Institute of Animal Science, v.v.i

&

Charles University in Prague

Faculty of Science

IRENA BARNETOVÁ

Doctoral Thesis

Genome reprogramming during the first cell cycle of embryonic development

Supervisor: Ing. Josef Fulka, DrSc., Jr.

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Declaration:

I declare that I wrote the thesis independently and I quote all the sources. I do declare that I have not used this thesis or any part of it to obtain any other academic titles.

Prague, 29th April 2011

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Irena Barnetová

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ABSTRACT

The sperm head contains highly compacted genome. This compaction is mediated by protamines. Sperm protamines are replaced by cytoplasmic histones after the sperm entry into the oocyte. Beside protein replacement, also particular epigenetic remodeling occurs. One of the most studied epigenetic remodeling in early zygotes is DNA demethylation. This phenomenon was observed in some mammals (mouse, rat, monkey) but not in other mammals (sheep) and what is more, about some of them quite inconsistent data were published (pig, human, goat, rabbit).

In our work we were mostly concentrated on porcine zygotes and attempted to explain the reason of inconsistency in observed data. Three factors were evaluated in our work – the technique of embryo production, sperm factors, and the oocyte quality. In the first part of the study (the technique of embryo production) we compared the zygotes produced by conventional *in vitro* fertilization and zygotes produced by intracytoplasmic sperm injection. The epigenetic remodeling was evaluated by immunolabeling. There was no difference between zygotes produced by both mentioned techniques. The paternal genome was not demethylated in any zygote. The labeling with anti-H3/K9-me2 (anti dimethyl group on lysine 9 of histone 3) showed the positive labeling of both pronuclei in about half of zygotes. In the second part of the study (sperm factors) we aimed at sperm factors. The technique of interspecific intracytoplasmic sperm injection (iICSI) was used to experimentally separate the oocyte and sperm factors. We injected boar spermatozoa into mouse oocytes, human spermatozoa into mouse oocytes, and mouse sperm heads into porcine oocytes. All the injected spermatozoa (or sperm heads) formed the paternal pronucleus in oocytes of different species. The paternal genome of porcine and human origin was demethylated in mouse oocytes. In contrast, the paternal genome of mouse origin was not demethylated in porcine oocytes. These results suggest that oocyte cytoplasm mainly affects the paternal genome remodeling. Moreover, boar spermatozoa, which are not demethylated in porcine zygotes, are able to undergo the active demethylation in cytoplasm of another species. In the third part of the study (the oocyte quality) we compared mouse and porcine ovulated and *in vitro* matured oocytes. The oocytes were used for embryo production by intracytoplasmic sperm injection techniques (ICSI and iICSI). We observed a difference between ovulated and *in vitro* matured oocytes

in both species. The difference was more evident in activation capabilities of oocytes then in epigenetic remodeling capabilities. Mouse ovulated oocytes are able to form the paternal pronucleus of porcine origin. In contrast, mouse *in vitro* matured oocytes were not able to form the paternal pronucleus of porcine origin. Similarly, the paternal pronucleus of mouse origin was formed in porcine ovulated oocytes, but *in vitro* matured oocytes formed the paternal pronucleus only after additional activation with the electrical pulse. Only a small difference was observed in epigenetic remodeling in mouse control zygotes (intraspecies) produced from ovulated and *in vitro* matured oocytes (extent of DNA demethylation). In addition, no difference was observed in porcine control zygotes (intraspecies) produced from ovulated and *in vitro* matured oocytes. Moreover, we did not observe the DNA demethylation of paternal pronucleus in porcine zygotes which is in agreement with some reported studies but in contrast with some other studies.

From these results we concluded that the technique used for embryo production, sperm factors, or the quality of oocytes did not affect the final epigenetic remodeling in zygotes. We suppose that the problem of inconsistent data observed in pig is much more complicated. It seems that the reason is not mainly in the quality of oocyte, as is often quoted, but in some other not defined factors.

CONTENTS

ABBREVIATIONS

5-hmc	5-hydroxymethylcytosin
5-MeC	5-methylcytosin
ADP	adenosine diphosphate
ATP	adenosine triphosphate
DNA	deoxyribonucleotic acid
Dnmt	DNA methyltransferase
Elp	elongator complex protein
FISH	fluorescent in situ hybridization
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H3	histone 3
H3/K4-me (2, 3)	(mono, di, tri) methylation of lysine 4 on histone 3
H3/K9-me (2, 3)	(mono, di, tri) methylation of lysine 9 on histone 3
H4	histone 4
HP1	heterochromatin protein 1
IAP	inhibitor of apoptosis protein
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
iICSI	interspecies intracytoplasmic sperm injection
iPS cells	induced pluripotent stem cells
IVF	in vitro fertilization
LOS	large offspring syndrome
MBD2	methyl binding domain 2
me	methyl group
MI	metaphase I oocyte
MII	metaphase II oocyte
mtDNA	mitochondrial deoxyribonucleotic acid
SAM	S-adenosylmethionine
SCNT	somatic cell nuclear transfer
S-S	disulphide bonds
TE	trofectoderm

TET protein	ten-eleven-translocation protein; oxidase
ZP	zona pellucida

1. INTRODUCTION

A better understanding of early mammalian development is important for embryonic biotechnology. High-quality embryos must be produced for successful application. A number of studies were made on mouse model where the techniques of embryo production are well developed. However, the mouse is biologically very different from human or livestock and the knowledge are hardly applicable in biotechnology. More studies must be therefore done in other animal models, such as bovine, rabbit, and pig.

