***In vivo* (n) (Flushing)**	***In vitro* (n) (OPU-ICSI)**
Collections		2,222	195
Produced embryos		1,575	141
Transferred embryos	**Fresh**	1,559	8
	Frozen	8	75

1.2 EVALUATION OF EMBRYO QUALITY

Embryo quality determination is essential to produce healthy offspring. Many techniques can be used to evaluate embryo quality, and each of them provides different valuable information. These techniques can be invasive or non-invasive. Invasive techniques mostly imply the death of the embryo, and the quality assessments made can only be extrapolated to other embryos produced under the same conditions. These techniques are mostly used in research, to test the safety and performance of ARTs, especially of new media formulations. Non-invasive methods (morphological evaluation) are always used *in vitro* and prior to embryo transfer. Traditionally, embryo quality determination was performed exclusively by morphological evaluation combined with determination of cell number and apoptotic cell ratio. Despite the valuable information provided by these traditional methods, they are blind to alterations in the transcriptome or epigenome of the embryos. As such, embryos evaluated as being of good quality were transferred and resulted in offspring with LOS. Therefore, the evaluation of the effects of ARTs on the transcriptome and the epigenome of the embryos is highly recommended before using the tested methods to produce embryos for transfer. However, embryo transfer and evaluation of the offspring´s health are the ultimate confirmation of the safety of new IVP procedures/media.

1.2.1 Morphological evaluation

This is a non-invasive method of embryo quality evaluation that allows subsequent embryo transfer. It is performed by using a stereomicroscope. The parameters to evaluate embryo quality by visual evaluation are timing of the development, presence of extruded blastomeres, color and cytoplasmic granulation, and intactness of the ZP. The Manual of the International Embryo Transfer Society contains a guide for bovine embryo quality evaluation (Stringfellow and Givens 2010). Embryos are divided in codes: 1) code 1, embryos considered excellent or good, with at least 85% of the cellular material intact, 2) code 2 rated as fair, with at least 50% of the cellular material intact, 3) code 3, rated as poor, with at least 25% of the cellular material intact, and 4) code 4 for the dead or degenerated embryos (Figure 1.5). Only embryos assigned to codes 1 and 2 are selected to be transferred.

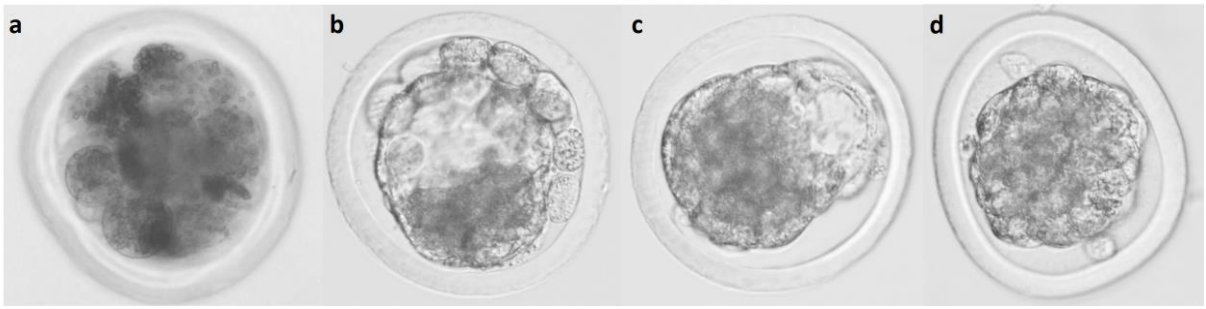

***Figure 1.5**. In vivo bovine embryos obtained by uterine flushing 6 days after insemination. Quality evaluation according to the IETS manual, (a) code 4 embryo (degenerated) arrested in 16 cells; (b) code 2 embryo (fair) presenting some extruded blastomeres; (c) and (d) code 1 embryos (excellent).*

A big advantage of IVP is that timing of development can be followed closely during the complete embryo culture period. However, the use of group culture can obstruct this task, because of the difficulty of identifying single embryos in a group. This problem has been overcome by the development of the WOW system (Vajta *et al*. 2008), in which embryos are cultured in group but each of them in a small well, which allows individual follow up. This system can be combined with the time-lapse embryo monitoring system (Primo vision), which takes images of the embryo at regular intervals within the incubator, avoiding their disturbance (Catteeuw *et al*. 2015).

1.2.2 Apoptotic cell rate and cell number

The evaluation of apoptosis ratio and embryo cell number belongs to the invasive methods that lead to the destruction of the embryo.

Cell number assessment is normally performed in blastocysts, where visual evaluation is not enough to determine the cell number. It is assumed that embryos of good quality will show a cell number similar to that of *in vivo* derived embryos of the same developmental stage. To count the cell number, DNA stains are regularly used to visualize the nuclei of the cells that then can be counted. Many DNA stains exist and can be used to this end. However, the traditionally most commonly used DNA dyes for cell number assessment are Hoechst (Figure 1.6) and DAPI, which stain the nuclei blue. Both of these dyes are cell-membrane permeable, so the embryo does not need to be permeabilized prior to incubation with the

DNA stains, simplifying the procedure. Another commonly used DNA dye is propidium Iodide (PI), which stains the nuclei red (Figure 1.6). This dye is cell-membrane impermeable, therefore the embryo needs to be permeabilized for cell counting. However, due to this condition, it can be used in combination with Hoechst or DAPI for determination of membrane integrity and for differentiation between ICM and TE. For the determination of the integrity of the membranes, the embryo is not previously permeabilized (Figure 1.6). Therefore, the cells that will be colored in pink are the ones with membrane damage, since PI was able to enter. On the other hand, blue cells be membrane intact.

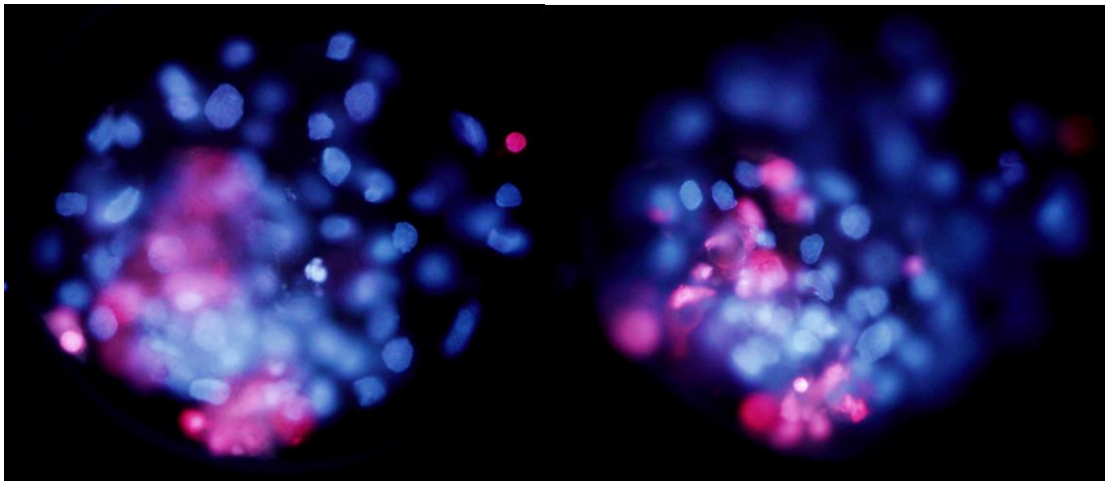

Figure 1.6. *Double staining with Hoechst and propidium iodide (PI) for membrane integrity determination. The cells colored pink have the plasma membrane damaged, because PI and Hoechst were able to enter them, the cells colored blue have the membrane intact and only Hoechst was able to enter them.*

For the differentiation between ICM and TE, a first short permeabilization step is necessary prior to incubation with both DNA dyes. With this short permeabilization step, the external cells (TE) will be permeabilized, so PI will be able to enter them, while the internal cells (ICM) will be not permeabilized, thus Hoechst will be the only dye to penetrate. In this way, the cells from the TE will be pink, while the cells from the ICM will be blue (George *et al*. 2008). A more reliable technique to differentiate between ICM and TE is based on the immunostaining of CDX2, a transcription factor that is only expressed in the TE cells, combined

with a nuclear dye (Figure 1.7a,b) (Wydooghe *et al*. 2011). The assessment of the ICM/TE ratio constitutes also an important embryo quality parameter, by comparing to embryos derived *in vivo* from the same stage.

Apoptosis is a natural process that takes place in all cell types. Its function during embryo development is to eliminate cells that are abnormal, misplaced, not functional or potentially dangerous. However, a high increase in the apoptotic cell ratio can lead to embryo death (Levy *et al*. 2001; Fabian *et al*. 2005). Still, no threshold of apoptotic rate leading to embryo death has been established yet.

The most commonly used methods to assess apoptotic ratios are based on immunostaining. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Fabian *et al*. 2005) is based on the presence of nicks in the DNA that can be identified by the terminal deoxynucleotidyl transferase. This enzyme catalyzes the addition of dUTPs that are secondary labelled with a marker. An alternative is the use of the active caspase-3 immunostaining (Wydooghe *et al*. 2011) (Figure 1.7c), which is an executioner caspase that plays a central role in all apoptotic pathways (Earnshaw *et al*. 1999).

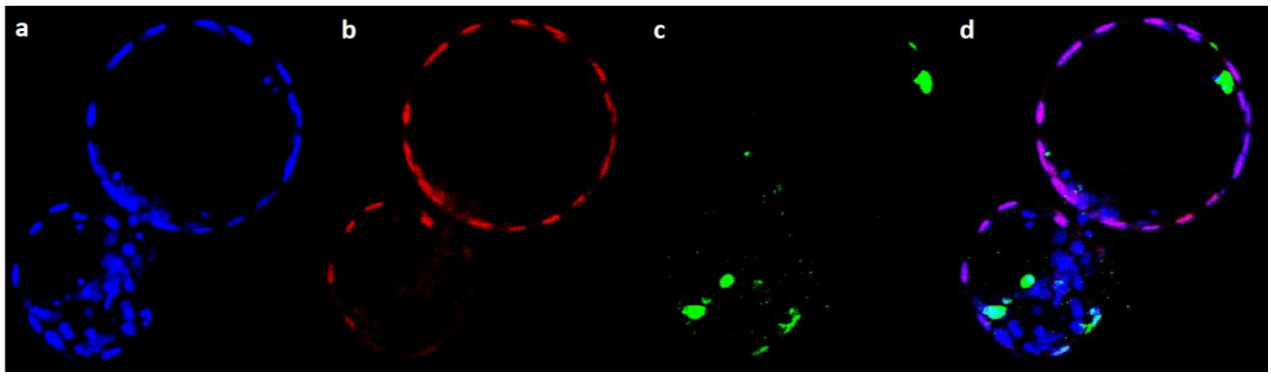

Figure 1.7. *Simultaneous assessment of total cell number, TE cells and apoptotic cells in a hatching bovine blastocyst produced in vitro. (a) total cell number, by Hoechst (b) TE cells by immunostaining of CDX-2 indirectly labelled with Texas red (c) apoptotic cells by immunostaining of active caspase-3 indirectly labelled with FITC (d) overlay of the three images. By the combination of (a) and (b) the ratio between ICM and TE cells can be established. Embryo kindly provided by drs Lynn Vandenberghe-RBU Ugent.*

1.2.3 Evaluation of gene expression patterns

Changes in mRNA transcription are amongst the first cell responses to developmental or environmental stimuli. Therefore, the analysis of the mRNA expression in oocytes and embryos allows to study their response to different ART. Even though the preimplantation embryo is known to be very plastic, the adaptation to certain challenging environmental conditions can result in aberrant embryonic development, eventually persisting in the adult life (Duranthon *et al*. 2008). Therefore, the study of changes of the mRNA expression pattern of embryos exposed to different conditions compared to *in vivo* derived embryos, gives an indication of how challenging these tested conditions are, and can help to improve the safety and performance of ART.

The sensitivity of transcriptomic techniques has greatly improved during the last years, and nowadays some of these techniques can even be used in single cells, and therefore also oocytes and embryos (Chitwood *et al*. 2013; Jiang *et al*. 2014). In the next pages, the most commonly used techniques in oocytes and preimplantation embryos will be described briefly.

- **Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

RT-qPCR is a modification of the conventional PCR technique developed by Mullis in 1983 that detects the targets during the exponential phase of amplification (Bustin 2000). It is based on the use of fluorescent reporters such as the SYBR Green dye or TaqMan probes. During the course of the reaction, fluorescence is generated, and it can be reliably quantified in the exponential phase of the amplification reaction (Bustin 2000).

This technique has been successfully applied to analyze gene expression in single oocytes, preimplantation embryos and even single blastomeres (Hartshorn *et al*. 2003; Lindeberg *et al*. 2004).

The principal advantage of RT-qPCR is that it has a high sensitivity, thus it can be used in low amounts of RNA input, which is the situation in oocytes and embryos. It also has a high reproducibility, and accurate quantitative comparisons can be performed. On the other hand, its limitations are mainly caused by the preceding RNA isolation method, by differences in reverse transcription (RT) and PCR efficiencies, and by the difficulty of proper normalization

and interpretation of the data. To overcome these issues, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were formulated (Bustin *et al*. 2009). In these guidelines, SOP design and data normalization are proposed to promote consistency between laboratories, helping to ensure the integrity of the scientific literature, and to increase experimental transparency. Another disadvantage of RT-qPCR is the high cost and labor intensity of the evaluation of multiple genes, which is partially overcome by digital PCR. Therefore, it is mainly used for the study of specific genes of interest, and, due to its accurate quantification, for validating of the results obtained by other techniques, such as microarray or RNA-sequencing.

- **DNA Microarray**

Microarray hybridization is a technique that allows the analysis of the expression of a large number of genes simultaneously in a single action. It is based on the hybridization of a mRNA molecule to the DNA template adsorbed to a solid support. This DNA template can be oligonucleotide probes or cDNA clones, and thousands of them can be included in one array.

For the analysis of differential gene expression, the test samples are chemically tagged with different fluorochromes (usually Cy3 and Cy5) or chemical conjugates and then hybridized to the array (Figure 1.8). The intensity of the signal of each spot is due to retention of complementary labelled nucleic acid. The hybridization signals are quantitatively recorded and the amount of complementary nucleic acid at each point is calculated with respect to local background. This technique can be used to determine the sequence or to detect variations in a gene sequence, also to evaluate gene expression of one or two different samples simultaneously, and for gene mapping.

The disadvantages of the microarray are possible cross-hybridization artifacts, in which the cDNA of a gene will bind to the probe of another closely related gene, poor quantification of lowly and highly expressed genes, and that only the genes contained in the chip will be analyzed, leaving out possible important genes. Importantly, a reference genome and transcriptome needs to be available before the microarray itself can be designed. Another main issue of microarray data analysis is how to normalize the dataset in order to remove systematic variation (Quackenbush 2002). Certain standards described as minimum

information required for microarray experiments are essential to guarantee consistent and reproducible databases (MIAME) (Brazma *et al*. 2001).

This technique has been successfully applied to study gene expression patterns in embryos from different species (Sirard *et al*. 2003; Hamatani *et al*. 2006; Bermejo-Alvarez *et al*. 2010b; Orozco-Lucero *et al*. 2014; Dalto *et al*. 2015; Dufort *et al*. 2015).

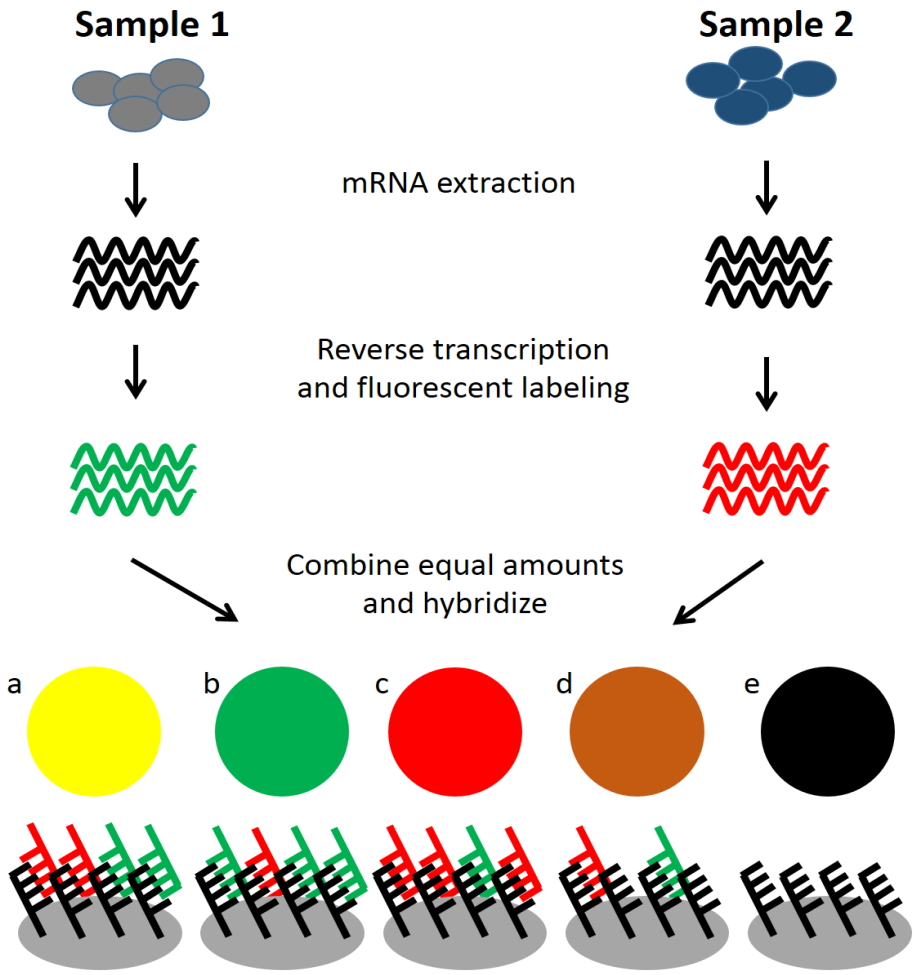

Figure 1.8. *Schematic representation of the use of a DNA microarray to detect differential gene expression between two samples. Sample 1 is labelled in green and sample 2 in red during reverse transcription. Each dot constitutes a gene of interest. (a) In both samples this gene is highly expressed, (b) this gene is mostly expressed in sample 1, (c) this gene is mostly expressed in sample 2, (d) this gene is equally low expressed in both samples, and (e) this gene is not expressed in either of the samples.*
Adapted from: http://www.ncbi.nlm.nih.gov/prove/docs/techmicroarray.

- **RNA sequencing (RNA-seq)**

RNA-seq uses next-generation sequencing (NGS), also known as high-throughput sequencing, to analyze the whole transcriptome of a given sample in one run. NGS includes several sequencing technologies, such as Illumina sequencing, Roche 454 sequencing, Ion torrent (proton/PMG) sequencing and SOLiD sequencing. For this process, RNA is extracted and either directly fragmented and then converted to cDNA or first converted to cDNA and then fragmented. RNA-seq focused on mRNA, small RNA, noncoding RNA or micro RNA can be achieved by including additional isolation or enrichment steps before fragmentation or cDNA synthesis. The length of the fragments depends on the particular sequencing machinery used. Subsequently, adaptors are ligated to one or both ends of this cDNA fragments, constituting the cDNA library. Each molecule is then sequenced in a high-throughput manner to obtain short sequences that can be originated from one end (single-end sequencing) or both ends (pair-end sequencing). The resulting reads are then aligned to a reference genome (Figure 1.9), to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene.

The main advantages of this technique are that it allows for identification and quantification of common and rare transcripts, it can be used in the study of SNPs and alternative splice variants, as well as detection of isoforms, novel transcripts and gene fusions. It can also be used in single cells. The drawbacks are the bias introduced by RNA/cDNA fragmentation for originating the libraries, the issues of handling non-unique and duplicated reads, and the bioinformatics challenges that encounters to handle and correctly analyze such a large amount of information (Wang *et al*. 2009).

In recent years, RNA-seq has been successfully applied to the study of the changes in gene expression during embryo development (Huang *et al*. 2010; Graf *et al*. 2014) and to study the effect of ART on gene expression (Chitwood *et al*. 2013)

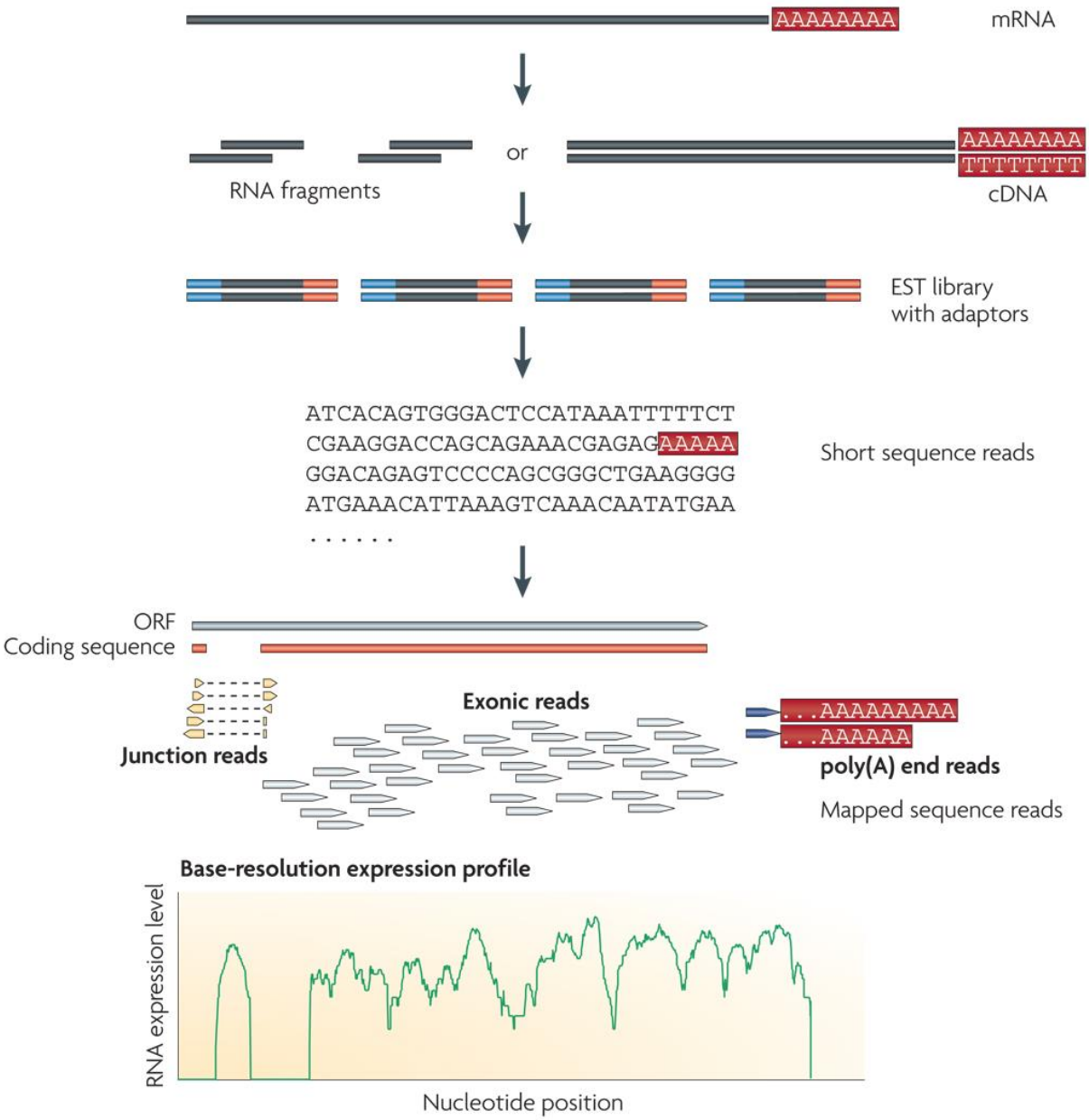

Figure 1.9. *Schematic representation of a typical RNA-seq experiment. mRNA are converted into a library of cDNA fragments through either RNA or DNA fragmentation. Subsequently, adaptors (blue and red) are added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned to the reference genome and used to generate a base-resolution expression profile for each gene. From Wang et al. 2009.*

1.2.4 Evaluation of epigenetic marks

Epigenetic marks are the changes in chromatin structure that occur independently of changes in the underlying DNA base sequence. They are the ultimate responsible of gene expression regulation and cell differentiation, since all cells within an individual contain the same DNA sequence. The pattern of the different epigenetic marks, or epigenome, changes to adapt to modifications in the environment. These changes in the epigenome can be transitory or can become permanent, and may lead to alterations in the individual, such as particular syndromes or cancer. Preimplantation embryo development is a critical stage for the epigenome, since, as it will be explained later, a global epigenetic reprogramming takes place. Therefore, changes in the epigenome induced by ARTs during this critical period can have consequences in the offspring because epigenetic marks can be inherited mitotically and meiotically. In 1953, the theory of "non-genetic transmission" was developed by Waddington based on the observation of genetic assimilation of phenotypic characteristics in the offspring of Drosophila exposed to heat. However, by that time, there was no insight in the underlying molecular mechanisms. This phenomenon, when phenotypic alterations are caused by transfer of chromosome/chromatin modifications other than DNA base sequence modifications through the gametes, is called transgenerational epigenetic inheritance (van Montfoort *et al*. 2012).

There are many different epigenetic marks, cytosine modifications, post translational histone tail modifications and RNA-mediated chromatin modifications, and it is shown that an interplay exists between them (van Montfoort *et al*. 2012) (Figure 1.10). However, the most studied is DNA methylation, which is involved in genomic imprinting, X chromosome inactivation, genome stability, silencing of retrotransposons and inactivation of genes in cancer (Dean *et al*. 2001).

Before describing the techniques that can be used to evaluate the effects of ARTs on the epigenome of the embryo, and therefore the quality of the embryos produced under these conditions, some important concepts will be briefly introduced.

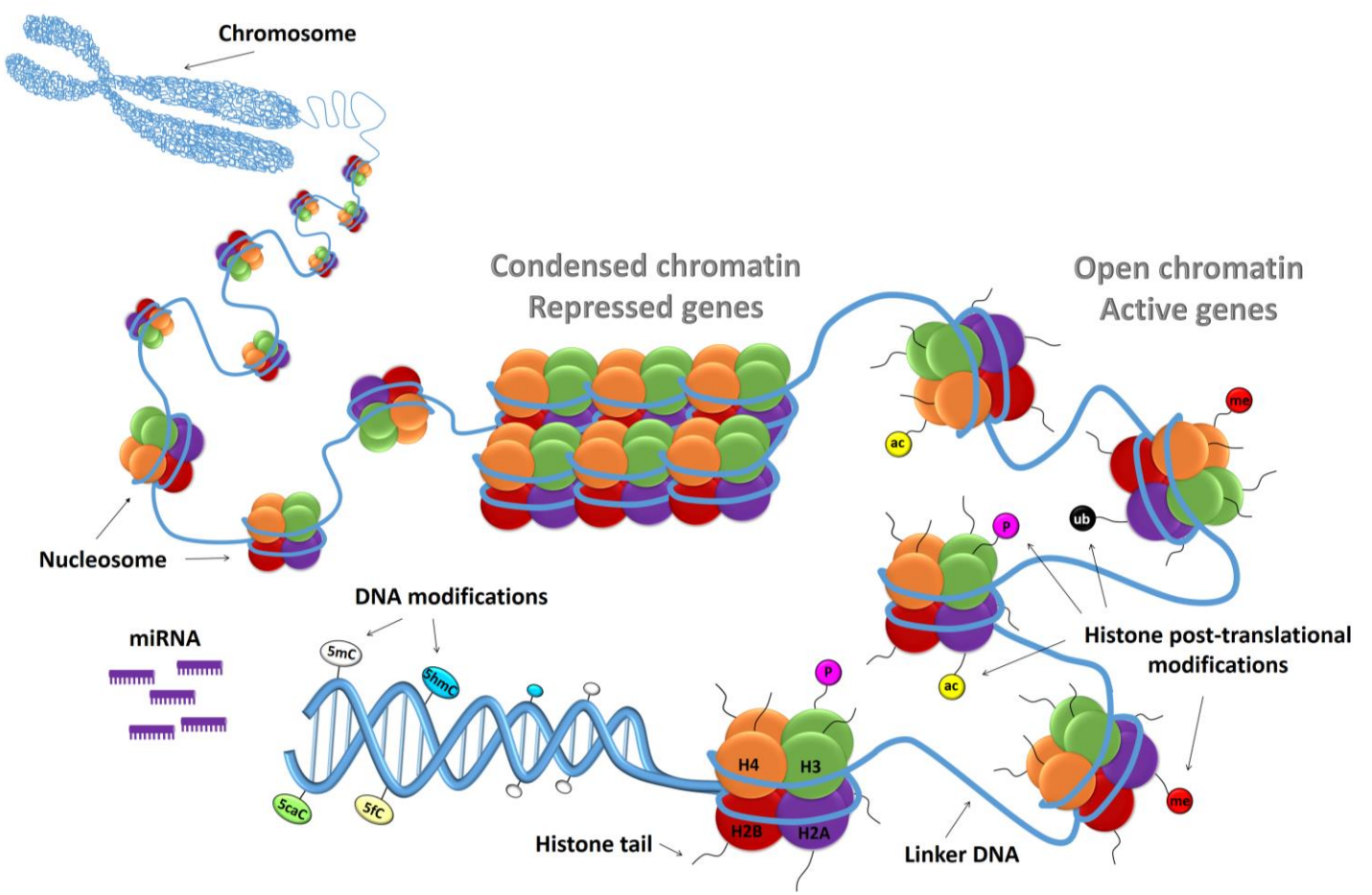

Figure 1.10. *Schematic representation of the different epigenetic modifications and their localization. The histone tails can be post translationally methylated, acetylated, phosphorylated, ubiquitinated, crotonylated and sumoylated. On the DNA itself, the cytosines are frequently methylated on C5, but also hydroxymethylated, formylated and carboxylated in lesser extent. Finally, non-codding RNAs pay an epigenetic role. The combination of the different epigenetic marks determines the conformation of the chromatin, when it is condensed the genes are repressed, and when it is open the genes are active.*

1.2.4.1 The epigenetic players

DNA methylation (5mC) represents the best studied epigenetic modification. It is a covalent modification consisting in the transfer of a methyl group to cytosine residues of mainly CpG dinucleotides (Shi and Wu 2009). The position of this epigenetic modification in the gene determines its expression or silencing. Methylation at promotor regions or CpG island shores (regions of lower CpG density that at ˜2kb of CpG islands) is associated with transcriptional inactivation. On the contrary, methylation in the gene body is linked with transcriptional activity, and it is intended to prevent aberrant transcriptional initiation inside the gene and therefore to help in avoiding the production of truncated mRNAs (reviewed by Portela and Esteller 2010 and Jurkowska *et al*. 2011) (Figure 1.11). Highly methylated CpG sites are also found in repetitive elements, where they are needed to protect chromosome integrity by preventing reactivations of endoparasitic sequences (reviewed by Portela and Esteller 2010). DNA methyltransferase enzymes (Dnmts) are responsible for the establishment and maintenance of DNA methylation. Dnmts transfer methyl groups from s-adenosyl-L-methionine onto the C5 positions of the cytosine primarily of CG dinucleotides, and only occasionally at non-CG sites (Young and Beaujean 2004; Jurkowska *et al*. 2011). The Dnmt3 family (Dnmt3A and Dnmt3B), the so called de novo methyl transferases, is responsible for the establishment of the DNA methylation. On the other hand, Dnmt1 is responsible for maintenance of DNA methylation, by the methylation of hemi-methylated DNA resulting from DNA replication (Jurkowska *et al*. 2011). However, an active role of Dnmt1 in de novo methylation has also been documented (Athanasiadou *et al*. 2010). Finally, Dnmt3L lacks catalytic activity but it is essential for the establishment of genomic imprints in the oocytes, and for the silencing of dispersed repeated sequences in the male germ cells (Jurkowska *et al*. 2011).

In 2009 another modified form of cytosine was discovered: 5-hydroxymethylcytosine (5hmC). At the same time, the family of enzymes responsible for oxidation of 5mC into 5hmC, the ten-eleven translocation family (TETs) was discovered (Kriaucionis and Heintz 2009; Tahiliani *et al*. 2009). Three members compose the TET family: TET1, TET2 and TET3. All three TETs can further oxidize 5hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani *et al*. 2009). Initially, 5hmC, 5fC and 5caC, were believed to be mere intermediaries for DNA demethylation. However, now evidence is available that they play a specific role in

the epigenetic landscape, being involved in chromatin and transcription regulation (Salvaing *et al*. 2012; Yu *et al*. 2012; Iurlaro *et al*. 2013; Li and O'Neill 2013; Delatte *et al*. 2014).

Besides cytosine modifications, there are many other less studied epigenetic marks. Histone modifications constitute a miscellaneous group of epigenetic marks involved in gene expression regulation, DNA repair, DNA replication, alternative splicing and chromosome condensation (reviewed by Portela and Esteller 2010). The N- and C-terminal histone tails protrude from the nucleosome core and can interact with adjacent nucleosomes and linker DNA. All histones can be post-translationally modified, even at different sites simultaneously. The most common are covalent modifications of residues of histone tails including methylation, acetylation, phosphorylation, ubiquitination, crotonylation and sumoylation. These modifications can regulate chromatin structure directly, and frequently act as binding sites for the recruitment of other non-histone proteins to chromatin. Some histone modifications are associated with an active chromatin state and others with a repressive state (Figure 1.11). However, a single histone mark does not determine the outcome alone, it is the combination of all marks in a nucleosome or region that specifies the outcome. Active genes typically carry high levels of lysine acetylation on the H3 and H4 tails, tri-methylation of H3 lysine 4 (H3K4me3), tri-methylation of H3 lysine 79 (H3K79me3), ubiquitination of H2B on lysine 120 (H2BK120ub1) and tri-methylation of H3 lysine 36 (H3K36me3). Marks associated with repressed genes include tri-methylation of H3 lysine 9 (H3K9me3), tri-methylation of lysine 27 (H3K27me3) and ubiquitination of H2A on lysine 119 (H2AK119ub1), (reviewed by Meehan *et al*. 2005; Quina *et al*. 2006; Zhang *et al*. 2015).

Histone modifications are recognized as an epigenetic code, since chromatin-mediated gene expression states can be heritable (Meehan *et al*. 2005). Interplay between DNA methylation and histone modifications exist whereby the acquisition of one may be dependent, or mutually exclusive with the other. In recent years, long non-coding RNAs (lncRNAs) and micro RNAs (miRNAs) have been proposed to constitute additional layers of epigenetic regulation. Many lncRNAs bind to chromatin-modifying proteins, and recruit them to specific sites in the genome, thereby impacting gene expression (Mercer and Mattick 2013; Rivera and Ross 2013). Several miRNAs, such as miR-29 and miR-26 families, can repress epigenetic regulatory enzymes (all TETs and TDG) (reviewed by Delatte *et al*. 2014). Additionally, sperm transfer RNA-derived small RNAs (tsRNAs) from mice feed with a high-fat-

diet have been shown to induce metabolic disorders in the offspring (Chen *et al*. 2016). Therefore, sperm tsRNAs constitute a paternal epigenetic factor that can mediate intergenerational inheritance of diet-induced metabolic diseases.

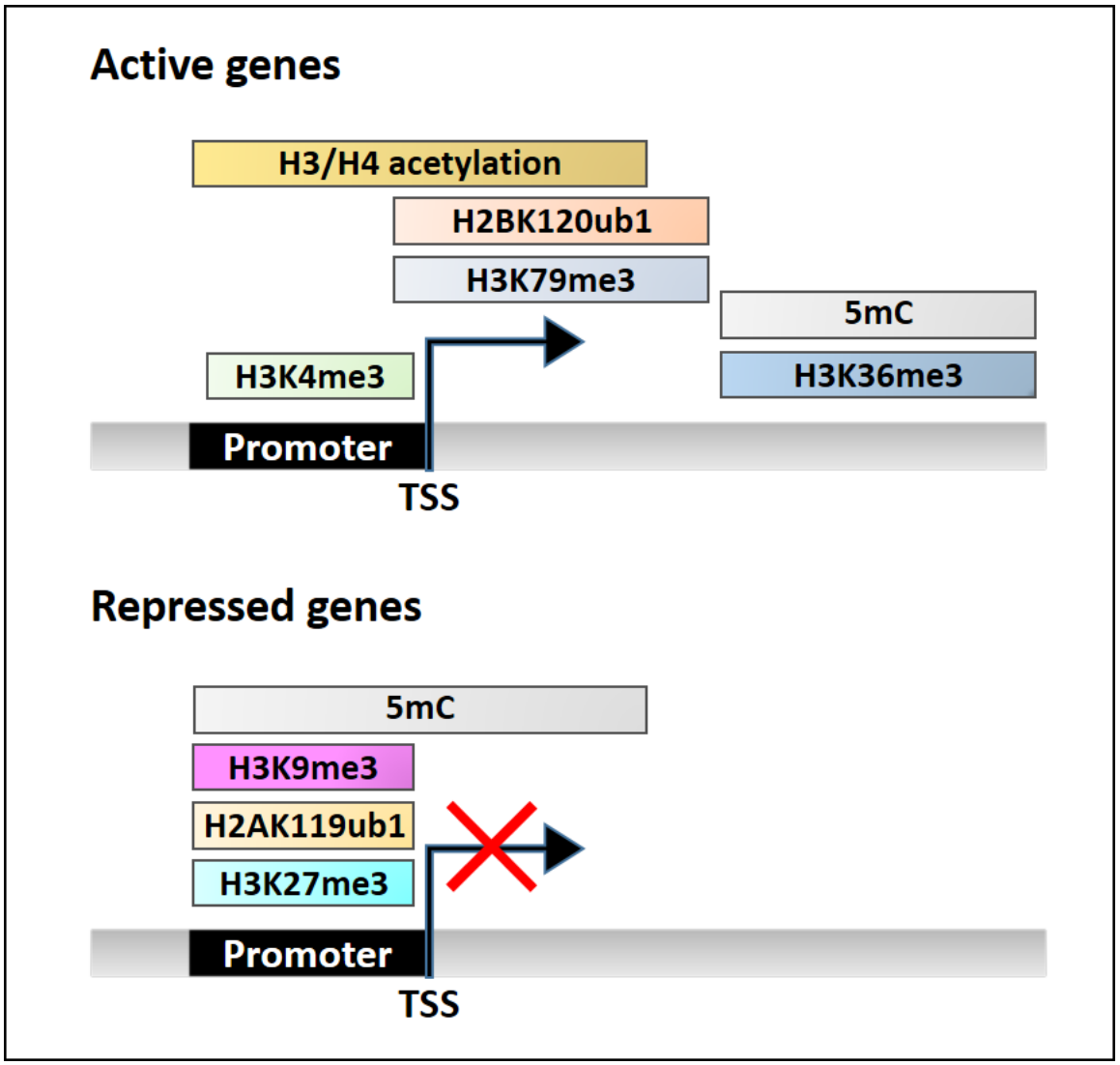

Figure 1.11. *The different epigenetic modifications and their position in the gene determine the transcriptional activity. Adapted from Zhang et al. 2015.*

1.2.4.2 Genomic imprinting

Genomic imprinting entails in parent-of-origin specific gene expression of a number of genomic loci. In this way, imprinted genes are transcribed from either the maternal or paternal allele while the other one is repressed.

Imprinted genes are usually arranged in clusters, with each cluster containing a local imprinting control region (ICR). Many factors are involved in the maintenance of this allele-specific expression, including microRNAs and insulator proteins. However, all these factors depend on differential DNA methylation at ICRs. This sex-specific DNA methylation of ICRs is established in the germ line, conserved throughout development, and generally maintained in adult tissues (reviewed by Messerschmidt 2012). During the epigenetic reprogramming that takes place during embryo development, H3K9me2 recruits the protein STELLA to the maternal genome and a subset of paternal ICRs, preventing TET-mediated active demethylation at these loci. Additionally, a heterochromatin-inducing complex, of which TRIM28 is the central scaffolding component, confer reprogramming resistance to imprinted regions. The DNA binding specificity of the complex is provided by the binding of TRIM28 with ZFP57. Finally, Dnmt1 together with TRIM28 and ZFP57 are involved in the further maintenance of the methylation at the imprinted regions (reviewed by Messerschmidt 2012).

Importantly, in the germ line of the next generation, these imprints will undergo erasure and subsequent re-establishment based on the sex of the individual. This de novo establishment of all-paternal or all-maternal ICR methylation patterns at the germ line is mediated by Dnmt3A. This enzyme depends on the enzymatic inactive regulatory factor Dnmt3L, which enables binding and methylation of the DNA (Bourc'his and Proudhon 2008). The establishment of the sex-specific DNA methylation at ICR occurs during late fetal development in males, and postnatally in growing oocytes in females (Kaneda *et al*. 2004; Lucifero *et al*. 2004).

1.2.4.3 Epigenetic reprogramming

The epigenetic reprogramming consists in the removal of the existing epigenetic marks in the nucleus, and the subsequent establishment of a different set of marks. Two major waves of epigenetic reprogramming of the genome are described to occur during normal mammalian

development; one during germ line differentiation and the other one during preimplantation development (Reik *et al*. 2001) (Figure 1.12). Epigenetic remodeling in these crucial stages includes cytosine modifications, histone modifications as well as other epigenetic marks (Meehan *et al*. 2005).

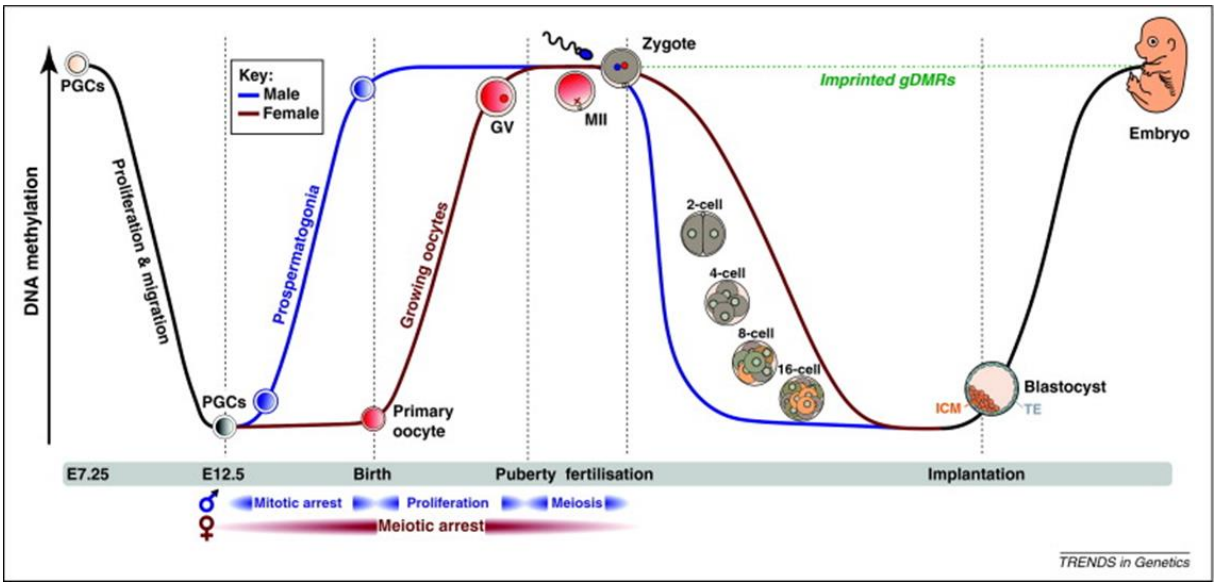

Figure 1.12. *Graphic representation of the two epigenetic reprogramming waves. In the first wave, primordial germ cells (PGCs) undergo a global erase of epigenetic marks, including methylation of imprinted genes during migration towards the genital ridge. The second wave of epigenetic reprogramming takes place during preimplantation development. Here, the paternal genome undergoes an active DNA demethylation before the first cleavage division, while the maternal genome undergoes a passive DNA demethylation, with the cleavages. In the second wave imprinted genes escape epigenetic reprogramming. GV, germinal vesicle; MII oocyte arrested in metaphase II; ICM, inner cell mas, TE trophectoderm. From Smallwood and Kelsey 2012.*

- **Germ line**

The primordial germ cells (PGCs) are the precursors of the female and male gametes and arise from the epiblast during gastrulation. At that stage, PGCs show the same epigenetic marks as the rest of the cells from the epiblast, including significant levels of DNA methylation (Seisenberger *et al*. 2013). However, during their migration towards the genital ridge, PGCs will undergo a genome wide epigenetic reprogramming that will erase most of the DNA

methylation marks (Morgan *et al*. 2005) (Figure 1.12). This is the case for the imprinted genes, which lose their DNA methylation marks, for later on gain all-maternal or all-paternal sex-specific marks (Seisenberger *et al*. 2013). This loss of DNA demethylation is also accompanied by a global erasure of histone modifications (Hajkova *et al*. 2008). Still, the DNA methylation marks of the most active retrotransposons will escape reprogramming. Interestingly, the acquisition of new epigenetic marks happens at different times in male and female (Messerschmidt 2012). The resulting DNA patterns are also different between male and female germ cells, with the male germ cells being heavily methylated with an 85% of global CG methylation, while oocytes are moderately methylated, with levels around 30% (Seisenberger *et al*. 2013).

- **Early development**

The second wave of epigenetic reprogramming takes place during preimplantation development, between fertilization and formation of the blastocyst (Dean *et al*. 2001).

Soon after the formation of the pronuclei and the replacement of protamines by histones, the paternal genome undergoes an active DNA demethylation process. In mammals, it is believed that this active DNA demethylation of the paternal genome is important for reprogramming of subsequent embryonic development (Mayer *et al*. 2000; Oswald *et al*. 2000; Dean *et al*. 2001). This active DNA demethylation is initiated by TET3, through the oxidation of 5mC into 5hmC (Iqbal *et al*. 2011). After its initiation, DNA demethylation can be completed through several routes: 1) in a replication-dependent way, with the 5hmC being diluted with the subsequent cell cycles, 2) by DNA repair enzymes (the base excision repair (BER) machinery), which enzymatically remove the modified base and replace it with a cytosine, 3) by the direct removal of 5fC and 5caC by the thymine-DNA glycosylase (TDG) followed by DNA repair, 4) by deamination of 5hmC to 5-hydroxymethyluridine (5hmU) by AIS/APOBEC enzymes followed by BER, and 5) by the dehydroxymethylase activity of Dnmt3A and Dnmt3B (reviewed by Delatte *et al*. 2014) (Figure 1.13).