The pig is used as a model in medicine research. Due to anatomical and physiological similarities of human and porcine organs, pig is used as a disease model in cardiology, pulmonary, and internal medicine. The pig is similar to human also on biochemical and immunological bases. Especially because of immunological similarities, the pig has a high potential for human regenerative medicine (porcine epidermal stem cell). Moreover, it could be useful model for stem cell biotechnology testing previous the application in human medicine (neural stem cells). Besides the above mentioned facts, the pig is also used as model for studies of aneuploidy in adult human oocytes. What is more, there is a possibility to use genetically modified pigs for xenotransplation. All these presumptions become more realistic in connection with the fact that some components of pig are already used in medicine practice (derma).

However, the biotechnology of pig still faces with many problems. First, the quality of ovulated and *in vitro* matured oocytes is largely different. Ovulated oocytes are difficult to obtain and only small number of them is derived from the stimulated animal. In spite of plentiful sources of ovaries from prepubescent gilts in slaughter houses, the seasonal effect is reflected in the quality of isolated oocytes. Moreover, maturation of isolated porcine oocytes *in vitro* takes extremely long time (40 - 44 hours) and the media for maturation are not optimized. The *in vitro* matured oocytes have therefore a lower quality. Second, the techniques used for embryo production are also problematical. For example IVF is accompanied with a high rate of polyspermy which is not easy to regulate (Funahashi, 2003). The problem of polyspermy is solved by ICSI, but this method faces with problem of insufficient sperm head decondensation (Kren et al., 2003). Third, the *in vitro* produced blastocysts contain much less cells compared to *in vivo* produced blastocysts. The embryo cultivation

condition must be improved to get developmentally capable embryos. By now, only a few research groups were able to give birth to live animals derived from *in vitro* produced embryos. The pig was also one of the latest species cloned by SCNT (somatic cell nuclear transfer). Finally, only putative embryonic stem cell lines were derived from porcine blastocyst (Vackova et al., 2007). The cultivation conditions are not developed either for porcine stem cells. Cells differentiate spontaneously within first few passages. The similar problems are expected in porcine iPS cells (induced pluripotent stem cells). So, in spite of high possibilities of biotechnology application of pig, there are still many aspects which have to be solved.

One of the aspects how to improve the quality of embryos is to identify the mechanisms of epigenetic remodeling. In the pig, the mechanism of epigenetic remodeling is unclear. A number of contrasting data has been published about the active demethylation of paternal genome in porcine zygotes (more details below).

Epigenetic remodeling reflects in many aspects of possible biotechnologies. For example epigenetic remodeling plays a crucial role in SCNT and in human assisted reproduction. The somatic cell nucleus has to be remodeled in the recipient oocyte after SCNT and this remodeling is considered to be on similar basis as remodeling of paternal pronucleus after fertilization. SCNT faces extremely low efficiency and individuals born after SCNT have often similar disorders called “large offspring syndrome” (LOS). It is supposed that LOS is a subsequence of insufficient remodeling of donor nucleus, especially in the area of epigenetics. Except of application of SCNT for a new individual production, the SCNT could be used even in human medicine. In this context, there has been speculated about so called “therapeutic cloning”. The therapeutic cloning means the SCNT embryos production with the aim to derive embryonic stem cells. The proper stem cell differentiation in a tissue is a precondition for human stem cells technologies. When there is an incorrect epigenetic remodeling in embryo, the derived embryonic stem cells could have problems with the proper differentiation and function.

Besides, epigenetic remodeling is important also for human assisted reproduction. Here a possible impact of *in vitro* cultivation on epigenetic remodeling of human embryos must be evaluated. The negative effect of human assisted reproduction on embryos has not been proved directly; however, due to the knowledge concerning other species we are not able to disprove it absolutely. It seems that in *in vitro* conditions the pronuclei formation and epigenetic remodeling are delayed compared

with *in vivo* conditions. The delay is especially obvious after the intracytoplasmic sperm injection (Katayama et al., 2002; Ajduk et al., 2006). These facts must also be taken into consideration.

All above mentioned statements acknowledge that a more detailed awareness of the mechanisms of epigenetic remodeling makes it possible to better understand and predict the disorders connected with the epigenetics. Our study evaluates factors which could affect the final epigenetic remodeling in porcine zygotes. Except of common techniques for embryo production, we have used iICSI (interspecies intracytoplasmic sperm injection) – a technique which allows us experimentally to divide the oocyte and sperm factors. A definition of negative factors affecting the quality of embryos will help us to produce embryos usable in biotechnologies.

1.1 Epigenetic remodeling

Epigenetic remodeling covers genome modifications which do not lead to a change of DNA sequence but it affects the development of the organism. The epigenetic modifications have an important role in preservation of structural integrity and gene regulation. Moreover, it was found that epigenetic processes have an impact on cell differentiation and also on cancer origin. Nevertheless, epigenetics plays an important role in early mammalian development.

Both gametes – the sperm and the oocyte, are highly specialized cells. In contrast, embryonic cells are at first totipotent and later, in blastocyst, they are pluripotent. Thus the genomes of gametes must be remodeled to make a functional diploid genome of zygote.

In the text below it is briefly discussed chromatin remodeling mechanisms in the context of early mammalian development; namely the text focused on covalent histone modifications and DNA methylation.

1.1.1 Sperm chromatin before fertilization

Sperm head contains highly condensed and inactive chromatin. This condensation is mediated by specific spermatic proteins called protamines. Incorporation of

protamines into the spermatic genome is the last step of spermatogenesis. According to the model of Balhorn (1982), arginine-rich core lies along minor groove of DNA helix and it fills one helical turn. There are two known types of protamines – protamine 1 and the family of protamine 2 proteins. Protamine 1 is found in all vertebrates; family of proteins protamine 2 are formed by protamine 2, 3, 4 components and they are found only in mice and human spermatozoa (Oliva, 2006).

In spite of the appearance of protamines in the sperm head, there are still some minority rests of histones. For example in human sperm, it was shown that 85% of the nucleus structure is organized with protamines, whereas 15% is organized with histones or other proteins. Thus some parts of chromatin of spermatozoa remain arranged into nucleosomes (Gatewood et al., 1987).

1.1.2 Fertilization

The entry of sperm into the oocyte is an activation signal for inactive metaphase II oocyte. Oocyte chromatin re-enters meiotic cell cycle; it progresses from metaphase into telophase and second polar body is extruded. Oocyte chromatin is then changed into maternal (female) pronucleus. Similarly, highly condensed sperm chromatin is decondensed in oocyte cytoplasm and it gets formed as paternal (male) pronucleus. Both genomes (the maternal and paternal) are important for successful development. Gynogenetic or androgenetic embryos are not viable in mammals (McGrath and Solter, 1984; Barton et al., 1984; Surani et al., 1984).

1.1.3 Morphological and molecular remodeling of chromatin after fertilization

A number of morphological and molecular changes of original sperm head precede the paternal pronucleus formation. Sperm chromatin is highly compacted and inactive therefore a remodeling is necessary for successful activation. There are three main steps of morphological remodeling of sperm head (Wright and Longo, 1988; Adenot et al., 1991). In the first step, when the oocyte completes anaphase II, the sperm chromatin is dispersed. The size of sperm chromatin enlarges about three-times.

In the second step, when the oocyte reaches the telophase II, sperm chromatin recondenses into a smaller mass. The size is reduced into a half. In the third step, when both pronuclei are formed, the area covered by sperm chromatin is ten times larger.

The crucial step of remodeling is the removal of protamines from the sperm genome. Protamines are completely removed from original sperm head at the end of anaphase II of oocyte cell cycle. It means that the replacement occurs shortly after sperm penetration, before the formation of nuclear membrane. For example in the pig, the replacement is finished within 2-3 hours after fertilization, at the time when the sperm head is still condensed (Shimada et al., 2000). The reduction of disulfide (S-S) bonds is necessary for protamines replacement. An important regulator of disulfide bonds reduction is cytoplasmic protein glutathione (Yoshida, 1993; Perreault et al., 1988).

Sperm protamines are replaced by oocytes cytoplasm proteins - histones. The protamine-histone exchange is mediated by cytoplasmic proteins called nuclear chaperones (Philpott et al., 2000). For example in amphibian, a chaperon called nucleoplasmin removes protamines from sperm and deposits H2A-H2B histone dimer. As the human sperm head decondenses even in amphibian extract, the decondensation factors do not seem to be species specific (Ohsumi et al., 1986). However, little is known about the replacement process in mammals. The nucleoplasmin-like protein in mammals has not been detected so far (Nakazawa et al., 2002).

Although both pronuclei are formed in the same cytoplasm, several epigenetic differences are established between them. The first difference is histone hyperacetylation of paternal pronucleus. This difference disappears before DNA replication. The reason is that histones H3 and H4 are incorporated into the pronucleus in their acetylated form; this modification is removed afterwards (Adenot et al., 1997; Verreault, 2000). Another difference is in residual transcription activity in pronuclei. The transcription activity in zygote is generally low; however, as was confirmed by genes microinjection, the residual transcription is higher in the paternal pronucleus (Aoki et al., 1997). Further, one of the most studied differences between pronuclei is the active demethylation of paternal pronucleus. This phenomenon appears shortly after fertilization and persists up to pronuclei apposition. The process of active demethylation is more described below.