On the contrary, the maternal genome is protected from this active demethylation by STELLA. This protein maintains normal DNA methylation levels in the maternal genome and imprinted genes by binding to H3K9me2 and repelling TET3 (Szabo and Pfeifer 2012). The loss of methylation of the maternal genome is associated with cell division (replication-

dependent). In this way, the maternal genome will lose half of its methyl groups with each cell division, in a process called passive demethylation (Figure 1.12). This passive loss of methylation takes place between the 2-cell and morula stages, with somatic cell levels being re-established at or after the blastocyst stage when differential lineages are first formed (Mayer *et al*. 2000; Bourc'his *et al*. 2001; Dean *et al*. 2001). This methylation re-establishment is mediated by de novo methyltransferases (Dnmt3).

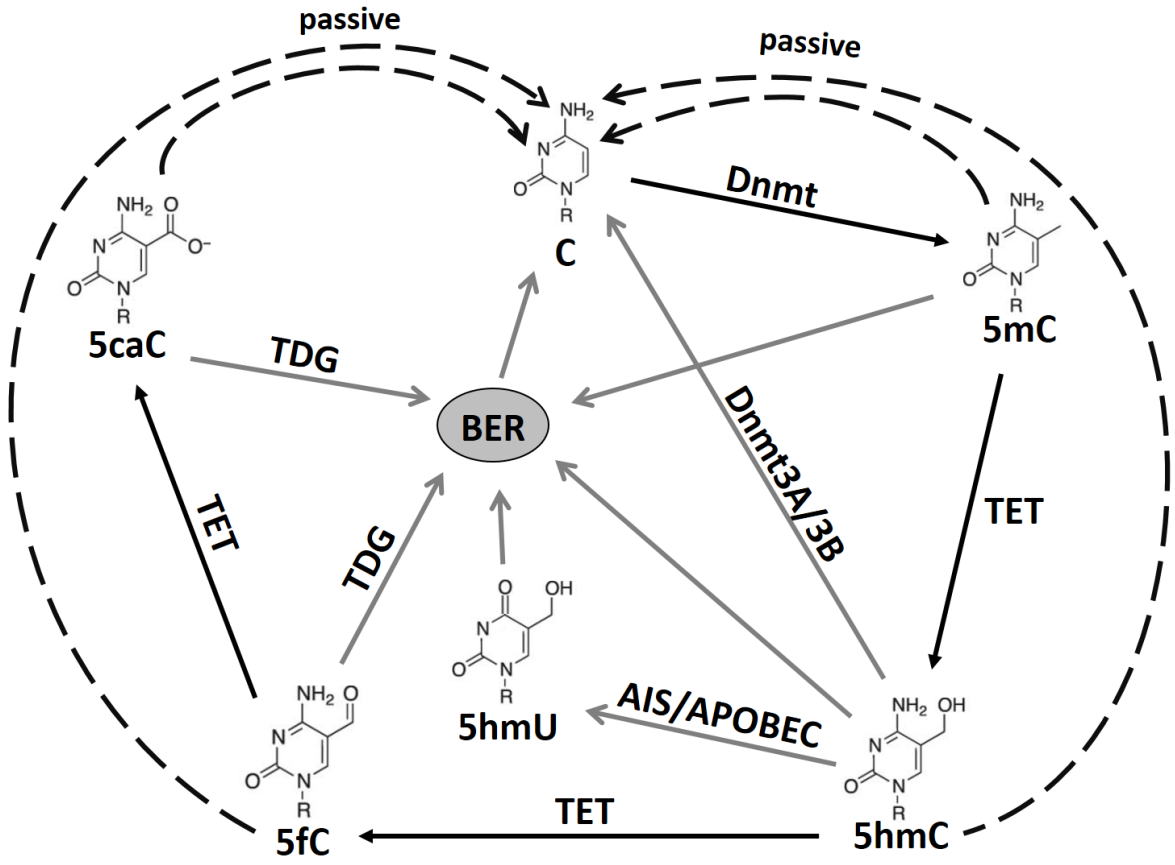

Figure 1.13. *Different cytosine modifications and their interactions. Cytosine (C) is methylated by the Dnmt enzymes at the 5´carbon position, generating 5-methylcytosine (5mC). TET enzymes can oxidize 5mC into 5-hydroxymethylcytosine (5hmC) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5hmC can also be deaminated to 5-hydroxymethyluracil (5hmU) by AIS/APOBEC enzymes, or directly be dehydroxymethylated by the dehydroxymethylase activity of Dnmt3A and Dnmt3B. The base excision repair machinery (BER) can convert 5mC, 5hmC, 5hmU, 5fC and 5caC into C. TDG can remove 5fC and 5caC, which are replaced by C by DNA repair. And finally, all cytosine modifications can be passively diluted through DNA replication. Adapted from Seisenberger et al. 2013.*

This pattern of epigenetic reprogramming was established in mouse (Oswald *et al*. 2000; Santos *et al*. 2002), and assumed to be conserved in all mammalian species (Dean *et al*. 2001). This assumption was supported by the results obtained in rat (Dean *et al*. 2001; Zaitseva *et al*. 2007) and human (Xu *et al*. 2005), which also showed this loss of methylation. However, the conservation of this active loss of methylation of the paternal genome was questioned when several other species, such as sheep (Beaujean *et al*. 2004a), goat (Hou *et al*. 2005), pig (Jeong *et al*. 2007), and rabbit (Reis Silva *et al*. 2011), failed to show it. Some other species, such as cow (Beaujean *et al*. 2004a), showed an intermediate pattern, with partial demethylation of the paternal pronucleus in the zygote. These findings suggest that active demethylation of the paternal genome is not an obligate requirement for normal early mammalian development. Controversially, in 2012, an article showing persistence of the DNA methylation in the paternal genome of the mouse zygote was released (Li and O'Neill 2012). By the use of a stronger epitope retrieval process, they were able to detect DNA methylation in the paternal pronuclei of mouse zygotes, which showed no differences in intensity with the maternal pronuclei.

Interestingly, an interspecific study of paternal DNA active demethylation showed that sheep sperm can be demethylated in mouse oocytes, and mouse sperm can also be demethylated to a limited extent in sheep oocytes (Beaujean *et al*. 2004c). Thus, the degree of demethylation of the male pronucleus is primarily determined by the oocyte environment. However, the fact that mouse sperm can be at least partially demethylated in all environments suggests that an undefined difference in the properties of the sperm also contributes to the demethylation process.

Furthermore, differences between species are also observed concerning subsequent passive demethylation. Sheep and rabbit embryos fail to show passive demethylation throughout preimplantation development (Beaujean *et al*. 2004a). In these two species, high levels of methylation of the two parental genomes are present throughout preimplantation development and suggest that genome-wide demethylation is not necessary for normal development. The timing of re-methylation also differs among species. It is reported to occur at the 8- to 16-cell stage in bovine embryos (Dean *et al*. 2001), whereas in mouse, it occurs only at the blastocyst stage (Dobbs *et al*. 2013). Finally, higher levels of methylation are reported either in ICM or TE depending on the species. In mouse and sheep embryos, higher

levels of DNA methylation are found in the ICM compared to TE (Santos *et al*. 2002; Beaujean *et al*. 2004a). In contrast, it has been observed that in cattle, human and rabbit blastocyst the ICM is less methylated than the TE (Dean *et al*. 2001; Fulka *et al*. 2004; Dobbs *et al*. 2013). The biological significance of these interspecies differences is not known yet.

Importantly, some DNA regions, including imprinted genes and some retrotransposons, escape this second wave of reprogramming (van Montfoort *et al*. 2012).

1.2.4.4 Techniques for the evaluation of epigenetic marks

There are many techniques developed in recent years for the study of epigenetics. These techniques can be divided in three groups: 1) affinity based strategies, such as immunofluorescent staining, techniques based on immunoprecipitation with 5mC antibodies (methylated DNA immunoprecipitation, MeDIP) and techniques based on proteins that bind to methylated CpG sequences (e.g. methylated-CpG island recovery assay, MIRA), 2) bisulfite-based strategies, such as bisulfite sequencing, reduced representation bisulfite sequencing, reduced bisulfite sequencing (redBS-Seq), and oxidative bisulfite sequencing, and 3) restriction enzymes-based strategies, such as *Hpa*II tiny fragment enrichment by ligation-mediated PCR (HELP) and methylation sensitive cut counting (MSCC). With the exception of the immunofluorescent staining, the techniques are focused on the study of cytosine modifications, especially 5-methylcytosine. The most commonly used techniques in embryos and gametes are described below. A detailed description of all the available methods for the study of DNA methylation and hydroxymethylation is reviewed by Olkhov-Mitsel and Bapat 2012.

- **Immunofluorescent staining**

Immunofluorescent staining is the most frequently used technique for the study of epigenetics in embryos. It has several advantages: 1) it can distinguish between the various cytosine modifications by the use of specific antibodies, 2) it can detect 5mC and the other cytosine modifications throughout the whole genome, and it is not restricted to the CGI (CpG islands), and 3) the epigenetic status of single embryos can be studied. Moreover, in zygotes, the dynamics of the epigenetics status of the male and the female pronuclei can be followed independently. This is especially relevant for the study of the epigenetic reprogramming

processes. The main disadvantage of the immunofluorescent staining is that it does not provide sequence-specific data, and it is not readily quantifiable (Salvaing *et al*. 2014).

To perform the immunofluorescent staining, the embryo has to be incubated with a specific antibody against the epigenetic modification of interest, which is subsequently indirectly labelled. Antibodies targeting many epigenetic modifications have been developed in the last years. Antibodies against 5-methylcytosine (5mC) are the most commonly used, and have been successfully applied in many species, such as rabbit (Reis Silva *et al*. 2011), cattle (Rahman *et al*. 2014), human (Efimova *et al*. 2015), mouse (Li and O'Neill 2012; Salvaing *et al*. 2012), sheep (Beaujean *et al*. 2004a), pig (Jeong *et al*. 2007), goat (Hou *et al*. 2005) and rat (Yoshizawa *et al*. 2010). The study of 5-hydroxymethylcytosine has gained more attention in the last years, and immunostaining of 5hmC has been successful in many species (Li and O'Neill 2013; Efimova *et al*. 2015). In 2011, the specific antibodies against the other cytosine modifications, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), were developed and applied in mouse embryos (Inoue *et al*. 2011a). Antibodies targeting many different histone modifications have also been successfully used in many species. Additionally to the use of histone modification labelling to study their epigenetic pattern, the labelling of several histone modifications, such as H3K9me3, is commonly used to determine the parental origin of the pronuclei in zygotes (Reis Silva *et al*. 2011; Heras *et al*. 2015). As such, it is commonly used in combination with 5mC and 5hmC immunostaining.

Importantly, for the immunofluorescent staining to be reliable and properly used, several steps need to be followed. First, a proper solvent exposure of the epitope is essential for its correct visualization and quantification. The characteristics of this process are epitope dependent and, in case of 5mC, a correct permeabilization of the embryo followed by a treatment with 2-4N HCL and trypsin are necessary for its correct exposure in the mouse (Li and O'Neill 2012). Additionally, the temperature and length of incubation with the primary antibodies need to be sufficient to reach thermodynamic equilibrium (i.e. saturation). Finally, a correct image acquisition and quantification are of major importance for the reliability of the results (Salvaing *et al*. 2014).

- **Bisulfite sequencing**

Besides the immunofluorescent staining, bisulfite sequencing is the most commonly used technique to study 5-methylcytosine patterns. This technique involves the treatment of the DNA with bisulfite (HSO3-). Bisulfite converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing.

Its advantage over immunofluorescent staining is that it can be used to evaluate locus specific 5mC status, and its changes after different treatments. However, its main disadvantage is that it cannot distinguish between different cytosine modifications (Jin *et al*. 2010). Therefore, cytosine modifications will always be assigned as 5mC, while they could be also 5hmC, which have a different biological meaning. This issue was addressed by modifications of the technique, as described below. Another concern about bisulfite sequencing is that incomplete denaturation or reannealing leads to incomplete conversion, since bisulfite converts single-stranded DNA. Thus, the presence of unconverted cytosines can also be a consequence of this artifact. Other potential problems are depurination, strand breakage and DNA degradation caused by the harsh reaction condition (Meissner *et al*. 2005).

- **Reduced representation bisulfite sequencing (RRBS)**

This technique was developed in 2005 by Meissner (Meissner *et al*. 2005) and it combines the use of restriction enzymes with the bisulfite treatment. The advantage of the use of restriction enzymes is that areas of the genome with high CpG content can be enriched, reducing the amount of nucleotides that need to be sequenced for an entire genome coverage to 1% of the genome, which limits the costs. The fragments that comprise the reduced genome still include the majority of promoters, as well as regions such as repeated sequences that are difficult to profile using conventional bisulfite sequencing approaches. Still, this technique fails to discriminate between 5mC and 5hmC (Jin *et al*. 2010).

RRBS consists in the digestion of the genomic DNA with a restriction enzyme and the selection of fragments between 500-600 bp. After ligation with adaptors, the fragments are treated with bisulfite, amplified and sequenced.

A disadvantage of these high covering techniques is the large amount of gDNA (5-100 µg) necessary for the process, making them difficult if not impossible when working with embryos and gametes.

- **Oxidative bisulfite sequencing (oxBS-seq)**

This technique was developed in 2013 by Booth (Booth *et al*. 2013). It is a modification of bisulfite sequencing that can quantitatively locate 5mC and 5hmC.

In oxBS-seq two DNA samples are prepared. One will directly be fragmented and bisulfite sequenced, while the other sample will be fragmented and oxidized to convert 5hmC into 5fC prior to bisulfite sequencing. During bisulfite sequencing, cytosine and 5fC will be converted to uracil, and so, the unconverted cytosines will correspond to 5mC. To determine the presence of 5hmC, data from the oxidation+bisulfite sequencing must be subtracted from the data from the single bisulfite sequencing (Figure 1.14).

The limitations of this technique are the high coverage needed to sequence 5hmC quantitatively. The reasons for that are the relative low levels of 5hmC in the genome, plus the fact that a subtraction is needed to obtain the actual levels of 5hmC, which increases the noise and so the need of a high number of replicates. Therefore, between 100 ng and 1 µg of gDNA are needed for this technique.

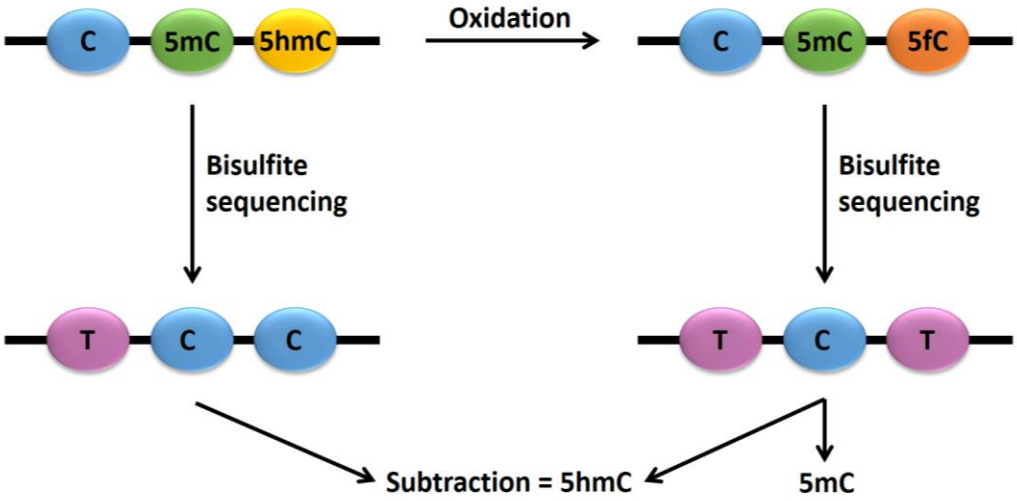

Figure 1.14. *Representation of the oxBS-seq process. In the gDNA samples treated with bisulfite sequencing, cytosines will be converted to uracil while 5mC and 5hmC will remain as cytosine. However, after oxidation of gDNA 5hmC will be converted into 5fC, which can be converted to uracil by bisulfite treatment. Finally to determine the presence of 5hmC, data from oxidation+bisulfite sequencing needs to be subtracted from the data obtained from single bisulfite treatment.*

- ***Hpa*II tiny fragment enrichment by ligation-mediated PCR assay (HELP)**

This technique is based on the use of restriction enzymes for the study of DNA methylation. One aliquot of gDNA is digested with the methylation-sensitive restriction enzyme *Hpa*II, which cuts 5'-CCGG-3' sites when the central cytosine is unmethylated. In parallel, a second aliquot is digested with the methylation-insensitive isoschizomer *Msp*I, which cuts at the same site irrespectively of its methylation status. The digestion products of both enzymes are ligation-mediated PCR amplified and analyzed by microarrays or by sequencing.

An application of this technique is the EmbryoGENE DNA methylation array. This array is used for the study of 5mC and 5hmC in bovine. It uses the same restriction enzymes as HELP but sequentially instead of in parallel. It starts with gDNA digestion using the MseI restriction enzyme, followed by a methylation-sensitive digestion and ligation mediated amplification PCR (LMA-PCR). To this end, adapters are ligated to the gDNA fragments previously obtained, which are then subjected to methyl-sensitive restriction enzymes (*Hpa*II). Unmethylated

fragments are cut, and thus cannot be amplified in the following PCR (Figure 1.15). Finally, the products are analyzed by microarray hybridization. The disadvantage of this method is that it only gives a prediction of the methylated/unmethylated sites, since in each fragment several methylated and unmethylated sites can be present, leading to erroneous results. Therefore results obtained with this technique need to be confirmed (EmbryoGENE).

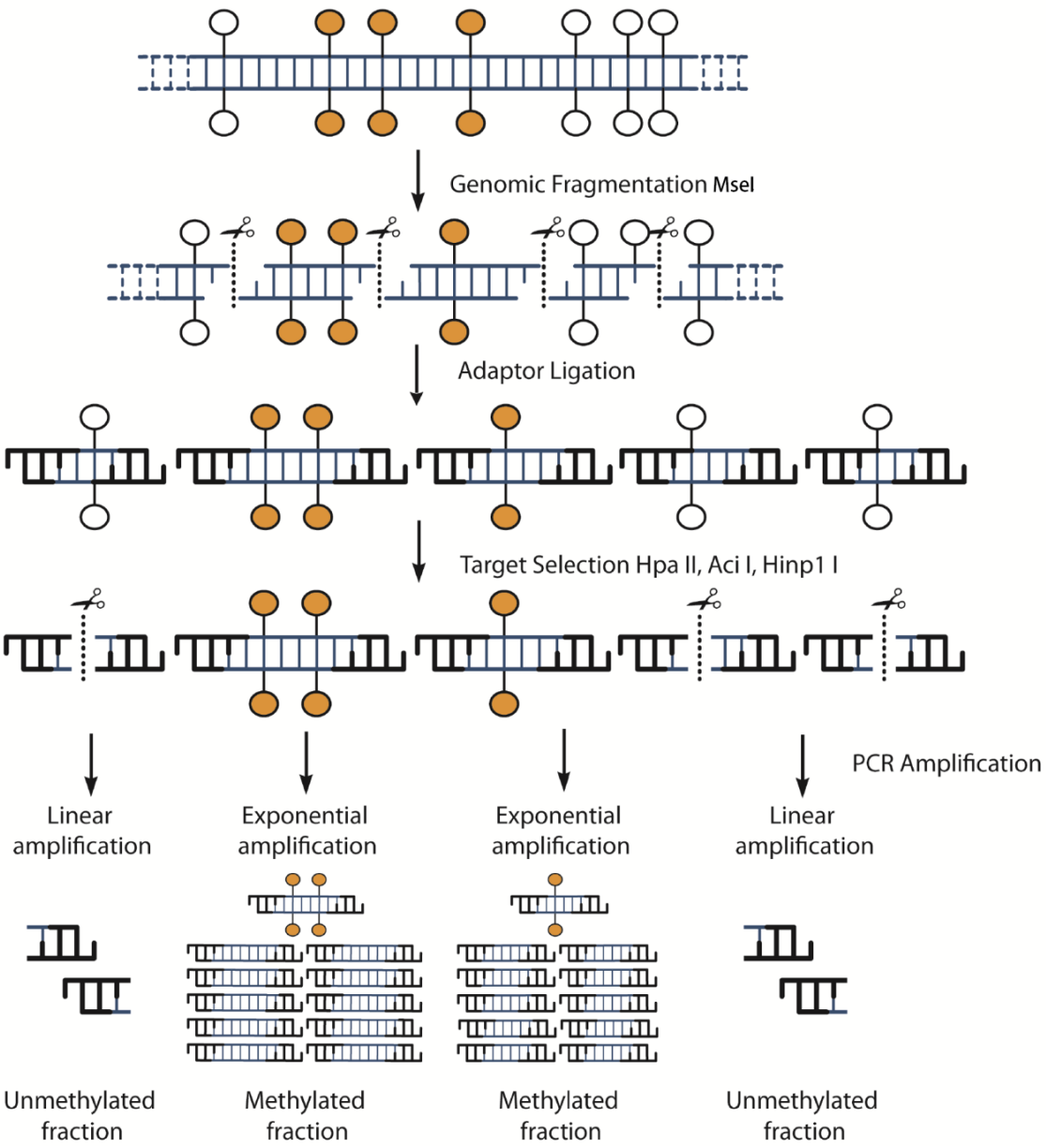

Figure 1.15. *Diagram of the gDNA fragmentation and subsequent selection of the methylated fragments by methylation-sensitive digestion and ligation mediated amplification PCR (LMA-PCR). The specific restriction enzymes used are Hpa II, Aci I and Hinp1 I. Adapted from de Montera et al. 2013.*

1.3 EFFECTS OF ART ON EMBRYO DEVELOPMENT AND QUALITY

Since the development of ARTs, a large number of studies has been conducted to test the performance and safety of the different developed techniques. For the evaluation of the performance, cleavage and blastocyst rates are the main parameters, with higher cleavage and blastocyst rates denoting better performance. In the early days of ARTs, cleavage and blastocysts rates were the main evaluation parameters of each newly developed technique, with laboratories selecting the techniques and media composition that resulted in higher cleavage and blastocyst rates. However, throughout the years, it became evident that higher cleavage/blastocysts rates do not necessarily correlate with better quality of the produced embryos.

ARTs have proven to induce alterations in many embryonic characteristics. IVP embryos showed a darker cytoplasm due to their higher lipid content (Pollard and Leibo 1994), a more fragile ZP (Duby *et al*. 1997), differences in metabolism (Khurana and Niemann 2000), a reduced intracellular communication (Boni *et al*. 1999), higher incidence of chromosome abnormalities (Viuff *et al*. 1996; Lonergan *et al*. 2004), errors of imprinting (Doherty *et al*. 2000), slower growth rate, higher thermal sensitivity, lower ICM/TE cell ratio (Van Soom *et al*. 1997a; Van Soom *et al*. 1997b), and differences in gene expression compared to their *in vivo* counterparts (Driver *et al*. 2012). Additionally, higher apoptotic rates have been reported in IVP embryos compared to their *in vivo* counterparts (Gjorret *et al*. 2003), with an increased incidence of apoptosis as the culture time increases (Vandaele *et al*. 2006). Surprisingly, in cattle, some media used for *in vitro* culture are reported to have an influence on the sex ratio of the produced embryos, with a shift towards male embryos (Behboodi *et al*. 1996; Massip *et al*. 1996; Gutierrez-Adan *et al*. 2001). Superovulation is reported to induce changes in gene expression and DNA methylation patterns in several species. In addition, the use of high dosages of gonadotropins induced spindle and chromosomal abnormalities in bovine oocytes (Liu *et al*. 2011). However, not all ARTs perform the same, and some specific techniques were shown to have a more detrimental effect on the developing embryo than others. For instance, in humans, the rate of abnormalities at birth is higher for ICSI than for conventional IVF pregnancies (Kent-First *et al*. 1996; Hewitson *et al*. 2000; Terada *et al*. 2000).

1.3.1 Effects of ARTs on gene expression

A large amount of research has been conducted studying the influence of ARTs on the gene expression pattern of preimplantation embryos. Before the development of wide screening techniques (such as microarray and RNA-seq), the effort was focused on genes known to play important roles during pre- and post-implantation development.

The expression of Dnmt1, 3A and 3B was up-regulated in bovine oocytes after IVM compared to *in vivo* matured oocytes (Heinzmann *et al*. 2011). IVC was shown to have a major effect on gene expression, which is logical since embryos spend 7-8 days in that environment. A microarray study showed that approximately 85% of differentially expressed genes was down-regulated in IVP bovine blastocysts compared to their *in vivo* counterparts (Corcoran *et al*. 2006). Most of these genes are involved in transcriptional and translational events suggesting that a deficient machinery associated with transcription and translation is behind the inferior quality of IVP embryos (Corcoran *et al*. 2006). Furthermore, different culture media had a different impact on genes associated with transcription and translation (Corcoran *et al*. 2007). This is not a unique case, genes involved in blastocyst formation such as cell-to-cell adhesion (E-cadherin, connexins, TJ genes), cell communication (gap junctions), differentiation marks (Boni *et al*. 1999; Lonergan *et al*. 2003b; Lonergan *et al*. 2003a; Miller *et al*. 2003) and genes related to apoptosis and oxidative stress (*Bax*, *SOX*, *Hsp70*) had different expression between different culture media (Rizos *et al*. 2002; Sagirkaya *et al*. 2006).

When comparing *in vivo* derived with IVP bovine embryos, genes related to metabolism, growth and differentiation (*GLUT-5*, *CX43*, *IGF-II*, *LIF*) were up-regulated in embryos derived *in vivo*, while genes related to stress (*SOX*, *MnSOD*, *BAX*, *Hsp70*, *PRDX5*) were up-regulated in IVP embryos. The significant increase in expression of those genes supports the hypothesis that current *in vitro* culture systems are associated with a considerable amount of oxidative stress (Lazzari *et al*. 2002; Rizos *et al*. 2003; Gutierrez-Adan *et al*. 2004). Additionally, in embryos produced *in vitro* in the absence of FBS, the expression of genes involved in the cholesterol biosynthesis pathway was up-regulated compared to *in vivo* derived embryos (Driver *et al*. 2012). In addition to culture media composition, culture conditions such as oxygen concentrations were shown to have an impact on gene expression (Harvey *et al*. 2004).

Other ARTs also induce alterations on the transcriptome. Some studies reported differences in the mRNA expression profile of several genes between embryos produced with sex-sorted and unsorted semen (Morton *et al*. 2007), while other studies failed to find differences (Bermejo-Alvarez *et al*. 2010a). Nevertheless, offspring from sex-sorted spermatozoa did not display more abnormalities than the controls (Seidel and Garner 2002). Vitrification of mouse oocytes arrested at MII induced down-regulation of Dnmt1, 1o, 3A, 3B and 3L in MII and of Dnmt3B in blastocysts (reviewed by Anckaert and Fair 2015). Furthermore, blastocyst vitrification had impact on the microRNA transcriptome of mouse embryos (Zhao *et al*. 2015). Additionally, superovulation induced alterations in the gene expression of bovine oocytes (Chu *et al*. 2012).

Despite all this, a similar expression of developmentally important genes was observed between *in vivo* and IVP embryos carried to term (Ghanem *et al*. 2011).

1.3.2 Effects of ARTs on epigenetic marks

The study of the effects of ARTs on the epigenetic pattern of the embryos and resulting offspring has gained more attention in recent years. The effects of ARTs on the global epigenetic status of preimplantation embryos has been observed in many species. IVP increased the levels of DNA methylation compared to embryos derived *in vivo*, in rats and mice (Zaitseva *et al*. 2007). In bovine blastocysts, IVP altered the DNA methylation profile, with longer *in vitro* culture being translated in higher alteration compared to *in vivo* derived embryos (Salilew-Wondim *et al*. 2015). Cloned embryos also showed aberrant DNA methylation patterns in several species, including cattle (Dean *et al*. 2003) and sheep (Beaujean *et al*. 2004b). However, in rabbits no differences in DNA methylation status were observed between cloned and IVP embryos (Shi *et al*. 2004).

Alterations of imprinting have been observed in embryos, placenta and offspring produced by ARTs. A loss of DNA methylation in *Igf2R* and *Peg1*, and a gain of methylation in *H19* were found after IVM compared to *in vivo* maturation in mice. This gain of methylation in *H19* was also reported in human after IVM in five out of 20 oocytes analyzed (reviewed by Ventura-Junca *et al*. 2015).

In mouse, loss of DNA methylation was reported in *H19*, *Snrpn* and *Peg3* in IVP embryos using different culture media (Doherty *et al*. 2000; Market-Velker *et al*. 2010a). These three genes and *Peg1* also showed loss of DNA methylation in mouse blastocysts after superovulation, and this alteration was dose-dependent, with aberrant methylation more frequent at high hormone dosage (Market-Velker *et al*. 2010b). Importantly, the loss of DNA methylation of *H19*, *Snrpn* and *Peg1*, could be observed in the sperm of the mouse offspring during two generations (Stouder *et al*. 2009). Superovulation in mice also resulted in biallelic expression of *Snrpn* and *H19* imprinted genes in the placenta (Fortier *et al*. 2008). In human, superovulation caused a loss of methylation of *KCNQ1OT1* imprinted gene in immature oocytes (Khoueiry *et al*. 2008). Additionally, serum supplementation induced alterations on the DNA methylation pattern of various imprinted genes (*H19*, *Igf2*, *Grb7*, *Grb10*, and *Peg1*), faster rates of development and long-term behavioral consequences in mouse embryos (reviewed by Velker *et al*. 2012). Vitrification also altered the methylation status of imprinted genes, causing a loss of methylation in *H19* in murine embryos (Wang *et al*. 2010). In mice, loss of DNA methylation in *H19* and *Snrpn* was observed in SCNT blastocysts (Mann *et al*. 2003). Moreover, altered allele specific DNA methylation and/or gene expression was found for *H19* in liver tissue and for *Peg3* in brain tissue in one and four out of 12 SCNT mice, respectively (de Waal *et al*. 2012).

The imprinting disorders, Beckwith-Wiedemann syndrome (BWS) and Angelman syndrome (AS), have been reported in human. BWS is characterized by highly variable clinical features such as prenatal and postnatal overgrowth, ear creases and predisposition to childhood tumors. AS is characterized by severe mental retardation, delayed motor development and absence of speech, among other features. Superovulation has been linked to BWS and AS in children conceived by ART (Denomme and Mann 2012). Many studies have reported an increased prevalence of IVP (IVF and ICSI) children among children with BWS compared with those in general population. Many of these BWS children display maternal *KCNQ1OT1* loss of methylation. Similarly, AS children born after IVF and ICSI have a greater prevalence of S*NRPN* imprinting defects than AS children in general (reviewed by Denomme and Mann 2012). The absolute risk of developing a genomic imprinting disorder in children born through ART as a result of an epigenetic defect is low. However, the relative risk when compared with non-ART children is significantly higher (Market-Velker *et al*. 2010b).

A similar condition of overgrowth can also be induced by ART in ruminants and is referred to as Large Offspring Syndrome (LOS). It is characterized by large size at birth, breathing difficulties, reluctance to suckle and sudden perinatal death (Young *et al*. 1998). LOS is caused by the exposure of pre-elongation ruminant embryos to unusual environmental conditions. It is not exactly clear what environmental changes are important but a major cause is the use of serum in the culture media (Sinclair *et al*. 1999). Recent studies provide evidence for epigenetic similarities between BWS and LOS as these syndromes share misregulation of several similar imprinted genes such as *IGF2R*, *KCNQ1OT1* or *CDKN1C* (Chen *et al*. 2013; Chen *et al*. 2015). Furthermore, loss of maternal-specific *SNRPN* methylation was found in the placenta from *in vitro* fertilized and cultured bovine embryos, similar to AS in humans, (reviewed by Velker *et al*. 2012).

Finally, oxidative stress reduces the global hydroxymethylation level in mouse somatic cells *in vivo* and *in vitro*, this effect is hypothesized to be caused by the inactivation of TETs by hydrogen peroxide treatment (reviewed by Delatte *et al*. 2014). Similar studies are still lacking in gametes and embryos.

In conclusion, a lot of progress has been made in the last decades in the development and refinement of ARTs. However, these techniques still have negative effects on the produced embryos and even on the resulting offspring. As demonstrated before, special attention has to be paid to the medium used for embryo culture, since it has a major impact on the transcriptomics and epigenetics of the embryos, and in most cases having no effect on the parameters evaluated by the traditional methods. Therefore, there is still a need for further optimization of these techniques, and to this end, the evaluation of the transcriptome and the epigenome of the embryos produced under different conditions constitutes a valuable tool.

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CHAPTER 2

AIMS OF THE THESIS

The development of assisted reproductive technologies (ARTs) has represented a revolution for the treatment of human infertility and for livestock production, maximizing the offspring of genetically valuable animals. However, the use of ARTs implies that gametes and embryos are exposed to suboptimal conditions during a crucial developmental period, which can induce alterations in the embryo that can have consequences in the offspring. Indeed, an increased incidence of particular syndromes has been reported in human and animals, many of them related to epigenetic alterations. Traditional methods for embryo quality evaluation, which focus on rates and speed of development, cell number and incidence of apoptosis, are unable to detect if particular ARTs are inducing alterations in the transcriptome or the epigenome of the embryo. As such, the use of fetal bovine serum (FBS) as media supplementation for *in vitro* embryo production yields excellent results when evaluated by the traditional methods. However, its use has increased the incidence of an imprinting syndrome, the large offspring syndrome (LOS), in ruminants. So far, no cases of LOS have been reported in horses even though FBS is also used in the culture medium. Still, the use and study of *in vitro* embryo production in horses is much more limited by now than in ruminants.

Therefore, in order to improve ARTs to produce more *in vivo*-like embryos, the effect of these techniques on the embryos needs to be evaluated in terms of gene expression and epigenetic modifications, in addition to the traditional methods.

The specific aims of this thesis were:

- To evaluate the effect of *in vitro* embryo production on the gene expression of early bovine blastocysts, using two different culture conditions, i.e. serum-containing and serum-free medium. To this end, the whole transcriptome of *in vitro* produced embryos in both culture conditions was compared to the transcriptome of *in vivo*-derived bovine embryos using RNA sequencing (Chapter 3).

- To optimize an immunofluorescent staining to evaluate 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in bovine and equine embryos during preimplantation development (Chapter 4). To this end, we first assessed a proper DNA counterstaining that can be combined with the epitope retrieval treatment needed for 5mC and 5hmC immunostaining (Chapter 4.1). Subsequently, marks to determine the

parental origin of the pronuclei were identified in equine (Chapter 4.2) and bovine (Chapter 4.3) zygotes.

- To use the previously optimized immunostaining to determine the dynamics of 5mC and 5hmC in equine (Chapter 5) and bovine embryos, with focus on the zygote stage, and to evaluate the effects of ARTs on the 5mC and 5hmC patterns of equine and bovine *in vitro* produced embryos.

CHAPTER 3

EFFECT OF *IN VITRO* PRODUCTION ON THE GLOBAL GENE EXPRESSION PATTERN OF BOVINE BLASTOCYSTS

Adapted from:

Heras S, De Coninck D, Van Poucke M, Goossens K, Bogado Pascottini O, Van Nieuwerburgh F, Deforce D, De Sutter P, Leroy J, Gutierrez-Adan A, Peelman L, Van Soom A. ***Suboptimal culture conditions induce more deviations in gene expression in male than female bovine blastocysts. BMC Genomics. (2016)*** *17(1):72.*

SUMMARY

Since the development of *in vitro* embryo production in cattle, different supplements have been added to culture media to support embryo development, with serum being the most popular. However, the addition of serum during embryo culture can induce high birthweights and low viability in calves (Large Offspring Syndrome). Analysis of global gene expression in bovine embryos produced under different conditions can provide valuable information to optimize culture media for *in vitro* embryo production.

We used RNA sequencing to examine the effect of *in vitro* embryo production, in either serum-containing or serum-free media, on the global gene expression pattern of individual bovine blastocysts. Compared to *in vivo* derived embryos, embryos produced in serum-containing medium had five times more differentially expressed genes than embryos produced in serum-free conditions (1,109 vs. 207). Importantly, *in vitro* production in the presence of serum appeared to have a different impact on the embryos according to their sex, with male embryos having three times more genes differentially expressed than their female counterparts (1,283 vs. 456). On the contrary, male and female embryos produced in serum-free conditions showed the same number (191 vs. 192) of genes expressed differentially; however, only 44 of those genes were common in both comparisons. The pathways affected by *in vitro* production differed depending on the type of supplementation. For example, embryos produced in serum-containing conditions had a lower expression of genes related to small molecule metabolism while embryos produced in serum-free conditions showed aberrations in genes involved in lipid metabolism.

Serum supplementation had a major impact on the gene expression pattern of embryos, with male embryos being the most affected. The transcriptome of embryos produced in serum-free conditions showed a greater resemblance to that of *in vivo* derived embryos, although genes involved in lipid metabolism were altered. Male embryos appeared to be most affected by suboptimal *in vitro* culture, i.e. in the presence of serum.

INTRODUCTION

Since the initial development of *in vitro* embryo production, the technique has been applied successfully to many species for clinical, commercial, and research purposes. In the early days, it was common practice to supplement culture media with serum to support embryo development in many species. But subsequently serum has been associated with fetal overgrowth in ruminants to give the so-called Large Offspring Syndrome at birth (Young *et al.* 1998). In cattle, serum supplementation is still being used in many laboratories (Corcoran *et al.* 2006; Bermejo-Alvarez *et al.* 2010; Chitwood *et al.* 2013) probably because it increases blastocyst rates and generally gives more consistent results (George *et al.* 2008). We recently adopted a robust serum-free culture system, consisting of Synthetic Oviduct Fluid (SOF), bovine serum albumin (BSA), and insulin-transferrin-selenium (ITS), which yields comparable blastocyst rates (~ 40%) to media supplemented with serum (George *et al.* 2008; Wydooghe *et al.* 2014). However, bovine embryos produced in these serum-free conditions have a much lower hatching rate than embryos produced in serum-containing medium (George *et al.* 2008; Wydooghe *et al.* 2014). Nevertheless, in many other aspects, the quality of the embryos produced in serum-free conditions is superior to that of embryos produced in the presence of serum. Embryos produced in serum-free conditions showed increased freezability and, after transfer, the birthweight and incidence of abnormalities of the resulting calves was in line with that of *in vivo* derived embryos (Young *et al.* 1998; George *et al.* 2008). In addition, cattle embryos produced in serum-free conditions scored higher when using traditional parameters to evaluate embryo quality (George *et al.* 2008; Wydooghe *et al.* 2014). These quality parameters are based on blastocyst development, blastocyst cell number, ratios of inner cell mass (ICM) and trophectoderm cells, apoptotic cell ratios (Wydooghe *et al.* 2014), and also the gene expression pattern of a limited number of selected genes analyzed by RT-qPCR (Goossens *et al.* 2007; Market-Velker *et al.* 2010a). The selection of only a few genes to check embryo quality was based on the fact that the evaluation of expression of all the genes in the genome by RT-qPCR was a daunting task. However, this no longer represents an obstacle with recently developed RNA sequencing techniques, which have proven to be a very powerful tool for evaluating and comparing the global gene expression pattern of even single cells, and therefore also of single embryos (Chitwood *et al.* 2013; Jiang *et al.* 2014). This new technique

also allows the study of associated pathways that may ultimately be involved in affecting embryo quality.

We hypothesized that the similarities between embryos derived *in vivo* and those produced in serum-free culture conditions would also translate to the global gene expression pattern and, hence, embryos produced in serum-free conditions would resemble *in vivo* derived embryos more than embryos produced in the presence of serum. Nevertheless, when evaluating the global gene expression pattern of embryos, their sex needs to be taken into account, since embryos of different sexes can respond differently to stress situations (Perez-Crespo *et al.* 2005) and this might be reflected in changes in their gene expression pattern.

Therefore, the aim of the present study was to evaluate the effect that *in vitro* production, either in serum-containing or serum-free conditions, might have on cattle embryos by comparing their global gene expression pattern to that of the gold standard; namely, embryos derived *in vivo*. Also, to determine which *in vitro* condition produces more *in vivo*-like blastocysts. Additionally, we wanted to assess the impact of *in vitro* culture on embryos depending on their sex.

To our knowledge, this is the first study that uses RNA sequencing to evaluate the effect of *in vitro* embryo production, both in serum-containing and serum-free conditions, while at the same time taking embryonic sex into account. The results provide insight into the effects that different supplementations used for *in vitro* production may have on cattle embryos, show clearly how male and female embryos respond differently to suboptimal culture conditions, and offer valuable information on how to improve serum-free culture systems.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

In this study, the transcriptome of 16 individual bovine early blastocysts produced *in vitro* under two different culture conditions (eight in serum and eight in serum-free conditions) was compared to the transcriptome of eight embryos derived *in vivo*. Each blastocyst constituted one replicate and its sex was determined by RT-PCR prior to RNA

sequencing. Blastocysts produced under each *in vitro* condition were only compared with the blastocysts derived *in vivo*. Three comparisons were made between the embryos derived *in vivo*, and those generated *in vitro* by the two different methods. First comparing all the embryos of both groups; second, comparing only the female embryos of both groups and third, comparing only the male embryos of both groups. In addition, male and female embryos of each condition were compared with each other. Consequently, a total of 9 comparisons were made (Figure 3.1).

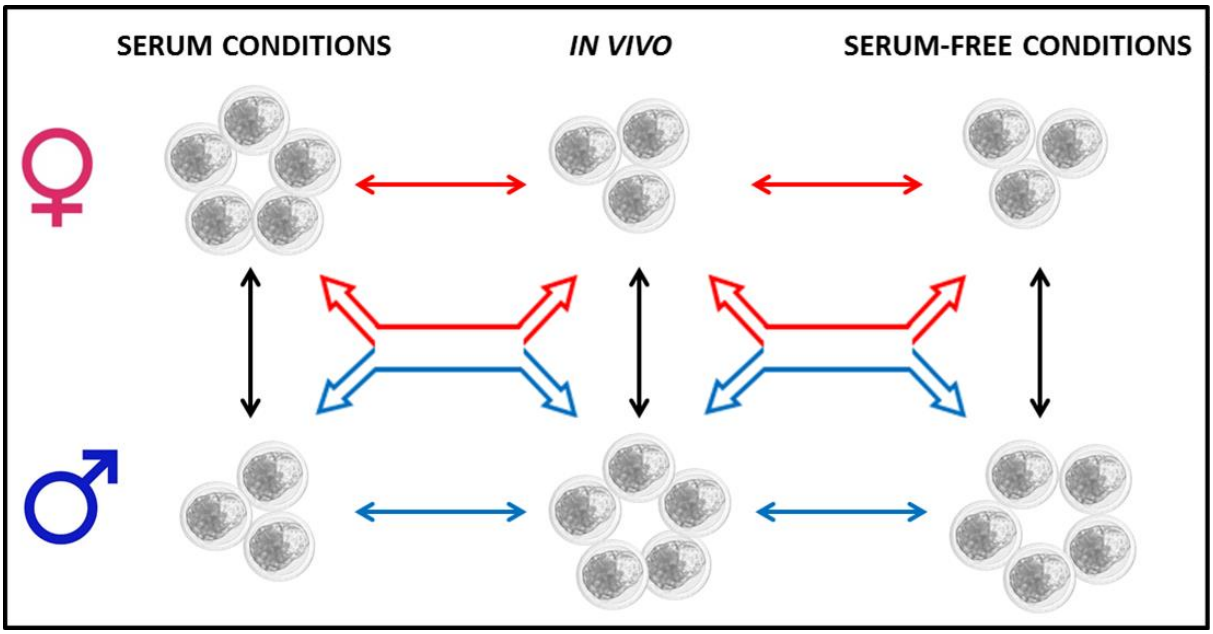

Figure 3.1. *Graphic representation of the experimental design. The number of embryos present in the figure represent the number of embryos included in the study per sex and condition. The arrows symbolize the 9 comparisons performed: red arrows represent the comparisons between female embryos; blue arrows depict the comparisons between male embryos; empty arrows represent the comparisons which included all the embryos, male and female, together; finally, black arrows are used for the comparisons between the sexes within each condition.*

IN VITRO EMBRYO PRODUCTION

Early bovine blastocysts (n=16) were produced by routine *in vitro* methods (Wydooghe *et al.* 2014). Briefly, ovaries were collected from Holstein cows at a local slaughterhouse and processed within 2h. Cumulus oocyte complexes were aspirated from follicles of 4–8 mm in diameter and matured in groups of 60 in 500 µL of maturation medium consisting of modified TCM-199 (GIBCO-BRL Life Technologies) supplemented, depending on the experimental design, with either 20 ng/mL EGF (Epidermal Growth Factor; Sigma E4127) and 50 µg/mL gentamycin (serum-free conditions) or 20% heat inactivated FBS (Fetal Bovine Serum; GIBCO, Invitrogen 10108-165), 50 µg/mL gentamycin, 0.4 mM L-Glutamine and 2 mM Na-pyruvate (serum conditions) for 22h at 38.5°C in 5% CO_2-in-air. Frozen-thawed spermatozoa from the same Holstein bull of proven fertility used to obtain *in vivo* derived embryos (to minimize variation throughout the experiments) were passed through a discontinuous Percoll gradient (45 and 90% (v/v); VWR International). A final sperm concentration of $1x10^6$ spermatozoa/mL was adjusted in IVF-TALP medium, consisting of bicarbonate buffered Tyrode solution supplemented with 6 mg/mL BSA (Sigma A8806) and 20 µg/mL heparin (Sigma). Matured oocytes were washed with WAS-TALP medium consisting of HEPES buffered Tyrode solution supplemented with 0.34 mg/mL BSA (Sigma A6003) before being incubated with the spermatozoa for 21h. Presumptive zygotes were vortexed for 3 min to remove the remaining cumulus cells and spermatozoa, washed with WAS-TALP and cultured in 50 µL drops of SOF supplemented with essential and non-essential amino acids and, depending upon the experimental design, 5% heat inactivated FBS (serum-containing conditions) or 4 mg/mL BSA (Sigma A9647) and ITS (5 µg/mL Insulin + 5 µg/mL Transferrin + 5 ng/mL Selenium; serum-free conditions). In both cases the embryos were held under mineral oil in groups of 25 at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2. Blastocysts were harvested, 6 and 7 days post insemination, from three independent *in vitro* experiments.