1.1.4 Epigenetic remodeling in zygote

1.1.4.1 Covalent histone modifications

The covalent histone modifications are complex and they involve many histone-modification enzymes. Core histones are covalently modified at lysine, arginine and serine residues. The most common modifications are methylation, acetylation, phosphorylation, ubiquitination and ADP ribosylation. For example some lysine residues on histone 3 (H3/K9, 14, 18 and 23), some lysine residues on histone 4 (H4/K5, 8, 12 and 16), and lysine residues on histones H2A and H2B are acetylated. Other lysine (H3/K4, 9, 27) and arginine residues (H3/R2, 17, 26) on histone 3 and histone 4 (H4/K20, H4/R3) are methylated (Li, 2002).

As mentioned above, histones are incorporated into the male pronucleus in hyperacetylated form. However, shortly after their incorporation, H3/K9-me, H3/K4-me and H3/K29-me are detectable (Morgan et al., 2005). It means that histones are immediately deacetylated and monomethylated by an appropriate histone methyltransferase. Dimethyl and trimethyl forms of these residues become detectable later (Morgan et al., 2005). Whereas histone acetylations and methylations of some histones (H3/K4, H3/K36, H3/K79) are transcriptionally permissive, methylations of other histones (H3/K9, H3/K27, H4/K20) are trascriptionally repressive modifications (Struhl, 1998). This possibly explains the higher residual transcription activity of paternal pronucleus. It is possible that the difference in transcription activity between pronuclei is the result of different acetylation level rather than its cause. It is unknown how the histone acetylation causes higher transcription activity. It is supposed that highly acetylated histones do not bind DNA so strongly, they loosen the nucleosome and DNA is then more accessible to transcription factors.

The role of histone modifications is that they can be recognized by various proteins, which influence the structure of chromatin (eu- vs. heterochromatin) or transcription. The example is HP1 (heterochromatin protein 1). HP1 binds with high affinity to methylated H3/K9 and through oligomerization HP1 maintains heterochromatin. Besides, in Arabidopsis HP1 bound on H3/K9 recruits DNA methyltransferase to its target CpG sites (Jackson et al., 2002). In spite of not knowing how histone modifications affect DNA methylation in mammals, it is expected that the mechanism is similar to Arabidopsis.

1.1.4.2 DNA methylation

DNA methylation is the most studied epigenetic difference between the maternal and paternal pronuclei. Mammalian DNA becomes methylated at the fifth position of cytosin; thus 5- methylcytosin (5-MeC) is formed from simple cytosin. Enzymes responsible for DNA methylation are various types of DNA methyltrasferases (Dnmts). DNA methylation occurs mainly in CpG dinucleotides of the genome. DNA methylation is mostly associated with the repression of gene transcription. The methods for study of DNA methylation are the indirect immunofluorescence and bisulfite sequencing.

Before fertilization, the maternal (oocyte) and paternal genomes (spermatozoa) are heavily methylated. The parental methylation is partly removed during early mammalian development. There are two types of demethylation in preimplantation mammalian development – the active and passive demethylation (Rougier et al., 1998). In the early development, shortly after fertilization, the paternal pronucleus becomes demethylated whilst the maternal pronucleus remains highly methylated. As the process is quite rapid and it starts before the first replication, we call this phenomenon the active demethylation. A factor responsible for active demethylation (a demethylase) is not fully known so far (more details below). Later in the development the methylation level passively declines and it reaches the lowest level in morula stage (Dean et al., 2001). The reason is that the *de novo* methyltransferase (Dnmt1) is not present, so, during following replication new replicated strands are not methylated (Bestor, 2000). In mouse blastocyst, when the first differentiation event occurs, the inner cell mass (ICM) becomes methylated again and trofectodermal cells (TE) are undermethylated (Dean et al., 2001; Santos et al., 2002). However, it seems that this phenomenos is species specific.