IN VIVO EMBRYO COLLECTION

The 8 *in vivo* derived blastocysts were obtained from three Holstein cows. They were superovulated with a total of 480 µg of FSH (Follicle-stimulating hormone; Stimufol) administered in eight decreasing doses every 12h over 4 days. An injection of prostaglandin (37.5 mg; Enzaprost) was administered 48h after the start of the superovulatory treatment.

Two inseminations with frozen-thawed semen from the same Holstein bull of proven fertility used for the *in vitro* experiments were performed 12h apart starting 8–12h after the onset of the estrous behavior. Seven days after insemination, both uterine horns were flushed non-surgically for embryo recovery. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine of Gent University (EC 2012/196 and EC 2013/161).

EMBRYO COLLECTION AND RNA EXTRACTION

The developmental stage and quality of the blastocysts, according to IETS standards, was determined for all the embryos recovered by the same three trained individuals. Only early blastocysts of quality 1 were selected for the study. The blastocysts were washed three times in RNase-free PBS (Phosphate buffered saline; Ambion), placed individually in 2 µL of lysis buffer consisting in 5 mM DTT (DT-Dithiothreitol; Promega), 4 U/µL RNasin Plus RNase inhibitor (Promega), and 0.64 µM Igepal (Sigma) in RNase free water (Sigma) and immediately stored at -80°C. RNA was extracted using the RNeasy micro kit (Qiagen); DNase treatment was omitted in embryos used for RNA-seq, but not in embryos used for RT-qPCR. Manufacturer´s instructions were followed with a single modification performed in the elution step, when 14 µL of RNase-free water was passed through the column twice.

EMBRYO SEXING

Sexing of the embryos used for RNA-seq was performed as previously described by Li *et al.* (Li *et al.* 2014). For sexing purposes, 2 µL of the eluted total RNA were used. RNA was reverse transcribed with the iScriptTM cDNA Synthesis Kit. Next, the cDNA was amplified using the following primer pairs: DDX3Y_F, 5´-GGACGTGTAGGAAACCTTGG-3´; DDX3Y_R, 5´-GCCAGAACTGCTACTTTGTCG-3´; HPRT1_F, 5´-TGCTGAGGATTTGGAGAAGG-3´; HPRT1_R, 5´-CAACAGGTCGGCAAAGAACT-3´, and the following PCR parameters; initial denaturation at 95°C for 3 min, 40 cycles at 95°C for 15s, 60°C for 15s and 72°C for 30s, followed by final elongation at 72°C for 5 min. PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and visualized under UV illumination. The *DDX3Y* gene is present on the Y chromosome, while *HPRT1* was used as the reference gene. Therefore, when one band was present, the embryo was classed as female while two bands denoted a male (Figure 3.2).

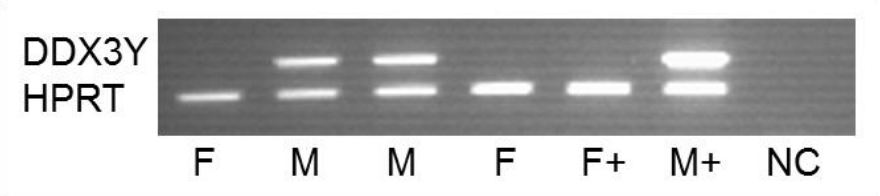

***Figure 3.2**. PCR products of the transcripts used for sexing the blastocysts. For embryo sexing, DDX3Y, a gene present on the Y chromosome, and HPRT, as reference gene, were used. The embryos that expressed only the HPRT transcript were considered to be female, while those that expressed the two transcripts were considered to be males.*

RNA AMPLIFICATION AND PREPARATION OF THE SEQUENCING LIBRARY

Concentration and quality of the total RNA extracted were examined using a Quant-iT RiboGreen RNA Assay kit (Life Technologies) and an RNA 6000 Pico Chip (Agilent Technologies), respectively. Subsequently, 1 ng of RNA was used to start the library preparation. First, cDNA was synthesized using the "SMARTer Ultra low input RNA for the Illumina, High Volume Kit" (Clontech) mostly following the manufacturer's instructions. For the PCR reaction, 12 cycles were chosen. Second, the "Low Input Library Prep Kit" (Clontech) was used to prepare the libraries for sequencing. Libraries were prepared according to the manufacturer's instructions and 4+6 cycles were chosen during the PCR reaction, as in the protocol. Libraries were quantified by qPCR, according to the February 2011 Illumina's protocol "Sequencing Library qPCR Quantification protocol guide." A high sensitivity DNA chip (Agilent Technologies) was used to control the size, distribution and quality of the libraries. Sequencing was performed by a rapid run on 2 lanes of the Illumina Hiseq-2500 sequencer using 2x100 bp paired-end reads; 12 samples were run per lane in equimolar quantities.

READ ALIGNMENT AND DIFFERENTIAL GENE-EXPRESSION ANALYSIS

After quality trimming and trimming of 12 nucleotides from the 5' terminal end of the reads, the latter were mapped to the *Bos taurus* UMD 3.1.75 bovine genome build (Ensembl). Specific imprinted genes of interest were added: *USP29* (NCBI Gene ID: 788661), *PEG3* (444864), *APEG3* (100169896), *MEG3* (100335527), *H19* (100126192), and *XIST* (338325), using the CLC Genomics Workbench 7.0.4 software (CLC Bio). Introns and exons were defined

in the annotation. For protein-coding genes, introns and exons were distinct while the whole sequence of the ncRNAs was considered as an exon. In this study, only uniquely mapping exon reads were considered in the analysis. The quality trimming was performed based on the Phred base quality scores and a limit setting equal to 0.03. To determine if a gene was expressed, a reads per kilobase per million (RPKM) >0.4 threshold was used for normalization. To quantify gene expression, the RNA-seq Analysis Tool from the CLC software was used, employing standard settings and mapping to gene regions only. Differentially expressed genes in the comparisons described in the experimental design were determined using the empirical analysis of the DGE tool employing standard settings. This Tool implements the 'Exact Test' for two-group comparisons developed by Robinson and Smyth (Robinson and Smyth 2008) and incorporated in the EdgeR Bioconductor package (Robinson *et al.* 2010). The EdgeR package was used to validate the differential expression results obtained from the CLC software. To minimize false positives, within each two-group comparison, genes that did not have an exon read count of at least 15 for all the replicates in at least one of the groups under consideration were filtered out prior to differential expression analysis; this cut-off of 15 exon read counts corresponds to the inflexion point of the read frequency distribution for most samples. To assess the validity of the EdgeR´s assumption of similar library distributions, boxplots of the raw read counts were rendered for the genes withheld during the previous filtering step. Genes were considered to be differentially expressed if they had a Benjamini-Hochberg corrected p-value <0.05 (Benjamini and Hochberg 1995) and an absolute fold change (FC) of ≥2.

HIERARCHICAL CLUSTERING AND PRINCIPAL COMPONENT ANALYSIS

Heatmap and Principal component analyses were performed using trimmed mean of M-values (TMM) normalized read counts of genes differentially expressed (Benjamini-Hochberg corrected p-value <0.05, absolute FC ≥2) in at least one of the comparisons. Both analyses were performed in the R statistical software package using the heatmap and prcomp functions respectively.

FUNCTIONAL ANNOTATION OF GENES

Ensembl gene IDs of differentially expressed genes (Benjamini-Hochberg corrected p-value <0.05, absolute FC ≥2) were analyzed with the ClueGO 2.1.5 plugin (Bindea *et al.* 2009) of the Cytoscape 3.1.1 software (Shannon *et al.* 2003) to obtain functional annotation of the genes in terms of enrichment of gene ontologies (levels 3–8) related to biological process, molecular function, and cellular component. In addition, a KEGG pathway enrichment analysis was performed. Only Gene Ontology (GO)-terms or pathways that contained at least 5 of the queried genes were considered. In addition, at least 5% of all genes associated with a GO-term or pathway needed to consist of genes in the query. Finally, results were considered statistically significant when the Benjamini-Hochberg corrected p-value was <0.01.

VALIDATION OF THE RNA-SEQ DATA

Reverse-transcription quantitative real-time PCR (RT-qPCR) was used to validate the differential expression of 4 selected genes in 10 individual early blastocysts from each condition. All RT-qPCR experiments were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009).

RNA from individual early blastocysts was extracted as described previously. A minus RT control was then performed with primers for *GAPDH* to check the removal of all the contaminating genomic DNA (Goossens *et al.* 2005). First-strand cDNA was generated from the total amount of RNA using the iScript cDNA synthesis kit (BioRad) which uses oligo(dT) and random hexamer primers, according to the manufacturer´s instructions. After reverse transcription, the cDNA was diluted 3-fold and used for downstream PCR. Combined with embryo sexing, cDNA quality control was performed based on Verbeke *et al.* (Verbeke *et al.* 2015). For this assay, a primer pair of *HPRT1* (reference gene) and a primer pair of *DDX3Y* (present in chromosome Y), which could amplify respectively 421 and 196 bp, were used. Only embryos that could amplify the 421 bp amplicon were included in the study. Good quality cDNA from male embryos showed two bands, one of the reference gene and one specific for the Y chromosome, while good quality cDNA from female embryos showed only the highest band, that of the reference gene. PCR reactions were performed in 10 µL reaction volumes with the following program: initial denaturation at 95°C for 5 min, 40 cycles at 95°C for 45s,

64°C for 45s and 72°C for 90s, followed by final elongation at 72°C for 5 min. PCR products were electrophoresed on 2% agarose gel containing ethidium bromide, visualized under UV illumination and sequenced for verification.

Reference genes used for normalization (*GAPDH*, *YWHAZ*, and *SDHA*) were selected according to previous studies (Goossens *et al.* 2005; Li *et al.* 2014) and confirmed by geNorm, with M-values ranging from 0.671 to 0.628, as described by Vandesompele *et al.* (Vandesompele *et al.* 2002). The primer pairs for the four selected genes (*PHGDH*, *HMGCS1*, *IDI1*, and *SFN*) and the specific primer annealing temperatures are given in Table 3.1.

Table 3.1. *Primer sequence, amplicon size, annealing temperature, and PCR efficiency of the primers of the genes used for RNA-seq validation by RT-qPCR, reference genes and genes used for the sexing and quality control assay.*

Gene	Primer sequence 5´-3´	Amplicon size (bp)	Annealing Ta (°C)	PCR Efficiency
HMGCS1	Forward: CCTCAGTGCATTAGACCGCTGCT Reverse: CTGAACCAGTTTACAATAGGGTGAGTGGA	142	65	100%
IDI1	Forward: ACGCTAAGATTACCTTCCCAGGGTGT Reverse: CTCTGTGCTGCTCTTCTTACTCCAATAGC	115	66	100%
PHGDH	Forward: AGGCCGCAACCAGAAAGGGCAT Reverse: TTCCGCTCCCACTTGCCATCCTT	151	67	110%
SFN	Forward: AAAGTCGGGTCTTCTACCTGAAAATGAAG Reverse: GGCATCTCCTTCTTGCTGATGTCC	145	66	100%
GAPDH	Forward: TTCAACGGCACAGTCAAGG Reverse: ACATACTCAGCACCAGCATCAC	119	62	96%
YWHAZ	Forward: GCATCCCACAGACTATTTCC Reverse: GCAAAGACAATGACAGACCA	120	60	104%
SDHA	Forward: GCAGAACCTGATGCTTTGTG Reverse: CGTAGGAGAGCGTGTGCTT	185	60	108%
HPRT1	Forward: CCCAGCGTGGTGATTAGCGATG Reverse: AAGTCTGCATTGTCTTCCCAGTGTC	421	64	
DDX3Y	Forward: AAGGCAGTTCAGGGTGGAGTTGTA Reverse: CGCTCAAATCTGCCAAAGCCAGT	196	64	

PCR reactions were performed in a 10 µL reaction volume on a BioRad CFX 96 PCR Detection system, including 5 µL Sso Advanced SYBR Green Supermix (BioRad), 600 nM of each primer (with the exception of *SFN* for which 60 nM was used) and 2 µL of diluted embryo cDNA. The PCR program consisted of an initial denaturation step at 95°C for 3 min, 40 cycles of denaturation for 5s at 95°C and a combined primer annealing-extension step for 30s at the specific primer annealing temperature, during which fluorescence was measured. A melting curve was produced afterwards by heating the samples from 70°C to 95°C in 0.5°C increments for 5s while fluorescence was measured. Each reaction was run in duplicate. PCR efficiencies were calculated by a relative standard curve of 5 points with ¼ dilution, derived from cDNA of pooled bovine blastocysts. All PCR efficiencies were between 90% and 110%, and the correlation coefficient (R^2) between 0.990 and 1. The geometric mean of the reference genes was used to calculate the normalization factor. The mean of the duplicates and the exact PCR efficiencies were used to calculate the raw data, which, for each gene and sample was divided by the respective normalization factor to obtain a normalized value according to the method described by Hellemans *et al.* 2007). The normalized values were used to determine differential expression between *in vivo* vs. serum-containing and *in vivo* vs. serum-free conditions. Normality of the data was studied and a log transformation was applied when the data were not normally distributed. The normally distributed data, before or after log transformation and with homogeneity of variances, were analyzed by One-Way ANOVA combined with Hochberg Post Hoc correction. When data was not normally distributed, the non-parametric Mann-Whitney test was performed. Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

GENERAL EXPRESSION PROFILE OF BLASTOCYSTS PRODUCED BY DIFFERENT CULTURE CONDITIONS

The global gene expression pattern of 24 early blastocysts was analyzed using the Illumina HiSeq 2500 system. Eight blastocysts produced *in vitro* in serum-containing medium and eight other blastocysts produced *in vitro* in serum-free medium were compared with eight blastocysts derived *in vivo*, each blastocyst constituting one replicate. In addition, between three and five blastocysts of each sex were represented in every culture condition, to avoid

the possibility that a sex bias could interfere with the interpretation of the results, as highlighted by Bermejo-Alvarez *et al.* who reported that the expression of about one third of the genes of a blastocyst are influenced by the sex (Bermejo-Alvarez *et al.* 2010).

On average, 28 million reads were generated per embryo. Of the total sequenced fragments, 51% (*in vivo*), 52.1% (serum-free) and 49.4% (serum-containing) could be mapped to the Ensembl UMD 3.1 reference genome and, of these, 94% (*in vivo*), 93% (serum-free) and 92% (serum-containing) were uniquely mapped to specific regions in the bovine genome (Table 2, Table S1). All the uniquely mapped fragments corresponded to annotated genes and of, an average of 62% (*in vivo*), 67% (serum-free), and 71% (serum-containing) mapped to annotated exons; the remainder overlapped with annotated introns (Tables 3.2, S3.1 and Figure 3.3D). Only reads uniquely mapped to exons were considered in this study since focus was placed on the expression of known annotated genes. The higher prevalence of intronic reads (~30%) is not uncommon in bovine RNA-seq experiments, when both random priming and/or oligo-dT primers are used to prepare libraries. For instance, Chitwood *et al.* 2013 (Chitwood *et al.* 2013) reported up to 40% intronic reads, Graf *et al.* 2014 (Graf *et al.* 2014) around 30%, and Huang and Khatib 2010 (Huang and Khatib 2010) some 20%. Moreover, these numbers are also in line with the 30-40% of intronic reads reported by Clontech Laboratories, manufacturers of the SMARTer Ultra Low RNA Kit-HV used for library preparation (Sara Gonzalez-Hilarionm Takara-Clontech, personal communication. A number of factors could explain the presence of intronic reads 1) the presence of pre-mRNA or unspliced RNA; 2) alternatively spliced forms, where an entire or partial intron sequence in one transcript may serve as an exon in another transcript, and 3) the presence of antisense non-coding RNAs overlapping the intronic regions; reads obtained with SMARTer Ultra Low Kits are not strand-specific so that expression coming from each strand cannot be differentiated.

Table 3.2. *Summary of sequence read alignments to the reference genome. The numbers correspond to the mean ± standard deviation (SD) of all the replicates per condition.*

Sample	***In vivo* derived embryos (mean ± SD)**	**Serum-free produced embryos (mean ± SD)**	**Serum-containing produced embryos (mean ± SD)**
Paired end reads	14,701,822.4 ± 1,066,098.2 x 2	12,731,192.8 ± 615,768.7 x 2	14,670,296.3 ± 2,890,016 x 2
Total sequenced fragments	14,701,822.4 ± 1,066,098.2	12,731,192.8 ± 615,768.7	14,670,296.3 ± 2,890,016
Total mapped fragments	7,508,158 ± 709,020.2	6,636,857.8 ± 552,752.8	7,240,822.8 ± 2,076,161.5
Uniquely mapped fragments	7,028,287.1 ± 662,984.5	6,174,226.4 ± 523,270.2	6,691,366.4 ± 1,939,025.2
Fragments mapped to annotated genes	7,028,287.1 ± 662,984.5	6,174,226.4 ± 523,270.2	6,691,366.4 ± 1,939,025.2
Fragments mapped to annotated exons	4,353,683.6 ± 619,988.6	4,119,767.6 ± 760,316.3	4,730,978.5 ± 1,683,623.3
Fragments overlapped with annotated introns	2,674,603.5 ± 157,817.1	2,054,458.8 ± 430,089.5	1,960,387.9 ± 465,486.1

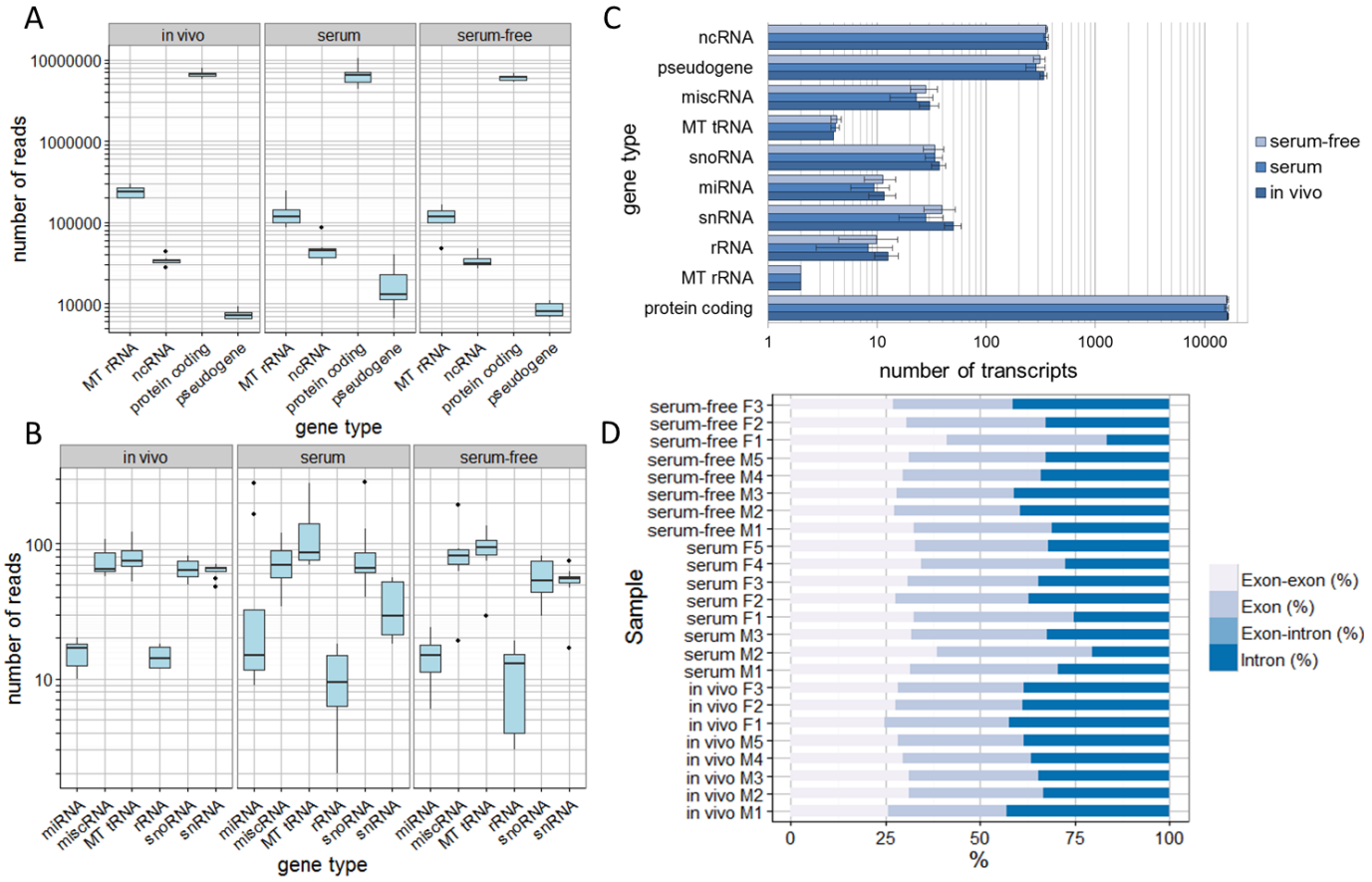

Figure 3.3. *Distribution of reads and transcripts among gene types and regions. Total reads belonging to, (A) highly represented RNA species and, (B) low represented RNA species per culture condition. The total reads correspond only to total exon reads in protein-coding genes, while for the rest of the RNA species correspond to the total reads of all the gene regions. The variability of the number of reads belonging to every RNA species among the different embryos of each condition was larger in the in vitro embryos, especially in the presence of serum, compared to in vivo embryos. (C) Transcript distribution among the different RNA species in the different conditions. (D) Distribution of reads uniquely mapped among gene regions in the different blastocysts (each blastocyst constitutes a replicate).*

The distribution of total reads among different RNA species per culture condition is depicted in figure 3.3A/B. In summary, an average of 6,359,679 (96.4%) of the total reads corresponded to protein-coding genes as a result of mRNA enrichment performed during library preparation. The next most represented RNA species was mitochondrial rRNA to which only 2.5% of the reads corresponded. Finally, the remaining 1.1% of the total reads was distributed among miscellaneous RNAs, including pseudogenes and different non-coding RNAs. The approximately 6 million total exon reads located in protein-coding genes corresponded to an average of 16,185 protein coding transcripts, with very similar values between the different culture conditions (Figure 3.3C). The total number of genes detected in the analysis ranged from 9,560 in a male embryo produced in serum-containing medium to 11,290 in a male embryo produced in serum-free medium with an average of 10,717 genes all round (Table 3.3); this represents almost 50% of the 22,000 protein-coding genes estimated to be present in the cattle genome (Elsik *et al.* 2009). The results were also in line with those of Chitwood *et al.* using RNA sequencing in individual blastocysts (with 11,039 genes detected) (Chitwood *et al.* 2013). In contrast, they were lower than those found in other studies where 11,924 (Jiang *et al.* 2014), 13,724 (Graf *et al.* 2014) and 17,634 genes were detected (Driver *et al.* 2012). This disparity might be due to technical differences such as: 1) the sequencing depth, which usually correlates with the number of genes detected, 2) the RPKM threshold used for normalization to determine when a gene is expressed and, 3) the alignment parameters that determine which reads were mapped and how non-uniquely mapping reads were dealt with.

Table 3.3. *Number genes expressed in each embryo with RPKM >0.4, each embryo constituting a replicate.*

Condition	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean ± SD
Male *in vivo*	11,022	10,750	10,414	10,370	10,654	10,642 ± 256.6
Female *in vivo*	10,919	10,785	10,467	-	-	10,724 ± 232.2
Male serum	10,276	9,560	10,168	-	-	10,001 ± 386
Female serum	10,300	11,009	11,205	11,073	11,113	10,940 ± 364.8
Male serum-free	10,733	11,115	10,451	11,290	10,776	10,873 ± 331.5
Female serum-free	10,651	11,122	10,985	-	-	10,919 ± 242.3

To verify if the embryos produced within the same condition were more similar to each other than to those produced under different conditions, a hierarchical clustering and a principal component analysis (PCA) were performed (Figure 3.4). In the hierarchical clustering two clusters were formed thereby separating the embryos produced in the presence of serum from those embryos produced under the other two conditions. This cluster further divided into two minor clusters, which separated embryos derived *in vivo* from embryos produced in serum-free medium. Within each minor cluster the embryos were grouped by sex, except in serum conditions where female and male embryos were mixed. In the PCA, the first three principle components of the differentially expressed (DE) genes were represented and, here, blastocysts produced under the same conditions were plotted together. In the PCA, as had been already observed in the hierarchical clustering, embryos produced in serum-free conditions showed a pattern closer to *in vivo* derived embryos than to those produced in serum. It was also observed that the variability of gene expression within the embryos produced in serum-containing medium was greater than under the other two conditions, even though all the embryos selected for the study were early blastocysts with a score of 1 according to the International Embryo Transfer Society (IETS) guidelines. A similar observation was made by Cote *et al.* who compared 10 different *in vitro* culture conditions and in which embryos produced in the presence of serum (in both maturation and culture) presented the largest variability in the pattern of gene expression among the replicates (Cote *et al.* 2011).

The results of the hierarchical clustering and the PCA confirmed the rigor of the study and showed that embryos produced under the same conditions were more similar to each other than to the rest. It also gave the first indication that the global gene expression pattern of embryos produced under serum-free conditions is more similar to embryos derived *in vivo* than those produced *in vitro* in the presence of serum.

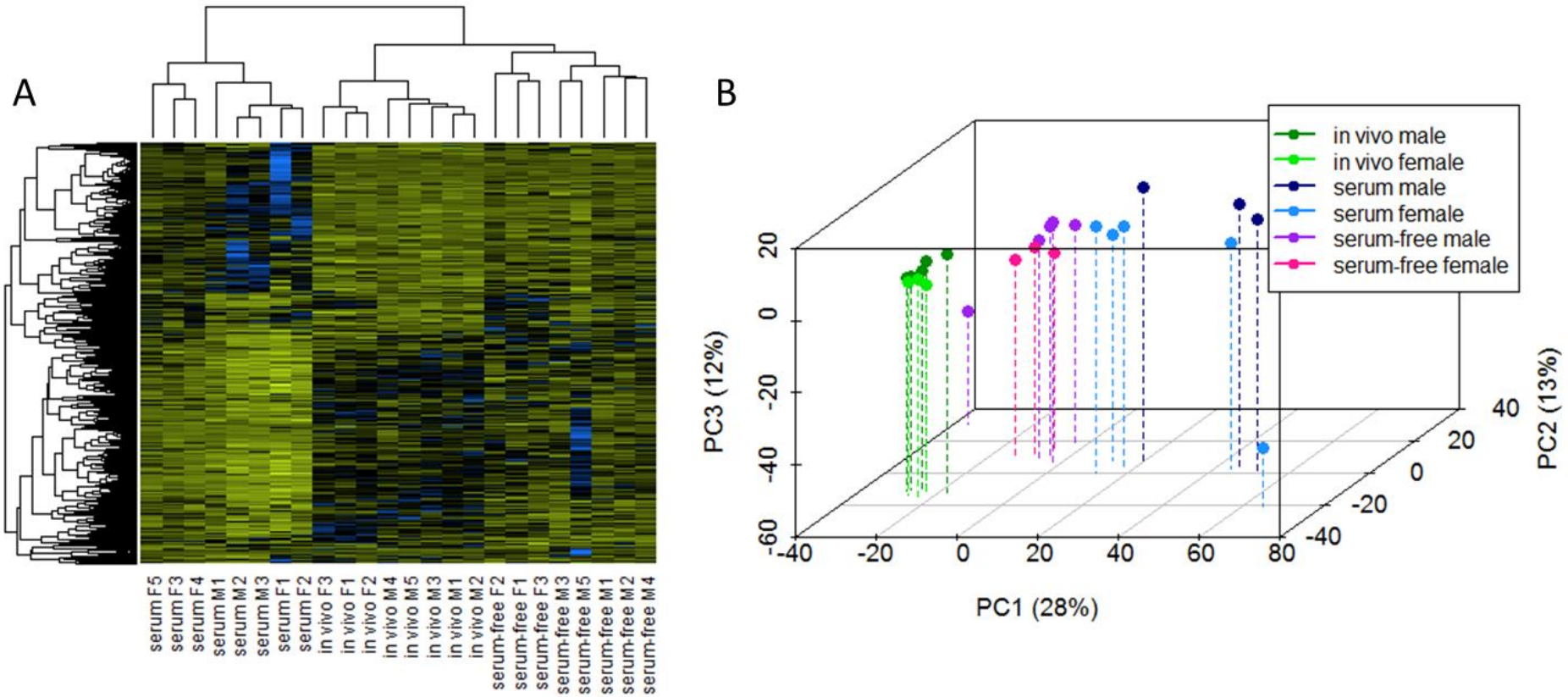

Figure 3.4. *Hierarchical clustering and PCA of differentially expressed genes among the different blastocysts. (A) Heatmap including all the differentially expressed genes. The color spectrum, ranging from yellow through black to blue, represents TMM normalized expression values scaled between -4.5 and 4.5, indicating low to high expression. Two main clusters were formed, with embryos cultured in the presence of serum in one cluster and in vivo derived and serum-free embryos in the other. (B) PCA of the 24 embryos used in the study considering all the differentially expressed genes. Each dot represents one blastocyst.*

GENE EXPRESSION DEVIATIONS COMPARED TO *IN VIVO* DERIVED EMBRYOS

The number of genes differentially expressed between *in vitro* produced, *in vivo* derived embryos, with a False Discovery Ratio (FDR) corrected p-value of <0.05 and an absolute fold change (FC) value of ≥2, were calculated. The aim was to determine if embryos produced in serum-free conditions showed a gene expression pattern closer to that of *in vivo* derived embryos than embryos produced in the presence of serum (Table 3.4, S3.2, S3.3, S3.4, S3.5, S3.6 and S3.7).

Considering female and male embryos together, those produced in serum-free conditions were more similar to *in vivo* derived embryos, having five times fewer DE genes (207) than embryos produced in serum-containing medium (1,109). Remarkably, this large difference was even greater when only male embryos were compared. In male embryos produced in the presence of serum, 1,283 genes were differentially expressed while the equivalent figure was only 191 in male embryos produced in serum-free conditions. When only female embryos were compared with *in vivo* derived embryos, the number of DE genes was drastically reduced for embryos produced in serum-containing medium, while it was maintained in embryos produced under serum-free conditions. Nevertheless, the number of DE genes in female embryos produced in serum-containing medium (456) was more than twice than that of female embryos produced in serum-free medium (192). These results indicated that serum supplementation had a greater impact on embryos than serum-free conditions, as was suggested by the clustering and PCA, with the greatest impact affecting male embryos.

Table 3.4. *Number of differentially expressed genes and number of up- or down regulated genes in Group 1 vs. Group 2, with an FDR corrected p-value of <0.05 and an absolute fold change (|FC|) ranging from ≥0 to ≥100.*

Comparison		**|FC|≥0**	**|FC|≥2**	**|FC|≥5**	**|FC|≥10**	**|FC|≥20**	**|FC|≥50**	**|FC|≥100**
All *in vivo* vs. all serum-containing generated	**Total**	**2,186**	**1,109**	**148**	**51**	**24**	**8**	**5**
	Up	1,080	624	78	22	5	1	1
	Down	1,106	485	70	29	19	7	4
All *in vivo* vs. all serum-free generated	**Total**	**534**	**207**	**18**	**3**	**1**	**0**	**0**
	Up	223	55	1	0	0	0	0
	Down	311	152	17	3	1	0	0
Male *in vivo* vs. male serum-containing generated	**Total**	**1,801**	**1,283**	**329**	**136**	**68**	**25**	**11**
	Up	919	760	236	91	46	15	6
	Down	882	523	93	45	22	10	5
Male *in vivo* vs. male serum-free generated	**Total**	**311**	**191**	**28**	**6**	**1**	**1**	**1**
	Up	124	59	4	1	0	0	0
	Down	187	132	24	5	1	1	1
Female *in vivo* vs. female serum-containing generated	**Total**	**458**	**456**	**203**	**85**	**41**	**14**	**7**
	Up	231	229	85	24	9	2	1
	Down	227	227	118	61	32	12	6
Female *in vivo* vs. female serum-free generated	**Total**	**221**	**192**	**77**	**27**	**11**	**2**	**1**
	Up	80	65	21	5	3	0	0
	Down	141	127	56	22	8	2	1
Male *in vivo* vs. female *in vivo* generated	**Total**	**225**	**119**	**25**	**18**	**13**	**12**	**11**
	Up	69	40	17	14	11	11	11
	Down	156	79	8	4	2	1	0
Male serum-containing vs. female serum containing generated	**Total**	**54**	**54**	**47**	**35**	**29**	**17**	**14**
	Up	17	17	15	14	12	11	11
	Down	37	37	32	21	16	6	3
Male serum-free vs. female serum-free generated	**Total**	**54**	**48**	**19**	**13**	**9**	**8**	**8**
	Up	14	14	12	10	8	8	8
	Down	40	34	7	3	1	0	0

In murine embryos it was reported that, after exposure of morulae to heat stress *in vitro* for 24h followed by subsequent transfer to pseudopregnant recipients, only 28% of the fetuses obtained on day 14 were males, compared to 55% in the control group (Perez-Crespo *et al.* 2005). Also in humans, maternal stress during early pregnancy, or at the time of conception, led to a lower male-female ratio at term (Hansen *et al.* 1999). Therefore, it seems that male embryos are more susceptible to suboptimal environmental conditions than female ones and, as demonstrated in the present study, this susceptibility might be reflected in their gene expression pattern. Surprisingly, studies from previous decades repeatedly reported more male than female calves born after *in vitro* embryo production (using mostly media supplemented with serum) (Van Soom *et al.* 1994; Massip *et al.* 1996; Camargo *et al.* 2010), suggesting that more male than female embryos survive after *in vitro* production. To explain this apparent contradiction, it has been hypothesized that, in previous days, a bias towards more male calves was introduced by the practice of selecting fast cleaving embryos for transfer (Gutierrez-Adan *et al.* 2014). Since male embryos develop faster than female embryos, more males would therefore have been transferred (Gutierrez-Adan *et al.* 2014).

Not only male embryos produced in the presence of serum showed a more deviant transcriptome than their female counterparts compared to embryos derived *in vivo*; moreover, only 275 of their DE genes were also differentially expressed in females. Similarly, even though male and female embryos produced in serum-free conditions showed the same number of differentially expressed genes compared to embryos derived *in vivo* (191 and 192 respectively), only 44 of those genes were common in both sexes. This generates additional evidence to support the concept that male and female embryos respond differently to the environment. However, for the two conditions, the common DE genes of both sexes were also equally up- or down-regulated. Moreover, 38 out of the 44 common DE genes in both sexes were down-regulated in *in vivo* derived embryos compared to those generated in serum-free medium. In the case of embryos generated in serum-containing medium, 127 out of 275 common genes were down-regulated *in vivo*.

Interestingly, more DE genes (FC ≥2) were up-regulated in embryos produced in serum-free conditions compared with *in vivo* derived embryos, irrespective of whether all embryos, male, or female, were compared. Surprisingly, more DE genes were up-regulated in *in vivo* derived embryos compared to embryos produced in serum-containing medium when all and

only the male embryos were considered. When all the embryos were considered, this tendency was reversed to give an FC ≥20, while when only male embryos were considered, the tendency was maintained for all FC. When female embryos were compared, the same number of DE genes were up-regulated in both groups with FC ≥2. From FC ≥5 onwards, more genes were up-regulated in embryos produced in serum-containing medium than in embryos derived *in vivo* (Table 3.4).

In a comparable study, Driver *et al.* reported that between embryos derived *in vivo* and those produced *in vitro* most of the DE genes were up-regulated *in vivo* for all the FC. Their study differed in a few important points from the present experiments. For example, they used a hybrid serum-IVM/serum-free-IVC *in vitro* conditions and *in vivo* derived embryos from non-superovulated cows, whereas we used superovulated cows. It is known that the superovulatory treatment can have an effect on the gene expression pattern of embryos recovered (Market-Velker *et al.* 2010b). Moreover, no replicates were performed in the study by Driver *et al.*, in which only one pool of *in vivo* derived and one pool of *in vitro* produced embryos were compared (Driver *et al.* 2012). Therefore, whether the differences in the results between Driver *et al.* and the present study are due to the effect of the superovulation/*in vitro* production conditions on the gene expression pattern of the embryos, or to the lack of replicates in the Driver *et al.* study needs to be investigated further.

GENES DIFFERENTIALLY EXPRESSED BETWEEN MALE AND FEMALE EMBRYOS

Only a few genes were differentially expressed when male and female embryos were compared under the same conditions (Table 3.4, S3.8, S3.9, and S3.10). In *in vivo* derived embryos, 119 genes were differentially expressed. Under *in vitro* conditions, the number of DE genes between male and female embryos decreased dramatically to less than half, with only 54 and 48 genes differentially expressed in serum-containing and serum-free medium, respectively. Of those, more genes (79, 37, and 34 respectively) were up-regulated in females than in males under all conditions.

The low number of genes found to be differentially expressed between male and female embryos contrasts with previous reports. Bermejo-Alvarez *et al.* showed one third (2,921) of the expressed genes to be differentially expressed between male and female bovine blastocysts produced in serum-containing medium (Bermejo-Alvarez *et al.* 2010). We found

only 225 DE genes between sexes in *in vivo* derived embryos and 54 in both serum-free and serum-containing *in vitro* conditions (Table 3.4), considering in both studies FDR corrected p-value <0.05 and all fold changes. These differences may be due to the large sample size used in the Bermejo-Alvarez *et al.* study increasing the statistical power to detect smaller differences between groups. However, when only genes with absolute fold changes of ≥2 were considered, the results of both studies became very similar. Bermejo-Alvarez *et al.* found that 55 genes were differentially expressed between male and female embryos produced in serum-containing medium, which corresponds almost exactly with the 54 genes that we found to be different according to sex in embryos produced in serum-containing medium. Similarly, in our serum-free medium, the number of DE genes between the sexes was 48. Only in embryos derived *in vivo* did we find a larger number of DE genes (119). On the other hand, Chitwood *et al.* found 168 genes to be differentially expressed between male and female bovine blastocysts (p-value <0.05 and absolute FC ≥2) produced *in vitro* in a hybrid serum-free/serum containing culture system, in which serum was added to the medium after three days of culture (Chitwood *et al.* 2013). However, in this last study, only one female embryo was compared with four male embryos. Surprisingly, when comparing our present results with the list of DE genes provided by Bermejo-Alvarez *et al.*, the similarities are very few. For example, out of the 85 genes with common identity to ours, 20 of them (24%) were common with those in *in vivo* derived embryos, 8 (9%) were common with embryos produced in serum-free conditions and only 2 genes (*XIST* and *BDH2*) with embryos produced in the presence of serum.

Furthermore, the chromosomal distribution of the DE genes between the sexes was studied (Figure 3.5). Here, a χ^2 analysis was performed to test for significant differences in chromosome location between genes up-regulated in each sex and expressed genes. The only chromosome that displayed significant differences between DE genes up-regulated in female embryos (FDR corrected p-value <0.05 and absolute FC value ≥2) and expressed genes was the X chromosome under all conditions. It accounted for 54.4% *in vivo*, 62.2% in serum-containing, and 61.7% in serum-free conditions of the total up-regulated DE genes in female embryos, while only 3.6% *in vivo*, 4% in serum-containing, and 3.8% in serum-free embryos of the expressed transcripts were X-linked. This number of X-linked expressed transcripts is very similar to the 2.8% reported by Bermejo-Alvarez *et al.* 2010). However, the percentage of X-linked genes among the up-regulated DE genes in female embryos was much higher in the

present study than the level of 18.1% noted by Bermejo-Alvarez *et al.* (Bermejo-Alvarez *et al.* 2010). Moreover, in contrast to Bermejo-Alvarez *et al.*, we found chromosome 17 to display significant differences between DE genes up-regulated in male embryos and expressed genes under all conditions. In fact, 12.5% for *in vivo* derived, 29.4% for serum-containing, and 35.7% for serum-free of the total up-regulated DE genes in male embryos belonged to chromosome 17, while only 2.9% of the expressed transcripts were from chromosome 17 under all conditions. Surprisingly, the X chromosome also displayed significant differences between DE genes up-regulated in male embryos produced in serum-containing medium and expressed genes, with 23.5% of the DE genes up-regulated in males being X-linked.

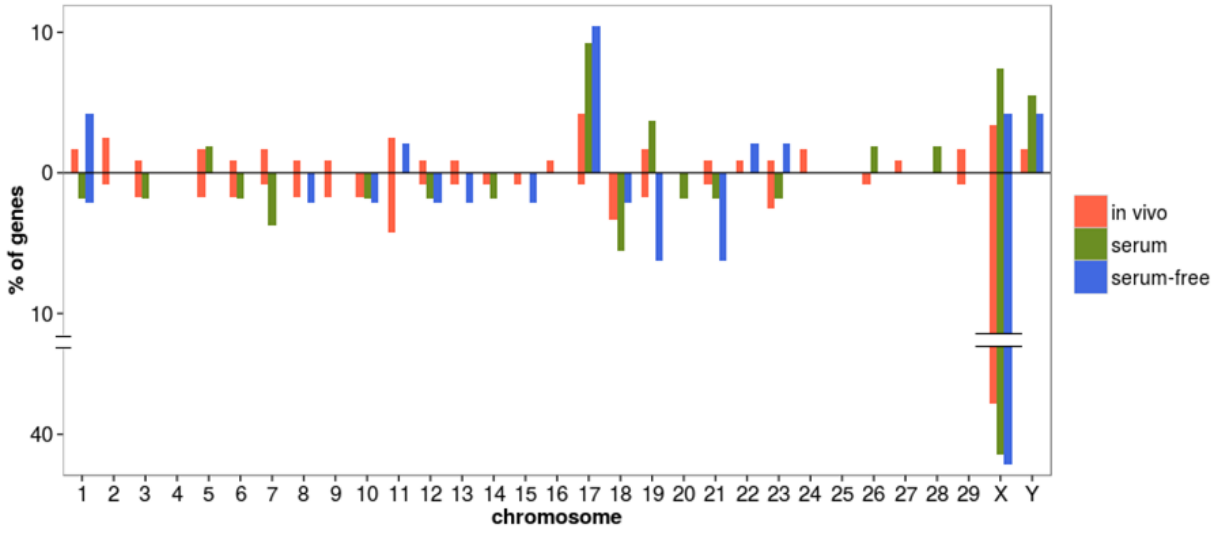

***Figure 3.5**. Chromosome distribution of the differentially expressed genes between male and female embryos. The bars represent the percentage of genes differentially expressed (FDR corrected p-value <0.05 and |FC| ≥2) up-regulated (above) and down-regulated (below) in males vs. females belonging to each chromosome in each of the three conditions studied.*

RNA-SEQ DATA VALIDATION

RNA-seq data was validated by RT-qPCR using 4 genes (*PHGDH*, *HMGCS1*, *IDI1*, and *SFN*) in 10 individual early embryos per condition, performing a total of 8 comparisons. The values obtained were normalized with 3 stable reference genes (*GAPDH*, *YWHAZ*, and *SDHA*). Three genes, *IDI1*, *HMGCS1*, and *PHGDH*, showed higher expression in embryos produced in

serum-free medium compared to those derived *in vivo*, with averages of 4.02-,7.43-, and 3.31-fold differences, respectively, using RNA-seq and 2.94- (p-value <0.1), 6.14-, and 3.47- fold differences, respectively, when measured using RT-qPCR, (p-value <0.05; Figure 3.6). *HMGCS1* showed higher expression in embryos produced in serum-containing medium than in those derived *in vivo*, with an average of 1.6-fold differences when using RNA-seq and 1.67-fold differences when measuring by RT-qPCR (p-value <0.1). Finally, *SFN* showed higher expression in embryos derived *in vivo* compared to those produced in serum-containing medium, with an average of 10.27-fold differences using RNA-seq, and 8.56-fold differences when measuring with RT-qPCR (p-value <0.05). The rest of the comparisons did not show significant differences, either when analyzed by RNA-seq, or by RT-qPCR, (p-values in all cases >0.2). Therefore, these 4 genes showed similar patterns of mRNA abundance in RNA-seq and RT-qPCR.

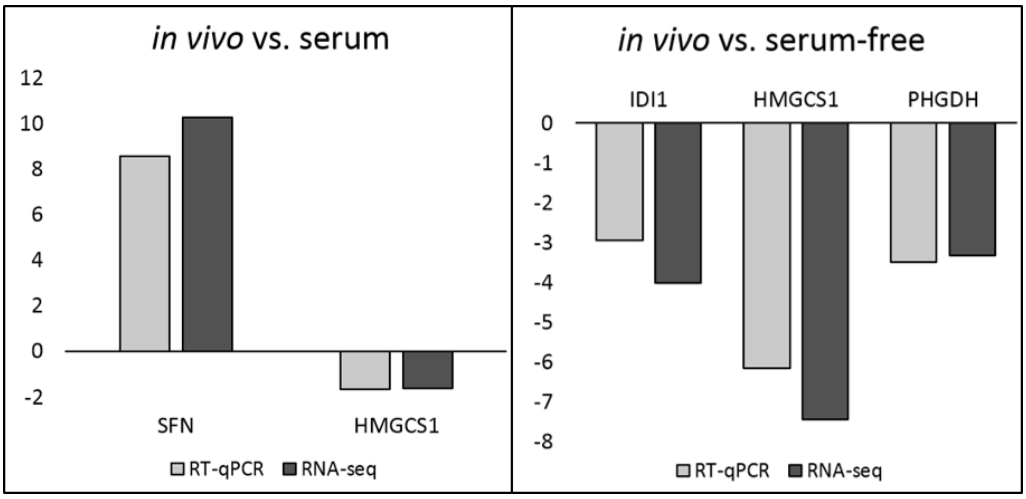

***Figure 3.6**. Comparison of the differential expression of 4 genes (SFN, HMGCS1, IDI1, and PHGDH) between in vivo derived vs. serum-containing produced (left) and in vivo derived vs. serum-free produced (right) embryos analyzed by RNA-seq (dark grey) vs. RT-qPCR (light grey). Only comparisons that showed differences at p-value <0.1 are depicted.*

FUNCTIONAL ANALYSIS

The functional analysis of the differentially expressed genes between the groups was performed using the Cytoscape 3.1.1 software and considering significant only annotations with a Benjamini-Hochberg corrected p-value of <0.01.