The role of active demethylation is not fully determined so far. First it seemed that the active demethylation of paternal pronucleus is a common phenomenon between all mammals (Dean et al., 2001). The paternal pronucleus in mouse zygote is extensively demethylated, whilst the maternal pronucleus remains highly methylated (Santos et al., 2002; Oswald et al., 2000). Similar results were observed in the rat and monkey (Yang et al., 2007; Zaitseva et al., 2007). However, later on, this assumption was challenged

by some additional experiments. For example, only partial demethylation was observed in bovine (Beaujean et al., 2004) and no active demethylation at all was observed in the sheep and rabbit (Beaujean et al., 2004; Shi et al., 2004). What is more, in some species quite inconsistent data were published. Examples of such species are the pig, goat and human. The demethylation of paternal pronucleus in the pig was observed by Dean et al. (2001) and Fulka et al. (2006a) whereas Jeong et al. (2007a) and Deshmukh et al. (2011) did not detect any demethylation at all. In the goat there was observed only partial (Park et al., 2007) demethylation together with absence of any demethylation (Hou et al., 2005). Similarly, only partial demethylation (Fulka et al., 2004) and complete demethylation (Beaujean et al., 2004; Xu et al., 2005) were observed in human zygotes. In addition, the latest published results of methylation in rabbit zygotes proved again the original assumption. Lepikhov et al. (2008) observed that even in the rabbit, in progressed stage of zygote, there is the active demethylation of paternal pronucleus (Table I).

Table I: <u>The DNA methylation patterns observed in various mammalian species</u>

Animal species	Paternal pronucleus demethylation		
	Extensive demethylation	Partial demethylation	No demethylation
mouse	(Oswald et al., 2000) (Santos et al., 2002)		
rat	(Zaitseva et al., 2007)		
monkey	(Yang et al., 2007)		
bovine		(Beaujean et al., 2004)	
sheep			(Beaujean et al., 2004)
rabbit	(Lepikhov et al., 2008)		(Beaujean et al., 2004) (Shi et al., 2004)
goat		(Park et al., 2007)	(Hou et al., 2005)
pig	(Fulka et al., 2006a) (Dean et al., 2001)		(Jeong et al., 2007a) (Deshmukh et al., 2011)
human	(Beaujean et al., 2004) (Xu et al., 2005)	(Fulka et al., 2004)	

1.1.4.3 Mechanism of active DNA demethylation

The mechanism of active DNA demethylation in the early development is not fully understood. Initially it has been speculated that a mechanism of DNA repair participates also in the process of active DNA demethylation (Ooi and Bestor, 2008). Three possibilities are considered in the connection of demethylation by DNA repair: (i) direct removal of methyl group from 5-MeC (Bhattacharya et al., 1999); (ii) base excision repair; and (iii) nucleotide excision repair (Gehring et al., 2009). First, a protein called MBD2 (methyl binding domain 2) that binds methylated DNA and directly removes methyl group from 5-MeC was reported by Bhattacharya et al. (1999). However, this results were not confirmed by other researchers (Ooi and Bestor, 2008) and what is more, DNA demethylation was still observed in knock-out mice for MBD2 (Hendrich et al., 2001). Second, the DNA repair by base excision repair means either direct removal of 5-MeC, which is common in plants (a glycosylase), or deamination of 5-MeC to thymine followed by T-G mismatch repair and specific replacement of thymine with cytosine. A glycosylase like enzyme was not reported in mammals so far (Gehring et al., 2009). A cytosine deaminase (Morgan et al., 2004) and DNA methyltrasferase (Dnmt) were reported as to be able of deamination of 5-MeC (Gehring et al., 2009). However, even here, although the maternal Dnmt3a was found in pronuclei of zygote, there is no difference in quantity of Dnmt3a between the maternal and paternal pronuclei (Hirasawa et al., 2008). Third, the mechanism of nucleotide excision repair seems to be improbable (Gehring et al., 2009; Okada et al., 2010), in spite of some reported pathways of DNA demethylation by nucleotid excision repair (Barreto et al., 2007). It must be noted here that base/nucleotide excision repair makes DNA strand breaks which can be detrimental especially in these critical stages of development (Gehring et al., 2009).

Later on, some other mechanisms of DNA demethylation were reported. Some components of elongator complex were identified (Elp1, 3, 4) to have a crucial effect on active demethylation in zygotes. What is quite interesting is that the SAM domain (S-adenosylmethionine; a donor of methyl group within methylation) was found to be necessary for the process (Okada et al., 2010). Another important finding was the presence of 5-hydroxymethylcytosin (5-hmc) in pronuclei. 5-hmc, which is derived from 5-MeC through oxidation by TET proteins, was found in high amounts in progressed pronuclei of zygote (Iqbal et al., 2011). In addition, anti-5-hmc antibody

labeled the pronuclei of zygote asymmetrically and the labeling was detectable also in later stages (2-, 4-, 8-cells). The mechanism of the DNA demethylation by oxidation is not fully understood; however, it seems that 5-hmc may work as a neutralizer of 5-MeC gene suppression. Simultaneously, 5-hmc is not a substrate for Dnmt1 (maintaining methyltransferase) and it leads to reduction of methylation in later stages of development. Moreover, Tet3 oxidase was confirmed to be expressed at high levels in oocytes and zygotes (Iqbal et al., 2011). However, some other experiments must be done to confirm this assumption.