When the gene ontology (GO) of the DE between all the embryos produced in serum-containing medium and all the embryos derived *in vivo* was examined, 17 biological processes, such as "lipid metabolic process" and "DNA repair" (Figure 3.7, Table S3.11), 8 molecular functions, such as "anion binding" and "actin binding," and 21 cellular components were over-represented. Additionally, 6 KEGG pathways, including "lysosome" and "metabolic pathways" were over-represented. The vast majority of the genes included in the GO terms and KEGG pathways were up-regulated in *in vivo* derived embryos and only two biological processes ("DNA repair" and "histone ubiquitination") and two cellular components ("organelle and intracellular organelle lumen") had more genes up-regulated in embryos produced in serum-containing medium than in *in vivo* derived embryos. A reduced number of terms and pathways were over-represented when only male embryos were taken into account. In particular, 10 biological processes (Figure 3.7, for the full list of over-represented terms and pathways see Table S4 of the published version), 4 molecular functions, 28 cellular components, and 3 KEGG pathways were over-represented, most of them in common with the previous comparison, but with a few differences such as "alpha-amino acid metabolic process" biological process, "enzyme binding" molecular function, "mitochondrion" cellular component, and "cysteine and methionine metabolism" KEGG pathway. When only female embryos were considered, 8 biological processes (Figure 3.7, for the full list of over-represented terms and pathways see Table S4 of the published version), 4 molecular functions, and "mitochondrial matrix" cellular component were over-represented. Surprisingly, only "oxidoreductase activity, acting on CH-OH group of donors" molecular function was common in the comparison of all the embryos. When only male or female embryos were considered, most of the terms and all the pathways had more genes up-regulated in *in vivo* derived embryos.

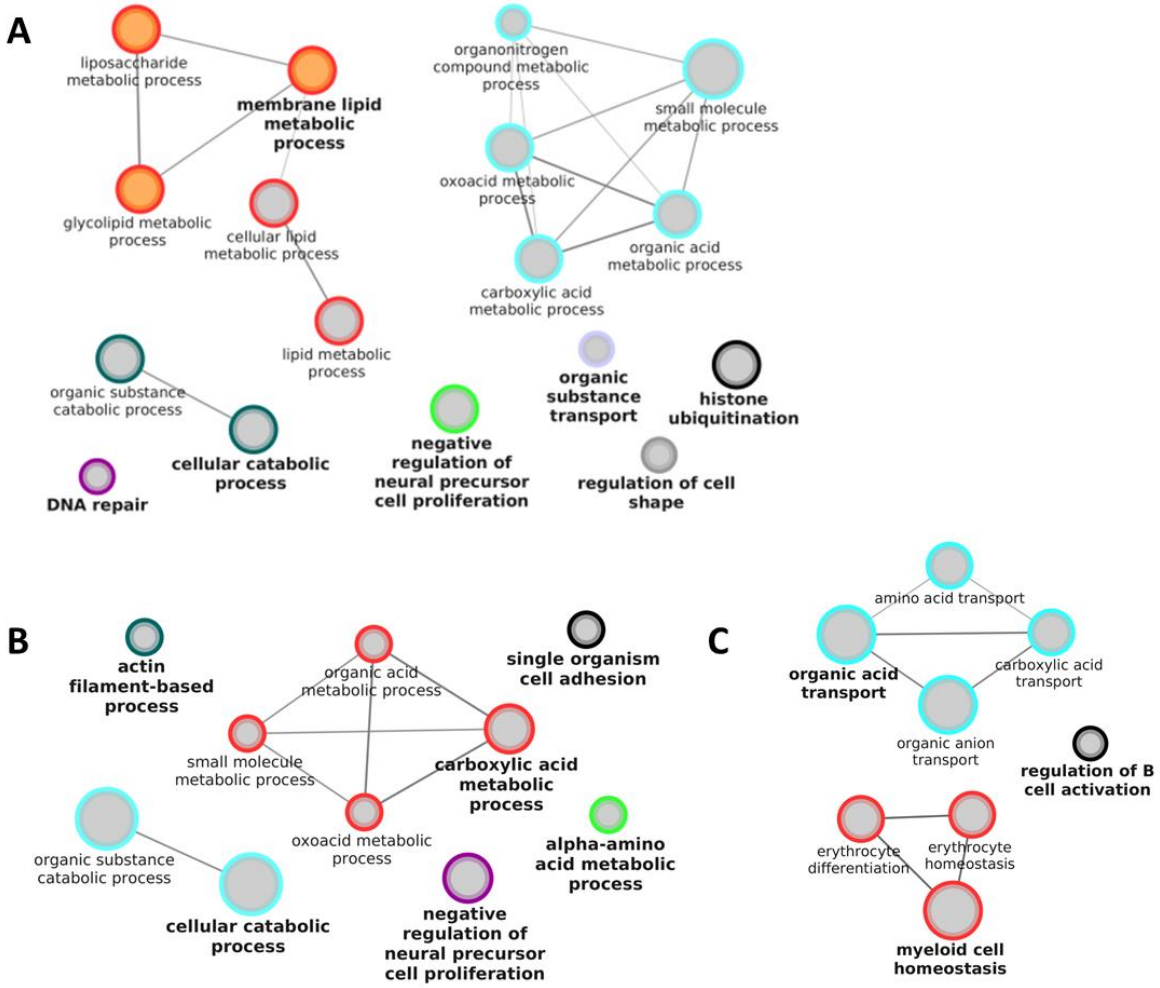

Figure 3.7*. GO biological processes enriched in genes differentially expressed in in vivo vs. serum. Enriched GO biological processes with Benjamini-Hochberg corrected p-value <0.01, genes per term/pathway ≥5, Goterm levels 3–8, in the genes differentially expressed (FDR corrected p-value <0.05, |FC| ≥2) in, (A) all the embryos, (B) only male embryos and, (C) only female embryos. The analysis was performed with the ClueGO 2.1.3 plugin of the Cytoscape 3.1.1. The size of the nodules represents their significance; orange nodules are only composed of genes up-regulated in vivo, grey nodules are composed of at least 5 genes up- and 5 genes down-regulated in vivo.*

On the other hand, the study of the gene ontology of the DE genes between all the embryos produced in serum-free medium and all the embryos derived *in vivo*, led to 2 molecular functions “oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor” and “tetrapyrrole binding”, and 23 biological processes being over-represented. These biological processes were related, among other things, to cholesterol and amino acid metabolism and biosynthesis (Figure 3.8, Table S3.12). Furthermore, 3 KEGG

pathways, such as the “p53 signaling pathway” and “glycine, serine, threonine metabolism” were also over-represented. If only male embryos were considered, 18 biological processes mostly common to the previous comparison (Figure 3.8, for the full list of over-represented terms and pathways see Table S4 of the published version), two molecular functions and two KEGG pathways were over-represented. Finally, when only the female embryos were considered, 10 biological process (Figure 3.8, for the full list of over-represented terms and pathways see Table S4 of the published version) and one KEGG pathway (“Glycine, serine, threonine metabolism”) were over-represented, all in common with the ones over-represented when all the embryos were considered. In contrast to the situation observed when comparing *in vivo*-derived embryos with those produced *in vitro* in the presence of serum, all the GO biological processes and KEGG pathways contained mostly, or even only, genes down-regulated in *in vivo*-derived embryos, when compared to embryos produced in serum-free medium.

In the mouse, it has been reported that during the first days of pregnancy, the mother supplies most of the cholesterol needed by the embryo (Tint *et al.* 2006). Therefore, embryos derived *in vivo* will not have to synthetize cholesterol, in contrast to embryos produced *in vitro* in serum-free medium. Embryos produced *in vitro* in the presence of serum do not show over-representation of these pathways, indicating that serum supplementation during embryo culture provides the lipids necessary for development. In addition, the over-representation of cholesterol biosynthesis and sterol synthesis in cattle embryos produced *in vitro* (with serum present during maturation but the subsequent culture in serum-free conditions) compared to *in vivo* derived embryos was described previously by Driver *et al.* (Driver *et al.* 2012). These results also suggest that the over expression of genes involved in lipid biosynthesis depends on the embryonic culture conditions, but is independent of the oocyte maturation conditions used, indicating that the oocytes matured in the presence of serum do not accumulate all the lipids needed for their development. Interestingly, biological processes involved in “DNA repair,” the “p53 signaling pathway” and “response to reactive oxygen species” had more genes up-regulated in embryos produced *in vitro* than embryos derived *in vivo*. This indicates that *in vitro* production is a source of stress for embryos regardless of culture conditions. No functional categories were over-represented when male and female embryos were compared.

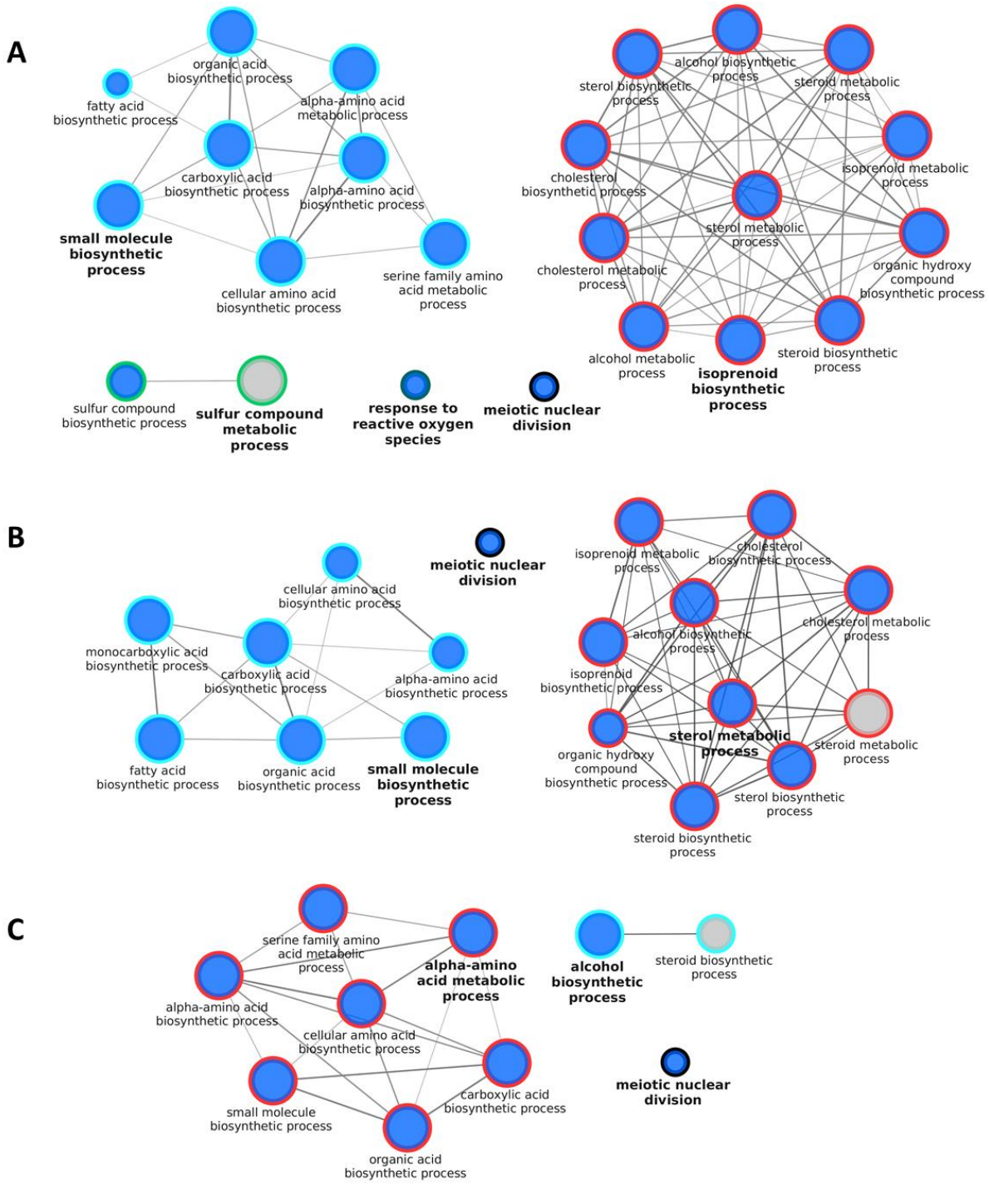

***Figure 3.8**. GO biological processes enriched in genes differentially expressed in the in vivo derived embryos vs. those cultured in serum-free medium. Enriched GO biological processes with a Benjamini-Hochberg corrected p-value of <0.01, genes per term/pathway ≥5, Goterm levels 3–8, in the genes differentially expressed (FDR corrected p-value <0.05, |FC| ≥2) in, (A) all the embryos, (B) male embryos only and, (C) female embryos only. The analysis was done with the ClueGO 2.1.3 plugin of the Cytoscape 3.1.1. The size of the nodules represents their significance; blue nodules are composed only of genes down-regulated in vivo, grey nodules are composed of at least 5 genes up- and 5 genes down-regulated in vivo.*

CONCLUSIONS

Embryos produced under serum-free conditions showed gene expression patterns that were more similar to those derived *in vivo* than embryos produced *in vitro* in the presence of serum. This was true regardless of the sex of the embryos. Importantly, male embryos were most affected by suboptimal *in vitro* conditions, (i.e. serum supplementation) and they showed a more deviant gene expression pattern than their female counterparts. Embryos produced in the presence of serum showed reduced expression of genes related to small molecule metabolism, and an enhanced expression of genes related to DNA repair. However, embryos produced under serum-free conditions had a deviant lipid and amino acid metabolism gene expression pattern compared to *in vivo* derived embryos, indicating that the serum-free conditions used in this study require further optimization to fulfill the needs of the embryo during preimplantation development.

All the results of this study provide evidence for the strong and abnormal effects of adding serum to embryo culture medium. Therefore, the formulation of the culture media should move towards serum-free supplementations, even for research purposes, since experiments performed on embryos produced in the presence of serum may lead to erroneous conclusions.

AVAILABILITY OF SUPPORTING DATA

The data sets supporting the results of this article are available in the NCBI Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE74675.

ACKNOWLEDGEMENTS

The authors thank Petra Van Damme and Isabel Lemahieu for their excellent technical assistance and CRV Belgium for kindly providing the *in vivo* derived blastocysts. This work was funded by the IWT (grant no. 111438 and GOA - project no. 01G01112). The authors are members of EPICONCEPT Cost Action FA1201.

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CHAPTER 4

OPTIMIZATION OF IMMUNOFLUORESCENT STAINING TO STUDY EPIGENETICS IN HORSE AND CATTLE ZYGOTES

SUMMARY

In this chapter, the immunofluorescent staining required to study the dynamics of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in horse and cattle embryos, with especial attention to zygotes, is optimized. Those two species were chosen because fetal bovine serum (FBS) is used as medium supplementation for *in vitro* embryo production in both cases. Interestingly, the use of FBS increases the incidence of large offspring syndrome, an epigenetic disorder, in cattle but not in horse. In future experiments, we want to determine the different effect of FBS, and other medium supplementations, on 5mC and 5hmC patterns of those two species, and to see if this effect could be observed by immunofluorescence.

First, several DNA dyes were first tested in order to select the best performing dye after the strong epitope retrieval treatments necessary to expose the 5mC and 5hmC epitopes (Chapter 4.1).

Subsequently, a proper technique to determine the parental pronuclear origin was optimized based on the asymmetric pattern of two histone 3 modifications. For equine zygotes, the asymmetric pattern histone 3 lysine 9 tri-methylation (H3K9me3) was studied (Chapter 4.2), while the pattern of H3K9me3 and histone 3 lysine 27 di-tri-methylation (H3K27me2-3) was described for bovine zygotes (Chapter 4.3). In both chapters, the possibility of combining the immunostaining of H3K9me3/H3K27me3 with the 5mC and 5hmC immunostaining was discussed as well, with focus on the epitope retrieval treatments.

In this chapter, we only used equine and bovine zygotes. Nevertheless, according to our observations, the best DNA counterstaining (Ethidium homodimer 2) can be used in every species, as the epitope retrieval treatments applied to bovine and equine zygotes are similar or even stronger to those used in other species. The immunofluorescent staining optimized here can also be applied to all preimplantation developmental stages in cattle and horse. However, since the asymmetric pattern of histone modifications is not completely conserved between species, it should be tested before applying it in other species.

CHAPTER 4.1

DNA COUNTERSTAINING FOR METHYLATION AND HYDROXYMETHYLATION IMMUNOSTAINING IN BOVINE ZYGOTES

Adapted from:

Heras S, Forier K, Rombouts K, Braeckmans K, Van Soom A. ***Anal Biochem. (2014)*** *454:14-6.*

SUMMARY

Immunostaining is the preferred technique to assess differences in methylation and hydroxymethylation status of both pronuclei in single zygotes. DNA counterstaining is needed to delineate the pronuclear area for quantification purposes. For a correct epitope retrieval of 5-methylcytosine and 5-hydroxymethylcytosine in bovine zygotes, 1h denaturation with 4N HCl is needed. However, DNA stains are sensitive to denaturation. Therefore, four DNA stains were tested after 1h of denaturation with 4N HCl in this study. After this treatment, DAPI and Hoechst failed to bind DNA, but both Propidium Iodide and Ethidium homodimer 2 successfully bound it and both pronuclei were stained.

INTRODUCTION

Immunofluorescent staining is a technique commonly used to evaluate the presence of 5-methylcytosine (5mC), and recently also 5-hydroxymethylcytosine (5hmC), in preimplantation embryos of different mammalian species. It is especially used in zygotes because at that stage differences between methylation and hydroxymethylation patterns in maternal and paternal pronuclei can be evaluated (Dean *et al.* 2001; Fulka *et al.* 2004; Reis Silva *et al.* 2011; Salvaing *et al.* 2012; Li and O'Neill 2013). This technique has been used to assess the dynamics of DNA methylation and hydroxymethylation in pronuclear stages (Fulka *et al.* 2004; Reis Silva *et al.* 2011; Salvaing *et al.* 2012; Li and O'Neill 2013) and to compare the effect of different Assisted Reproductive Technology (ART) conditions on the epigenetic status of zygotes (Dean *et al.* 2001; Abdalla *et al.* 2009). For revealing the dynamics of the methylation and hydroxymethylation pattern of bovine zygotes, correct epitope retrieval is essential and, as such, a "deep denaturation" step is needed. If the epitope retrieval is only partial, the results may be biased because of this technical drawback (Li and O'Neill 2012). At the same time, DNA counterstaining is essential to perform a correct analysis; it is necessary to assess the total pronuclear area in order to quantify the presence of methylation and hydroxymethylation. DNA dyes are very sensitive to denaturation because this step causes changes in the structure of the DNA that totally or partially hamper the binding of the dyes. For bovine zygotes, different DNA dyes have been used within different immunostaining protocols, producing controversial results (Dean *et al.* 2001; Beaujean *et al.* 2004; Park *et al.* 2007). Therefore there is a need for evaluation of different DNA dyes to assess which is the superior stain for delimitation of the pronuclear DNA after the denaturation step.

In this study, four different fluorescent nuclear stains - Propidium Iodide (PI), Ethidium homodimer 2 (EthD-2), Hoechst and DAPI (4´,6-diamidino-2-phenylindole) - were tested as DNA counterstaining for methylation and hydroxymethylation immunostaining in bovine zygotes. PI and EthD-2 are orange-red fluorescent nuclear stains that bind to DNA by intercalating between bases with no sequence preference (Markovits *et al.* 1979; Suzuki *et al.* 1997), while Hoechst and DAPI fluorescence in blue and bind to the minor groove of the DNA with preference for regions rich in adenine and thymine (Portugal and Waring 1988). DAPI can also bind to RNA by adenine-uracil selective intercalation. PI, Hoechst and DAPI are the DNA

stains most commonly used; EthD-2 has been previously used as DNA counterstaining for methylation immunostaining in rabbit zygotes (Reis Silva *et al.* 2011).

MATERIALS AND METHODS

EMBRYO PRODUCTION

Bovine zygotes were obtained by routine *in vitro* production methods (Wydooghe *et al.* 2014). Briefly, ovaries from Holstein cows were collected at a local slaughterhouse and processed within 2h. Cumulus oocyte complexes were aspirated from follicles between 4 and 8 mm in diameter and matured in groups of 60 in 500 µL of maturation media consisting in modified TCM-199 medium (GIBCO-BRL Life Technologies) supplemented with 20 ng/mL EGF (Epidermal Growth Factor; Sigma E4127) and 50 µg/mL gentamicin for 22h at 38.5°C in 5% CO_2-in-air. Frozen-thawed sperm from a Holstein bull was separated through a discontinuous Percoll gradient (45 and 90% (v/v); VWR International). The final sperm concentration of $1x10^6$ spermatozoa/mL was adjusted in IVF-TALP, consisting of bicarbonate buffered Tyrode solution, supplemented with 6 mg/mL BSA (bovine serum albumin; Sigma A8806) and 20 µg/mL heparin (Sigma). Matured oocytes were washed and incubated with the sperm for 22h. Presumptive zygotes were collected 22h after fertilization, fixed in 4% paraformaldehyde for 20 min at room temperature (RT) and kept in 2% paraformaldehyde at 4°C for a maximum of 4 days until immunostaining was performed.

At least three replicates were performed, and a total of 296 zygotes were stained in this study, including test groups, negative controls and non-immune immunoglobulin controls. Of those, 64 zygotes were stained with PI, 92 with EthD-2, 95 with Hoechst and 45 with DAPI.

IMMUNOFLUORESCENT STAINING

The collected zygotes were washed in phosphate-buffered saline (PBS, Gibco) containing 0.5% bovine serum albumin (Sigma-Aldrich) (PBS-BSA) for 1h at RT. After washing, the embryos were subsequently permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and 0.05% Tween 20 (Sigma-Aldrich) in PBS for 1h at RT, and then washed 3 times for 2 min in PBS-BSA. Epitope retrieval was performed by treating the embryos with 4N HCl for 1h at RT and with 100 mM TrisHCl (pH 8.5) for 10 min at RT, followed by washing three times with PBS-BSA

for 5 min. The zygotes were treated with 1 mg/mL RNase A (Affymetrix) for 30 min at 37°C to avoid the binding of PI, EthD-2 and DAPI to RNA (Suzuki *et al.* 1997) and were washed three times with PBS-BSA for 5 min. The zygotes were subsequently incubated with the nuclear stain - either PI (25 µg/mL) in PBS-BSA for 30 min at RT, EthD-2 (0.5 nM) in PBS-BSA for 30 min at RT, Hoechst 33342 (0.1 mg/mL) in PBS-BSA for 20 min at RT or DAPI (5 µg/mL) in PBS-BSA for 20 min at RT and washed four times with PBS-BSA for 2 min. All nuclear stains were purchased from Molecular Probes. Unspecific binding of DAPI occurs when the pH is different from 7.0. Therefore, an equilibration step of 5 min with McIlvaine's buffer (pH 7.0) was performed before the incubation with DAPI. After washing, the zygotes were incubated in 30% goat serum (Gibco) and 0.05% Tween 20 in PBS (blocking solution) at 4°C overnight. Embryos used as negative control remained in blocking solution until the time of incubation with the secondary antibodies. Before incubation with the antibodies, the zygotes were washed three times with PBS-BSA for 2 min.

EVALUATION OF METHYLATION

After washing, test zygotes were incubated with 0.01 mg/mL mouse anti 5mC (Epigentek) in blocking solution overnight at 4°C. Part of the zygotes were incubated with mouse IgG1 (non-immune) control antibody (0.01 mg/mL; cat. no. M7894 Sigma-Aldrich) in blocking solution overnight at 4°C. After incubation with the primary antibodies, all of the zygotes, including negative controls were washed three times for 10 min in PBS-BSA and subsequently incubated with the secondary antibody goat anti-mouse FITC (9 µg/mL; Life technologies) for 1h at RT.

EVALUATION OF HYDROXYMETHYLATION

After washing, the test zygotes were incubated with 1:150 rabbit anti 5hmC (Active Motif) in blocking solution overnight at 4°C. Part of the zygotes were incubated with rabbit IgG (non-immune) control antibody (0.01 mg/mL; cat. no. 011-0102 Rockland) in blocking solution overnight at 4°C. After incubation with the primary antibodies, all of the zygotes, including negative controls, were washed three times for 10 min in PBS-BSA and subsequently incubated with the secondary antibody goat anti-rabbit FITC (10 µg/mL; Molecular Probes) for 1h at RT.

FLUORESCENCE MICROSCOPY

To avoid fading, evaluation of the embryos was performed the next day by fluorescence microscopy with a Leica DM 5500 B microscope with excitation filters of BP 360/40 nm, BP 450/90 nm, BP 560/40 nm and a 100 W mercury lamp. The emission spectra were respectively detected by BP 470/40 nm (Blue), LP 515 nm (Green) and BP 645/75 nm (Red) filters. Images were acquired by Image Database program (Leica). The results were confirmed using a Nikon C1si confocal microscope (Nikon) using a Plan Apo VC 60X oil immersion objective (Nikon). On this system, laser lines of 408 nm SS, 488 nm SS, 561 nm DPSS or 636 nm SS were respectively combined with a BP 440/40 nm (Blue), BP 525/50 nm (Green), BP 596/50 nm (Orange) and LP 660 nm (Red) filters. For each wavelength digital optical sections were collected using Z-series acquisition every 0.35 µm.

RESULTS AND DISCUSSION

The incubation with the DNA stains was performed before blocking since preliminary experiments showed that incubation with blocking solution was impeding the binding of PI and EthD-2 to the DNA. The HCl treatment was found to be critical for the immunostaining process. Different times of denaturation and different HCl concentrations dramatically affected the visualization of 5mC and 5hmC in bovine zygotes. Previous experiments (data not shown) revealed that treating the embryos with 2N HCl for 1h was not enough to achieve the complete epitope retrieval and hence 5mC and 5hmC could not be visualized. When HCl normality was increased to 4N, 1h of denaturation was found to be optimal.

Incubations with the non-immune immunoglobulins for both 5mC (mouse) and 5hmC (rabbit) were performed in all replicates, and no signal was observed, showing that the blocking step was enough to avoid unspecific bindings of the antibodies.

The binding of all the tested DNA stains was shown to be sensitive to denaturation treatment. After application of such a strong denaturation agent for 1h (Figure 4.1.1), DAPI and Hoechst were no longer able to bind DNA, and no staining of the pronuclei was observed in any zygote. The other two DNA stains tested, PI and EthD-2, could still bind to the DNA after the denaturation treatment, and although variation in the intensity of the counterstaining was

observed between zygotes, both pronuclei were stained and correctly visualized in all the zygotes.

The affinity of PI for the DNA after denaturation appears to be much lower than of EthD-2, with the latter showing a stronger signal that was more resistant to fading. This can be due to the fact that EthD-2 is also able to bind single-stranded DNA (ssDNA). Therefore, DNA intercalators appear to be superior for use as DNA counterstaining than dyes that bind to the minor groove of the DNA under these immunostaining conditions. Other commercial DNA stains such as YOYO-1, a bis-intercalator member of the TOTO family, have been used as counterstaining for 5mC in mouse embryos (Fabian *et al.* 2009; Santos *et al.* 2013). In mice, the denaturation conditions were different, with shorter incubation times and reduced HCl normality. If YOYO-1 is to be applied as counterstaining for 5mC and 5hmC in bovine zygotes, it needs to be properly tested under the conditions described in the current article. Anti ssDNA antibodies have been previously used as counterstaining for 5mC and 5hmC (Wossidlo *et al.* 2011; Salvaing *et al.* 2012) and constitute an alternative to DNA stains. An advantage is that they can bind DNA after strong denaturation treatments, but an important disadvantage is that the immunostaining process takes longer to perform and the use of three antibodies simultaneously makes triple staining (5mC, 5hmC and ssDNA) more difficult.

In conclusion, for epitope retrieval in 5mC and 5hmC immunostaining of bovine zygotes, 1h of denaturation with 4N HCl is needed. In these conditions, both PI and EthD-2 can be used as DNA counterstaining because they still bind DNA, with EthD-2 being the preferred one because it gives the strongest signal. Importantly, DAPI and Hoechst cannot be used because they fail to bind DNA after the denaturation conditions needed for the correct visualization of 5mC and 5hmC.

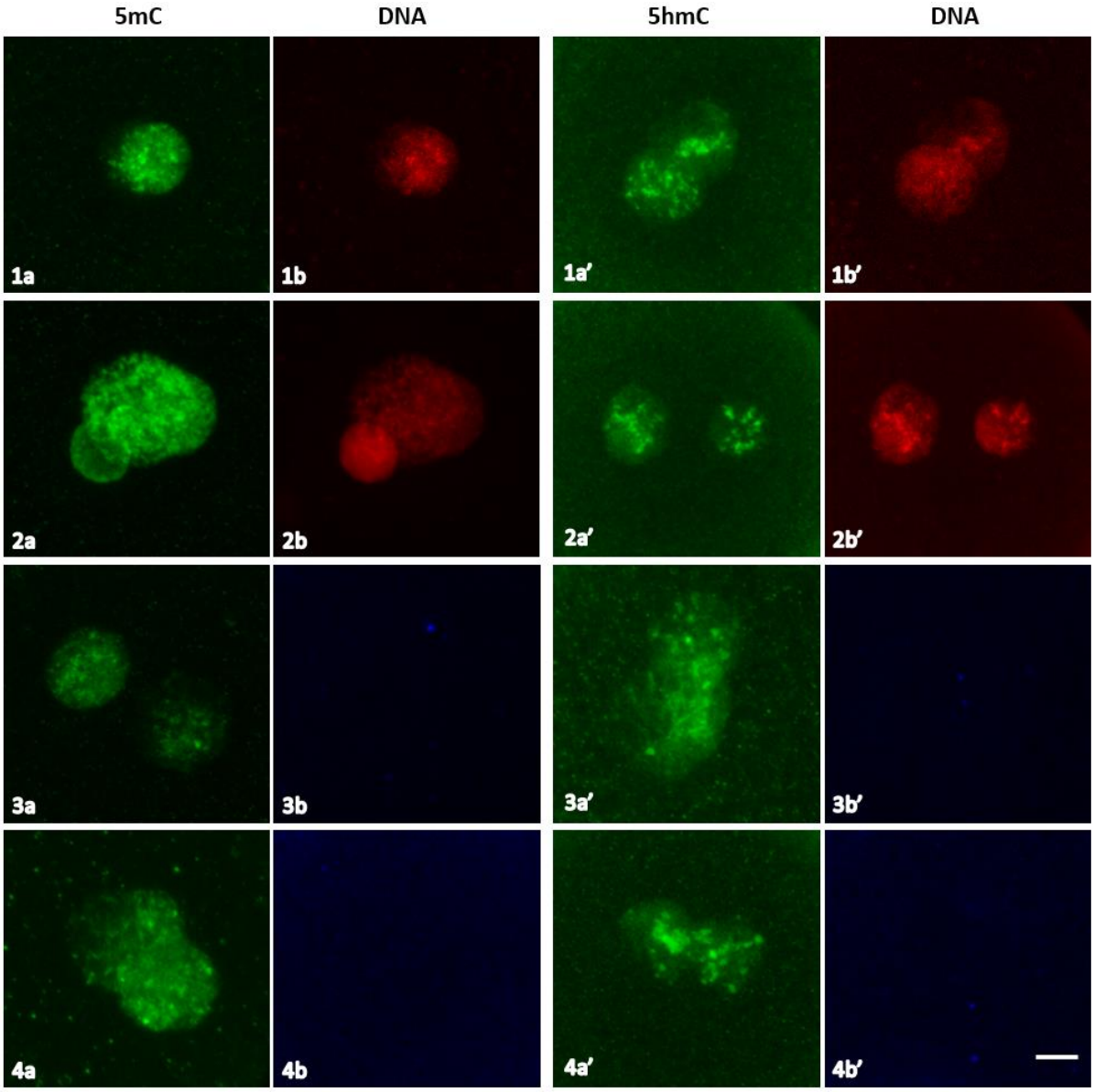

Figure 4.1.1. *Immunofluorescent localization of 5mC (a-b) and 5hmC (a'-b') after 1h denaturation with 4N HCl in pronuclear stage bovine zygotes by confocal microscopy. Both 5mC and 5hmC antibodies were indirectly labeled with FITC. DNA was counterstained with PI (1), EthD-2 (2), Hoechst (3) or DAPI (4). All the images were taken at the same magnification (600x). The scale bar represents 10 µm.*

ACKNOWLEDGEMENTS

The authors thank Petra Van Damme and Isabel Lemahieu for their excellent technical assistance. This work was funded by the Agentschap voor Innovatie door Wetenschap en Technologie (IWT) grant number 111438 and GOA - project no. 01G01112 – Pathways to pluripotency and differentiation in embryos and embryonic stem cells.

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CHAPTER 4.2

ASYMMETRIC HISTONE 3 METHYLATION PATTERN BETWEEN PATERNAL AND MATERNAL PRONUCLEI IN EQUINE ZYGOTES

Adapted from:

Heras S, Smits K, Leemans B, Van Soom A. ***Anal Biochem. (2015)*** *471:67-69.*

SUMMARY

Hoechst staining has traditionally been used to evaluate fertilization and parental origin of pronuclei. However, prevalence of parthenogenetic activation cannot be distinguished accurately by this protocol, and variation of relative pronuclear size and position makes it impossible to determine parental origin. We demonstrate that in equine zygotes, the epigenetic modification histone 3 lysine 9 tri-methylation (H3K9me3) shows an asymmetric pattern between maternal and paternal pronuclei. H3K9me3 immunostaining appears to be a robust technique to identify the parent of origin of equine pronuclei; it can be used in combination with 5-methylcytosine and 5-hydroxymethylcytosine immunostaining, and applied to evaluate fertilization.

INTRODUCTION

In many domestic mammals, including the horse, the traditional method to evaluate if fertilization has occurred is the presence or absence of pronuclei. Because the cytoplasm of domestic animals is too lipid rich and opaque to visualize the pronuclei, the DNA needs to be stained for pronuclear evaluation, with Hoechst being the most commonly used fluorescent DNA dye (Hinrichs *et al.* 2002; McPartlin *et al.* 2009). However, a major disadvantage is that this method is not able to differentiate between normal fertilization and parthenogenesis, after which two pronuclei are also formed. Furthermore, it does not allow to evaluate whether monopronuclear zygotes have a mono- or bi-parental origin (van der Heijden *et al.* 2009).

To evaluate DNA methylation and hydroxymethylation patterns in pronuclear stages, it is necessary to identify the parental origin of the pronuclei. The most commonly used criteria to distinguish between maternal and paternal pronuclei are the relative pronuclear size (with the paternal pronucleus being larger) and the relative distance to the polar body (with the maternal pronucleus being closer), but these criteria cannot be applied in all species (Young and Beaujean 2004; Jeong *et al.* 2007). As an alternative, the differential pattern of the epigenetic modification H3K9me3 between paternal and maternal pronuclei, in which H3K9me3 is only present in the maternal pronucleus while being absent in the paternal pronucleus, has been successfully used in several species, such as rabbit (Reis Silva *et al.* 2011), human (van der Heijden *et al.* 2009) and mouse (van der Heijden *et al.* 2009) to determine the parental origin of the pronuclei. In pigs (Jeong *et al.* 2007) and cattle (Park *et al.* 2007), this asymmetric distribution of H3K9me3 is present during the early stages of pronuclear development, where the paternal pronucleus displays a weaker H3K9me3 level, whereas with the progression of the pronuclear development, the levels of H3K9me3 increase until they are similar in both pronuclei.

The aim of the current study was to evaluate whether the differential pattern of H3K9me3 in the maternal and paternal pronuclei is conserved in the horse and ultimately to develop a robust system to determine the parental origin of the pronuclei, which can also be applied to evaluate fertilization. This is especially important in the horse because in this species conventional *in vitro* fertilization (IVF) is not successful and *in vitro* embryos are generated by means of intracytoplasmic sperm injection (ICSI). Differentiation between

fertilization and parthenogenesis is very useful to evaluate the effect of the injection process during ICSI or the effect of different capacitation and activation media while studying IVF. In order to combine H3K9me3 immunostaining with 5-methylcytosine (5mC) or 5-hydroxymethylcytosine (5hmC), to determine the pattern of expression of 5mC and 5hmC between maternal and paternal pronuclei, we needed to test whether the H3K9me3 signal is present after the denaturation conditions required for 5mC and 5hmC epitope retrieval.

MATERIALS AND METHODS

EMBRYO PRODUCTION

Equine zygotes were produced *in vitro* by piezo drill-assisted ICSI as described previously (Smits *et al.* 2012). In brief, equine oocytes were matured in DMEM-F12 based medium for 24h. After maturation cumulus cells were removed, and only oocytes with a visible polar body were fertilized by ICSI. Equine mature oocytes were parthenogenetically activated by incubation with 5 µM Ionomycin (Sigma-Aldrich) in non-capacitation medium (McPartlin *et al.* 2008) for 5 min, holding in non-capacitation medium for 20 min followed by incubation in 2 mM 6-DMAP (Sigma-Aldrich) in DMEM/F12 for 4h (Hinrichs, personal communication). Presumptive zygotes and parthenotes were cultured in DMEM-F12 with 10% fetal bovine serum in 90% N_2, 5% CO_2 and 5% O_2 at 38.2°C, and collected 22-24h after production.

After collection, zygotes and parthenotes were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and kept in 2% PFA at 4°C for a maximum of 4 days until immunostaining was performed.

Three immunostaining replicates were performed using ICSI zygotes obtained in three independent experiments. A total of 50 equine ICSI zygotes and 10 parthenotes were stained in this study, including test groups, negative controls and non-immune controls.

IMMUNOFLUORESCENT STAINING

The zygotes and parthenotes were subsequently washed in phosphate-buffered saline (PBS, Gibco) containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) (0.5% PBS-BSA) for 1h at RT. After washing, they were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and 0.05% Tween 20 (Sigma-Aldrich) in PBS for 1h at RT, and washed three times for 5 min in 0.5% PBS-BSA.

Zygotes and parthenotes were blocked in PBS containing 2% BSA (2% PBS-BSA) for 1h at RT and washed three times for 2 min in 0.5% PBS-BSA.

After washing, zygotes and parthenotes were incubated with the primary antibody rabbit anti-H3K9me3 (1:100, Active Motif) in 2% PBS-BSA overnight at 4°C. Part of them were incubated with the non-immune control antibody rabbit IgG control antibody (0.01 mg/mL; Rockland 011-0102) in 2% PBS-BSA overnight at 4°C. Zygotes that served as negative control remained in 2% PBS-BSA without the primary antibody.

Subsequently, all zygotes and parthenotes including the non-immune and the negative control were washed three times for 10 min in 0.5% PBS-BSA and treated with 1 mg/mL RNase A (Affymetrix) for 30 min at 37°C to avoid the binding of the nuclear stain Ethidium homodimer 2 (EthD-2; Molecular Probes) to RNA (Suzuki *et al.* 1997). They were subsequently washed three times in 0.5% PBS-BSA for 5 min and incubated with the nuclear stain 0.5 nM EthD-2 in 0.5% PBS-BSA for 30 min at RT. The zygotes and parthenotes were washed four times for 2 min in 0.5% PBS-BSA and incubated with the secondary antibody goat anti-rabbit FITC (20 µg/mL; Molecular Probes) for 1h at RT in 30% goat serum.

In order to combine H3K9me3 with 5mC or 5hmC immunostaining, we tested whether anti H3K9me3 antibody could be used in combination with the treatment necessary for 5mC and 5hmC epitope retrieval. Therefore, zygotes were incubated with the primary antibody rabbit anti H3K9me3, washed three times for 10 min with 5% PBS-BSA, postfixed with 4% PFA for 25 min and subsequently washed three times for 10 min in 5% PBS-BSA. It was observed that before the epitope retrieval treatment, a postfixing step was needed to correctly visualize H3K9me3. Epitope retrieval was performed by treating the zygotes with 4N HCl for 30 min at RT and 100 mM TrisHCl (pH 8.5) for 10 min at RT, followed by three times washing with 5% PBS-BSA for 5 min and 20s treatment with 0.25% (w/v) trypsin at 37°C (Sigma). Tryptic digestion was stopped by incubation with 30% goat serum in PBS for 3 min at RT (Heras *et al.* 2014b). After epitope retrieval zygotes followed the protocol as described previously.

FLUORESCENCE MICROSCOPY

To avoid fading, evaluation of the embryos was performed the next day by fluorescence microscopy with a Leica DM 5500 B microscope with excitation filters of BP 450/90 nm, BP 560/40 nm and a 100 W mercury lamp. The emission spectra were respectively detected by LP 515 nm (green) and BP 645/75 nm (red) filters. Images were acquired by Image Database program (Leica, Belgium). The results were confirmed using a Leica TSC SPE-II confocal microscope (Leica, Belgium) using an ACS APO 63X oil immersion objective (Leica). On this system, laser lines of 488 nm or 561 nm were respectively combined with a BP 525/50 nm (green) and BP 596/50 nm (orange) filters. For each wavelength digital optical sections were collected using Z-series acquisition every 0.5 µm.

RESULTS AND DISCUSSION

Incubations with the non-immune control were performed in all replicates, and no signal was observed, showing that the blocking step was enough to avoid unspecific bindings of the antibodies. No signal was observed in the negative control.

When no epitope retrieval step is performed, any DNA stain can be used as counterstaining, taking into account the label of the secondary antibody. However, when the epitope retrieval treatment was performed, the best DNA stain to be used as counterstaining was EthD-2, as demonstrated previously (Heras *et al.* 2014a).

In this study, late pronuclear stages were selected in equine zygotes since in bovine and pig zygotes, the difference in H3K9me3 pattern between parental origin is reduced in late stages until it finally disappears (Jeong *et al.* 2007; Park *et al.* 2007).

In parthenotes, H3K9me3 was present in both pronuclei as expected since both are from maternal origin (Figure 4.2.1). On the contrary, in ICSI produced zygotes, H3K9me3 was intensively present in the maternal pronucleus, whereas the presence of H3K9me3 varied from absent to slightly present in the paternal pronucleus (Figure 4.2.1). In all cases, the difference in H3K9me3 pattern between maternal and paternal pronuclei could be clearly observed.

It is also evident from figure 4.2.1, that the pattern of expression of H3K9me3 is evident no matter whether there was epitope retrieval or not. Epitope retrieval did not affect the

intensity of the H3K9me3 signal, but it slightly reduced the intensity of the counterstaining, as expected (Heras *et al.* 2014a).

In conclusion, equine zygotes display an asymmetric H3K9me3 pattern. The H3K9me3 epigenetic modification is present in the maternal pronucleus, whereas it is absent or practically absent in the paternal pronucleus. H3K9me3 immunostaining therefore constitutes an easy and reliable tool to determine the parental origin of the pronuclei, with possible applications for evaluation of fertilization and in 5mC and 5hmC pronuclear pattern determination.

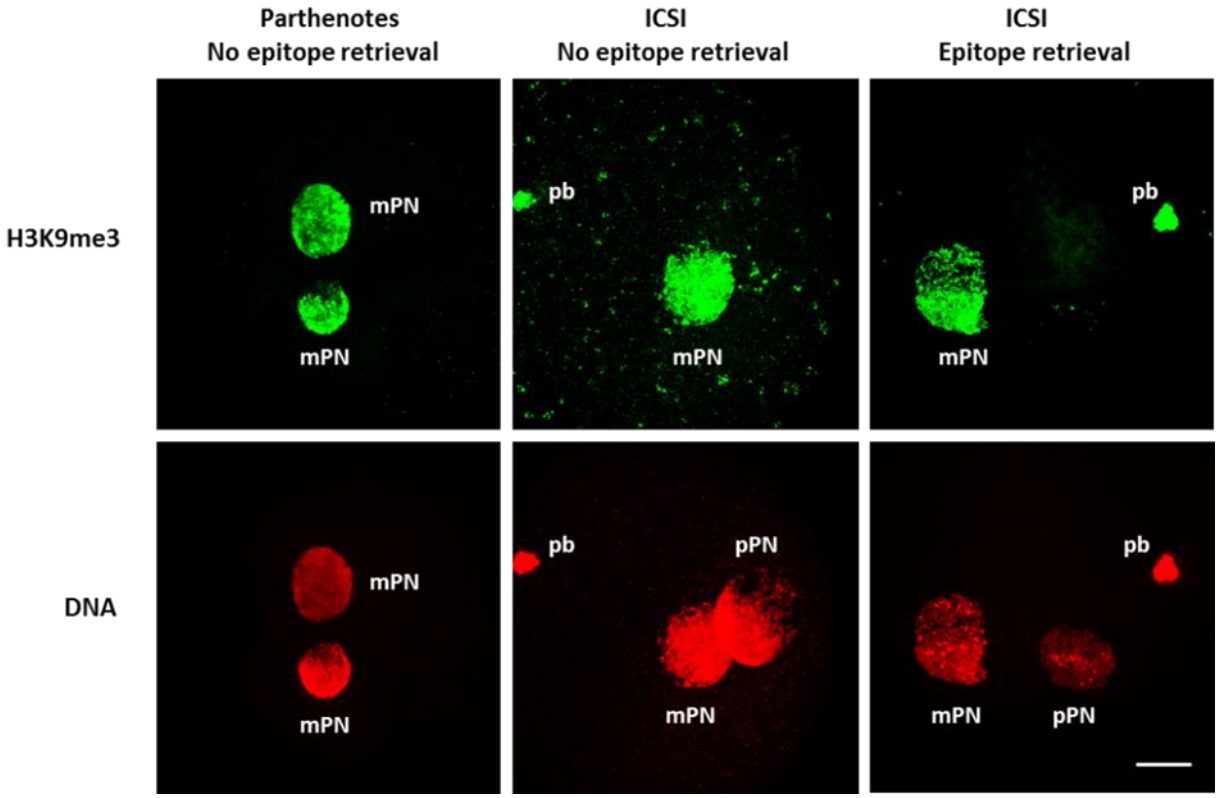

***Figure 4.2.1**. Immunofluorescent localization of H3K9me3 in pronuclear stage ICSI zygotes and parthenotes by confocal microscopy. Parthenotes display two maternal pronuclei, while in ICSI zygotes one maternal and one paternal pronucleus are seen. Parthenotes were stained following the protocol without epitope retrieval whereas ICSI zygotes were stained following both protocols, with and without epitope retrieval. Rabbit anti-H3K9me3 antibody was indirectly labeled with goat anti-rabbit FITC. DNA was counterstained with EthD-2. All the images were taken at 630X. mPN, maternal pronucleus; pPN, paternal pronucleus; pb, polar body. The scale bar represents 20 µm.*

ACKNOWLEDGEMENTS

The authors thank Petra Van Damme and Isabel Lemahieu for their excellent technical assistance. This work was funded by the Agentschap voor Innovatie door Wetenschap en Technologie (IWT) grant number 111438 and GOA - project no. 01G01112 – Pathways to pluripotency and differentiation in embryos and embryonic stem cells.

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CHAPTER 4.3

DETERMINATION OF THE PARENTAL PRONUCLEAR ORIGIN IN BOVINE ZYGOTES: H3K9ME3 VERSUS H3K27ME2-3

Adapted from:

Heras S, Vandenberghe L, Van Soom A. ***Anal Biochem, submitted****.*

SUMMARY

To study the dynamics of 5-methylcytosine and 5-hydroxymethylcytosine in zygotes, the parental origin of the pronuclei needs to be determined. To this end the use of the asymmetric distribution of histone modifications in pronuclei is becoming more popular. Here, we demonstrated that histone 3 lysine 27 di-tri-methylation shows a stable pattern being present in the maternal but not in the paternal pronucleus of bovine zygotes, even in late stages of pronuclear development. In contrast, the pattern of histone 3 lysine 9 tri-methylation is very variable, and therefore cannot be used to reliably determine the parental origin of bovine pronuclei.

INTRODUCTION

The 5-methylcytosine (5mC) pattern in preimplantation embryos has become a topic of research since it was first determined in mouse zygotes in 2000 (Mayer *et al.* 2000). Changes in methylation patterns are important during the global epigenetic reprogramming that takes place during preimplantation development and leads to the establishment of totipotent state (Reik *et al.* 2001). Recently, this research has been extended to other 5-cytosine modifications (Inoue *et al.* 2011), especially 5-hydroxymethylation (5hmC) as they are believed to be intermediate stages to demethylation (Wossidlo *et al.* 2011; Zhang *et al.* 2012; Li and O'Neill 2013). To study the dynamics of 5mC and 5hmC, particularly in the pronuclear stages, immunofluorescent staining is still the most common technique. Indeed, as only a limited amount of DNA can be obtained from a single embryo, a large number of embryos should be pooled to perform alternative techniques such as oxidative bisulfite sequencing (Booth *et al.* 2013). Besides, a major advantage of immunofluorescent staining is that the dynamics of 5mC and 5hmC can be studied separately in the maternal (mPN) and paternal (pPN) pronucleus of the zygotes. Traditionally, the parental origin of the pronuclei is identified based on the relative distance to the polar body (with the mPN being closer) and the relative size (with the pPN being larger) (Dean *et al.* 2001). However, this method is not accurate in all species (Young and Beaujean 2004; Jeong *et al.* 2007). Alternatively, the asymmetric pattern of different histone modifications between the mPN and pPN is being applied more and more to determine their parental origin. To this end, histone 3 lysine 9 tri-methylation (H3K9me3) has been successfully used in horse (Heras *et al.* 2015), rabbit (Reis Silva *et al.* 2011), human (van der Heijden *et al.* 2009) and mouse (van der Heijden *et al.* 2009), in which it is consistently present only in the mPN. In pig (Jeong *et al.* 2007) and cattle (Park *et al.* 2007), this asymmetric distribution of H3K9me3 is only detected in the early pronuclear stages with the pPN exhibiting a weaker H3K9me3 signal. However, when the pronuclear development progresses the levels of H3K9me3 in the pPN increase until similar levels as in the mPN. The asymmetric pattern of histone 3 lysine 27 tri-methylation (H3K27me3) in human (van der Heijden *et al.* 2009) and mouse (van der Heijden *et al.* 2005) zygotes, and of histone 3 lysine 27 di-methylation (H3K27me2) in mouse zygotes (van der Heijden *et al.* 2005) have been used to determine the parental origin of the pronuclei, since both modifications are only present in

the mPN. To our knowledge, the pattern of these histone modifications has not been studied in bovine zygotes yet.

Therefore, the aim of the present study was to evaluate if H3K9me3 and H3K27me2-3 are asymmetrically distributed in the mPN versus the pPN, also in late pronuclear stages, and as such, can be used to determine the parental origin of the pronuclei in bovine zygotes. Besides, it was evaluated if the H3K9me3 and H3K27me2-3 immunostaining, respectively, can be combined with the 5mC or 5hmC immunostaining considering the harsh epitope retrieval treatment which is necessary for the latter.

MATERIALS AND METHODS

EMBRYO PRODUCTION

Bovine zygotes were obtained by routine *in vitro* production methods (Wydooghe *et al.* 2014). Mature bovine oocytes were parthenogenetically activated by incubation with 5 µM Ionomycin (Sigma) for 5 min followed by 2 mM 6-DMAP (6-(Dimethylamino)purine; Sigma) in DMEM/F12 (Gibco) for 4h (Heras *et al.* 2015). Subsequently, they were cultured in SOF supplemented with 4 mg/mL BSA (bovine serum albumin; Sigma A9647) and ITS (5 µg/mL insulin + 5 µg/mL transferrin + 5 ng/mL selenium). Presumptive zygotes and parthenotes were collected 22h after fertilization or activation, respectively, fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and stored in 2% PFA at 4°C for a maximum of 4 days until immunostaining was performed.

Three immunostaining replicates with H3K27me2-3 and four with H3K9me3 were performed using bovine zygotes and parthenotes produced in independent experiments. A total of 150 zygotes and 35 parthenotes were stained, including test groups, negative controls (n=14 zygotes) and non-immune controls (14 zygotes and 7 parthenotes). Incubations with the non-immune and negative controls were performed in all replicates and no signal was observed.

IMMUNOFLUORESCENT STAINING

Both zygotes and parthenotes were washed in phosphate-buffered saline (PBS, Gibco) containing 0.5% BSA (0.5% PBS-BSA) for 1h at RT. Subsequently, they were permeabilized with 0.5% Triton X-100 and 0.05% Tween 20 in PBS for 1h at RT, and washed 3 times for 5 min in

0.5% PBS-BSA. Zygotes and parthenotes were blocked in PBS containing 2% BSA (2% PBS-BSA) for 1h at RT and washed 3 times for 2 min in 0.5% PBS-BSA. Subsequently, they were incubated with the primary antibody, i.e. rabbit anti-H3K9me3 (1:100, Active Motif) or mouse anti-H3K27me2-3 (0.01 mg/mL; Abcam) respectively, in 2% PBS-BSA overnight at 4°C. In each replicate, 2 zygotes and 1 parthenote were incubated with the non-immune control antibody, i.e. rabbit IgG control antibody (0.01 mg/mL; Rockland 011-0102) or mouse IgG1 control antibody (0.01 mg/mL; M7894 Sigma-Aldrich) respectively, in 2% PBS-BSA overnight at 4°C. Zygotes used as negative control remained in 2% PBS-BSA without adding any primary antibody. Subsequently, all zygotes and parthenotes including non-immune and negative controls were washed 3 times for 10 min in 0.5% PBS-BSA and treated with 1 mg/mL RNase A (Affymetrix) for 30 min at 37°C to avoid binding of the nuclear stain Ethidium homodimer 2 (EthD-2; Molecular Probes) to RNA (Suzuki *et al.* 1997). Next, they were washed 3 times in 0.5% PBS-BSA for 5 min and incubated with the nuclear stain 0.5 nM EthD-2 in 0.5% PBS-BSA for 30 min at RT. EthD-2 was chosen as DNA counterstaining as we have previously demonstrated that it is the best dye to use in combination with the epitope retrieval treatment (Heras *et al.* 2014). After 4 washing steps for 2 min in 0.5% PBS-BSA, they were incubated with the secondary antibody, i.e. goat anti-rabbit Alexa fluor 488 (20 µg/mL; Abcam) or goat anti-mouse Alexa fluor 488 (20 µg/mL; Abcam) respectively, for 1h at RT in 30% goat serum.

To evaluate if H3K9me3 and H3K27me2-3 antibodies can be used in combination with 5mC or 5hmC immunostaining in bovine zygotes, we tested these antibodies after the epitope retrieval treatment necessary for 5mC and 5hmC, as previously described (Heras *et al.* 2015). Briefly, zygotes were incubated with the respective primary antibody, washed 3 times for 10 min with 5% PBS-BSA, post-fixed with 4% PFA for 25 min and subsequently washed 3 times for 10 min in 5% PBS-BSA. For epitope retrieval, the bovine zygotes were first treated with 4N HCL for 30 min at RT and 100 mM TrisHCl (pH 8.5) for 10 min at RT, followed by 3 washing steps with 5% PBS-BSA for 5 min and 20s treatment with 0.25% (w/v) trypsin at 37°C (Sigma). Tryptic digestion was stopped by incubation with 30% goat serum in PBS for 3 min at RT. After epitope retrieval, zygotes followed the protocol as previously described.

FLUORESCENCE MICROSCOPY

The immunofluorescent staining of zygotes and parthenotes was evaluated the next day using a Leica TSC SPE-II confocal microscope using an ACS APO 63X oil immersion objective (Leica). Laser lines of 488 nm or 561 nm were combined with BP 525/50 nm (Green) and BP 596/50 nm (Orange) filters, respectively. For each wavelength, digital optical sections were collected using Z-series acquisition every 0.5 µm.

RESULTS AND DISCUSSION

In this study, we focused on late pronuclear stages as it has been demonstrated in bovine and porcine zygotes that the difference in H3K9me3 pattern between parental origin was reduced in late stages and even disappeared eventually (Jeong *et al.* 2007; Park *et al.* 2007). As expected, H3K9me3 (Figure 4.3.1) and H3K27me2-3 (Figure 4.3.2) were present in both pronuclei in parthenotes, as both are from maternal origin. However, in zygotes, different expression patterns of H3K9me3 and H3K27me2-3 were observed. H3K9me3 was intensively present in the mPN while its presence in the pPN was very variable, from absent (in a few cases) to present at the same level as in the mPN. When H3K9me3 was present in both pronuclei though, the staining was in most cases more intense in the mPN. Nevertheless, it was not possible to reliably distinguish between both pronuclei using this staining, which confirms previous reports (Park *et al.* 2007) (Figure 4.3.1). In contrast, H3K27me2-3 showed a stable pattern, and was only present in the mPN (Figure 4.3.2). Rarely, a weak aspecific signal was observed in the pPN. In addition, the binding of both antibodies was not affected by the epitope retrieval treatment and could be visualized correctly in all the zygotes (Figures 4.3.1 and 4.3.2). Interestingly, the signal of H3K9me3 was more intense in the mPN than of H3K27me2-3, in zygotes and parthenotes irrespective of the epitope retrieval treatment. Still, the intensity of H3K27me2-3 was sufficient to clearly identify the parental origin of the pronuclei. In case of polyspermy, H3K9me3 was present in the 3 pronuclei (albeit stronger in the mPN; Figure 4.3.1c) while H3K27me2-3 was only present in the mPN (Figure 4.3.2c).

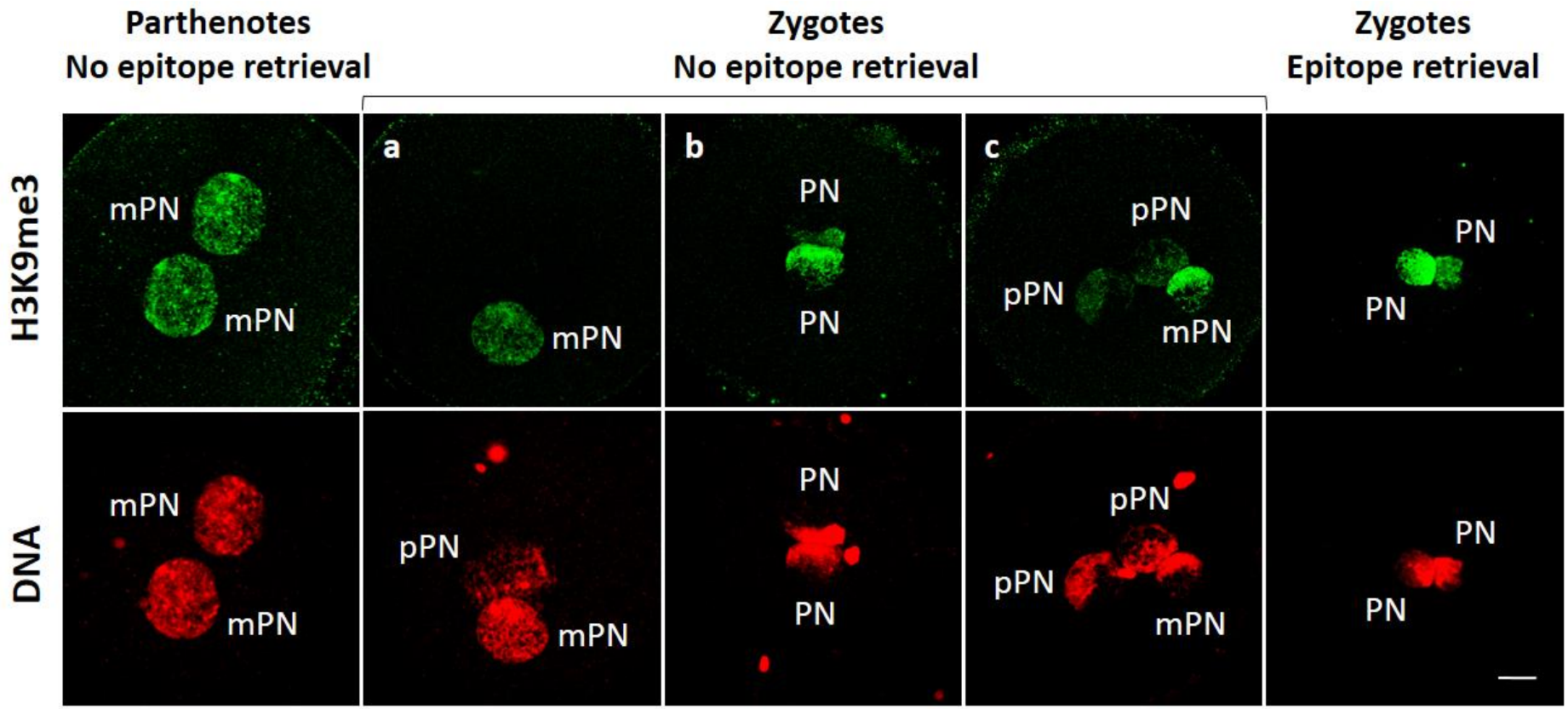

Figure 4.3.1. *Immunofluorescent localization of H3K9me3 in bovine zygotes and parthenotes by confocal microscopy. Rabbit anti-H3K9me3 antibody was indirectly labeled with goat anti-rabbit Alexa Fluor 488 and DNA was counterstained with EthD-2. In the parthenotes, H3K9me3 was present in the two mPN. However, in zygotes, H3K9me3 was present in the mPN while its presence in the pPN was variable (a-c). All the images were taken at 630X. The scale bar represents 20 µm.*

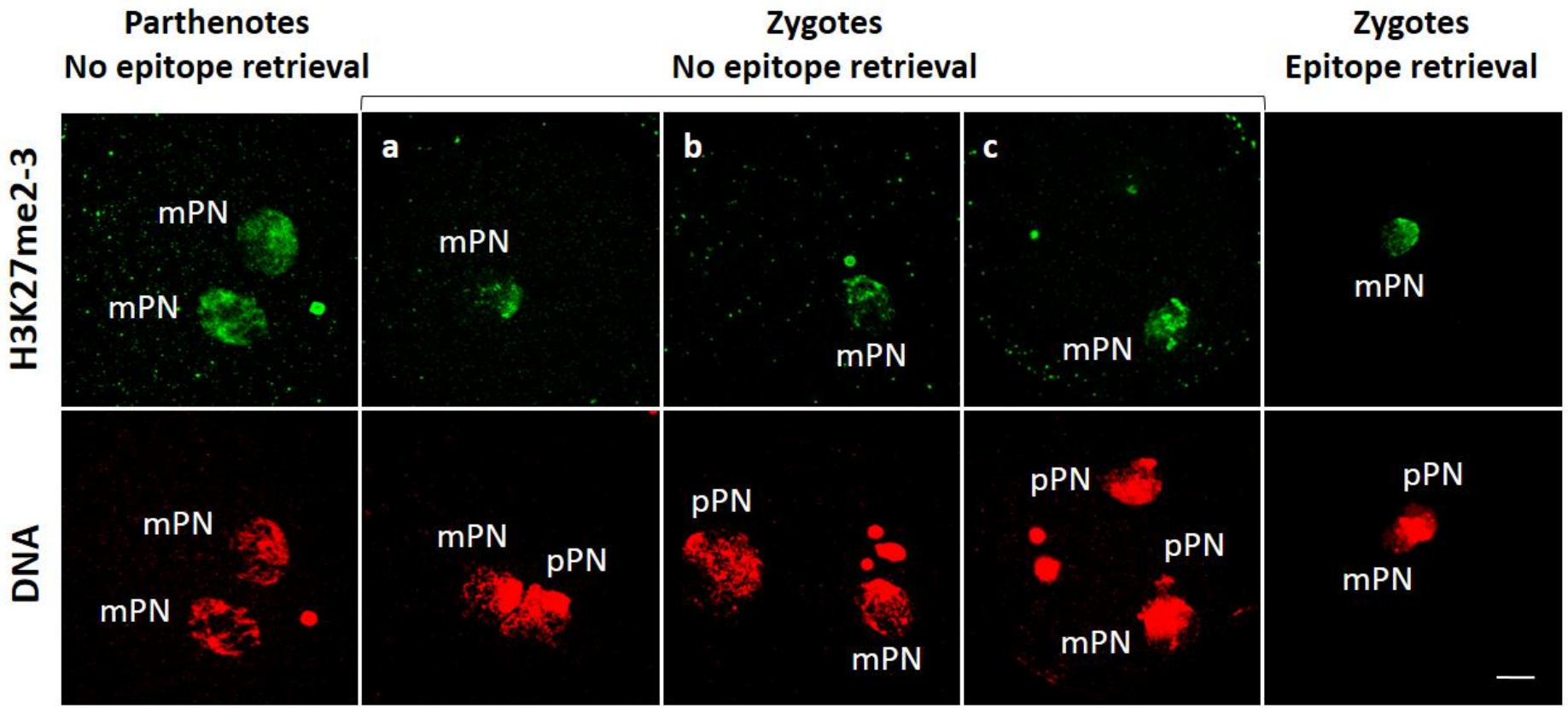

Figure 4.3.2. *Immunofluorescent localization of H3K27me2-3 in bovine zygotes and parthenotes by confocal microscopy. Mouse anti-H3K27me2-3 antibody was indirectly labeled with goat anti-mouse Alexa Fluor 488 and DNA was counterstained with EthD-2. In the parthenotes, H3K27me2-3 was present in both mPN. In contrast, in zygotes, H3K27me2-3 was present in the mPN but absent in the pPN. All the images were taken at 630X. The scale bar represents 20 µm.*

In conclusion, H3K27me2-3 showed a stable pattern in all the zygotes analyzed being only present in the mPN. In contrast, the presence of H3K9me3 in the pPN, was variable between zygotes. Therefore, H3K27me2-3 immunostaining is the preferred tool to reliably determine the parental origin of the pronuclei in bovine zygotes. Additionally, this immunostaining can be used in combination with 5mC and 5hmC immunostaining to determine the dynamics of these two cytosine modifications in the pPN and mPN.

ACKNOWLEDGEMENTS

The authors thank Petra Van Damme and Isabel Lemahieu for their excellent technical assistance. This work was funded by the Agentschap voor Innovatie door Wetenschap en Technologie (IWT) grant number 111438 and GOA - project no. 01G01112 – Pathways to pluripotency and differentiation in embryos and embryonic stem cells.

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CHAPTER 5

DYNAMICS OF 5-METHYLCYTOSINE AND 5-HYDROXYMETHYLCYTOSINE DURING PRONUCLEAR DEVELOPMENT IN *IN VITRO* PRODUCED EQUINE ZYGOTES

Adapted from:

Heras S, Smits K, Peelman, L and Van Soom A. In preparation.

ABSTRACT

Global epigenetic reprogramming is essential during embryo development for the establishment of totipotency. In the classic model first described in the mouse, the genome-wide DNA demethylation is asymmetric between the paternal and the maternal genome. The paternal genome undergoes TET-mediated active DNA demethylation, which is completed before the end of the first cell cycle. 5-hydroxymethylcytosine was postulated to be an intermediate stage towards DNA demethylation, since TET enzymes oxidize 5-methylcytosine to 5-hydroxymethylcytosine. In contrast, the maternal genome is protected from active demethylation and undergoes replication-dependent DNA demethylation. However, so far several species have failed to show the described DNA demethylation process, and 5-methylcytosine and 5-hydroxymethylcytosine are present during the first cell cycle in both parental genomes. In this study, the patterns of both cytosine modifications were evaluated in *in vitro* produced horse zygotes. A significant reduction in the levels of 5-methylcytosine was reported between PN2 and PN4 in both parental genomes, and this reduction was replication-dependent. Despite this finding, 5-methylcytosine was obviously present in both parental genomes throughout pronuclear development. Additionally, no differences in 5-hydroxymethylcytosine were observed between pronuclear stages regardless the parental origin of the pronuclei, and it was highly present during the complete first cell cycle. In conclusion, the horse is, together with rabbit, goat and sheep, one of the species that does not follow the classical genome-wide DNA demethylation process, at least during the first cell cycle.

INTRODUCTION

During mammalian development, two major waves of epigenetic reprogramming take place, one at the level of the germ cells and the other one during preimplantation embryo development. Epigenetic reprogramming during embryo development is found to be of major importance, because it has been suggested to be necessary for the establishment of a totipotent state (Reik *et al*. 2001). The methylation of the fifth carbon of cytosine, 5-methylcytosine (5mC), was the first epigenetic modification discovered in the DNA, and is the best studied DNA epigenetic modification nowadays. It plays a key role in gene expression regulation, X chromosome inactivation, gene imprinting and the control of endogenous retrotransposons (Dean *et al*. 2001). The genome-wide DNA demethylation during embryo development was proposed to be asymmetric between the maternal and the paternal genome, based on studies in mouse embryos (Mayer *et al*. 2000; Oswald *et al*. 2000). The complete demethylation of the paternal DNA is achieved before the end of the first cell cycle by active demethylation (replication-independent). In contrast, the maternal DNA is protected from this active demethylation by STELLA (Szabo and Pfeifer 2012), and undergoes passive demethylation with the cell divisions (replication-dependent). However, this asymmetric demethylation pattern between the DNA from paternal and maternal origin is not conserved in all species, in the rabbit (Shi *et al*. 2004), the pig (Jeong *et al*. 2007), the goat (Hou *et al*. 2005) and the sheep (Beaujean *et al*. 2004) no DNA demethylation has been observed during the first cell cycle, regardless of the parental origin of the genome.

In 2009, the existence of a new modified form of cytosine, 5-hydroxymethylcytosine (5hmC), was reported. This new modification is generated by the oxidation of 5mC by the ten-eleven translocation (TET) enzymes (Kriaucionis and Heintz 2009; Tahiliani *et al*. 2009). The TET family is able to further oxidize 5hmC into to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani *et al*. 2009). In this way, the TETs were proposed to be the initiators of active DNA demethylation in the paternal pronucleus, and 5hmC was considered a DNA demethylation transient. However, the high presence of 5hmC in several tissues, including the nervous system, indicates that this epigenetic modification of the DNA plays its own epigenetic role (Kriaucionis and Heintz 2009). Yet, the presence of 5hmC has been reported in preimplantation embryos of several species, including mice (Salvaing *et al*. 2012), rabbits (Wossidlo *et al*. 2011) and cattle (Wossidlo *et al*. 2011). Some studies support the

transient character of 5hmC to DNA demethylation (Wossidlo *et al*. 2011), while others indicate it plays its own epigenetic role, being involved in chromatin and transcription regulation (Salvaing *et al*. 2012; Li and O'Neill 2013).

As mentioned before, the dynamic patterns of 5mC and to a lesser extent, of 5hmC during the first cell cycle have been already described in many species but, to our knowledge, not yet in the horse. Therefore, the aim of the present study was to characterize for the first time the dynamics of 5mC and 5hmC throughout pronuclear development in both the maternal (mPN) and the paternal (pPN) pronucleus of the equine zygote, by using an immunofluorescent staining protocol optimized previously (Heras *et al*. 2014a; Heras *et al*. 2015).

MATERIALS AND METHODS

IN VITRO EMBRYO PRODUCTION

Equine zygotes were produced *in vitro* by piezo drill-assisted intracytoplasmic sperm injection (ICSI) as described previously (Smits *et al*. 2012). Briefly, ovaries were collected from slaughtered mares and processed within 4h. Cumulus oocyte complexes (COCs) were aspirated from follicles larger than 5mm using a 16 gauge needle attached to a vacuum pump (-100 mm Hg) and matured in groups of maximum 30 in 500 µL of Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) based maturation medium (Galli *et al*. 2007) for minimum 25h at 38.5°C in a humidified atmosphere of 5% CO_2-in-air. After maturation, COCs were denuded by gentle pipetting in 0.05% bovine hyaluronidase diluted in HEPES buffered TCM199 medium. Only oocytes with an extruded polar body were used for piezo drill-assisted ICSI. Frozen and fresh sperm of two different stallions was used for ICSI; after Percoll, the sperm was washed and held in calcium-free TALP and manipulated in 9% polyvinylpyrrolidone in phosphate buffered saline (PBS). All manipulations were performed on the heated stage (37 °C) of an inverted microscope; a progressively motile sperm was immobilized by piezo pulses and subsequently injected into the cytoplasm of a mature oocyte using a piezo drill. The injected oocytes were cultured in groups of 10 to 15 in 20 µL drops of DMEM-F12 supplemented with 10% fetal bovine serum (FBS) at 38.2°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Presumptive zygotes were collected after 8, 11, 15, 19 and 23h in culture in order to obtain all the pronuclear stages.

The pronuclear stages were classified as follows; PN0: the sperm head is decondensing, meiosis II is finished, the second polar body is extruded and the chromosomes start to decondense, and the nuclear envelope of both pronuclei is starting to form; PN1: the DNA of the mPN and pPN are decondensed and the nuclear envelope is completed forming two small pronuclei; PN2: the pronuclei have increased sizes and are starting to migrate towards the center; PN3: the pronuclei have reached their maximum size and they are in apposition; PN4: there is a nuclear membrane break down.

IMMUNOFLUORESCENT STAINING

After collection, presumptive zygotes were vortexed for 1 min to remove any remaining cumulus cell, fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and kept in 2% PFA at 4°C for a maximum of four days until immunostaining was performed. The zygotes were subsequently washed in phosphate-buffered saline (PBS, Gibco) containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) (0.5% BSA-PBS) for 1h at RT. After washing, they were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and 0.05% Tween 20 (Sigma-Aldrich) in PBS for 1h at RT, and washed three times for 5 min in 0.5% BSA-PBS. Zygotes were blocked in PBS containing 2% BSA (2% BSA-PBS) for 1h at RT and washed three times for 2 min in 0.5% BSA-PBS. After washing, they were incubated with the primary antibody rabbit anti-H3K9me3 (1:100, Active Motif) in 2% BSA-PBS overnight at 4°C. Simultaneously, 4 zygotes were incubated with the non-immune control antibody rabbit IgG (0.01 mg/mL; Rockland 011-0102) in 2% BSA-PBS overnight at 4°C. Zygotes which served as negative control remained in 2% PBS-BSA without adding any primary antibody. Next, all zygotes, including the non-immune and the negative control, were washed three times for 10 min in 0.5% BSA-PBS, post-fixed in 4% PFA for 25 min and washed again three times in 0.5% BSA-PBS for 10 min. Epitope retrieval was performed as described previously (Heras *et al*. 2015) by treating the zygotes with 4N HCl for 30 min at RT and 100 mM TrisHCl (pH 8.5) for 10 min at RT, followed by 3 times washing with 5% BSA-PBS for 5 min and 20s treatment with 0.25% (w/v) trypsin at 37°C (Sigma). Tryptic digestion was stopped by incubating the zygotes with 30% goat serum in PBS (blocking solution) for 2 min at RT and subsequently washing them with 0.5% BSA-PBS 3 times for 5 min. After epitope retrieval, zygotes were treated with 1 mg/mL RNase A (Affymetrix) for 30 min at 37°C to avoid the binding of the nuclear stain Ethidium homodimer 2 (EthD-2; Molecular Probes) to RNA (Suzuki *et al*. 1997). They were

subsequently washed three times in 0.5% BSA-PBS for 5 min and incubated with the nuclear stain 0.5 nM EthD-2 in 0.5% BSA-PBS for 30 min at RT. After 4 washing steps of 2 min in 0.5% BSA-PBS, zygotes were incubated in blocking solution overnight at 4°C. Subsequently, zygotes previously incubated with rabbit anti H3K9me3 were incubated with either mouse anti 5mC (0.01 mg/mL; Gentaur) or mouse anti 5hmC (0.01 mg/mL; Active Motif) primary antibodies overnight at 4°C. At the same time, zygotes previously incubated with rabbit IgG control antibody were incubated with mouse IgG control antibody (0.01 mg/mL; Sigma-Aldrich) overnight at 4°C. Zygotes used as negative control remained in blocking solution. After incubation with the primary antibodies, zygotes were washed and serially incubated with the two secondary antibodies, for 1h at RT each. For the H3K9me3-5mC immunostaining, goat anti rabbit FITC (0.02 mg/mL; Life Technologies) and goat anti mouse Alexa Fluor 405 (0.02 mg/mL; Abcam) secondary antibodies were used, respectively. For the H3K9me3-5hmC immunostaining goat anti rabbit Alexa Fluor 405 (0.02 mg/mL; Abcam) and goat anti mouse Alexa Fluor 488 (0.02 mg/mL; Abcam) secondary antibodies were used, respectively.

Specificity of the primary antibodies was tested by the providing companies, 5mC by MeDIP, 5hmC by dot blot and MeDIP, and H3K9me3 by dot blot and peptide array analysis.

FLUORESCENCE MICROSCOPY AND IMAGE ANALYSIS

To avoid fading, evaluation of the embryos was performed 2 days after immunostaining was completed, using a Leica TSC SPE-II confocal microscope (Leica, Belgium) with an ACS APO 63X oil immersion objective (Leica) and laser lines at 405-, 488- and 561 nm wavelengths.

For each wavelength, single images were taken independently of the middle section of each pronucleus. Additionally, digital optical sections of the complete embryo were taken using Z-series acquisition every 0.5 µm.

Quantitative analysis was performed using the ImageJ software. The area of the middle section of each pronucleus was manually outlined, and the mean fluorescence intensity was measured for 5mC/5hmC and EthD-2. The mean fluorescence intensities were then multiplied by the pronuclear areas to obtain the total fluorescence of 5mC/5hmC and EthD-2. Finally, for

each pronucleus, the total fluorescence of 5mC/5hmC was divided by the total fluorescence of EthD-2 to obtain the normalized fluorescence.

It has to be noted that when a small part of the mPN and pPN was overlapping, the overlapping area was not measured. Additionally, when most of the area of the pronuclei were overlapping and the analysis of both pronuclei could not be made independently, these zygotes were excluded from the study.

STATISTICAL ANALYSIS

Three replicates were performed for the study of 5mC and four for 5hmC. When the data were not normally distributed, a log10 transformation was performed. Additionally, the Levene´s Test for equality of variances was made. The dependent T-Test was used to compare the normalized fluorescence between the mPN and the pPN. On the other hand, ANOVA, combined with Bonferroni post Hoc test, was used to compare the total and normalized fluorescence between the different pronuclear stages, independently for the mPN and pPN, when the equality of variances was fulfilled. The non-parametric test Kruskal-Wallis was used when variances were not equal. All the analyses were performed with SPSS Statistics 23 and p-values <0.05 were considered significant.

RESULTS AND DISCUSSION

We analyzed for the first time the dynamic patterns of 5mC and 5hmC independently for the pPN and the mPN during pronuclear development in *in vitro* produced equine zygotes. To this end, equine zygotes were produced and collected at five time points ranging from 8 to 23h after ICSI to obtain zygotes in all pronuclear stages. Due to the lipid-rich cytoplasm of equine oocytes and zygotes, the determination of the pronuclear stages could only be performed after immunostaining. The distribution of the different pronuclear and cleavage stages among the time points of collection is illustrated in figure 5.1. Since the embryos were produced by ICSI, the timing after the penetration of the spermatozoon could be established precisely. There was a lot of variability in the stage of development of the embryos within each time point of collection, indicating that the activation of the oocyte and the further pronuclear formation is not following the same timing schedule in the individual embryos. The oocyte activation rate was increasing with the time in culture. The shortest activation rate was found

at 8h after ICSI, with only around 40% of the injected oocytes activated. The activation rate increased until 90% at 23h after ICSI, the last time point of collection. At 8h after ICSI, most zygotes were at PN1 and PN2 stages. The majority of zygotes collected at 11h after ICSI were at PN2 stage, while at 15h after ICSI, an increase of PN3 zygotes was found. At 19h after ICSI, zygotes were equally divided over PN2, PN3 and PN4 stages, while at 23h after ICSI, the most common pronuclear stages were PN3 and PN4. In the last two time points of collection, 19 and 23h after ICSI, some embryos already reached the 2-cell stage. Furthermore, 23h after ICSI, some 4-cell embryos were collected. Only 2 embryos were collected at PN0, showing that this is a very transient stage, and after oocyte activation, the formation of the pronuclei happens quickly. In contrast, the PN2 stage, during which the pronuclei are increasing in size and migrating towards the center, seems to be the longest stage.

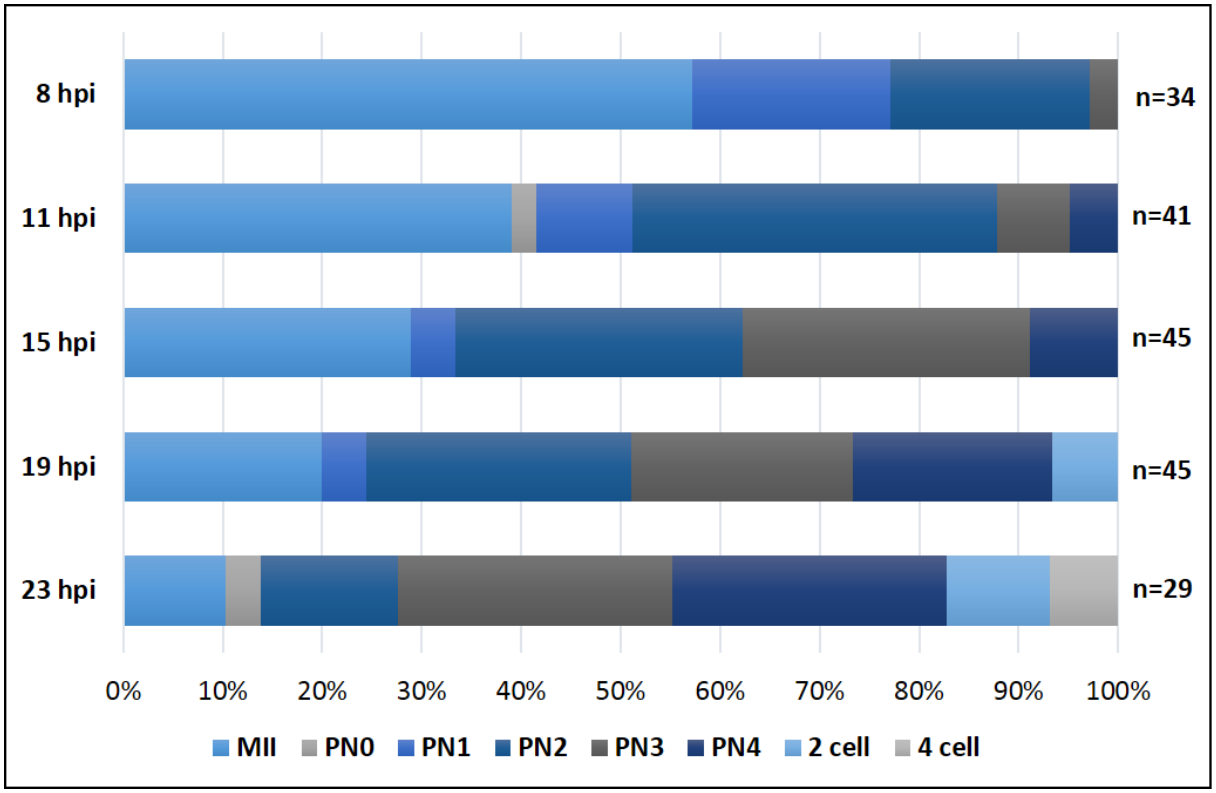

Figure 5.1. *Distribution of ICSI produced equine embryos among stages of development in each time point of collection. The time points of collection are measured in hours after ICSI (hpi). The oocytes in metaphase II (MII) did not activate after the injection of the spermatozoon, which was found intact in the cytoplasm of the oocyte. The number of oocytes used for each time point are indicated on the right. Degenerated oocytes and zygotes were excluded from the analysis.*

To differentiate between the mPN and pPN, the asymmetric pattern of Histone 3 lysine 9 tri-methylation (H3K9me3) was used. This histone modification is only present in the mPN throughout pronuclear development in many mammalian species, and it has been successfully used in horse (Heras *et al*. 2015), rabbit (Reis Silva *et al*. 2011), human (van der Heijden *et al*. 2009) and mouse (van der Heijden *et al*. 2009). In the mouse, the relative pronuclear size is used to determine the parental origin of the pronuclei (Santos *et al*. 2002; Li and O'Neill 2012). However, this is not possible in the horse because the relative size of the pronuclei is variable, and frequently both pronuclei have the same size (Figures 5.2 and 5.3).

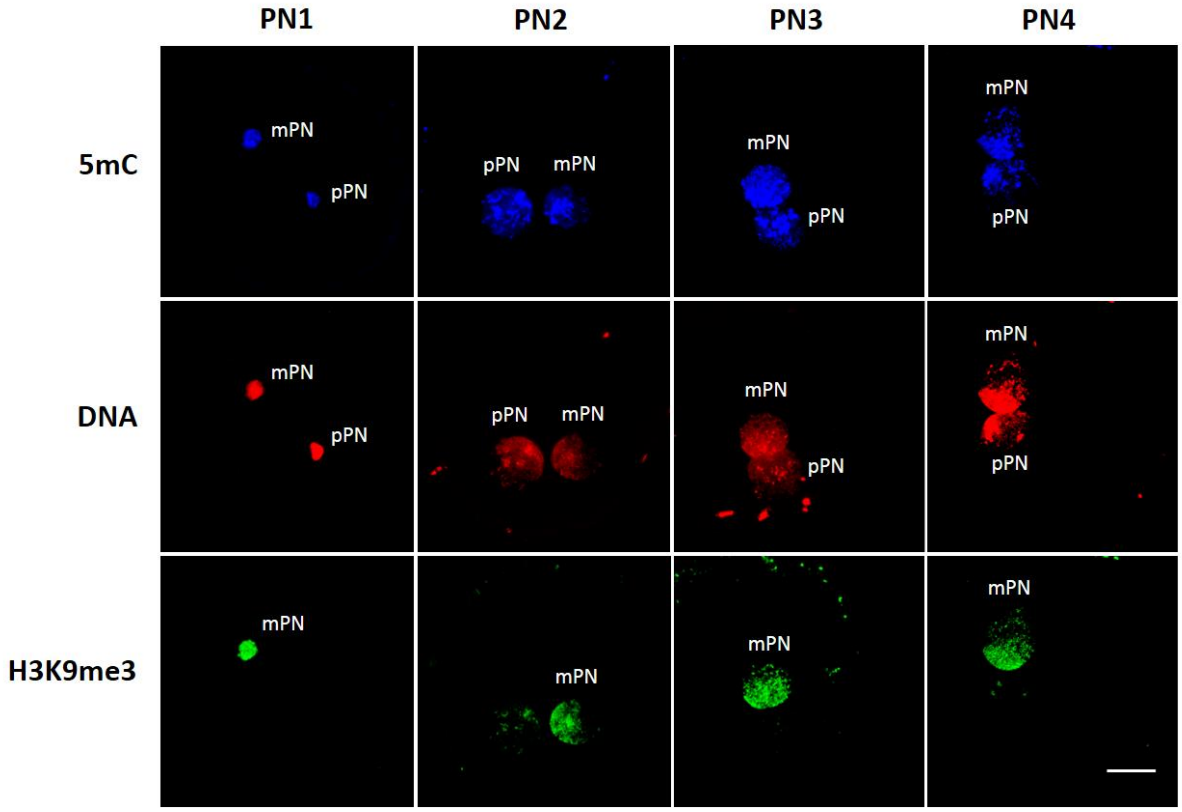

Figure 5.2. *Patterns of 5-methylcytosine (5mC) in the maternal (mPN) and paternal (pPN) pronucleus during pronuclear development. 5mC was present throughout pronuclear development in both the mPN and the pPN. The parental origin of the pronuclei was determined by H3K9me3 immunostaining, with this histone modification only being present in the mPN. The DNA was stained by EthD-2. All the images were taken at 630X. The scale bar represents 20 µm.*

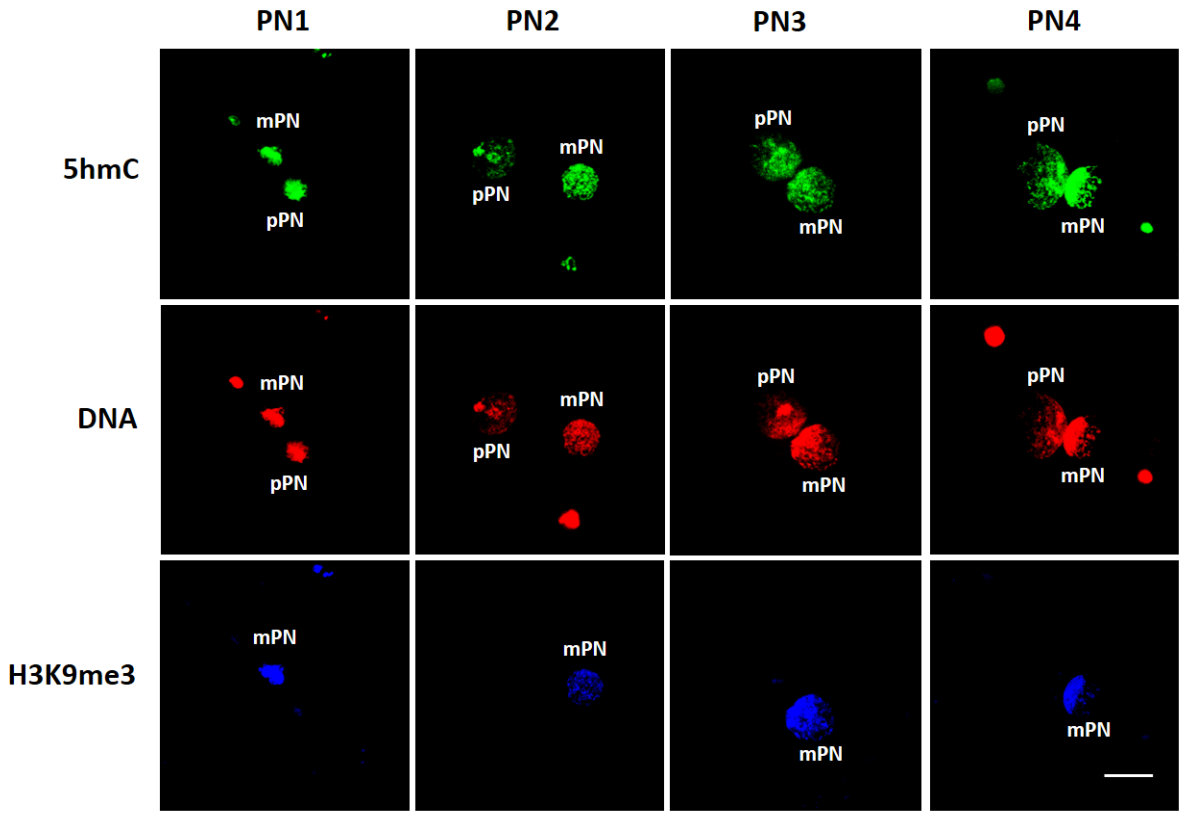

Figure 5.3. *Patterns of 5-hydroxymethylcytosine (5hmC) in the maternal (mPN) and paternal (pPN) pronucleus during pronuclear development. 5hmC was present throughout the pronuclear development in both the mPN and the pPN. The parental origin of the pronuclei was determined by H3K9me3 immunostaining and the DNA was stained by EthD-2. All the images were taken at 630X. The scale bar represents 20 µm.*

Once the stage and parental origin of the pronuclei were determined, the patterns of 5mC and 5hmC were studied. To this end, the total fluorescence of 5mC or 5hmC and of DNA was calculated for each pronucleus of each zygote, by multiplying the fluorescence intensity by the corresponding pronuclear area. Furthermore, the normalized fluorescence of 5mC or 5hmC of each pronucleus was calculated by dividing the total 5mC or 5hmC fluorescence by the corresponding total DNA fluorescence, in order to correct for the DNA replication that takes place during pronuclear maturation (Hyttel *et al*. 1989). When comparing the total DNA fluorescence between the different pronuclear stages independently for the mPN and the pPN (Figure 5.4), a significant increase was found between PN2 and PN3 in both the mPN (p-value =0.002) and the pPN (p-value =0.005). This indicates that, in the horse, the DNA replication (S phase) takes place during PN2, which could be the reason for the long duration of the PN2

stage. In rabbit, the DNA replication was proven to occur during PN2 and PN3 by injecting DIG-11dUTP in the zygotes (Reis Silva *et al*. 2011). In our study, no differences in the total DNA fluorescence were found between PN3 and PN4 nor in the mPN or pPN in equine zygotes, which indicates that the S phase is restricted to PN2.