1.1.5 Oocyte remodeling capabilities

The conflicting data of DNA demethylation observed in some species are difficult to explain. Nevertheless, there are some points that might play a significant role. The protocol used for evaluation is the first point. Most of results are concluded from immunofluorescence where two antibodies are used. The extent of active demethylation can be particularly influenced by the antibody dilution thus the complete *vs.* partial demethylation or partial *vs.* no demethylation observed in some species may be caused by different antibody concentration (Fulková, 2007). Another point is connected with the time of demethylation occurrence which is different between species and probably even between strains of the animal species. For example in the mouse, some researches reported complete demethylation already in four hours after fertilization (Santos et al., 2002) whereas another authors did not observe complete demethylation until eight hours (Mayer et al., 2000). The authors used different mouse strains. Similarly in the pig, according to our previous results, it seems that active demethylation occurs shortly after fertilization in the miniature pig (Fulka et al., 2006a); however, no demethylation was observed in recently published results from the breeding pig (Deshmukh et al., 2011). Next point is connected with methylation/demethylation dynamics during the first cell cycle. As described in bovine, demethylation and remethylation in zygotes occur in waves (Park et al., 2007). Therefore the timing of sample preparation must also be taken into consideration. Further point is the quality of oocytes. This fact may become important in those species where oocytes are matured *in vitro*.

The quality of oocytes is reflected in oocyte remodeling capabilities. Not only maturation condition but also the method of oocyte isolation may affect oocyte quality (Wang et al., 2007). Maturation of porcine oocytes takes about 44 hours and it is one

the longest maturation among species used in common research. The comparison of *in vitro* and *in vivo* matured porcine oocytes reported that these oocytes differ in many aspects. For example, the polyspermy is lower in *in vivo* matured oocytes (Gioia et al., 2005); the formation of paternal pronucleus is delayed in *in vitro* matured oocytes; asynchronous pronuclei formation is typical for zygotes after use of *in vitro* matured oocytes (Laurincik et al., 1994). Beside these mentioned aspects the quality of oocyte may influence also the epigenetic remodeling. The different occurrence of methylation and demethylation was observed between *in vivo* and *in vitro* matured porcine oocytes. Oocytes matured completely *in vivo* or just partly *in vitro* were able to actively demethylate the paternal pronucleus. In contrast, in oocytes matured completely *in vitro* there was no demethylation observed. An interesting point concluded from this study is that the key point of maturation is the time of germinal vesicle breakdown (GVBD; Gioia et al., 2005). However, in contrast to this statement no active demethylation was observed recently in naturally fertilized zygotes of breeding pig (Deshmukh et al., 2011). Therefore it seems that there are many aspects which are reflected in oocyte remodeling capabilities and the importance of each aspect must be determined more.

1.2 Interspecies intracytoplasmic sperm injection (iICSI)

Species specific binding proteins on the sperm and oocyte protect the oocyte against fertilization with a sperm of other species. The method of intracytoplasmic sperm injection (ICSI) allows us to inject sperm interspecifically. As mentioned above, the mammalian sperm head decondenses and forms the paternal pronucleus in cytoplasm of amphibian oocyte (Ohsumi et al., 1986). Similarly, it forms the paternal pronucleus in oocytes of different mammals (Yanagida et al., 1991; Wakayama et al., 1997; Kimura et al., 1998). Sperm remodeling factors (glutathione, chaperones) are therefore universal, not species specific.

All these mentioned facts cause that the iICSI method is useful for the study of factors contributing to the pronuclei formation. The iICSI allows us to divide experimentally the maternal and paternal contribution to the zygote and to study remodeling capabilities of oocytes. Especially, it seems to be a beneficial method in epigenetic studies of early development.

What is more, the iICSI could be used as a tool for sperm analysis of patients undergoing the assisted reproduction. The remodeled paternal pronucleus could be used for chromosomes number analysis. Together with the FISH method (fluorescence *in situ* hybridization) we might be able to analyze specific chromosomes for a deletion or amplification. The extreme genome condensation of the sperm does not allow us to use the intact sperm head for similar detection (H. Fulková, personal communication).

2. AIMS OF THE STUDY

The general aim of the study was to evaluate factors which could affect the final epigenetic remodeling of zygotes and which could lead to production of high quality embryos. The study characterizes the epigenetic reprogramming capabilities of oocytes. A special attention was paid to the DNA and histone methylation. The laboratory animal species (mouse) and breeding animal species (pig) were used for evaluation. The study attempted to specify the reason of inconsistent data of epigenetic remodeling observed in porcine zygotes.

Specific aims of the study were:

1. to characterize the potential role of techniques (IVF and ICSI) used for embryo production on epigenetic remodeling of porcine zygote

2. to evaluate the paternal genome remodeling in iICSI (interspecies intracytoplasmic sperm injection) embryos, namely the remodeling of paternal genome of human and porcine origin in mouse oocytes and the remodeling of paternal genome of mouse origin in porcine oocytes

3. to characterize the role of maturation quality in remodeling capabilities of oocytes, to compare remodeling capabilities of ovulated and *in vitro* matured oocytes of mouse and pig

3. COMMENTS AND DISCUSSION ON SPECIFIC PUBLICATIONS AND UNPUBLISHED RESULTS

The list of publications is not arranged chronologically but according to the relevance to a given topic.

3.1 Comments and discussion on specific publications

3.1.1 Research paper I

Barnetova I, Okada K
Genome reprogramming during the first cell cycle in in vitro produced porcine embryos
CZECH JOURNAL OF ANIMAL SCIENCE 2010; 55 (2): 49-57

Specific contribution to the article: conventional *in vitro* fertilization, intracytoplasmic sperm injection, immunolabeling, image analysis, manuscript preparation

As mentioned above quite inconsistent data about DNA methylation has been published in the pig. Some authors observed the active demethylation of paternal pronucleus (Dean et al., 2001; Fulka et al., 2006) whereas the other authors exclude this notion (Jeong et al., 2007a).

In this work we aimed at the method for embryo production (IVF and ICSI). Both methods are quite problematic in the pig (polyspermy *vers*. aberrant sperm head decondensation). We therefore test, if there is a difference in epigenetic remodeling between embryos produced by these two mentioned techniques.

Both types of porcine zygotes were fixed at 22 hpf (hours post fertilization). Zygotes were then used for the antibody labeling with anti-5-MeC (anti-5-methylcytosin) and anti-H3/K9-me2 (anti-dimethyl group on lysine 9 of histone 3). No difference between the two groups of zygotes was observed. The paternal genome was not demethylated on 5-MeC in any types of embryos (IVF and ICSI). H3/K9-me2 of paternal pronucleus was positive (both pronuclei labeled) in 58% of IVF and 56% ICSI embryos (no statistically significant difference; χ2-test). Thus it seems that the method of embryo production does not affect the epigenetic remodeling of zygotes.

Similar experiment was done by Fulka et al. (2006b) on mice. No difference between the techniques was observed as well. What is more, the authors did not see any difference between zygotes produced *in vivo* and *in vitro*. In contrast, a different embryo quality was observed after IVF and ICSI in the rat (Yoshizawa et al., 2010). There, the methods used for embryo production affected the rate of demethylation between zygotes. It must be noted that the cultivation system and media composition in mouse biotechnology is well developed. This is not the case of rat biotechnology. The developmental rate up blastocyst is, in the rat, about 50% after IVF and 20-30% after ICSI. Therefore a media and system optimization would probably lead to the normal remodeling dynamics also in the rat (Yoshizawa et al., 2010).

The relation of active demethylation and H3/K9 methylation is not fully understood. Santos et al. (2005) suggested that H3/K9-me2 protects the maternal pronucleus from demethylation; so the absence of H3/K9-me2 in male pronucleus allows it to undergo active demethylation. Sega et al. (2007) also supposes that the demethylation is not possible when H3/K9-me2 is present. In our work we observed H3/K9-me2 in the paternal pronucleus in about half of the zygotes, whereas the demethylation was not seen at all. Jeong et al. (2007b) also saw the methylation on H3/K9. It seems that the methylation of H3/K9 is established during the pronucleus development and it prevents the male pronucleus from later demethylation.

To sum up these comments, the paternal pronucleus in porcine zygotes produced from *in vitro* matured oocytes remained methylated and this outcome was not affected by the method used for embryo production.

3.1.2 Research paper II

Barnetova I, Fulka H, Fulka J, Jr.
Epigenetic characteristics of paternal chromatin in interspecies zygotes
JOURNAL OF REPRODUCTIN AND DEVELOPMENT 2010; 56 (6): 601-606

Specific contribution to the article: intracytoplasmic sperm injection, immunolabeling, image analysis, manuscript preparation

The method of iICSI allows us to experimentally divide the oocyte and sperm contribution to the remodeling. We used this method to study the maternal and paternal contribution to the remodeling separately. Porcine sperm heads, which were not demethylated in porcine *in vitro* matured oocytes, were injected into mouse ovulated oocytes. The paternal pronucleus demethylation is a common phenomenon in mouse zygote (Dean et al., 2001; Santos et al., 2005). We therefore interested in whether the porcine sperm head is able to undergo the active demethylation in mouse oocyte. Moreover, according to our knowledge, the oocytes mostly used for mouse zygote production are naturally ovulated and there are not more detailed studies of remodeling abilities of oocytes matured *in vitro*. To test the importance of maturation condition we have also used *in vitro* matured mouse oocytes for both iICSI and ICSI.