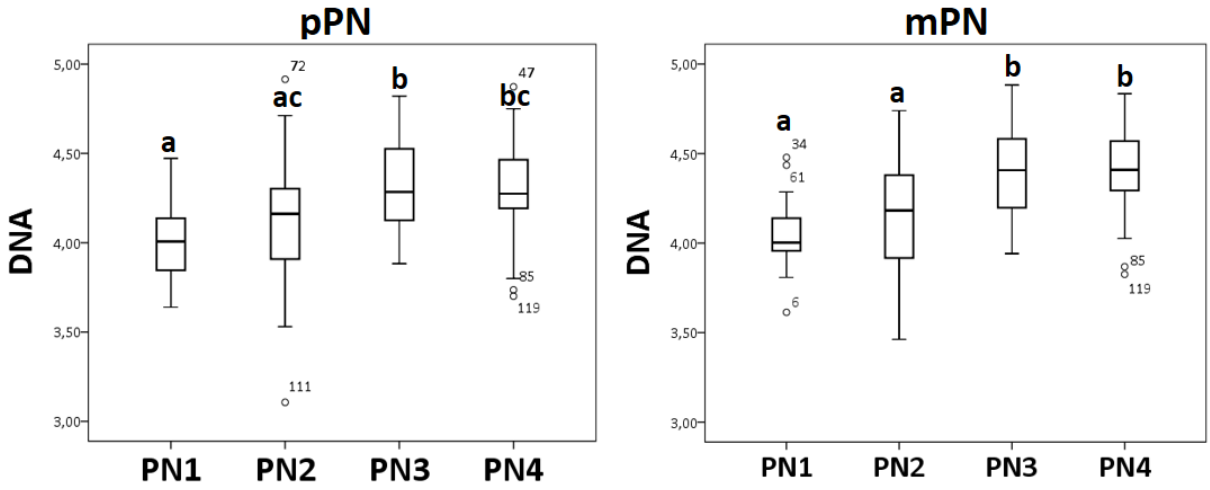

Figure 5.4. *Total DNA fluorescence of the paternal (pPN) and the maternal (mPN) pronucleus in each pronuclear stage. Comparisons were made between the different pronuclear stages independently for the pPN and the mPN. Different superscripts indicate significant differences. In the pPN, significant differences were observed between PN1 vs. PN3-PN4, and PN2 vs. PN3. In the mPN, significant differences were found between PN1-PN2 vs. PN3-PN4. The data was analyzed with SPSS Statistics 23, for it, the data was log10 transformed to achieve normal distribution and analyzed by ANOVA combined with Bonferroni post Hoc test. p-values <0.05 were considered significant.*

DYNAMICS OF DNA METHYLATION DURING PRONUCLEAR DEVELOPMENT

Throughout pronuclear development, 5mC was highly present in both the mPN and the pPN. Interestingly, its distribution in the pronuclei was not homogeneous, with higher concentrations in certain regions of the DNA (Figure 5.2).

When the dynamics of the normalized 5mC fluorescence between pronuclear stages was studied, a significant reduction was observed between PN2 and PN4 in both the pPN (p-value =0.011) and the mPN (p-value =0.031) (Figure 5.5). This reduction was around 40% in

the pPN and around 30% in the mPN. No differences were found between mPN and pPN from the same stage. Remarkably, when looking to the total 5mC fluorescence, no differences were found between pronuclear stages independently of the parental origin of the pronuclei. On the contrary, as observed when all the zygotes of the study were taken together, DNA replication caused an increase of the total DNA fluorescence between PN2 and PN3-PN4 in both the mPN and the pPN. Considering all these, the loss of methylation (normalized values) observed between PN2 and PN4 in both mPN and pPN is likely to be caused by DNA replication, and not by an active demethylation processes, in which case, a reduction of the total 5mC should be observed.

Active demethylation (replication-independent) of the pPN during the first cell cycle was first observed by immunostaining in the mouse, and it resulted in complete DNA demethylation of the pPN as early as 8h after fertilization (Mayer *et al*. 2000). This active demethylation of the pPN of murine zygotes was soon confirmed by other groups (Santos *et al*. 2002; Beaujean *et al*. 2004; Xu *et al*. 2005), and believed to be conserved in all mammalian species (Dean *et al*. 2001). As such, active demethylation of the pPN was also found in human (Beaujean *et al*. 2004; Xu *et al*. 2005) and rat (Zaitseva *et al*. 2007; Yoshizawa *et al*. 2010) by immunofluorescent staining.

However, in bovine zygotes, only partial loss of methylation of the pPN during the first cell cycle was found (Beaujean *et al*. 2004). In the rabbit, a loss of methylation in the pPN was observed between PN1 and PN3, but methylation levels increased again by PN4, so no erasure of the methylation in the pPN was observed in the first cell cycle (Reis Silva *et al*. 2011). Other studies found no loss of methylation in any of the pronuclei of rabbit zygotes (Shi *et al*. 2004). Furthermore, in sheep (Beaujean *et al*. 2004), pigs (Jeong *et al*. 2007) and goats (Hou *et al*. 2005), no loss of methylation was reported neither in the pPN or the mPN during the first cell cycle.

Surprisingly, in 2012, the use of a new method for 5mC epitope retrieval, which combined a short tryptic-digestion with the traditional acid-treatment, did not show active demethylation of the pPN of mouse zygotes, indicating that the previously reported asymmetric demethylation of the maternal and paternal DNA in zygotes might be due to an immunostaining artifact (Li and O'Neill 2012). Additionally, the use of round spermatid

injection (ROSI) to produce mouse embryos has demonstrated that active demethylation of the pPN is not necessary for normal embryo development in mouse. Since the embryos produced by ROSI showed similar high levels of methylation in pPN and mPN, and the zygotes developed to term (Polanski *et al*. 2008).

We compared the traditional acid-treatment (4N HCl) with the new epitope retrieval method (4N HCl + tryptic digestion) to evaluate the dynamics of 5mC in equine zygotes, and observed that both lead to the same results (Heras *et al*. 2014b). In the horse, using immunostaining, we found no evidences of active demethylation of the pPN in the zygotes analyzed, since the total levels of 5mC fluorescence remained constant in both pronuclei between the different pronuclear stages. However, when the total 5mC fluorescence was normalized against total DNA fluorescence to correct for DNA replication, a reduction in the normalized methylation levels between PN2 and PN4 was observed in both mPN and pPN. This indicates a loss of methylation by passive demethylation (replication-dependent) in both pronuclei, in the horse. This loss of methylation seems faster in the pPN (˜40%) than in the mPN (˜30%) in the horse, as it was observed in the mouse using reduced representation bisulfite sequencing (RRBS) (Guo *et al*. 2013). Recent studies using reduced representation bisulfite sequencing (RRBS) found active and passive DNA demethylation of both maternal and paternal genomes in the mouse zygote. Additionally, passive demethylation (replication-dependent) was the major contributor to DNA demethylation of both genomes (Guo *et al*. 2013; Guo *et al*. 2014; Shen *et al*. 2014).

The exclusion of Dnmt1 from the nucleus was postulated to be responsible for the passive demethylation (Howell *et al*. 2001). However, recent studies have shown methylation of the pPN in rabbit (Reis Silva *et al*. 2011) and bovine (Park *et al*. 2007), which indicates the presence of an active Dnmt.

In bovine, it was reported that the pattern of H3K9me3 was highly associated with the pattern of 5mC. Zygotes that presented high levels of 5mC in the pPN also had high levels of H3K9me3, while in embryos with low levels of 5mC in the pPN, the levels of H3K9me3 were also low (Park *et al*. 2007). This association was not found in horse, H3K9me3 was never present in the pPN, while the levels of 5mC remained high.

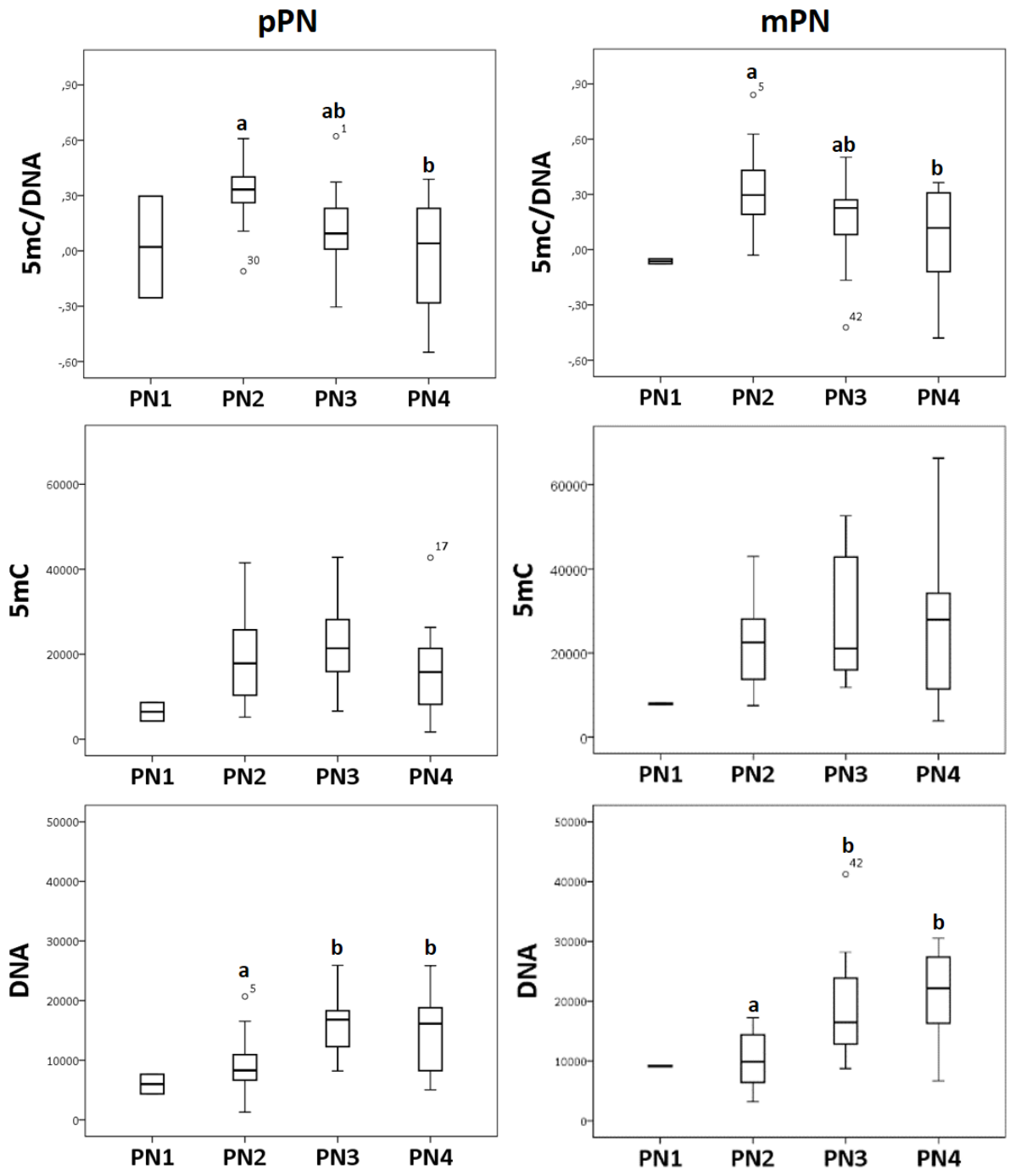

Figure 5.5. *Dynamics of 5mC and DNA levels in the paternal (pPN) and the maternal (mPN) pronucleus during pronuclear development in in vitro produced equine zygotes. In both the pPN and mPN, a significant reduction in the normalized levels of 5mC (5mC/DNA) from PN2 to PN4 was observed. No differences in the total 5mC fluorescence between pronuclear stages were found either in the pPN or the mPN. However, an increase in the DNA levels was observed between PN2 and PN3-PN4 in both pPN and mPN. Additionally, no differences in normalized levels of 5mC were found between mPN and pPN within pronuclear stage. PN1 (n=2), PN2 (n=18), PN3 (n=13) and PN4 (n=10). The analysis was performed with SPSS Statistics 23, different superscripts indicate significant differences, and p-values <0.05 were considered*

significant. The data was log10 transformed to achieve normal distribution when required. ANOVA, combined with Bonferroni post Hoc test, was used to compare total 5mC, DNA and normalized (5mC/DNA) fluorescence between the different pronuclear stages, independently for the mPN and pPN. Only in the comparison of the normalized fluorescence between different pronuclear stages in the pPN, the non-parametric test Kruskal-Wallis was used since the variances were not equal. Finally, to compare the normalized levels of 5mC between mPN and pPN within pronuclear stage, the data was analyzed by dependent T-Test. Due to the low sample number (n=2), no statistical conclusions can be drown about PN1 stage.

The embryos used in this study were produced by ICSI. In several species, such as rabbit (Reis Silva *et al*. 2011) and rat (Yoshizawa *et al*. 2010), the loss of methylation observed by immunostaining in the pPN of *in vivo* derived zygotes, was reduced after *in vitro* production, and especially after ICSI. However, in sheep (Beaujean *et al*. 2004) and cattle (Abdalla *et al*. 2009) no differences in DNA methylation patterns were observed between *in vivo* and *in vitro* zygotes using the same technique. In mouse, reduced loss of methylation of the pPN was observed after IVF when the acid-based epitope retrieval was used for immunostaining. Instead, when the acid-tryptic-based epitope retrieval was used instead, no differences in the DNA methylation pattern were observed between *in vivo*-derived and *in vitro*-produced mouse zygotes, with both mPN and pPN showing high levels of DNA methylation (Li and O'Neill 2012). Whether the observations made in this study truly reveal the dynamics of 5mC in equine zygotes or instead only the dynamics of *in vitro* produced equine zygotes, needs to be investigated further. The collection of equine zygotes *in vivo* is a daunting task, since the horse is a monovulatory species that does not respond to superovulation. The ICSI protocol used in this study yields 20% blastocysts rate, which is in line with the results obtained by other groups (Galli *et al*. 2007). Additionally, healthy foals have been born from embryos produced using the same *in vitro* production procedure of this study. Therefore, the active demethylation of the pPN during the first cell cycle does not seem to be essential for obtaining normal, healthy offspring in the horse.

No statistically relevant conclusions could be drawn from the PN1 stage, since the sample size was only two zygotes. However, 5mC was highly present in both pronuclei in the two zygotes analyzed.

DYNAMICS OF DNA HYDROXYMETHYLATION DURING PRONUCLEAR DEVELOPMENT

The pattern of 5hmC was evaluated in equine zygotes by immunostaining. The presence of 5hmC was found throughout pronuclear development in both the pPN and the mPN, and its distribution was very homogeneous (Figure 5.3). This contrasts with previous studies in the mouse reporting accumulation of 5hmC in partial rings around the nucleolar precursor bodies (Salvaing *et al*. 2012; Li and O'Neill 2013).

When first discovered, 5hmC was considered to be only an intermediate form for DNA demethylation through TET oxidation (Tahiliani *et al*. 2009). The inverse patterns between 5mC and 5hmC reported in mouse, rabbit and bovine zygotes (Iqbal *et al*. 2011; Wossidlo *et al*. 2011), in which 5hmC was only present in the pPN, support this hypothesis. However, indications are raising that 5hmC has its own epigenetic role (Salvaing *et al*. 2012; Hahn *et al*. 2013; Iurlaro *et al*. 2013; Li and O'Neill 2013).

In the present study, no significant differences were observed in the normalized 5hmC levels (5hmC/DNA) between pronuclear stages regardless the parental origin of the pronuclei (Figure 5.6). This indicates a constant level of 5hmC relative to DNA throughout pronuclear development. This persistent level of 5hmC relative to DNA throughout pronuclear development has also been reported in mouse (Li and O'Neill 2013).

Consequently, when studying the total 5hmC fluorescence between pronuclear stages independently in the pPN and the mPN, a significant increase was observed between PN1 and PN3 (p-value =0.022) in the pPN, and between PN1 and PN3-PN4 (p-value =0.017 and 0.019, respectively) in the mPN (Figure 5.6). Additionally, the total DNA fluorescence between pronuclear stages was significantly higher in the pPN and mPN between PN1 vs. PN3-PN4 (p-value <0.01), and PN2 vs. PN3 (p-value <0.05). These results indicate that there is an increase in the 5hmC associated to the increase of DNA (replication).

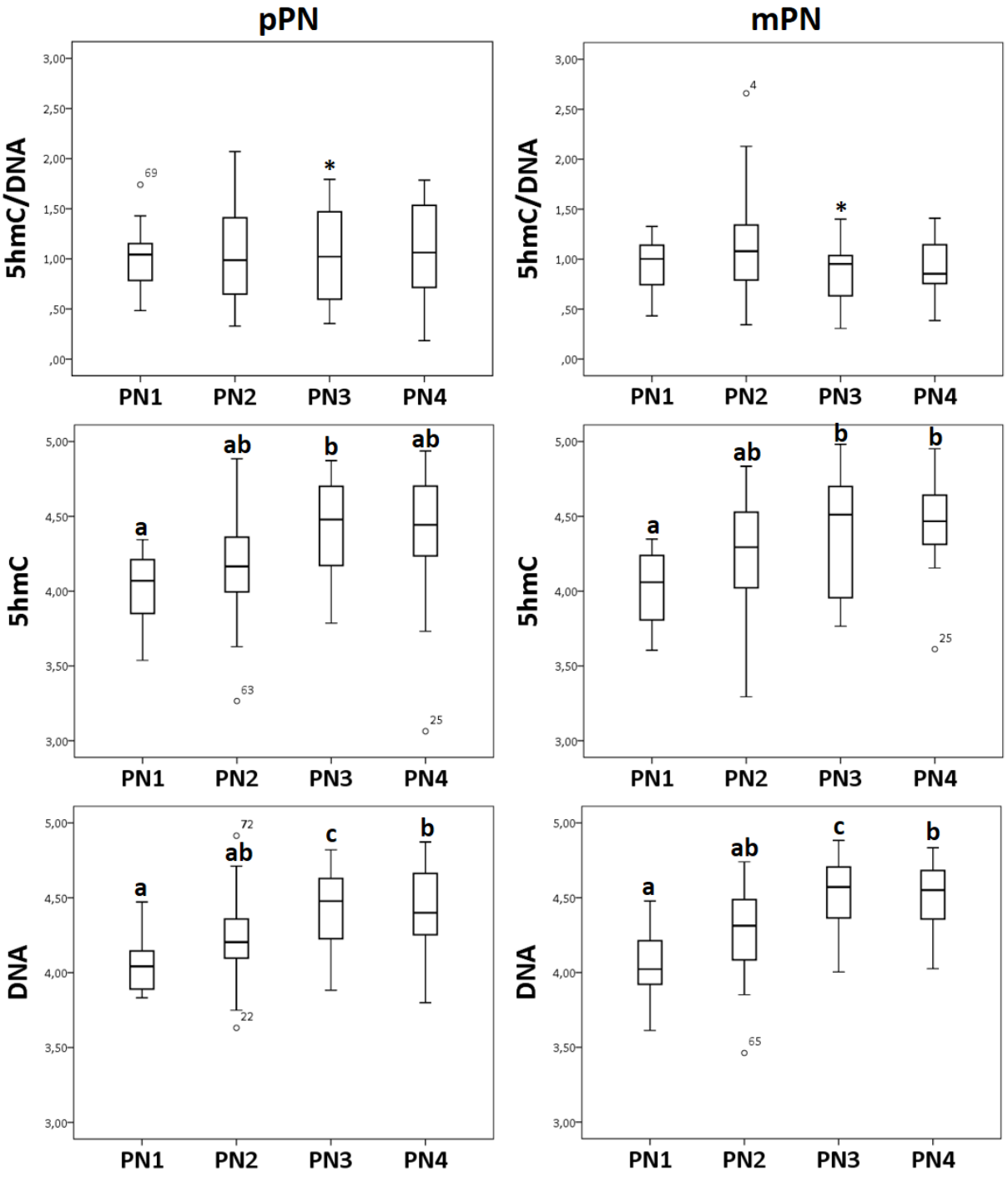

Figure 5.6. *Dynamics of 5hmC and DNA levels in the paternal (pPN) and the maternal (mPN) pronucleus during pronuclear development in in vitro produced equine zygotes. Different superscripts indicate significant differences. No differences in the normalized levels of 5hmC (5hmC/DNA) were observed between the developmental stages in pPN or the mPN. However, the levels of 5hmC were significantly lower in the maternal PN3 than the paternal counterpart (p-value =0.46). A significant increase in the levels of total 5hmC fluorescence was found in the pPN between PN1 vs. PN3, and in the mPN between PN1 vs. PN3-PN4. Additionally, a significant increase in the total DNA fluorescence was observed between PN1 and PN3-PN4, and PN2 vs. PN3 both in pPN and mPN. PN1 (n=12), PN2 (n=36), PN3 (n=20) and PN4 (n=13).*

The analysis was performed with SPSS Statistics 23, different superscripts indicate significant differences, and p-values <0.05 were considered significant. The data was log10 transformed to achieve normal distribution when required. ANOVA, combined with Bonferroni post Hoc test, was used to compare total 5mC, DNA and normalized (5mC/DNA) fluorescence between the different pronuclear stages, independently for the mPN and pPN. Only in the comparison of the normalized fluorescence between different pronuclear stages in the mPN, the non-parametric test Kruskal-Wallis was used since the variances were not equal. Finally, to compare the normalized levels of 5mC between mPN and pPN within pronuclear stage, the data was analyzed by dependent T-Test.

Significantly lower levels of normalized 5hmC were found in the mPN than the pPN in PN3, with a paternal/maternal ratio of 1.23 (p-value =0.046). This coincides with the significant increase of the paternal/maternal ratio of normalized 5hmC between pre- and early-replication found in the mouse (Salvaing *et al*. 2012). In our study, DNA replication appears to take place between PN2 and PN3 stages based upon the significant increase in total DNA fluorescence observed. Therefore, the increase of paternal/maternal 5hmC ratio seems to be also associated with replication in the horse. However, we did not observe differences between the pPN and the mPN in any of the other pronuclear stages, as has been reported in the mouse, rabbit and bovine (Iqbal *et al*. 2011; Wossidlo *et al*. 2011; Salvaing *et al*. 2012).

The inverse behavior of 5hmC and 5mC, with 5mC being present only in the mPN and 5hmC being present only in the pPN, as previously reported in mouse (Iqbal *et al*. 2011; Wossidlo *et al*. 2011; Zhang *et al*. 2012), was not observed in the present study conducted in the horse. Here, both 5mC and 5hmC were present in both the mPN and pPN throughout pronuclear development. Moreover, the pattern of distribution was totally different, with 5mC more concentrated in some regions, while 5hmC was homogeneously distributed in the pronuclei. Additionally, the significant reduction in normalized 5mC levels observed between PN2 and PN4 stages in both mPN and pPN, was not translated into an increase of the normalized 5hmC levels that remained unchanged, which further reinforce the hypothesis that no active DNA demethylation takes place in the horse zygote. Studies in neurons have also reported an increase in 5hmC without reduction in the 5mC levels (Hahn *et al*. 2013). Additionally, other studies in mouse zygotes also failed to observe an increase of 5hmC paired with the 5mC decrease (Salvaing *et al*. 2012; Li and O'Neill 2013).

In conclusion, in the present study the dynamics of DNA replication, DNA methylation and hydroxymethylation throughout pronuclear development are described for the first time in the horse. The significant increase in DNA observed between PN2 and PN3 in both the pPN and the mPN indicates that replication takes place at this time in equine zygotes. This coincides with a significant reduction of the normalized 5mC fluorescence (5mC/DNA) between PN2 and PN4 in both mPN and pPN, while the total 5mC fluorescence remains constant. There is no evidence of active DNA demethylation in the pPN but instead, it indicates a passive DNA replication-dependent loss of methylation in both the mPN and the pPN in the horse. On the other hand, an increase in the total 5hmC fluorescence was associated with the increase of DNA during replication. This resulted in constant normalized 5hmC fluorescence (5hmC/DNA) levels throughout pronuclear development in both the pPN and the mPN. Importantly, the disparity of results found in literature indicates that immunofluorescent staining protocols have a great impact on the results obtained. Therefore, a lot of caution needs to be taken into account before comparing different studies and drawing conclusions.

ACKNOWLEDGEMENTS

The authors thank Petra Van Damme and Isabel Lemahieu for their excellent technical assistance. This work was supported by a grant awarded to SH from the “Agentschap voor Innovatie door Wetenschap en Technologie” (IWT) grant number 111438. Experimental work was supported by a GOA grant - project no. 01G01112 – Pathways to pluripotency and differentiation in embryos and embryonic stem cells.

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CHAPTER 6

GENERAL DISCUSSION

When the general idea of this thesis was drafted in 2011, serum was the most commonly used medium supplementation for the support of *in vitro* development of cattle and horse embryos. In the nineties, an imprinting-related overgrowth syndrome, the so called large offspring syndrome (LOS), was reported in ruminants after the exposure of pre-elongating embryos to unusual conditions *in vitro* and *in vivo* (Young *et al*. 1998). Unfortunately the causes of LOS are not completely elucidated yet, but the use of co-culture and serum supplementation has been associated with increased rates of this syndrome in sheep and cattle (Young *et al*. 1998; Vajta *et al*. 2010). Additionally, serum supplementation was also reported to induce developmental abnormalities in mice (Khosla *et al*. 2001). For sanitary reasons and because of its association with LOS, serum started to be gradually replaced by other supplements, mainly bovine serum albumin (BSA), for the culture of cattle embryos used for transfer. In research, the replacement of serum for the production of cattle embryos *in vitro* was slower due to the high blastocyst rates obtained with this supplementation, so much so that serum is still currently used in some laboratories for bovine embryo production (Lopera-Vasquez *et al*. 2016). In horses, serum is nowadays the supplement used for embryo culture both for research and for transfer, and so far, no cases of overgrowth syndromes have been reported (Johnson *et al*. 2010; Johnson and Hinrichs 2015). In 2008, a serum-free culture medium for bovine *in vitro* embryo production, in which serum was replaced by BSA and insulin-transferrin-selenium (ITS), was applied with good blastocyst rates (George *et al*. 2008). Similar serum-free media intended for *in vitro* bovine embryo production were then introduced in several laboratories, including ours (Goovaerts *et al*. 2012; Wydooghe *et al*. 2014).

The first aim of this thesis was to evaluate the effect of this newly developed serum-free medium on the gene expression of cattle embryos produced, respect to that of *in vivo* derived embryos, and to use these findings to gain insights on how to improve *in vitro* embryo production in cattle. Secondly, since several assisted reproductive technologies (ARTs) are reported to induce epigenetic disorders (such as LOS), we aimed to evaluate the effect of *in vitro* production (especially of serum supplementation) on the epigenetic patterns of equine and bovine embryos, by means of immunofluorescent staining. And additionally, to study the global epigenetic pattern of these two species.

6.1 INFLUENCE OF THE ENVIRONMENT ON THE TRANSCRIPTOME AND THE EPIGENOME OF THE BOVINE EMBRYO

6.1.1 The *in vivo* environment: gold standard?

Physiologically, the embryo develops in the oviduct and the uterus and this maternal environment *in vivo* provides the optimal conditions to support the embryo. Therefore, *in vivo* derived embryos can be considered as a gold standard when embryo quality is assessed for embryos produced *in vitro* in different culture conditions. However, also *in vivo*, the embryos can be affected by changes in the environment, such as changes in nutrition and hormone treatments, which affect their transcriptomic and epigenetic fingerprint (van Montfoort *et al*. 2012).

The *in vivo* embryos used in chapter 3 were obtained from superovulated cows for practical reasons. Initially we tested if it was possible to obtain sufficient embryos after natural (single) ovulation, since superovulation has been associated with alterations of imprinting in the mouse (Market-Velker *et al*. 2010b), and of gene expression in bovine oocytes (Chu *et al*. 2012). However, after inseminating 16 cows, only 3 produced an embryo, each at different stages of development. In another study using single ovulating animals, 84 cows were necessary to obtain 5 grade 1 blastocysts of the same developmental stage, according to International Embryo Transfer Society (IETS) (Stringfellow and Givens 2010). This meant that a high number of animals would be necessary for our experiment, and it would take a long time so we decided to continue with superovulation. Most studies on transcriptomics using *in vivo* bovine embryos in literature have used superovulated cows (Kues *et al*. 2008; Vallee *et al*. 2009; Bermejo-Alvarez *et al*. 2010; Cote *et al*. 2011; Ghanem *et al*. 2011; Gad *et al*. 2012; Jiang *et al*. 2014). Nevertheless, we cannot exclude that superovulation could have influenced the results of this study.

6.1.2 Serum versus serum-free bovine embryo culture

The replacement of serum by BSA-ITS, was mainly motivated by the increased incidence of LOS reported after the use of serum (Young *et al*. 1998). Additionally, despite the fact that using serum was yielding high blastocyst rates, with high reproducibility of the experiments, individual embryo culture did not work under this condition (Wydooghe *et al*.

2014). Therefore, the first experiments of this thesis were focused on the establishment of this serum-free culture system. Soon, similar blastocyst rates were obtained with serum-free to the ones obtained with serum supplementation. Additionally, individual embryo culture was successful under this condition, yielding excellent blastocyst rates (Wydooghe *et al*. 2014). The embryos produced in group under serum-free conditions were of higher quality than the ones produced in the presence of serum, showing similar total cell number (TCN) and apoptosis, and higher inner cell mass/trophectoderm (ICM/TE) ratio. However, embryo development was slower, and hatching rates were significantly lower (Wydooghe *et al*. 2014).

Traditional parameters (developmental timing, blastocyst rates, TCN, ICM/TE rate and apoptosis) are valuable but not sufficient for the evaluation of embryo quality, since different culture conditions are reported to induce aberrations in gene expression and in epigenetic patterns that cannot be determined with the traditional methods (Market-Velker *et al*. 2010a; Driver *et al*. 2012). Embryos are very plastic, and morphologically normal blastocysts can develop under suboptimal conditions (Purpera *et al*. 2009). However, this capacity of adaptation can result in aberrant embryonic development (Duranthon *et al*. 2008). Whether the changes in the transcriptome and the epigenome of the resulting *in vitro* produced embryos are temporary adaptations to the suboptimal conditions or whether they will lead to aberrant embryonic development and even to long term effects, such as LOS, needs to be elucidated further. In any case, a high deviation from the *in vivo* transcriptome and epigenome indicates how challenging the environment is for the developing embryos. Therefore, we also wanted to determine transcriptomic changes and eventual epigenetic modifications, as visualized by changes in expression of genes encoding for enzymes responsible for DNA methylation. We investigated these changes in embryos produced in both *in vitro* conditions, and compared them to their *in vivo* counterparts.

In chapter 3 we determined that the transcriptome of the bovine embryos produced in serum-free culture conditions resemble more that of *in vivo* embryos than that of the embryos produced in serum conditions, independently of the sex of the embryos. Still, the embryos produced in serum-free medium showed up-regulation of genes involved in the biosynthesis of many molecules, including cholesterol, steroid, isoprenoid and carboxylic acid, compared to *in vivo* derived embryos. The alterations in the lipid pathways of embryos produced in serum-free conditions were also previously reported (Driver *et al*. 2012). Instead,

in the embryos produced in serum, the up-regulated pathways were mainly related to DNA repair, which is an indication of the detrimental effect that serum supplementation has on these embryos.

The gene expression of the enzymes responsible for the establishment and the maintenance of the DNA methylation, the DNA methyltransferases (Dnmts), was also affected by both *in vitro* conditions. However the alterations were greater in serum (Chapter 3). Dnmt1, responsible for the maintenance of the DNA methylation, was 1.6 and 1.8 folds up-regulated in serum and serum-free, respectively. On the contrary, the de novo methyltransferases were down-regulated *in vitro*. Dnmt3A was 1.6 folds down-regulated in serum-free and 5.7 folds down-regulated in serum, Dnmt3B was 5.2 folds down-regulated only in serum. No differences on the gene expression of the TET family was observed between the embryos produced *in vitro*, in neither of the conditions, compared to *in vivo* derived embryos. This illustrates again how embryos cultured in serum deviate more from their *in vivo* counterparts than embryos cultured in serum-free conditions, and that these deviations at the level of the transcriptome might have further implications at the epigenetic level. Considering the classic model of global epigenetic reprogramming, in cattle global re-methylation starts at the 8-cell stage and it is completed by the morula stage (Dean *et al*. 2003). In chapter 3 we used early blastocyst, in which global re-methylation should be already completed. We could hypothesize that the reduction of expression of de novo methyltransferases observed *in vitro* could indicate that global re-methylation was completed earlier *in vitro*, especially in serum. However, it is important to consider that even though the different epigenetic marks (epigenome) regulate the gene expression (transcriptome), comparisons between epigenome and transcriptome are not straightforward. Changes in mRNA levels are not necessary translated into changes at the protein level, which is especially true for mRNAs that specify proteins that are part of multifunction protein complexes (Schmidt *et al*. 2007). Furthermore, gene expression regulation by epigenetic marks is multifactorial, and not only DNA methylation (which is mostly studied) is involved, the other cytosine modifications, histone modifications and non-coding RNAs, and their interconnections, play also an important role.

Bearing in mind the dynamic character of the transcriptome in adaptation to the environment, most of the differences in gene expression observed in chapter 3 between *in vivo* derived and *in vitro* produced blastocysts are expected to be caused by *in vitro* culture

rather than by oocyte maturation. Supporting this hypothesis, in a comparable study, Driver *et al*. reported an over-representation of cholesterol biosynthesis and sterol synthesis in *in vitro* produced compared to *in vivo* derived blastocysts. In that study, they used serum supplementation for oocyte maturation and serum-free supplementation for embryo culture (Driver *et al*. 2012). Interestingly, in chapter 3, these two pathways were over-represented in the embryos produced in serum-free conditions compared to *in vivo*, but not in the embryos produced in serum conditions, showing the strong effect that embryo culture has on the transcriptome. Additionally, in a previous study comparing the effect of serum and serum-free supplementation for individual culture, in oocyte maturation or embryo culture, using the traditional methods, blastocysts rates were more similar according to the supplementation used for embryo culture. Higher blastocyst rates were obtained when serum-free supplementation was used for embryo culture, independently of the supplementation used for oocyte maturation compared to embryos cultured in the presence of serum, independently of the medium used for oocyte maturation (Wydooghe *et al*. 2014).

To gain further insight into the influence of the environment on the embryonic epigenome, we wanted to evaluate the global DNA methylation and hydroxymethylation status of embryos, of all developmental stages, produced *in vitro* in both conditions and to compare it to that of embryos derived *in vivo*. However, by that time, our laboratory did not have any experience with the study of epigenetics, and the optimization of a reliable immunofluorescent staining protocol was a time consuming task (Chapter 4.1 and 4.3). This left no time for the analysis of the effects of the two *in vitro* conditions on the methylation and hydroxymethylation status of the embryos, but it represents an interesting topic for further research.

Considering all this, serum-free culture is superior to serum for the production of bovine blastocysts with a transcriptome closer to that of *in vivo* and excellent embryo quality. Therefore, this strongly encourages the replacement of serum by serum-free conditions even for the production of bovine embryos intended for research. However, further optimization of this serum-free culture medium is still required.

6.2 IMMUNOFLUORESCENT STAINING FOR THE STUDY OF EPIGENETICS

Immunofluorescent staining and bisulfite sequencing are the most commonly used techniques for the study of epigenetics in gametes and preimplantation embryos (Stouder *et al*. 2009; Market-Velker *et al*. 2010a; Inoue *et al*. 2011; Salvaing *et al*. 2012; Li and O'Neill 2013). Recently, reduced representation bisulfite sequencing (RRBS) has also been applied to study DNA methylation in preimplantation embryos (Smith *et al*. 2012; Guo *et al*. 2013).

Immunofluorescent staining is a very powerful tool for the study of epigenetics because 1) it is very specific, 2) many epigenetic marks can be studied with this technique, even some simultaneously; 3) the epigenetic marks can be studied in single embryos and even independently in the paternal and the maternal genome of individual zygotes, and 4) global changes in the epigenetic patterns can be determined, which is useful to study the epigenetic reprogramming that takes place during preimplantation development, and to study the effect of *in vitro* production on epigenetic marks. As a drawback, immunofluorescent staining cannot be used for the study of epigenetic marks in specific genes, and cannot detect small epigenetic changes induced by different conditions. Bisulfite sequencing can only be used to study 5-methylcytosine (5mC), although, it cannot differentiate between 5mC and 5-hydroxymethylcytosine (5hmC). Additionally, it can be used for the study of single genes, and therefore, it is commonly used to study the methylation status of imprinted genes (Stouder *et al*. 2009; Market-Velker *et al*. 2010a; Market-Velker *et al*. 2010b). Reduced representation bisulfite sequencing (RRBS) also fails to differentiate between 5mC and 5hmC, however, its genome coverage is larger than that of bisulfite sequencing. Other techniques such as oxidative-bisulfite sequencing or methylated DNA immunoprecipitation (MeDIP) require large amounts of DNA. For all these reasons, immunofluorescent staining was the technique chosen for the study of epigenetic patterns in horse and cattle, in this thesis.

Despite all its advantages, the results obtained by immunofluorescent staining are highly influenced by the protocol used, and therefore, careful optimization of this protocol is required for its correct use and interpretation. In a recent paper, the technical requirements for the correct use of this technique were described (Salvaing *et al*. 2014), and special attention needs to be paid to three main steps.

- Proper epitope exposure
- Incubation length and concentration of primary antibodies
- Image acquisition and quantification

The immunofluorescent staining of 5mC and 5hmC is a complex process composed by many steps that need to be performed in a specific sequence. In figure 6.1 the specific protocol optimized in chapter 4 for equine and cattle zygotes is described. The most critical steps, namely epitope retrieval, DNA counterstaining and determination of the parental origin of the pronuclei, are discussed further in the following pages.

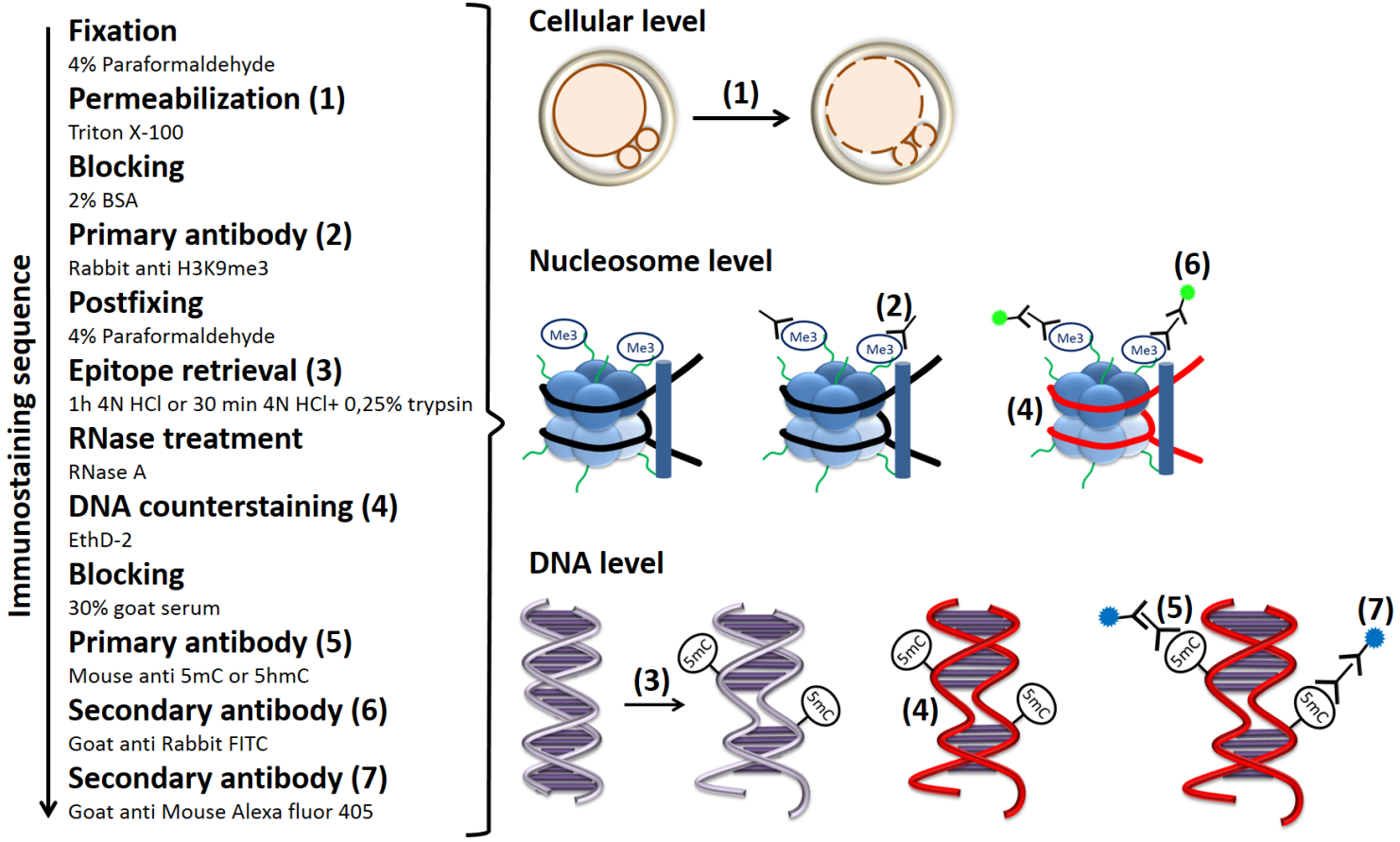

Figure 6.1. *Schematic representation of the protocol optimized for the immunofluorescent staining of equine and cattle zygotes. On the left the immunostaining sequence is described. The effects of several important steps of the immunostaining at cellular, nucleosome or DNA levels are graphically represented on the right.*

6.2.1 Epitope exposure and DNA counterstaining

Proper epitope exposure is essential for obtaining reliable results using immunofluorescent staining for the study of epigenetics. Unfortunately, the acid treatment required for it, completely or partially hampers the binding of DNA dyes. Therefore, the optimization of the epitope retrieval conditions required for each epigenetic mark in each species is necessarily associated to the optimization of the required DNA counterstaining.

The most commonly used epitope retrieval treatments are based on acid treatment and consist of 30 min to 1h incubation with 2 or 4N HCl (Santos *et al*. 2002; Yoshizawa *et al*. 2010; Reis Silva *et al*. 2011; Kurotaki *et al*. 2015). Shorter acid treatments have also been used, especially in mice (Li and O'Neill 2013). Alternative epitope retrieval treatments include DNAseI treatment simultaneous with incubation of 5mC primary antibody for 1h at room temperature, used in rat and mouse zygotes (Zaitseva *et al*. 2007); denaturation with 70% formamide at 80˚C for 1 min, used in mice and human zygotes (Xu *et al*. 2005), and brief tryptic digestion combined with acid treatment, used in mice (Li and O'Neill 2013).

It has been observed that the paternal and the maternal genome of mouse zygotes have different sensitivities to the different epitope retrieval treatments (Li and O'Neill 2012; Li and O'Neill 2013). After the traditional acid treatment, 5mC could only be observed in the maternal genome of mouse zygotes. However, when a brief tryptic digestion was combined to the acid treatment, stable levels of 5mC could be observed in both paternal and maternal pronuclei. We have determined that a correct exposure of 5mC and 5hmC epitopes in cattle and horse zygotes is achieved after 1h treatment with 4N HCl or 30 min treatment with 4N HCl combined with 20s tryptic digestion (Chapter 4.1 and Heras *et al*. 2014). The addition of a brief tryptic digestion in cattle and horse did not change the observed 5mC and 5hmC patterns, as previously described in mice (Li and O'Neill 2013). However, only 20s of tryptic digestion could shorten in half the length of the acid treatment. For bovine zygotes both protocols can be used indistinctly, but in equine zygotes, 30 min treatment with 4N HCl combined with 20s tryptic digestion is preferred, because it preserves better the integrity of the zona pellucida (ZP). Interestingly, despite the fact that the ZP of equine embryos is thicker and harder to penetrate mechanically than that of bovine embryos, it is more sensitive to acid

treatment, and 1h incubation with 4N HCl dissolves it almost completely. This is not the case in cattle, where after 1h incubation with 4N HCl the ZP is only partially dissolved.

As determined in chapter 4.1, under these epitope retrieval conditions, only intercalators, and especially Ethidium homodimer 2 (EthD-2), can be used as DNA counterstaining. Under different epitope retrieval conditions, other DNA dyes have been successfully used in different species. DAPI was successfully used in mouse but after incubation with 4N HCl for only 10 min (Li and O'Neill 2013). Additionally, anti-single stranded DNA antibodies (Wossidlo *et al*. 2011; Salvaing *et al*. 2012) and the intercalating dye YOYO-1 (Santos *et al*. 2002) have also been used.

The different requirements for epitope retrieval between species, and even between the maternal and the paternal pronuclei of the same zygote indicate different chromatin conformation between maternal and paternal pronuclei, and between different species (Salvaing *et al*. 2014). This chromatin conformation determines the different accessibility of antibodies to the DNA. The methyl group attached to 5-cytosine lies within the major groove of DNA, therefore, a linear conformation of the DNA is necessary for 5mC to be accessible to the antibodies (Salvaing *et al*. 2014).

6.2.2 Incubation length and concentration of primary antibodies

For the antibodies to correctly reflect the localization and levels of their target, the immunostaining conditions have to allow the antibody-antigen reaction to reach thermodynamic equilibrium. A reduction in the concentration of 5hmC primary antibody to 1:1000 resulted in a reduced signal at the mPN in mouse zygotes (Salvaing *et al*. 2014). Incubation with 5hmC primary antibody for longer than 1h at room temperature or shorter than 6h at 4˚C showed higher levels of 5hmC in the paternal than the maternal pronucleus; these differences disappeared when saturation binding conditions were used (Li and O'Neill 2013). We have determined that the most stable results were achieved after 24h incubation with each primary antibodies in both equine and bovine zygotes.

6.2.3 Determination of the parental origin of the pronuclei

One of the main advantages of the use of immunofluorescent staining for the study of epigenetics is that epigenetic patterns can be independently determined in the maternal and the paternal genome during pronuclear development in single zygotes. This is essential for the study of the asymmetric global epigenetic reprogramming that is believed to take place during the first cell cycle. To this end, the parental origin of the pronuclei needs to be unequivocally determined. In mouse zygotes, the size (with the pPN being the larger) and the relative position (with the mPN being the closest to the polar body) of the pronuclei are enough to determine their parental origin (Li and O'Neill 2013). However, in many other species, such as cattle and horse, the size of both pronuclei is very similar and variable, and the position of the pronuclei changes during migration. Despite this fact, several studies have used relative size and position to determine the parental origin of the pronuclei in bovine zygotes with questionable results (Hou *et al*. 2005; Abdalla *et al*. 2009). The asymmetric pattern of some histone modifications, being allocated only in the mPN, is frequently used as an alternative for the determination of the parental origin of the pronuclei. In chapter 4.2 we demonstrated that histone 3 lysine 9 tri-methylation (H3K9me3) was only present in the mPN of the equine zygote, which was confirmed in chapter 5 during all pronuclear development. This asymmetric pattern has also been observed in rabbit (Reis Silva *et al*. 2011), human (van der Heijden *et al*. 2009) and mouse (van der Heijden *et al*. 2009). In contrast, in the late pronuclear stages of bovine zygotes, H3K9me3 is also variably present in the pPN, as demonstrated in chapter 4.3, and therefore cannot be used for parental origin determination of the pronuclei. The same observations were made previously in pig zygotes (Jeong *et al*. 2007). Instead, histone 3 lysine 27 two-tri-methylation (H3K27me2-3) is only present in the pPN of bovine zygotes, and therefore can be used for determination of parental origin. This asymmetric pattern is conserved in human (van der Heijden *et al*. 2009) and mouse (van der Heijden *et al*. 2005), and H3K27me2 in mouse (van der Heijden *et al*. 2005). The reason for the non-conservation of the pattern for histone modifications is not known.

In human, the asymmetric pattern of 5mC and 5hmC between the maternal and the paternal pronuclei has also been used to determine the parental origin of the pronuclei (Kai *et al*. 2015). However, one study conducted in human zygotes reported that only half of the zygotes showed an asymmetric pattern between the pPN and the mPN (Fulka *et al*. 2004).

In conclusion, several methods can be used to determine the parental origin of the pronuclei. Nevertheless, these methods cannot be applied to every species, and testing is required before their application to every new species.

6.2.4 Image acquisition and quantification

Single image vs. z-stack. A z-stack consists in a series of images taken at different focal planes of a selected region of interest. It thus provides a better general idea of the region of interest, in this case the pronuclei. However, during z-stack acquisition, the intensity of the fluorescence gets reduced due to bleaching, introducing bias between the first and the last image acquired.

The choice of the secondary antibody for fluorescence quantification is also essential to reduce bias due to bleaching. As an example, fluorescein isothiocynate (FITC) is not recommended for immunofluorescence quantification because it is prone to photobleaching and its signal is sensitive to pH changes. Instead, derivatives of fluorescein such as Alexa fluor 488 have a greater photostability.

Proper correction for pronuclear size and DNA content is also required. The size of each pronucleus in a zygote is often different. Therefore, lower fluorescence intensity in the larger pronucleus does not necessarily mean lower presence of the epigenetic mark, because it can be just distributed in a larger area. Similarly, correction for DNA content is essential for correct image quantification, as it was observed in chapter 5. DNA replication takes place during pronuclear migration. Therefore, an increase in the total fluorescence intensity of the epigenetic mark after DNA replication does not indicate an increase of the mark between pronuclear stages. Instead, it indicates that the levels of the epigenetic mark remain constant relative to the DNA content during pronuclear development, and that these levels are maintained after DNA replication.

All in all considered, it is fundamental to keep in mind that immunofluorescence quantification is not absolute but relative, and that it is subjected to technical issues, such as over-exposed areas in the region of interest, which is very frequent in heterogeneous targets such as DNA, and can lead to wrong conclusions.

6.3 GLOBAL EPIGENETIC REPROGRAMMING DURING PRONUCLEAR DEVELOPMENT, FACT OR ARTIFACT?

In the last part of this thesis, we used the 5mC and 5hmC optimized immunofluorescent staining for horse zygotes (Chapter 4) to evaluate if our species of interest followed the classic model of global epigenetic reprogramming (Chapter 5).

The classic model of global epigenetic reprogramming consists in a global erasure of epigenetic marks during preimplantation embryo development for the establishment of totipotency. The global loss of DNA methylation, excluding imprinted genes and retrotransposons, is asymmetric between the maternal and the paternal genome (Figure 6.2). The paternal genome undergoes active demethylation (replication-independent) before the onset of the first cell cycle. This active demethylation is initiated by the ten-eleven translocation 3 (TET3) enzyme that oxidizes 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (Kriaucionis and Heintz 2009; Tahiliani *et al*. 2009). Therefore, a reduction in the levels of 5mC, combined with a rise in the levels of 5hmC, which will reach their minimum and maximum, respectively, are observed during the first cell cycle in the paternal genome. In contrast, the maternal genome is protected from active demethylation and undergoes passive demethylation by the dilution of the DNA methylation marks with each cell division (replication-dependent). The protein STELLA protects the maternal genome from active demethylation by binding to the histone modification H3K9me3, excluding TET3 and thus avoiding the oxidation of 5mC to 5hmC (Szabo and Pfeifer 2012; Kang *et al*. 2013). Therefore in the maternal genome, a reduction of 5mC after DNA replication, and no 5hmC are observed during the first cell cycle.

This classic model was established based on studies conducted in the mouse (Mayer *et al*. 2000; Oswald *et al*. 2000) and at first, it was believed to be conserved in all species (Dean *et al*. 2001). However, several of the species studied did not follow this model, and three big groups of species were created according to their DNA demethylation behavior during the first cell cycle: 1) species that followed the classic model, showing a complete demethylation of the pPN, 2) species that did not show any demethylation of neither the paternal or the maternal pronuclei, and 3) species that showed only a partial demethylation of the pPN (Table 6.1).

Table 6.1. *Species belonging to each of the three groups according to their DNA methylation reprogramming model during the first cell cycle. When controversy in the literature was found, the species were assigned to the globally most accepted group. The controversy between the studies in rabbit and pigs made it impossible include them in any of the groups. Additionally, the observations found in horse in chapter 5 are also not compatible with any of the groups.*

Reprogramming model	Species	Reference
Classic model: Asymmetric complete demethylation	Mouse	(Mayer *et al*. 2000; Santos *et al*. 2002)
	Rat	(Zaitseva *et al*. 2007; Yoshizawa *et al*. 2010)
	Human	(Beaujean *et al*. 2004a; Xu *et al*. 2005; Guo *et al*. 2014b)
Partial asymmetric demethylation	Cattle	(Beaujean *et al*. 2004a; Hou *et al*. 2005; Abdalla *et al*. 2009)
No demethylation	Sheep	(Beaujean *et al*. 2004a; Hou *et al*. 2005)
	Goat	(Hou *et al*. 2005)

The mere fact that not all the species studied follow the classic epigenetic reprogramming model raises questions about the necessity of this epigenetic reprogramming for normal development, but it could be explained by species specific differences. However, the use of different techniques, and more importantly of different immunostaining conditions, data evaluation and processing has led to contradictory results, even within the same species.

In the mouse, the classic model has been confirmed in several studies by immunostaining (Barton *et al*. 2001; Santos *et al*. 2002; Beaujean *et al*. 2004a; Xu *et al*. 2005; Inoue *et al*. 2011; Iqbal *et al*. 2011; Wossidlo *et al*. 2011; Zhang *et al*. 2012; Kurotaki *et al*. 2015) and reduced representation bisulfite sequencing (RRBS) (Smith *et al*. 2012). But other studies conducted in mice did not confirm the classical model. In 2012, Salvaing observed, using immunostaining, presence of 5mC and 5hmC in both pronuclei during pronuclear development, even though the levels of 5mC were lower in the pPN, and the levels of 5hmC were lower in the mPN. Additionally, no complementary pattern between 5mC and 5hmC was observed in the pronuclei (Salvaing *et al*. 2012). Furthermore, by changing the epitope retrieval conditions, Li observed the same levels of 5mC and 5hmC in both pronuclei during the entire pronuclear development (Li and O'Neill 2012; Li and O'Neill 2013). Passive

demethylation of the mPN and a combination of passive and active demethylation of the pPN was observed by single-cell RRBS (Guo *et al*. 2013). And a combination of passive and active demethylation in both pronuclei was observed by RRBS (Guo *et al*. 2014a; Shen *et al*. 2014) and hairpin bisulfite sequencing (DHBS) (Arand *et al*. 2015). Despite the assumption that human follows the classic model (Beaujean *et al*. 2004a; Xu *et al*. 2005; Guo *et al*. 2014b), in one study, in half of the zygotes 5mC remained at the same level in both pronuclei during pronuclear development (Fulka *et al*. 2004). In the rabbit, the observations ranged from the model proposed in the mouse (Wossidlo *et al*. 2011), to only partial demethylation of the pPN (Reis Silva *et al*. 2011) to no loss of methylation in any of the pronuclei (Beaujean *et al*. 2004a). In pigs, active demethylation of the pPN has been reported in some studies (Dean *et al*. 2001; Fulka *et al*. 2006), while in others no demethylation was observed (Jeong *et al*. 2007). Cattle is considered to display partial demethylation of the pPN. In the different studies, partial demethylation of the pPN (Beaujean *et al*. 2004a; Hou *et al*. 2005; Abdalla *et al*. 2009) but also partial active demethylation followed by immediate remethylation of the pPN (Park *et al*. 2007) have been reported. The technique used in all these studies was immunofluorescent staining, however, in each study different protocols were used. In chapter 4.1 of this thesis, we demonstrated that the optimal epitope retrieval for 5mC and 5hmC exposure in bovine zygotes was 1h treatment with 4N HCl or 30 min treatment with 4N HCl combined with a brief tryptic digestion, both at room temperature. Only in one of these studies 1h treatment with 4N HCl was used, but it was performed at 37˚C (Hou *et al*. 2005). Additionally, the determination of the parental origin of the pronuclei in two of the studies was based on relative pronuclear size and position (Hou *et al*. 2005; Abdalla *et al*. 2009), which is not reliable for bovine zygotes as it was established in chapter 4.3. Furthermore, in the mentioned studies, the guidelines proposed by Salvaing for image acquisition and analysis were not followed (Salvaing *et al*. 2014), and no corrections for pronuclear size and DNA content were applied, which we demonstrated in chapter 5 to be of major importance. Therefore, the results reported in cattle need to be interpreted with caution, and it is advisable to perform a re-analysis using the protocol optimized in chapter 4. Unfortunately, time constraints prevented us to analyze the 5mC and 5hmC patterns in bovine zygotes with the optimized immunostaining protocol. Accordingly in chapter 5, equine zygotes also did not follow the classic model of global epigenetic reprogramming, and no asymmetric pattern of DNA demethylation during the first cell stage was observed (Figure 6.2).

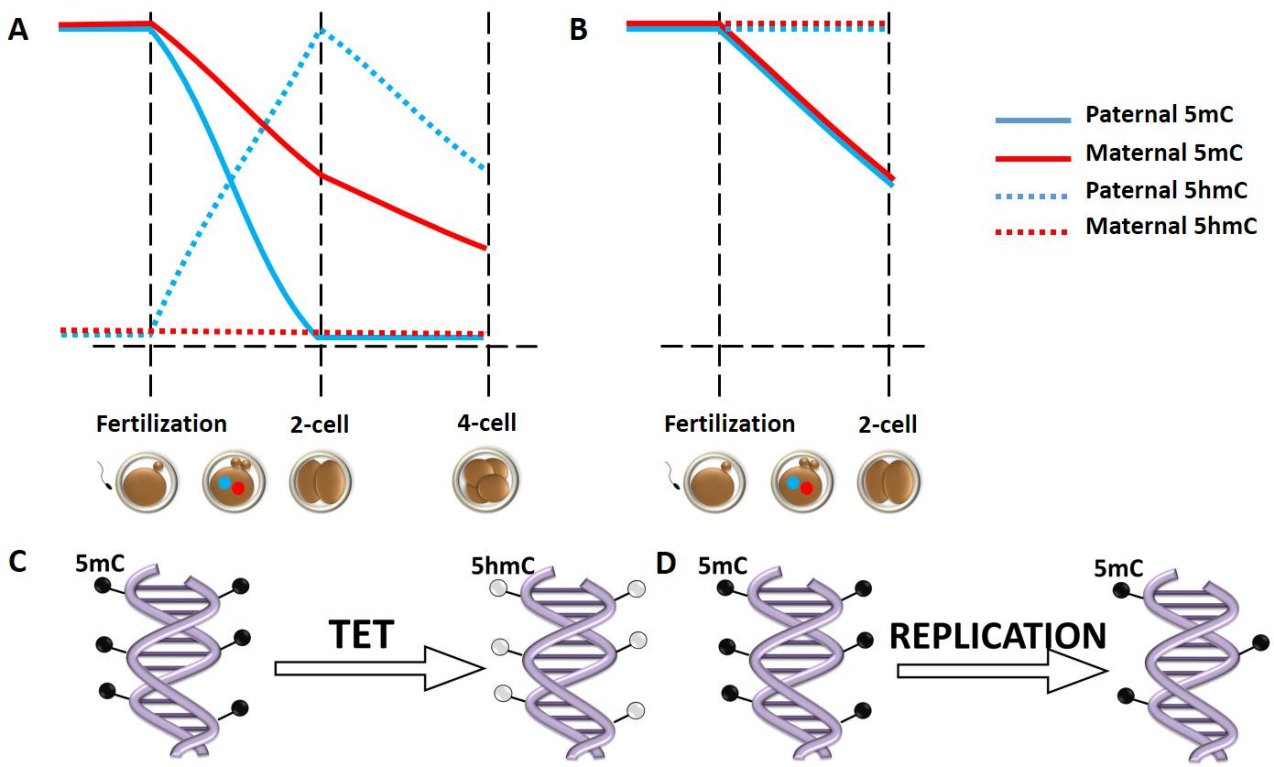

Figure 6.2. *Epigenetic reprogramming at preimplantation embryo development. (A) Classic epigenetic reprogramming model established in the mouse. After fertilization, the paternal genome undergoes active demethylation that is completed before the end of the first cell cycle. Simultaneously, an increase in 5hmC is observed in the paternal genome, which reaches its maximum at the 2-cell stage, and decreases thereafter. In contrast, the maternal genome undergoes passive demethylation through DNA replication, losing half of its methyl groups after each cell division. The level of 5hmC remain low during preimplantation embryo development. (B) Epigenetic reprogramming in the horse. Both the paternal and maternal genomes undergo passive demethylation in the first cell cycle. The levels of 5hmC remain high in both genomes during the first cell cycle. (C) The active demethylation is mediated by the TET enzymes, which oxidize 5mC into 5hmC. (D) In the passive demethylation with the DNA replication, the DNA becomes hemi-methylated.*

Instead, passive demethylation of both pPN and mPN during the first cell cycle, combined with high levels of 5hmC in both pronuclei was demonstrated. Considering the observations found in the horse in chapter 5, this species cannot be included in any of the proposed reprogramming groups. No other studies have been conducted in the horse to compare with ours, but the careful optimization of the immunostaining conditions performed in chapter 4 makes us confident of our findings. Nevertheless, it has to be noted that the zygotes used in this study were produced after ICSI, which is a technique that has been reported to impair active demethylation in rat (Yoshizawa *et al*. 2010). However, no effects of ICSI on the epigenetic dynamics have been observed in mouse (Polanski *et al*. 2008; Kurotaki *et al*. 2015), sheep (Beaujean *et al*. 2004b) and cattle (Abdalla *et al*. 2009). Therefore, the effect of *in vitro* production on the epigenetic dynamics of equine zygotes needs to be further elucidated.

The controversial results of studies conducted in the same species point out the sensitivity of the immunofluorescent staining to technical variations. Clearly, the use of different immunostaining protocols and different data processing led to different results within the same species. This further questions the reliability of the studies, and makes further comparisons between studies very difficult, even within the same species.

All these results, together with the observation that mouse embryos which are failing to display global demethylation can develop normally to term (Polanski *et al*. 2008), suggest that the global epigenetic reprogramming during preimplantation embryo development is not essential for normal development. This indicates that the global epigenetic reprogramming during embryo development might not occur as such, or be only restricted to certain genes.

6.4 SAMPLE SIZE: OPTIMAL VERSUS POSSIBLE

Finally, it is important to bear in mind that the use of the correct sample size in an experimental study is essential to draw valid conclusions.

Before performing any experiment in which the significance of the results will be established by statistics, it is essential to calculate the optimal experimental sample size, according to the magnitude of the expected differences between the groups. Differences that are actually biologically relevant may not be statistically significant if the sample size is too

small. On the contrary, if the sample size is too large, the opposite risk exists, meaning that significant differences will be found even when they do not have any biological meaning.

The absolute minimum number of biological replicates necessary for RNA sequencing to be able to perform reliable statistics is 3. In chapter 3, we used in total 24 embryos, 8 originated from each culture condition, with each embryo constituting a biological replicate. Therefore, the number of replicates included in each comparison varied from 8, when embryos of both sexes were included in the comparison, to 3, when embryos were segregated by sex. Therefore, the number of replicates included in chapter 3 is in every case above the minimum, and when all the embryos were included in the comparison, it is higher than most of the studies using RNA sequencing (Driver *et al*. 2012; Chitwood *et al*. 2013; Graf *et al*. 2014). The main limitation to increase the number of replicates is the high cost of each replicate in RNA sequencing.

On the other hand, since each embryo constitutes a replicate, the number of embryos per comparison is low, especially when the embryos were compared by sex. In some studies, to increase the number of embryos without increasing the costs, pools of several embryos constitute each replicate (Driver *et al*. 2012; Graf *et al*. 2014). In our experiment, pooling was not an option due to technical reasons. We wanted to compare the embryos by sex, and hence pooling of the embryos could be performed either before or after RNA extraction. For pooling before RNA extraction, either sex-sorted semen, or embryo biopsy is required for sex determination, and both of these techniques can alter the transcriptome of the embryos. For pooling after RNA extraction and sex determination, an extra step of RNA concentration is necessary to reduce the volume for library preparation, which lead in RNA loss and reducing its quality.

Whether the low amount of embryos included in chapter 3 is representative of the whole population of embryos produced under those conditions is debatable. However, the consistency of the hierarchical clustering and the principal component analysis, and the fact that some of the results obtained in chapter 3 were previously reported in a similar study (Driver *et al*. 2012) indicates it is indeed so.

In chapter 5 the main limitation was the scarce amount of equine oocytes. Additionally, some pronuclear stages are very transitory, as PN1, which combined with the low activation

rates of the first time point of collection (only around 40% of the oocytes are activated 8h after ICSI), leads to a low number of embryos at PN1 stage, being only 2 when studying 5mC patterns. In that situation, no conclusions about the pattern of 5mC at PN1 can be achieved.

In experiments in which no statistics are required to draw conclusions, such as the evaluation of the presence and/or location of a specific epigenetic mark by immunofluorescent staining, the necessary sample size depends on the consistency of the observations. If they are very variable, as the pattern of H3K9me3 in bovine zygotes (Chapter 4.3) a relatively high sample size is required to elucidate the real pattern of the mark. Instead, when the observations are consistent, as in chapter 4.2, once the pattern is established, and increase in the sample size will not increase the information obtained in the experiment.

6.5 FURTHER CONSIDERATIONS

The results obtained in chapter 3 constitute a first step towards the optimization of serum-free culture medium for *in vitro* production of bovine embryos. Here we demonstrated that serum must be replaced by serum-free culture conditions for bovine embryo production. However, the serum-free culture medium used in the study is still not optimal, and many genes involved in the biosynthesis of many molecules, such as cholesterol, sterol and alpha-amino acid, were up-regulated in the embryos produced in serum-free conditions compared to *in vivo*. This could be just a coping reaction of the embryo to this stressful environment, but also could indicate that *in vivo* these molecules are provided by the mother, while under these conditions the molecules had to be synthetized by the embryo. The addition of these molecules to the serum-free culture medium should be further tested for medium optimization. Additionally, the effect of *in vitro* production in serum-free conditions on the epigenetic pattern of bovine embryos during the complete embryo development, still needs to be assessed by using the immunostaining protocol optimized in chapter 4.

Furthermore, the optimized immunofluorescent staining protocol can be applied to gain new insights in the epigenetic reprogramming of bovine preimplantation embryos, and to determine if, with this new optimized protocol, an asymmetric pattern between pPN and mPN is also observed.

In chapter 5, the dynamics of 5mC and 5hmC during pronuclear development were determined, but the study of the complete pre-elongation development is still lacking. Moreover, the 5mC and 5hmC patterns of *in vivo* derived equine zygotes and embryos need to be studied and compared to the pattern of *in vitro* produced embryos, to elucidate if IVP induces global epigenetic changes in equine zygotes.

6.6 GENERAL CONCLUSIONS

The conclusions of this thesis are:

- The replacement of serum supplementation by bovine serum albumin and insulin-transferrin-selenium (BSA-ITS) for bovine *in vitro* embryo production is advisable based upon the data obtained by means of transcriptomics. Nevertheless, further optimization of this serum-free culture medium is required, since the expression of genes involved in the biosynthesis of many molecules is up-regulated in embryos produced under this condition, compared to *in vivo* derived embryos.

- The presence of serum during *in vitro* production appeared to have a different impact on the embryos according to their sex, with male embryos having three times more genes differentially expressed compared to *in vivo* derived embryos, than their female counterparts.

- A robust immunofluorescent staining protocol was optimized for the study of epigenetic patterns of bovine and equine zygotes and embryos. The validity of this protocol in other species needs to be evaluated further.

- For the correct exposure of 5mC and 5hmC epitopes in bovine zygotes, 1h denaturation with 4N HCl, or 30 min denaturation with 4N HCl combined with a brief tryptic digestion are essential. For equine zygotes, 30 min denaturation with 4N HCl combined with a brief tryptic digestion is optimal, since after 1h denaturation with 4N HCl, the fragility of the zygotes is highly increased.

- Under these epitope retrieval conditions, the use of DNA intercalating dyes, especially Ethidium homodimer 2 (EthD-2), is required, since DNA dyes that bind to the minor groove, such as Hoechst, can no longer bind to the DNA after the denaturation process.

- The asymmetric pattern of H3K9me3 can be used to determine the parental origin of the pronuclei in equine zygotes. In contrast, the pattern of H3K9me3 is not consistent between the paternal and the maternal pronuclei in bovine zygotes. Instead, the asymmetric pattern of H3K27me2-3 is recommended for the determination of the pronuclear parental origin of bovine zygotes.

- During pronuclear development in equine, a loss of DNA methylation associated with DNA replication (passive DNA demethylation) of both paternal and maternal pronuclei takes place. Additionally, the levels of 5hmC remain constant during pronuclear development regardless the parental origin of the pronuclei.

- Taking into account recent literature and the findings of this thesis, there is enough evidence to warrant a revision of the classic model of epigenetic reprogramming during preimplantation embryo development as earlier described in the mouse.

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SUMMARY

Assisted reproductive technologies (ARTs) are routinely used to produce transferable embryos for treatment of infertility in human and for economic reasons in livestock. In recent years, ARTs have become more sophisticated, yielding higher blastocysts rates, as we gained more in-depth knowledge on embryo development. Despite this positive evolution, the increased use of ART is exposing gametes and embryos to suboptimal conditions during a developmental period in which they are very susceptible to genetic and epigenetic modifications. Changes in the transcriptome and epigenetic pattern of zygotes and embryos have been reported in many species, including human. Special attention needs to be paid to the composition of the media used for embryo culture, as this has a high impact, not only on blastocyst rates, but also on the quality of the produced embryos.

Therefore, the evaluation of the effects of ARTs on the produced gametes and embryos, in terms of inducing transcriptomic and epigenetic alterations, is fundamental to test their safety. Additionally, the knowledge gained with this evaluation can help improving ARTs, resulting in the production of more *in vivo*-like embryos.

In chapter 1, the events that take place during preimplantation embryo development, and the techniques used to support this development *in vitro*, the so called assisted reproductive technologies are described. Additionally, an overview is given of the effects of ARTs on embryo quality and the different techniques used to evaluate the latter.

The general aim of this thesis was to study the impact that *in vitro* production has on bovine and equine embryos (Chapter 2). The first aim was to evaluate the effect that two different culture conditions have on the transcriptome of bovine blastocysts. The second aim was to determine the epigenetic patterns of *in vitro* produced equine and bovine zygotes, and to evaluate if changes in the epigenetic patterns could already be detected at this early stages.

First, we used RNA sequencing to examine the effect of exposing embryos to different environments on the global gene expression pattern of bovine blastocysts (Chapter 3). To this end, we exposed bovine embryos either to a suboptimal environment known to induce alterations in the offspring, such as large and less viable calves (serum-containing medium), or to an optimized culture medium in which serum has been replaced by bovine serum albumin and insulin-transferrin-selenium (BSA-ITS; serum-free conditions). Then, we compared the transcriptome of the blastocysts produced under these two conditions to that

of *in vivo* derived blastocysts of the same stage. Serum supplementation had a major impact on the gene expression pattern of the embryos, with embryos produced in serum-containing medium expressing five times more genes differentially than embryos produced in serum-free medium, when compared to *in vivo* derived embryos (1,109 vs. 207). Importantly, the use of serum supplementation for *in vitro* production of bovine embryos appeared to have a different impact on the embryos according to their sex, with male embryos expressing three times more genes differentially than their female counterparts (1,283 vs. 456). In contrast, male and female embryos produced in serum-free conditions showed the same number (191 vs. 192) of genes expressed differentially. The pathways affected by *in vitro* production differed depending on the type of supplementation. Embryos produced in serum-containing medium had lower expression of genes related to metabolism, while embryos produced in serum-free conditions showed up-regulation of pathways related to biosynthesis of different molecules, especially lipids. This indicates that further optimization of the serum-free culture is required.

We concluded that serum-free supplementation is preferred for *in vitro* embryo production in cattle. However, the impact of serum-free embryo culture on the epigenetic pattern of such embryos needs to be further evaluated.

In a second part of this PhD thesis, an immunofluorescence staining to the study 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) patterns was introduced in the laboratory and optimized (Chapter 4).

First, the optimal conditions for both 5mC and 5hmC epitope exposure, and the dye used as DNA counterstaining were determined (Chapter 4.1). Based on literature, several epitope retrieval treatments were tested. For bovine embryos, 1h denaturation with 4N HCl, or 30 min denaturation with 4N HCl combined with a brief tryptic digestion were found to yield the best results. In the horse, both treatments successfully exposed 5mC and 5hmC epitopes, though only the latter method maintained embryo integrity. Subsequently, the ability of binding to DNA after these epitope retrieval conditions of several DNA was tested. Intercalating dyes, especially Ethidium homodimer 2 (EthD-2) could successfully bind to DNA after these conditions, and therefore, they are the appropriate dyes to use to study of 5mC and 5hmC patterns.

Next, a proper technique to determine the parental origin of the pronuclei in equine and bovine zygotes was optimized based on the asymmetric pattern of two histone modifications. In literature, it has been described in several species that histone 3 lysine 9 tri-methylation (H3K9me3) and/or histone 3 lysine 27 di-tri-methylation (H3K27me2-3) are only present in the maternal pronucleus, while absent in the paternal one. However, these histone modification patterns are not completely conserved between species. In equine zygotes, H3K9me3 indeed showed an asymmetric pattern between the maternal and paternal pronucleus in all pronuclear stages (Chapter 4.2). Instead, in bovine zygotes, H3K9me3 showed a variable pattern between the maternal and paternal pronucleus, while H3K27me2-3 was only present in the maternal pronucleus in all pronuclear stages (Chapter 4.3). Additionally, the double immunofluorescent staining for 5mC or 5hmC combined with H3K9me3 and H3K27me2-3 for equine and bovine zygotes, respectively, was optimized.

Finally, the optimized double immunofluorescent staining was applied to study the dynamics of 5mC and 5hmC in *in vitro* produced equine zygotes during global epigenetic reprogramming (Chapter 5). A significant reduction in the levels of 5mC was reported during pronuclear development in both parental genomes, and this reduction was replication-dependent. Nevertheless, 5mC was highly present in both parental genomes throughout pronuclear development. Additionally, 5hmC was highly present during the complete first cell cycle in both parental genomes, and the levels of 5hmC remained stable during pronuclear development. Therefore, the horse is one of the species, together with rabbit, goat and sheep that do not follow the classical genome-wide DNA demethylation model developed in mouse, at least not during the first cell cycle.

The general discussion and the conclusions of this thesis are presented in chapter 6:

- The replacement of serum supplementation by bovine serum albumin and insulin-transferrin-selenium (BSA-ITS) for bovine *in vitro* embryo production is advisable based upon our data obtained by means of transcriptomics. Nevertheless, further optimization of this serum-free culture medium is required, since the expression of genes involved in the biosynthesis of many molecules is up-regulated in embryos produced under this condition, compared to *in vivo* derived embryos.

- The presence of serum during *in vitro* production appeared to have a different impact on the embryos according to their sex, with male embryos having three times more genes differentially expressed compared to *in vivo* derived embryos, than their female counterparts.
- A robust immunofluorescent staining protocol was optimized for the study of epigenetic patterns of bovine and equine zygotes and embryos. The validity of this protocol in other species needs to be evaluated further.
- For the correct exposure of 5mC and 5hmC epitopes in bovine zygotes, 1h denaturation with 4N HCl, or 30 min denaturation with 4N HCl combined with a brief tryptic digestion are essential. For equine zygotes, 30 min denaturation with 4N HCl combined with a brief tryptic digestion is optimal, since after 1h denaturation with 4N HCl, the fragility of the zygotes is highly increased.
- Under these epitope retrieval conditions, the use of DNA intercalating dyes, especially Ethidium homodimer 2 (EthD-2), is required, since DNA dyes that bind to the minor groove, such as Hoechst, can no longer bind to the DNA after the denaturation process.
- The asymmetric pattern of H3K9me3 can be used to determine the parental origin of the pronuclei in equine zygotes. In contrast, the pattern of H3K9me3 is not consistent between the paternal and the maternal pronuclei in bovine zygotes. Instead, the asymmetric pattern of H3K27me2-3 is recommended for the determination of the pronuclear parental origin of bovine zygotes.
- During pronuclear development in equine, a loss of DNA methylation associated with DNA replication (passive DNA demethylation) of both paternal and maternal pronuclei takes place. Additionally, the levels of 5hmC remain constant during pronuclear development regardless the parental origin of the pronuclei.
- Taking into account the recently published articles and the findings of this thesis, there is enough evidence to warrant a revision of the classic model of epigenetic reprogramming during preimplantation embryo development as earlier described in the mouse.

SAMENVATTING

Geassisteerde voortplantingstechnieken worden routinematig gebruikt om embryo's te produceren om onvruchtbaarheid te behandelen bij de mens of omwille van economische of foktechnische redenen bij rundvee. Met de toenemende kennis over de embryonale ontwikkeling werden de geassisteerde voortplantingstechnieken in de laatste jaren meer ontwikkeld en verfijnd en worden hogere percentages blastocysten bekomen. Ondanks deze positieve evolutie leidt het toegenomen gebruik van geassisteerde voortplantingstechnieken tot een verhoogde blootstelling van gameten en embryo's aan suboptimale omstandigheden, en dat tijdens een periode in hun ontwikkeling waarin ze zeer vatbaar zijn voor genetische en epigenetische modificaties. Veranderingen in de genexpressie en in het epigenetisch patroon van zygoten en embryo's zijn beschreven bij verschillende diersoorten, inclusief de mens. Belangrijk hierbij is de samenstelling van de media die gebruikt worden tijdens de cultuur van de embryo's. Deze heeft ni*et al*leen een grote impact op het percentage blastocysten, maar ook op de kwaliteit van de geproduceerde embryo's.

Om de veiligheid van geassisteerde voortplantingstechnieken te testen, dienen daarom de effecten van deze technieken op eventuele veranderingen in de genexpressie en de epigenetica van de geproduceerde zygoten en embryo's geëvalueerd te worden. Daarbij kan de kennis die opgedaan wordt bij deze evaluatie helpen bij de verbetering van de geassisteerde voortplantingstechnieken, zodat uiteindelijk embryo's kunnen geproduceerd worden die meer lijken op embryo's die *in vivo* ontwikkelen.

In hoofdstuk 1 worden de gebeurtenissen die plaatsvinden tijdens de vroege embryonale ontwikkeling en de technieken die gebruikt worden om deze ontwikkeling *in vitro* te ondersteunen, de zogenaamde geassisteerde voortplantingstechnieken, beschreven. Verder wordt een overzicht gegeven over de effecten van geassisteerde voortplantingstechnieken op de kwaliteit van het embryo en over de verschillende technieken die gebruikt worden om dit te bepalen.

De algemene doelstelling van deze studie was om de impact van *in vitro* productie op runder- en paardenembryo's te bestuderen (hoofdstuk 2). De eerste specifieke doelstelling hierbij was om het effect van twee verschillende omstandigheden voor embryocultuur op de genexpressie van runderblastocysten te evalueren. De tweede specifieke doelstelling was om de epigenetische patronen van *in vitro* geproduceerde runder- en paardenzygoten te bepalen

en om na te gaan of veranderingen in epigenetische patronen tijdens deze vroege ontwikkelingsstadia kunnen worden vastgesteld.

In eerste instantie hebben we de techniek 'RNA-sequenering' gebruikt om het effect van de blootstelling aan verschillende cultuuromstandigheden op de globale genexpressie bij runderembryo's te bestuderen (hoofdstuk 3). Hiervoor hebben we runderembryo's blootgesteld aan ofwel een suboptimale omgeving waarvan men weet dat ze afwijkingen bij de nakomelingen veroorzaakt, zoals grote en weinig levensvatbare kalveren (medium met serum), ofwel werden de embryo's gekweekt in een geoptimaliseerd cultuurmedium waarbij serum vervangen werd door boviene serum albumine en insuline, transferrine en selenium (BSA-ITS, serumvrije omstandigheden). Vervolgens hebben we de genexpressie van de blastocysten die onder deze twee omstandigheden gekweekt werden vergeleken met deze van blastocysten die *in vivo* ontwikkelden. Het toevoegen van serum had een zeer grote impact op de genexpressie van de embryo's. Bij embryo's die in medium met serum waren geproduceerd kwamen vijf maal meer genen differentieel tot expressie in vergelijking met *in vivo* embryo's dan bij embryo's die geproduceerd werden in serumvrij medium (1109 vs. 207). Van belang hierbij was dat het gebruik van serum tijdens de *in vitro* productie van runderembryo's een andere impact bleek te hebben afhankelijk van het geslacht van de embryo's. Bij de mannelijke embryo's kwamen drie maal meer genen differentieel tot expressie dan bij hun vrouwelijke tegenhangers (1283 vs. 456). Mannelijke en vrouwelijke embryo's die onder serumvrije omstandigheden werden geproduceerd daarentegen vertoonden gelijkaardige aantallen genen die differentieel tot expressie kwamen (191 vs. 192). De metabole reactiepaden die door de *in vitro* productie werden beïnvloed, verschilden afhankelijk van de toevoegingen aan het cultuurmedium. Embryo's die geproduceerd werden in medium met serum vertoonden een lagere expressie van genen die gerelateerd waren aan het metabolisme, terwijl embryo's die geproduceerd werden in serumvrij medium een hogere expressie vertoonden van reactiepaden die gerelateerd waren aan de biosynthese van verschillende moleculen, voornamelijk vetten. Dit wijst erop dat een verdere optimalisatie van de serumvrije cultuuromstandigheden noodzakelijk is.

We concludeerden dat serumvrij medium verkiesbaar is voor de *in vitro* productie van rundsembryo's, maar dat de impact van deze serumvrije cultuur op het epigenetisch patroon van deze embryo's verder geëvalueerd dient te worden.

In een tweede deel werd een immunofluorescente kleuring voor het bestuderen van de 5-methylcytosine (5mC) en 5-hydroxymethylcytosine (5hmC) patronen in ons laboratorium geïntroduceerd en geoptimaliseerd (hoofdstuk 4).

Hiervoor werden eerst de optimale omstandigheden voor de blootlegging van de 5mC en de 5hmC epitopen bepaald, evenals de kleurstof die gebruikt kan worden om het DNA te visualiseren (hoofdstuk 4.1). Op basis van de literatuur werden verschillende behandelingen voor de blootlegging van de epitopen getest. Voor de runderembryo's was 1 uur denaturatie met 4N HCl of 30 minuten denaturatie met 4N HCl gecombineerd met een korte digestie met trypsine optimaal. Bij het paard resulteerden deze beide behandelingen in een succesvolle blootlegging van de 5mC en de 5hmC epitopen, maar de laatstgenoemde behandeling hield eveneens de integriteit van het embryo in stand. Vervolgens werd voor verschillende DNA kleurstoffen geëvalueerd of ze nog aan DNA konden binden na blootstelling aan bovengenoemde behandelingen. Intercalerende kleurstoffen, voornamelijk ethidium homodimeer 2 (EthD-2), konden succesvol binden aan DNA na blootstelling aan deze omstandigheden en zijn daarom optimaal voor het bestuderen van 5mC en 5hmC patronen.

Daarna werd een techniek op punt gesteld om de parentale oorsprong van de pronuclei te bepalen bij paarden- en runderzygoten, een techniek die gebaseerd is op de asymmetrische verdeling van twee histonenmodificaties. In de literatuur is beschreven dat histone 3 lysine 9 tri-methylatie (H3K9me3) en/of histone 3 lysine 27 di-tri-methylation (H3K27me2-3) alleen aanwezig is in de maternale pronucleus, terwijl dit type van histonenmethylatie bij verschillende diersoorten afwezig is in de paternale pronucleus. Toch zijn deze patronen van histonenmodificatie niet volledig bewaard tussen diersoorten. Bij paardenzygoten vertoont H3K9me3 inderdaad een asymmetrische verdeling tussen de maternale en de paternale pronucleus tijdens de vorming van de pronuclei (hoofdstuk 4.2). Bij runderzygoten echter vertoont H3K9me3 een variabele verdeling tussen de maternale en paternale pronucleus, terwijl H3K27me2-3 alleen aanwezig is in de maternale pronucleus tijdens de vorming van de pronuclei (hoofdstuk 4.3). De dubbele immunofluorescente kleuring werd geoptimaliseerd voor 5mC of 5hmC in combinatie met H3K9me3 en H3K27me2-3 voor paarden- en runderzygoten

Uiteindelijk werd deze dubbele immunofluorescente kleuring toegepast om de dynamiek van 5mC en 5hmC te onderzoeken bij *in vitro* geproduceerde paardenzygoten tijdens de globale epigenetische reprogrammering (hoofdstuk 5). Er werd een significante reductie in het gehalte van 5mC gerapporteerd tijdens de vorming van de pronuclei in beide parentale genomen, en deze reductie was afhankelijk van de replicatie. Ondanks deze bevinding was 5mC goed vertegenwoordigd in beide parentale genomen. Ook 5hmC was aanwezig tijdens de eerste celcyclus in beide parentale genomen en de gehalten ervan bleven stabiel tijdens de vorming van de pronuclei. Het paard is dus, samen met het konijn, de geit en het schaap, één van de diersoorten die het klassieke genoomwijde demethylatie model, dat eerst beschreven werd bij de muis, niet volgen, tenminste niet tijdens de eerste celcyclus.

De algemene discussie en de besluiten van de thesis worden voorgesteld in hoofdstuk 6:

- De vervanging van serum door boviene serum albumine en insuline-transferrine-selenium (BSA-ITS) voor productie van runderembryo's *in vitro* is een positieve evolutie, zeker wanneer men de embryo's analyseert via transcriptomics. Toch is een verdere optimalisatie van dit serumvrije cultuurmedium vereist, omdat er bepaalde genen die betrokken zijn bij de biosynthese van vele belangrijke molecules opgereguleerd zijn in embryo's die onder deze omstandigheden geproduceerd worden.
- De aanwezigheid van serum tijdens *in vitro* embryo productie heeft schijnbaar een verschillende impact op de embryo's naargelang hun geslacht. Mannelijke embryo's die *in vitro* geproduceerd werden brengen drie maal meer genen verschillend tot expressie dan *in vivo* embryo's, in vergelijking met hun vrouwelijke tegenhangers.
- Een robuust immunofluorescent kleuringsprotocol werd verfijnd voor de studie van de epigenetische patronen van runder- en paardenzygoten en -embryo's. De bruikbaarheid van dit protocol bij andere diersoorten moet nog verder onderzocht worden.
- Om de 5mC en 5hmC epitopen correct te kunnen blootleggen bij in boviene zygoten, is een denaturatie van 1 uur nodig met 4N HCl, of 30 minuten denaturatie met 4N HCl in combinatie met een korte digestie door middel van trypsine . Voor paardenzygoten is een denaturatie van 30 minuten met 4N HCl in combinatie met een korte digestie

door middel van trypsine optimaal; want na een denaturatie van 1 uur worden de zygoten te fragiel.

- Onder deze voorwaarden van epitoopblootlegging, is het gebruik van intercalerende DNA kleurstoffen nodig, en meer bepaald is Ethidium homodimer 2 (EthD-2) ideaal, omdat DNA kleurstoffen die binden op de kleine groeve van DNA, zoals Hoechst, niet langer kunnen binden aan het DNA na het denaturatieproces.
- Het asymmetrische patroon van H3K9me3 kan gebruikt worden om de parentale oorsprong van de pronuclei bij paardenzygoten aan te tonen. Dit patroon van H3K9me3 is niet consistent verdeeld tussen pronuclei bij runderzygoten en in plaats daarvan wordt het asymmetrische verdelingspatroon van H3K27me2-3 aanbevolen om de oorsprong van de pronuclei aan te tonen bij runderzygoten.
- Tijdens de vorming van de pronuclei bij het paard, wordt een verlies van DNA-methylatie geassocieerd met DNA-replicatie (passieve DNA-demethylatie) en die vindt plaats bij zowel paternale als maternale pronuclei. Ook blijven de gehalten van 5hmC constant tijdens de vorming van de pronuclei, zonder dat de oorsprong van de pronucleus hierop een invloed uitoefent.
- In het kader van de recente literatuur en de bevindingen in deze thesis, is er voldoende bewijsvoering aangebracht om een revisie van het klassieke model van de epigenetische reprogrammering, zoals die eerder beschreven werd tijdens de preimplantatie-ontwikkeling van de muis, te eisen.

Acknowledgements

Now that this period comes to an end, I cannot be but grateful to all the people that was part of my life during this time and contributed in a way or another to this PhD.

First of all I would like to thank my promoter, Prof. dr. Ann Van Soom to give me the opportunity to do the PhD at her lab. Thank you for giving us freedom to perform our experiments and for supporting us to attend to conferences. I would also like to thank my co-promoter, Prof. dr. Luc Peelman, to open me the doors of the department of genetics, and for the advice on the genetic experiments.

I would like to extend my gratitude to the exam committee (Prof. dr. Pilar Coy, Prof. dr. Alfonso Gutiérrez Adán, Prof. dr. Catherine Delesalle, Prof. dr. Jo Leroy, Dr. Katrien Smits, Dr. Mario Van Poucke, Dr. Catharina De Schauwer) for reading and critically reviewing my thesis, and make it improve with their valuable comments.

My deepest thanks goes also to all my colleagues from the IVF lab. To the ones that are still here, and to the ones that already left to make space for fresh blood!, for the help with the experiments and the chats during the many hours we spent in the lab puncturing, looking up the oocytes, fertilizing, SOFing….Hilde, Josine, Ruth, Mohammad, Leen, wish you all the best with you careers! Eline (thanks for teaching me in the lab when I first arrived!), Maaike, Feline, Nerea, very good luck finishing your PhDs!! Petra and Isabel, thanks for patiently teaching all of us the secrets of IVF, and for making the media and getting everything ready for our experiments. Katrien, thank you for doing ICSI for me, and for doing it much more times than we initially planned, but, as you know, the Murphy´s law drove my PhD. Also, thank you very much for correcting the book and helping a lot improving it!. Catharina, thank you also for the big corrections in the book, and in general for trying to improve things in the lab, and for fighting for what is fair.

Lynn, we had a bad beginning, but we finished as friends, so as we said in Spanish “bien está lo que bien acaba”. Thanks for the scientific discussions that we had in the office, where, with Bart, we felt free to say honestly our impressions about each other’s results and what to do to improve them. Thank you also for the laughs, the gossips and the stupidities that we had in the never-ending hours that we spend between that four walls. Very good luck with the end of your PhD, you will do great!!

To my favorite postdoc, Bart, what can I say….thank you very much for being by my side during these 5 years, I really miss you since you are gone. Thanks for listening, and for the support during all those moments when quitting looked like the only option. I am very glad to

have you as friend. Thank you for the scientific discussions, but also for the talks about our personal life, as you are learning now, when you are abroad that extra support is very important. We will celebrate very soon ☺.

To all the rest of the people from the department. Steven, thanks for fixing and installing things many times on my computer, and for being able to rescue more than one missing document; Els, thanks for helping binding the book; Sandra and Leila, thanks for helping with the logistics and organization of many things. Jenne and Osvaldo, thanks for the chats, lunches and Christmas dinners, keep smiling and good luck with the end of the PhD! Alfonso and Ruben, thanks for helping me out when I first arrived with all the administration. Also, thank you for always saying yes when I came asking for a favor. To all the people that were temporarily in the lab Nelly, Alberto, Olga, Aspasia, Sam, Joey, Marcin, Fenia, thank you all for enriching our time!

To the people from genetics, thanks for making your department my home for few months and for trusting me to leave the last and come during the weekends to speed up my research. Karen, thanks for the guidance, the discussions for setting up the experiments and the help producing the *in vivo* embryos. Mario, thank you very much for teaching me everything about qPCR, and for always having "a minute" to check my results and discuss about how to proceed. Also, as I said before, thanks for your critical comments that helped improving the book. Ruben and Linda, thank you for ordering and providing all the material that I needed during that period always with a smile. "little" Eline, very good luck with finishing your PhD, I cross my fingers that you get the grant! Sara, good luck with the end of your PhD, and enjoy the family! To my dear Wenwen, thank you for being always such an optimist. Thank you for being a restless traveler that made us have such a nice time in Canada, and for the support during my first oral presentation. Good luck with finishing, you are almost there!!!

To Prof. dr. Chris O´Neill for the inspirational meeting that we had in the lab few years ago, it opened my eyes and changed the course of my PhD! Also thank you for being always helpful and critical with my research since then.

This adventure did not start in Ghent, it started a couple of years earlier in Murcia when I decided to follow the master after master in Biology and Technology of Reproduction. My gratitude goes to all the professors of the master (Pilar Coy, Manuel Aviles, Joaquin Gadea, Carmen Matas, Raquel Romar, Octavio Lopez, Fran Garcia…) to initiate me in this world and make me love it!

But as not everything in life is work, I would like to thank to all the people that had shared my life during these 5 years.

To the “lost in gent” for the good moments, the barbecues, trips, Blaarmeersen, parties at Vlaanderenstraat and countless beers. To Celina, my first roomie, to first introduce me to the life in Ghent, and the endless chats over a cup of tea. Domenico, thanks for the support during really difficult moments.

Diego, gracias por tener paciencia conmigo (unas veces más que otras) en estos momentos de estrés, y en definitiva, ser mi mejor error.

Porque la distancia separa cuerpos, no corazones, gracias a mis amigos por estar siempre ahí cuando vuelvo de dar tumbos por el mundo. Javi, Raul, Nati, Andrea, Irene, Liz, os quiero!! Sito, más que un amigo, mi hermano. Gracias por tu apoyo a lo largo de los muchos años que hace que nos conocemos, y por hacerme un hueco en tu casa siempre que lo necesito. A las maravillosas personas que conocí durante mi año en Murcia: Ricardo, Jon, Angie, Octavio y Chema, me alucina el hecho de que sigamos siendo amigos después de todos estos años en los que nos hemos visto máximo dos veces al año. Espero seguir compartiendo muchos años más con vosotros, pero chavales, ya toca vernos en otro lado!

Quiero agradecer a toda mi familia su apoyo y cariño. A Mari, por preocuparse siempre por todos; A Mamen, por hacer de su casa la mía durante los veranos; A Fati, por cuidarme tantas veces y llevarme siempre de acá para allá a cada sitio que iba; A mi abuela, que nos dejó hace poco más de un año, por cuidarme tantas y tantas veces, y por creer siempre en mí, aunque tuviera la cabeza demasiado pequeña para estudiar tanto. A mis primos, por tantos momentos compartidos. A Andrés, por los incontables viajes al aeropuerto, incluso a horas intempestivas, durante todos estos años, y por hacer a mi madre feliz.

Finalmente, le dedico esta tesis a mi madre. Gracias por siempre creer en mí, apoyarme, y dejarme tomar mis propias decisiones. Gracias por tu paciencia, tus consejos…. y por todo, sin ti no sería la persona que soy hoy.

Thank you all for the things I said and for the ones I did not.

THANK YOU VERY MUCH!

¡MUCHISIMAS GRACIAS!

DANKUWEL!

CURRICULUM VITAE

Sonia Heras García was born on March 11th 1983, in Madrid, Spain. She finalized her secondary studies in Biomedical Sciences in 2001, in Madrid. She continued her academic formation at the Faculty of Biology of the Autonomous University of Madrid, where she obtained her MSc in Biology in 2006. After graduation, she worked 10 months as assistant researcher in the biotechnology-microbiology laboratory of the Chemical Engineering Department of the Complutense University of Madrid. In 2008 she went back to study to obtain a Master after Master in Biology and Biotechnology of Reproduction in Mammals in 2009 at the Faculty of Veterinary Medicine of the University of Murcia. In 2011 she started her PhD at Ghent University granted by IWT. She successfully completed the full curriculum of the Doctoral Training Program, organized by the Doctoral Schools of Ghent University.

Sonia is first author and co-author of several articles published in international peer reviewed journals. Her experimental work has been presented during different European and international congresses.

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Addendum

Table S3.1. *Details of the sequenced reads, fragments, and their mapping to the reference genome per embryo and condition.*

***In vivo* embryos**	**Female 1**	**Female 2**	**Female 3**	**Male 1**	**Male 2**	**Male 3**	**Male 4**	**Male 5**
Paired end reads	16,121,606 x2	14,409,617 x2	13,843,116 x2	13,620,506 x2	16,277,723 x2	15,350,609 x2	14,148,286 x2	13,843,116 x2
Total sequenced fragments	16,121,606	14,409,617	13,843,116	13,620,506	16,277,723	15,350,609	14,148,286	13,843,116
Total mapped fragments	7,610,073	7,446,981	7,090,972	6,480,699	8,769,114	8,209,054	7,367,399	7,090,972
Uniquely mapped fragments	7,110,780	6,986,045	6,642,570	6,077,412	8,243,423	7,636,560	6,886,937	6,642,570
Fragments uniquely mapped to annotated genes	7,110,780	6,986,045	6,642,570	6,077,412	8,243,423	7,636,560	6,886,937	6,642,570
Fragments uniquely mapped to annotated exons	4,102,468	4,270,863	4,087,575	3,454,351	5,481,012	4,985,662	4,359,963	4,087,575
Fragments uniquely overlapped with annotated introns	3,008,312	2,715,182	2,554,995	2,623,061	2,762,411	2,650,898	2,526,974	2,554,995

Serum-free embryos	**Female 1**	**Female 2**	**Female 3**	**Male 1**	**Male 2**	**Male 3**	**Male 4**	**Male 5**
Paired end reads	12,379,410 x2	12,271,016 x2	12,396,047 x2	13,699,027 x2	13,244,900 x2	12,986,278 x2	11,817,188 x2	13,055,676 x2
Total sequenced fragments	12,379,410	12,271,016	12,396,047	13,699,027	13,244,900	12,986,278	11,817,188	13,055,676
Total mapped fragments	7,030,434	6,702,281	5,852,157	7,537,374	6,697,231	6,225,558	6,103,602	6,946,225
Uniquely mapped fragments	6,547,162	6,209,519	5,451,078	7,039,712	6,249,430	5,765,092	5,673,586	6,458,232
Fragments uniquely mapped to annotated genes	6,547,162	6,209,519	5,451,078	7,039,712	6,249,430	5,765,092	5,673,586	6,458,232
Fragments uniquely mapped to annotated exons	5,461,255	4,187,439	3,188,923	4,860,264	3,773,491	3,397,218	3,749,514	4,340,037
Fragments uniquely overlapped with annotated introns	1,085,907	2,022,080	2,262,155	2,179,448	2,475,939	2,367,874	1,924,072	2,118,195

Table S3.1. *Continuation.*

Serum embryos	**Female 1**	**Female 2**	**Female 3**	**Female 4**	**Female 5**	**Male 1**	**Male 2**	**Male 3**
Paired end reads	11,062,303 x2	13,830,821 x2	14,529,681 x2	12,989,933 x2	13,951,124 x2	15,149,686 x2	21,084,638 x2	14,764,184 x2
Total sequenced fragments	11,062,303	13,830,821	14,529,681	12,989,933	13,951,124	15,149,686	21,084,638	14,764,184
Total mapped fragments	6,263,359	6,969,915	7,924,647	7,476,000	7,738,814	5,115,204	11,531,436	4,907,207
Uniquely mapped fragments	5,721,258	6,335,832	7,367,133	6,936,042	7,205,318	4,750,391	10,691,683	4,523,274
Fragments uniquely mapped to annotated genes	5,721,258	6,335,832	7,367,133	6,936,042	7,205,318	4,750,391	10,691,683	4,523,274
Fragments uniquely mapped to annotated exons	4,268,748	3,968,231	4,809,020	5,015,515	4,884,879	3,345,415	8,500,472	3,055,548
Fragments uniquely overlapped with annotated introns	1,452,510	2,367,601	2,558,113	1,920,527	2,320,439	1,404,976	2,191,211	1,467,726

Table S3.2. *List of the first 35 genes differentially expressed (p-value <0.05) between all the embryos derived in vivo and all the embryos produced in serum conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in in vivo and negative fold change DE genes up-regulated in serum conditions. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
ENSBTAG00000046720	-333.65	3.76 E-35	3
ENSBTAG00000037644	-277.57	5.13 E-15	15
GSTM3	-159.86	5.14 E-12	3
SDS	-137.03	1.46 E-09	17
MFSD2	-76.20	9.91 E-12	3
ENSBTAG00000007477	-73.91	1.19 E-23	27
QPCT	-70.40	1.52 E-12	11
ENSBTAG00000047356	-44.37	4.19 E-07	15
H2B_12	-38.82	5.57 E-08	23
IL2RG	-32.64	8.01 E-11	X
ENSBTAG00000024700	-29.51	2.81 E-08	26
ENSBTAG00000006093	-28.67	3.92E-13	15
ENSBTAG00000020849	-25.82	5.13 E-15	7
CT55	-24.76	1.84 E-18	X
ZSCAN4	-23.69	7.05 E-12	18
SLC28A2	-22.46	2.70 E-09	10
HIST1H2BJ	-22.19	7.87 E-12	23
SPOPL	-21.15	5.96 E-06	2
ENSBTAG00000009440	-21.02	2.46 E-11	15
ARL4D	-19.66	4.95 E-05	19
ARRDC2	-19.53	8.89 E-07	7
ENSBTAG00000024947	-19.22	1.13 E-09	19
SERPINA5	-18.30	3.17 E-05	21
PLS1	12.81	4.05 E-07	1
TMEM62	13.61	1.69 E-15	10
FCGR2	14.30	4.16 E-08	3
CRIP1	15.97	8.01 E-11	21
GCM-1	16.04	8.91 E-13	23
RPL12_2	16.53	1.72 E-08	11
PAG2	16.67	2.09 E-04	29
SH3YL1	22.97	1.82 E-14	
SLC28A3	32.85	5.13 E-15	8
HKDC1	33.06	1.02 E-10	28
DPEP1	34.02	5.92 E-10	18
PRSS8	209.73	4.20 E-34	25

Table S3.3. *List of the first 35 genes differentially expressed (p-value <0.05) between all the embryos derived in vivo and all the embryos produced in serum-free conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in in vivo and negative fold change DE genes up-regulated in serum-free conditions. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
BTG4	-26.62	1.13 E-03	15
ERP70	-12.26	6.67 E-08	4
LAMA1	-11.01	5.79 E-08	24
HMGCS1	-7.43	1.74 E-05	20
MGC137030	-7.20	6.20 E-07	7
GPX2	-6.48	1.51 E-03	10
S100A2	-6.24	3.17 E-08	3
DDX4	-6.21	2.85 E-04	20
ASZ1	-6.06	5.07 E-06	4
PCK2	-5.69	1.89 E-13	10
ENSBTAG00000023007	-5.61	2.08 E-05	17
FADS1	-5.59	2.80 E-05	29
PGHS-2	-5.39	9.54 E-05	16
FABP5	-5.22	1.32 E-04	14
ENSBTAG00000009440	-5.13	2.39 E-06	15
OOEP	-5.05	9.62 E-07	9
TM4SF1	-5.04	4.40 E-09	1
MST4	-4.78	8.65 E-04	X
DPYSL3	-4.74	5.65 E-05	7
RNF17	-4.62	1.30 E-05	12
MRGPRX2	-4.62	6.82 E-04	29
S100A11	-4.51	1.41 E-04	3
SLC28A2	-4.51	2.06 E-03	10
MAPRE2	-4.50	1.14 E-05	24
PSAT1	-4.49	6.23 E-08	8
TCEAL8	-4.47	9.41 E-05	X
ABCB1	-4.43	2.54 E-10	4
FDFT1	-4.21	5.07 E-07	8
IDI1	-4.02	2.06 E-03	13
MT2	3.09	7.40 E-03	18
SH3YL1	3.16	2.58 E-03	
KRTCAP3	3.18	7.12 E-03	11
GGNBP1	3.37	8.81 E-03	23
GARNL3	4.45	4.36 E-06	11
ENSBTAG00000035959	9.01	6.34 E-04	23

Table S3.4. *List of the first 35 genes differentially expressed (p-value <0.05) between male embryos derived in vivo and male embryos produced in serum conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in in vivo and negative fold change DE genes up-regulated in serum conditions. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
ENSBTAG00000046720	-205.60	1.35 E-49	3
ENSBTAG00000013398	-151.86	3.30 E-13	29
QPCT	-145.74	1.47 E-15	11
ACTA1	-121.00	3.48 E-07	28
ENSBTAG00000037644	-100.06	4.10 E-10	15
ENSBTAG00000047356	-83.55	1.17 E-07	15
MFSD2	-75.06	6.65 E-11	3
PRAME_2	-68.35	5.76 E-13	17
ENSBTAG00000024700	-65.60	2.00 E-09	26
ENSBTAG00000007477	-65.48	2.34 E-21	27
AHSG	-43.67	2.09 E-08	1
H2B_12	-38.78	2.20 E-08	23
ENSBTAG00000006093	-34.58	3.30 E-13	15
MAB21L3	33.68	3.07 E-07	3
A2M	33.94	1.08 E-07	5
FHL2	34.54	7.71 E-07	11
MGAT4A	35.89	2.77 E-07	11
SFN	37.56	6.94 E-13	2
SLC6A20	41.86	8.67 E-19	22
FCGR2	43.15	5.13 E-13	3
ANXA3	50.14	2.94 E-06	6
LY6E	51.31	2.80 E-10	14
PLS1	51.79	2.18 E-08	1
TAPBP	62.24	2.49 E-07	23
ANXA1	68.77	1.41 E-08	8
GCM-1	75.68	3.56 E-09	23
PFKL	83.19	7.27 E-21	1
SLC28A3	85.39	1.24 E-19	8
DPEP1	99.35	4.10 E-10	18
HKDC1	218.04	1.01 E-21	28
IFT172	349.04	3.35 E-09	11
PRSS8	438.89	1.44 E-17	25
VGLL1	471.66	2.67 E-09	X
SLC16A7	602.17	5.78 E-09	5
PAG2	1657.68	2.00 E-10	29

Table S3.5. *List of the first 35 genes differentially expressed (p-value <0.05) between male embryos derived in vivo and male embryos produced in serum-free conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in in vivo and negative fold change DE genes up-regulated in serum-free conditions. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
BTG4	-306.60	4.63 E-12	15
ERP70	-16.43	4.52 E-05	4
MGC137030	-10.84	8.86 E-06	7
LAMA1	-10.74	1.01 E-06	24
HMGCS1	-10.18	1.50 E-04	20
GPX2	-9.56	5.11 E-04	10
SYCP2	-8.33	6.77 E-04	13
TM4SF1	-7.57	2.26 E-10	1
ENSBTAG00000009440	-7.54	1.01 E-06	15
FADS1	-7.32	9.83 E-04	29
MRGPRX2	-7.17	9.90 E-04	29
IDI1	-6.37	2.13 E-03	13
ABHD4	-6.33	5.41 E-03	10
FABP5	-6.26	2.13 E-03	14
S100A11	-6.10	3.00 E-03	3
DDX4	-5.95	0.027	20
S100A2	-5.74	8.32 E-04	3
PCK2	-5.69	6.46 E-13	10
NR3C2	-5.38	3.00 E-03	17
DDX58	-5.23	7.77 E-04	8
FDFT1	-5.12	1.78 E-04	8
ENSBTAG00000023007	-5.12	2.22 E-05	17
TCEAL8	-5.09	2.24 E-04	X
TPI1	-5.06	0.023	5
PIM2	-4.83	8.32 E-04	X
ADAM19	-4.82	6.16 E-03	7
HRASLS	-4.77	0.013	1
UHRF1	-4.57	4.12 E-05	7
PGHS-2	-4.54	0.017	16
SC4MOL	-4.52	4.21 E-03	17
GARNL3	4.45	6.87 E-05	11
SLC12A8	6.69	4.00 E-04	1
FBXW9	7.14	1.99 E-04	7
ZNF514	8.15	1.05 E-04	11
ENSBTAG00000035959	12.46	3.09 E-04	23

Table S3.6. *List of the first 35 genes differentially expressed (p-value <0.05) between female embryos derived in vivo and female embryos produced in serum conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in in vivo and negative fold change DE genes up-regulated in serum conditions. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
GSTM3	-3497.31	6.38 E-09	3
ENSBTAG00000046720	-489.25	8.88 E-12	3
ENSBTAG00000037644	-307.71	1.10 E-08	15
SDS	-286.31	9.73 E-05	17
HSD3B	-167.02	1.26 E-05	3
ENSBTAG00000007477	-106.80	1.53 E-09	27
H2B_12	-93.07	1.13 E-04	23
ARL4D	-86.81	2.39 E-03	19
MGC148328_2	-73.36	3.16 E-03	X
ENSBTAG00000034940	-68.98	4.32 E-04	29
ENSBTAG00000046796	-62.29	6.59 E-04	X
MFSD2	-55.35	3.29 E-04	3
ENSBTAG00000024947	-42.78	4.04 E-08	19
HIST1H2BJ	-40.70	6.38 E-05	23
MEST	-38.88	4.86 E-05	4
ENSBTAG00000020522	-38.41	1.58 E-04	X
QPCT	-37.52	2.96 E-04	11
APOBEC3Z3	-34.54	1.94 E-03	5
SLC28A2	-34.19	4.86 E-05	10
MGC148328_1	-32.37	7.75 E-03	X
SPOPL	-29.93	0.019	2
ENSBTAG00000039540	-28.06	4.86 E-05	3
TRPM8	-27.55	1.34 E-06	3
ENSBTAG00000020849	-27.44	3.64 E-05	7
STAT5B	-26.12	1.69 E-05	19
ENSBTAG00000047356	-26.11	1.72 E-03	15
PTGIS	-25.51	2.59 E-04	13
MAGEB16	-24.82	1.80 E-04	X
ENSBTAG00000002355	23.98	1.17 E-05	5
ENSBTAG00000018481	24.21	7.01 E-05	8
RPL12_2	27.29	3.68 E-21	11
AIM1L	27.89	6.11 E-06	2
ENSBTAG00000047990	38.25	7.19 E-07	8
TMEM125	99.27	3.57 E-09	3
PRSS8	112.38	1.85 E-15	25

Table S3.7. *List of the first 35 genes differentially expressed (p-value <0.05) between female embryos derived in vivo and female embryos produced in serum-free conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in in vivo and negative fold change DE genes up-regulated in serum-free conditions. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
ENSBTAG00000021026	-216.36	1.17 E-06	X
PPFIBP1	-65.74	1.19 E-05	5
ENSBTAG00000046720	-41.40	7.99 E-06	3
SYT4	-40.26	2.61 E-05	24
STAT5B	-32.23	3.38 E-05	19
MEST	-24.18	1.93 E-05	4
ENSBTAG00000037644	-21.58	8.26 E-03	15
PTGIS	-21.15	7.16 E-06	13
ENSBTAG00000047547	-18.42	4.34 E-04	X
LUM	-16.59	1.04 E-03	5
ASZ1	-16.46	3.56 E-05	4
PINLYP	-15.63	2.69 E-06	18
TBC1D19	-15.30	2.15 E-04	6
SLC25A31	-14.78	4.05 E-04	17
SERPINA5	-13.66	0.032	21
LAMA1	-12.20	4.52 E-04	24
APOA1	-12.14	4.83 E-08	15
ENSBTAG00000017734	-12.12	1.17 E-03	8
RPL15_1	-12.05	0.040	2
MID1IP1	-11.12	0.021	X
CD40	-10.31	3.60 E-03	13
ARHGEF3	-10.22	1.67 E-03	22
MBD5	-9.86	3.41 E-03	2
DEPDC1	-9.03	7.95 E-03	3
HMGN5	-8.75	8.24 E-03	X
CYP51A1	-8.55	7.81 E-04	4
ENSBTAG00000023007	-8.54	0.011	17
QPCT	-8.38	0.027	11
DBNDD1	8.30	0.010	18
KRT1	9.32	0.033	5
CA6	10.02	5.25 E-03	16
MGST1	11.34	6.00 E-09	5
PRDM1	24.12	5.68 E-06	9
SLC30A1	32.03	3.18 E-06	16
RPL12_2	37.89	1.11 E-64	11

Table S3.8. *List of the first 35 genes differentially expressed (p-value <0.05) between male and female embryos derived in vivo, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in male embryos and negative fold change DE genes up-regulated in female embryos. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
TEX11	-54.01	7.21 E-13	X
LPO	-47.70	1.13 E-11	19
XIST	-13.99	9.08 E-26	X
PIM2	-10.80	2.11 E-13	X
ALAS2	-7.80	0.027	X
PARP16	-7.46	3.19 E-04	10
CPNE2	-6.85	3.76 E-03	18
JAKMIP2	-5.52	2.15 E-04	7
PRDM1	-4.86	8.29 E-03	9
MGC127538	-4.85	3.88 E-10	2
TXNIP	-4.76	0.011	3
ABHD4	-4.65	9.57 E-03	10
GGNBP1	-4.31	5.00 E-09	23
ACTB_1	-4.01	0.030	11
HOP	-3.95	0.025	6
MGC142781	4.23	0.019	11
MGC139228	4.43	0.015	11
RBBP9	4.92	0.034	13
PAG2	5.32	0.046	29
ENSBTAG00000046301	5.49	5.52 E-03	22
ARMC9	5.60	0.029	2
IFT172	12.34	4.90 E-05	11
UTY	12.87	1.82 E-09	Y
ENSBTAG00000035959	13.85	5.83 E-03	23
ENSBTAG00000033558	178.82	4.89 E-17	17
ENSBTAG00000048102	238.41	2.64 E-10	X
ENSBTAG00000035606	309.13	4.46 E-11	17
ENSBTAG00000036115	385.48	8.73 E-14	19
ENSBTAG00000036321	435.23	4.57 E-14	17
ENSBTAG00000039769	564.45	7.50 E-48	17
ENSBTAG00000048172	769.97	8.40 E-23	X
ENSBTAG00000034761	773.63	3.25 E-76	17
ENSBTAG00000045544	873.54	1.09 E-66	X
ENSBTAG00000040363	2695.55	3.88 E-54	X
DDX3Y	2935.13	1.05 E-51	Y

Table S3.9. *List of the first 35 genes differentially expressed (p-value <0.05) between male and female embryos produced in serum conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in male embryos and negative fold change DE genes up-regulated in female embryos. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
GALC	-190.70	1.00 E-05	10
ENSBTAG00000047709	-132.25	8.00 E-04	X
CT47B1	-104.81	9.31 E-10	X
BDH2	-74.51	9.04 E-06	6
MST4	-64.86	2.03 E-06	X
DMD	-57.16	2.50 E-04	X
MTMR8	-42.37	3.67 E-05	X
ENSBTAG00000037496	-41.45	1.30 E-04	X
ENSBTAG00000047804	-39.19	0.034	X
ADAM19	-34.82	9.96 E-04	7
TEX11	-31.55	0.016	X
GPR4	-30.84	2.37 E-04	18
TCL1B	-28.80	9.66 E-04	21
HSD3B	-26.00	2.25 E-03	3
L1CAM	-25.97	9.96 E-04	X
ENSBTAG00000046340	-23.71	0.012	X
SPICE1	-18.18	2.00 E-04	1
MBNL2	-18.04	5.07 E-04	12
ELK1	-14.87	1.18 E-03	X
ENSBTAG00000010463	-12.57	2.25 E-03	X
ZNF165	-11.65	0.032	23
ACTA1	17.96	2.88 E-03	28
ENSBTAG00000036321	21.05	1.31 E-06	17
ACTA2	33.15	4.75 E-05	26
ENSBTAG00000048102	179.59	2.80 E-10	X
ENSBTAG00000036115	217.68	2.37 E-09	19
ENSBTAG00000033558	297.86	5.81 E-16	17
ENSBTAG00000040363	299.78	1.83 E-16	X
ENSBTAG00000045544	575.30	1.26 E-29	X
DDX3Y	663.36	1.42 E-24	Y
EIF1AY	736.75	6.40 E-15	Y
ENSBTAG00000039769	749.42	4.91 E-33	17
PRAME_2	910.07	1.34 E-14	17
ENSBTAG00000048172	1307.40	5.09 E-21	X
ENSBTAG00000034761	1442.32	9.62 E-48	17

Table S3.10. *List of the first 35 genes differentially expressed (p-value <0.05) between male and female embryos produced in serum-free conditions, according to the absolute fold change. Positive fold change indicates DE genes up-regulated in male embryos and negative fold change DE genes up-regulated in female embryos. For the full list see table S3 of the published version.*

Gene symbol	Fold-change	FDR corrected p-value	Chromosome
XIST	-20.26	5.14 E-14	X
SLC6A14	-12.26	2.69 E-03	X
PARP9	-10.60	5.22 E-03	1
ZNF214	-7.18	0.019	15
SPIN2	-6.02	0.027	X
ENSBTAG00000037496	-5.41	5.56 E-03	X
MAGEH1	-5.39	7.10 E-04	X
YIF1B	-4.98	4.96 E-03	18
MMP14	-4.96	0.042	10
WNK3	-4.57	0.042	X
OTUB2	-4.16	0.042	21
RNF128	-4.03	0.022	X
AKAP6	-3.96	0.046	21
LMO6	-3.56	0.039	X
ATP1B2	-3.41	7.51 E-03	19
TP53RK	-3.39	0.021	13
UBQLN2	-3.15	0.046	X
MPP1	-2.92	0.014	X
ENSBTAG00000048049	-2.85	3.03 E-03	21
ENSBTAG00000001219	-2.76	4.16 E-03	8
PRPS1	-2.55	0.014	X
NT5DC2	3.84	0.042	22
DONSON	4.21	0.038	1
URB1	5.90	0.014	1
KIF13A	6.22	0.027	23
RPL12_2	12.38	0.022	11
UTY	14.57	7.02 E-07	Y
ENSBTAG00000039769	240.89	3.29 E-27	17
ENSBTAG00000035606	351.97	7.67 E-10	17
ENSBTAG00000040363	395.91	6.62 E-28	X
ENSBTAG00000034761	399.38	1.00 E-37	17
DDX3Y	440.18	1.33 E-38	Y
ENSBTAG00000036321	446.61	1.88 E-10	17
ENSBTAG00000033558	838.37	8.11 E-15	17
ENSBTAG00000045544	916.62	2.98 E-37	X

Table S3.11. *GO biological processes, and genes up-regulated in each of them, enriched in genes differentially expressed (DE) (FDR corrected p-value <0.05, |FC| ≥2) between all in vivo derived embryos and all the embryos cultured in serum conditions. Enriched GO biological processes with Benjamini-Hochberg corrected p-value <0.01, genes per term/pathway ≥5, Goterm levels 3–8.*

GO Term	p-value	Genes up-regulated *in vivo* (%)	Genes up-regulated in serum (%)
Organic acid metabolic process	4.88 E-03	**(72.3)** [AASS, ACADS, ACSS1, ALDH4A1, ALDH6A1, APLP2, ARRB1, ASL, ASRGL1, BCAT1, BRP44L, CBLC, CBS, CRTAP, CRYL1, CYP2D14, CYP2S1, DLAT, DPEP1, DTD1, ELOVL1, ENO1, ERBB3, ETFDH, FADS3, FASN, FH, GAPDH, GATA2, GATA3, GCAT, HADHA, HK1, HKDC1, IARS2, IDH3G, IL6ST, KIT, LONP2, LYN, MTHFR, MUT, MVP, NFE2, PDPN, PDXDC1, PECAM1, PEX7, PFKL, PPARGC1A, PRKCZ, PTGR1, PTGS2, PTPLB, SCD, SMAD7, SREBF1, ST6GAL1, SUCLG2, TP53]	**(27.7)** [ACSL1, AFAP1L2, ALDOC, CD40, CRAT, CSF1R, DDIT4, DUOXA2, GFPT2, GLDC, HAL, IER3, IL6, KITLG, MSMO1, NANP, NR5A2, PTGES, SDS, SHMT2, STAT5B, TDH, UGDH]
DNA repair	9.92 E-03	**(41.2)** [BRE, CDC45, DDB2, DTX3L, FANCC, FBXO6, KIAA0415, PRKCG, PSME4, RTEL1, SETMAR, TDG, TP53, TRPC2]	**(58.8)** [ALKBH1, CETN2, CUL4B, DEM1, FANCI, IER3, MLH3, NPM1, RAD1, REV3L, SETMAR, SOD1, TEX12, TP53BP1, TRIP12, TRIP13, UBE2A, UHRF1, USP7, WRN]
Lipid metabolic process	3.45 E-03	**(80)** [ABCA1, ABHD12, ACADS, ALDH3A2, APLP2, ASAH1, CHKB, CLN6, CRYL1, CYB5R3, CYP2D14, CYP2S1, DEGS2, ELOVL1, EPHX2, ETFDH, FADS3, FASN, FECH, GDPD5, GM2A, GPLD1, HADHA, HINT2, HSD17B8, KIT, LONP2, LYN, MAP7, ORMDL2, PCYT2, PDGFRA, PDPN, PEX7, PGAP2, PGAP3, PI4KA, PIGU, PIGV, PLD2, PLD3, PNPLA6, PPAPDC1B, PPARGC1A, PRDX6, PSAP, PTDSS1, PTGR1, PTGS2, PTPLB, SCD, SCP2, SERAC1, SMPD1, SMPDL3A, SRD5A3, SREBF1, TAMM41, TIPARP, UGCG]	**(20)** [ACSL1, ATG14, CSF1R, EPT1, FDFT1, FLT1, ID2, MSMO1, NR5A2, PI4KB, PTGES, RDH12, SOD1, STAT5B, TRPV1]
Membrane lipid metabolic process	2.86 E-03	**(100)** [CLN6, DEGS2, ELOVL1, GM2A, GPLD1, KIT, MAP7, ORMDL2, PGAP2, PGAP3, PIGU, PIGV, PSAP, PTPLB, SERAC1, SMPD1, SMPDL3A, UGCG]	**(0)**
Glycolipid metabolic process	4.12 E-03	**(100)** [CLN6, GM2A, GPLD1, KIT, MAP7, PGAP2, PGAP3, PIGU, PIGV, SERAC1, UGCG]	**(0)**
Regulation of cell shape	6.07 E-03	**(68.8)** [ANXA7, ARHGAP15, CDC42EP1, CDC42EP4, EZR, FBLIM1, FN1, KIT, MYH9, MYL9, VRK3]	**(31.3)** [BRWD3, CSF1R, CSNK1G1, FMNL3, PRPF40A]
Histone ubiquitination	4.46 E-03	**(33.3**) [DDB2, DTX3L, KDM2B]	**(66.7)** [CUL4B, PAF1, PCGF1, TRIP12, UBE2A, UHRF1]
Carboxylic acid metabolic process	8.70 E-03	**(72.3)** [AASS, ACADS, ACSS1, ALDH4A1, ALDH6A1, APLP2, ARRB1, ASL, ASRGL1, BCAT1, BRP44L, CBLC, CBS, CRTAP, CRYL1, CYP2D14, CYP2S1, DLAT, DPEP1, DTD1, ELOVL1, ENO1, ERBB3, ETFDH, FADS3, FASN, FH, GAPDH, GATA2, GATA3, GCAT, HADHA, HK1, HKDC1, IARS2, IDH3G, IL6ST, KIT, LONP2, LYN, MTHFR, MUT, MVP, NFE2, PDPN, PDXDC1, PECAM1, PEX7, PFKL, PPARGC1A, PRKCZ, PTGR1, PTGS2, PTPLB, SCD, SMAD7, SREBF1, ST6GAL1, SUCLG2, TP53]	**(27.7)** [ACSL1, AFAP1L2, ALDOC, CD40, CRAT, CSF1R, DDIT4, DUOXA2, GFPT2, GLDC, HAL, IER3, IL6, KITLG, MSMO1, NANP, NR5A2, PTGES, SDS, SHMT2, STAT5B, TDH, UGDH]

Table S3.11. *Continuation.*

GO Term	p-value	Genes up-regulated *in vivo* (%)	Genes up-regulated in serum (%)
Oxoacid metabolic process	3.46 E-03	**(72.3)** [AASS, ACADS, ACSS1, ALDH4A1, ALDH6A1, APLP2, ARRB1, ASL, ASRGL1, BCAT1, BRP44L, CBLC, CBS, CRTAP, CRYL1, CYP2D14, CYP2S1, DLAT, DPEP1, DTD1, ELOVL1, ENO1, ERBB3, ETFDH, FADS3, FASN, FH, GAPDH, GATA2, GATA3, GCAT, HADHA, HK1, HKDC1, IARS2, IDH3G, IL6ST, KIT, LONP2, LYN, MTHFR, MUT, MVP, NFE2, PDPN, PDXDC1, PECAM1, PEX7, PFKL, PPARGC1A, PRKCZ, PTGR1, PTGS2, PTPLB, SCD, SMAD7, SREBF1, ST6GAL1, SUCLG2, TP53]	**(27.7)** [ACSL1, AFAP1L2, ALDOC, CD40, CRAT, CSF1R, DDIT4, DUOXA2, GFPT2, GLDC, HAL, IER3, IL6, KITLG, MSMO1, NANP, NR5A2, PTGES, SDS, SHMT2, STAT5B, TDH, UGDH]
Cellular catabolic process	8.22 E-04	**(54.6)** [AASS, ABHD12, ACBD5, ALDH4A1, ALDH6A1, ANXA7, ARRB1, ARRB2, ASRGL1, BCAT1, BLVRA, CAPN1, CBLC, CLN6, CRYZ, CTSD, CYP2D14, CYP2S1, DAB2, DTD1, DUT, EDEM1, ENPP4, EPHX2, ETFDH, FBXL3, FBXO18, FBXO6, FOXRED2, GCAT, GM2A, GPLD1, HADHA, LONP2, NAALAD2, NBAS, NEDD4L, PEX7, PRDX6, PRICKLE1, PRKCG, PSME4, RNF146B, SAMHD1, SETMAR, SMAD7, SMPD1, SMPDL3A, SNX9, SRD5A3, TP53, UBR1, XBP1]	**(45.4)** [ALKBH1, ATG14, AURKA, BNIP3, CCRN4L, CLPX, CSNK2A2, CUL4B, DDIT4, DEM1, DIS3L, FBXO5, GABARAPL1, GK, GLDC, GSTM3, HAL, IER3, KLHL8, LGMN, LOC614531, LSM1, NCBP2, NPM1, PLK3, POLR2G, PON2, SDS, SETMAR, SIAH1, SKP1, SOD1, SPOPL, SRPX, TCEB1, TDH, TOB1, TRIP12, UBE2A, UBE2J2, UBE2K, UHRF1, USP7, ZNRF1]
Cellular lipid metabolic process	3.35 E-03	**(81.4)** [ABHD12, ACADS, ALDH3A2, CHKB, CLN6, CRYL1, CYP2D14, CYP2S1, DEGS2, ELOVL1, EPHX2, ETFDH, FADS3, FASN, GM2A, GPLD1, HADHA, KIT, LONP2, LYN, MAP7, ORMDL2, PCYT2, PDGFRA, PDPN, PEX7, PGAP2, PGAP3, PI4KA, PIGU, PIGV, PNPLA6, PPAPDC1B, PPARGC1A, PRDX6, PSAP, PTDSS1, PTGR1, PTGS2, PTPLB, SCD, SERAC1, SMPD1, SMPDL3A, SRD5A3, SREBF1, TAMM41, UGCG]	**(18.6)** [ACSL1, ATG14, CSF1R, EPT1, FDFT1, FLT1, MSMO1, PI4KB, PTGES, RDH12, STAT5B]
Small molecule metabolic process	2.75 E-04	**(70.7)** [AASS, ABCA1, ACADS, ACSS1, AHCYL2, ALDH2, ALDH3A2, ALDH4A1, ALDH6A1, APLP2, ARRB1, ASL, ASRGL1, ATP6V0A4, ATP6V1B1, ATPIF1, BCAT1, BRP44L, CBLC, CBS, CHKB, CLN6, CRTAP, CRYL1, CYB5R3, CYP2D14, CYP2S1, DCXR, DEGS2, DLAT, DPEP1, DTD1, DUT, ELOVL1, ENO1, ENPP4, EPHX2, ERBB3, ETFDH, FADS3, FASN, FECH, FH, GAPDH, GATA2, GATA3, GCAT, GDPD5, GMPS, GNAS, GPLD1, H6PD, HADHA, HK1, HKDC1, IARS2, IDH3G, IL6ST, KIT, LONP2, LRP2, LYN, MCEE, MTHFR, MUT, MVP, NADSYN1, NFE2, NME3, PDPN, PDXDC1, PECAM1, PEX7, PFKL, PNPLA6, PPARGC1A, PRKCZ, PTGR1, PTGS2, PTPLB, PTPMT1, SAMHD1, SCD, SCP2, SHPK, SMAD7, SMPD1, SMPDL3A, SRD5A3, SREBF1, ST6GAL1, SUCLG2, TP53, TP53RK]	**(29.3)** [ACSL1, AFAP1L2, ALDOC, AMPD2, ATP6V0A1, BAD, CBR1, CD40, CRAT, CSF1R, DDIT4, DUOXA2, FDFT1, GART, GFPT2, GK, GLDC, HAL, IER3, IL6, KITLG, MSMO1, NAMPT, NANP, NR5A2, PNP, PPAT, PRPS1, PTGES, RDH12, SDS, SHMT2, SLC25A13, SOD1, STAT5B, TBPL1, TDH, UCK2, UGDH]

Table S3.11. *Continuation.*

GO Term	p-value	Genes up-regulated *in vivo* (%)	Genes up-regulated in serum (%)
Organic substance transport	9.17 E-03	**(66.1)** [ABCA1, ABCB6, ANXA1, ANXA4, AP1G2, AP1M2, AP1S3, AP2A2, APOM, ARRB1, ARRB2, BRP44L, CADM1, CD63, COG3, DAB2, DAG1, DSCR3, F2RL1, FLNA, FOLR1, GATA3, GPLD1, JUP, KRT18, LMBRD1, LONP2, LY6E, LYN, MFF, MFN2, MYH9, MYL9, MYO18A, NDFIP2, NEDD4L, OSBPL1A, OSBPL2, PEX7, PFKL, PGAP2, PKIA, PQLC2, PRICKLE1, PRKCZ, PTTG1IP, RAB15, RAB25, RAB27A, RAB34, RABGEF1, RASEF, SCP2, SERAC1, SERGEF, SFN, SLC17A5, SLC19A2, SLC1A1, SLC26A6, SLC28A3, SLC29A1, SLC38A2, SLC38A7, SLC4A2, SLC5A11, SLC6A20, SLC7A4, SLC7A7, SLC9A3R1, SNX17, SNX8, SNX9, SREBF1, STEAP3, TOM1, TP53, VPS26A, VPS33B, VPS36]	**(33.9)** [ACSL1, ADRBK1, AQP3, ATP11C, BAD, BMP4, CD40, CRYM, CSF1R, DUOXA2, EIF5A, FKBP1B, FXC1, GGA3, GJA1, HPS4, IL18, IL6, KPNA6, MFSD2A, NCBP2, NDUFAF2, NXF1, PLIN2, PLK3, RANGRF, SAR1B, SEC61A1, SELK, SERPINA5, SLC1A5, SLC23A2, SLC25A13, SLC28A2, SLC33A1, SLC7A6, SLC7A6OS, TFRC, TIMM22, TOB1, TRPV1]
Organonitrogen compound metabolic process	7.62 E-03	**(72.7)** [AASS, ABCA1, ABCB6, ABHD12, AHCYL2, ALDH4A1, ALDH6A1, APEH, APLP2, ARRB1, ASL, ASRGL1, ATP6V0A4, ATP6V1B1, ATPIF1, BCAT1, BLVRA, CBLC, CBS, CHKB, CLN6, CRTAP, DCXR, DEGS2, DPEP1, DTD1, DUT, ELOVL1, ENPP4, ERBB3, FECH, GATA2, GATA3, GCAT, GM2A, GMPS, GNAS, GPLD1, H6PD, IARS2, IL6ST, KIT, LY6E, LYN, MAP7, MCEE, MTHFR, MUT, MVP, NADSYN1, NFE2, NME3, ORMDL2, PECAM1, PICALM, PNPLA6, PPARGC1A, PRKCZ, PSAP, PTDSS1, PTPLB, SAMHD1, SHPK, SMAD7, SMPD1, SMPDL3A, SMS, TMEM14C, TP53, TP53RK, UGCG, UROD]	**(27.3)** [AFAP1L2, AMPD2, ATP6V0A1, BAD, CD40, CSF1R, DDIT4, DUOXA2, GART, GFPT2, GLDC, GSTM3, HAL, IL6, KITLG, NAMPT, PNP, PPAT, SAT1, SDS, SHMT2, SLC25A13, SOD1, STAT5B, TBPL1, TDH, UCK2]
Organic substance catabolic process	8.37 E-04	**(57.8)** [AASS, ABHD12, ALDH2, ALDH4A1, ALDH6A1, ARRB1, ARRB2, ASRGL1, BCAT1, BLVRA, CAPN1, CBLC, CLN6, DAB2, DTD1, DUT, EDEM1, ENO1, ENPP4, EPHX2, ETFDH, FBXL3, FBXO18, FBXO6, FLNA, FOXRED2, GAPDH, GCAT, GM2A, GPLD1, H6PD, HADHA, HK1, HKDC1, LONP2, NBAS, NEDD4L, PEX7, PFKL, PGM1, PLD2, PLD3, PRDX6, PRICKLE1, PRKCG, PSME4, PTPMT1, RNF146B, SAMHD1, SETMAR, SHPK, SMAD7, SMPD1, SMPDL3A, SNX9, SRD5A3, TIPARP, UBR1, XBP1]	**(42.2)** [ALDOC, ALKBH1, AURKA, BAD, BNIP3, CCRN4L, CLPX, CSNK2A2, CUL4B, DDIT4, DEM1, DIS3L, FBXO5, GGA3, GJA1, GK, GLDC, HAL, IER3, KLHL8, LGMN, LSM1, NCBP2, NPM1, PLK3, POLR2G, SAT1, SDS, SETMAR, SIAH1, SKP1, SOD1, SPOPL, TCEB1, TDH, TOB1, TRIP12, UBE2A, UBE2J2, UBE2K, UHRF1, USP7, ZNRF1]
Liposaccharide metabolic process	4.75 E-03	**(100)** [CLN6, GM2A, GPLD1, KIT, MAP7, PGAP2, PGAP3, PIGU, PIGV, SERAC1, UGCG]	**(0)**
Negative regulation of neural precursor cell proliferation	7.92 E-04	**(71.4)** [GATA2, ILK, KDM2B, LIMS1, TP53]	**(28.6)** [ID2, KCTD11]

Table S3.12. *GO biological processes, and genes up-regulated in each of them, enriched in genes DE (FDR corrected p-value <0.05, |FC| ≥2) between all in vivo and all serum-free embryos. Benjamini-Hochberg corrected p-value <0.01, genes per term/pathway ≥5, Goterm levels 3–8.*

GO Term	p-value	Genes up-regulated *in vivo* (%)	Genes upregulated in serum-free (%)
Response to reactive oxygen species	5.40 E-03	**(0)**	**(100)** [IL6, PARK7, PYCR1, SESN1, SOD1]
Alcohol metabolic process	4.78 E-05	**(0)**	**(100)** [DHCR24, FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, PARK7, PCTP, RDH12, SOD1]
Fatty acid biosynthetic process	6.24 E-03	**(0)**	**(100)** [FADS1, GGT5, MSMO1, PTGES, PTGS2]
Steroid biosynthetic process	1.54 E-05	**(0)**	**(100)** [CYP17A1, FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, SOD1]
Cholesterol biosynthetic process	4.62 E-07	**(0)**	**(100)** [FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, SOD1]
Isoprenoid metabolic process	6.36 E-06	**(0)**	**(100)** [FDFT1, FDPS, HMGCR, HMGCS1, IDI1, MVD, RDH12]
Sulfur compound metabolic process	4.88 E-05	**(10)** [MGST1]	**(90)** [ADI1, CTH, GGCT, GGT5, MTR, MVD, PHGDH, SLC35D1, SOD1]
Meiotic nuclear division	6.82 E-03	**(0)**	**(100)** [ASZ1, DDX4, MAEL, PIWIL2, TEX12]
Steroid metabolic process	3.02 E-05	**(0)**	**(100)** [CYP17A1, DHCR24, FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, PCTP, SOD1]
Cholesterol metabolic process	7.25 E-07	**(0)**	**(100)** [DHCR24, FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, PCTP, SOD1]
Isoprenoid biosynthetic process	3.53 E-07	**(0)**	**(100)** [FDFT1, FDPS, HMGCR, HMGCS1, IDI1, MVD]
Cellular amino acid biosynthetic process	3.92 E-07	**(0)**	**(100)** [ADI1, ASS1, CTH, MTR, PARK7, PHGDH, PSAT1, PSPH, PYCR1]
Serine family amino acid metabolic process	2.70 E-05	**(0)**	**(100)** [CTH, PHGDH, PSAT1, PSPH, SHMT2]
Organic acid biosynthetic process	3.92 E-07	**(0)**	**(100)** [ADI1, ASS1, CTH, FADS1, GGT5, MSMO1, MTR, PARK7, PHGDH, PSAT1, PSPH, PTGES, PTGS2, PYCR1]
Sterol metabolic process	9.17 E-07	**(0)**	**(100)** [DHCR24, FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, PCTP, SOD1]
Sterol biosynthetic process	3.74 E-07	**(0)**	**(100)** [FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, SOD1]
Sulfur compound biosynthetic process	2.73 E-03	**(0)**	**(100)** [ADI1, CTH, GGCT, MTR, SLC35D1]
Small molecule biosynthetic process	6.46 E-10	**(0)**	**(100)** [ADI1, ASS1, CTH, FADS1, FDFT1, FDPS, GGT5, HMGCR, IDI1, MSMO1, MTR, MVD, NSDHL, PARK7, PHGDH, PSAT1, PSPH, PTGES, PTGS2, PYCR1, SOD1]
Alcohol biosynthetic process	1.15 E-05	**(0)**	**(100)** [FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, PARK7, SOD1]
Carboxylic acid biosynthetic process	3.92 E-07	**(0)**	**(100)** [ADI1, ASS1, CTH, FADS1, GGT5, MSMO1, MTR, PARK7, PHGDH, PSAT1, PSPH, PTGES, PTGS2, PYCR1]
Alpha-amino acid metabolic process	7.09 E-06	**(0)**	**(100)** [ADI1, ASS1, CTH, GFPT2, MTR, PARK7, PHGDH, PSAT1, PSPH, PYCR1, SHMT2]
Alpha-amino acid biosynthetic process	5.05 E-07	**(0)**	**(100)** [ADI1, ASS1, CTH, MTR, PARK7, PHGDH, PSAT1, PSPH, PYCR1]
Organic hydroxy compound biosynthetic process	1.97 E-04	**(0)**	**(100)** [FDFT1, FDPS, HMGCR, IDI1, MVD, NSDHL, PARK7, SOD1]
Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	5.43 E-03	**(0)**	**(100)** [DHCR24, HMGCR, NSDHL, PHGDH, RDH12]
Tetrapyrrole binding	3.74 E-03	**(0)**	**(100)** [CYP17A1, FADS1, HBA, MTR, PGRMC1, PTGS2]

Whether you think you can,
or think you can´t, you are right
Henry Ford

Tanto si piensas que puedes,
como si piensas que no, estás en lo cierto
Henry Ford