Both pronuclei (maternal and paternal) were formed after the porcine sperm head injection into mouse ovulated oocytes. The paternal pronucleus was larger that the maternal (12-14 hpf) – as it is typical for the mouse, but not for the pig. Both pronuclei were labeled with anti-Pan histone antibody which was used as a control of protamine-histone exchange. The labeling with the antibody against 5-MeC showed gradual demethylation of paternal pronucleus of porcine origin. The paternal pronucleus was also negative after the labeling with HP1, H3/K9-me2, H3/K4-me3 antibodies. From that arises that the porcine paternal genome was remodeled similarly to the mouse paternal genome. It seems that the porcine genome is able to undergo demethylation in appropriate cytoplasm and that the reason of absence of demethylation in porcine zygotes produced in previous work is not caused by sperm factors.

Consequently, we decided to look at the remodeling ability of *in vitro* matured mouse oocytes. First, we have used these oocytes for porcine sperm head injection

(iICSI). Here, to our surprise, the male pronucleus was not formed at all in spite of obvious activation of oocytes (second polar bodies extruded, MIII stage chromosomes). The paternal pronucleus was not formed neither after additional activation of oocyte by $SrCl_2$ nor after the treatment of boar spermatozoa (permeabilization with Triton X-100, freezing, sonication; Barnetova, additional experiments). Because these treatment techniques help us to remove the sperm membrane, the problem seems to be rather in protamine-histone exchange. Second, we have used the mouse *in vitro* matured oocytes for mouse sperm head injection (intraspecies ICSI). Methylation patterns (HP1, H3/K9-me2, H3/K4-me3, 5-MeC) of these zygotes was compared to the patterns typical for zygotes produced from ovulated oocytes. The methylation pattern was essentially the same except of 5-MeC. Paternal pronuclei in mouse zygotes derived from ovulated oocytes were extensively demethylated in 12 hpf, whereas the demethylation was not so extensive in zygotes derived from *in vitro* matured oocytes (Barnetova, additional experiments, Fig. 1). Thus it seems that the activation capability and remodeling dynamics of mouse ovulated and *in vitro* matured oocytes are different.

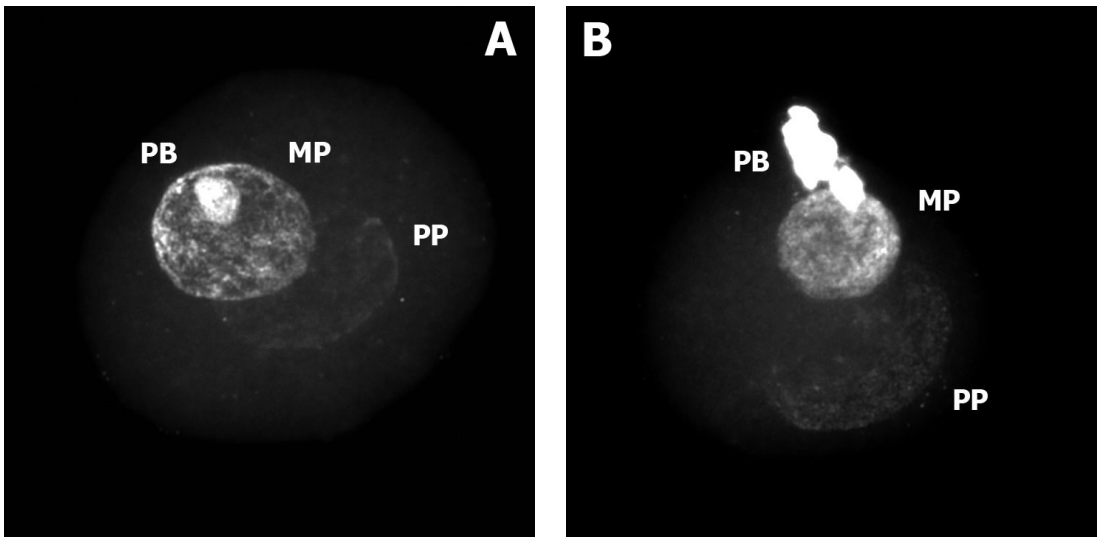

Figure 1: DNA methylation pattern in mouse intraspecies zygote 12 hpf. (A) Zygote produced with the use of ovulated oocyte. (B) Zygote produced with the use of *in vitro* matured oocyte.

MP – maternal pronucleus; PP – paternal pronucleus; PB – polar body

3.1.3 Research paper III

Fulka H, Barnetova I, Mosko T, Fulka J, Jr.
Epigenetic analysis of human spermatozoa after their injection into ovulated mouse oocytes
HUMAN REPRODUCTION 2008; 23 (3): 627-634

Specific contribution to the article: additional and supporting experiments

The role of active demethylation in human embryos is also not fully determined. Quite inconsistent data were observed even in human embryos. Almost complete demethylation was detected by Beaujean et al. (2004); in contrast Fulka et al. (2004) and Xu et al. (2005) observed that about half of embryos had the paternal genome less methylated than the maternal genome. A more detailed study is protected by ethical reasons.

In this work we have used the method of iICSI for sperm remodeling evaluation. Human spermatozoa were injected into mouse ovulated oocytes. The paternal pronucleus was larger than the maternal pronucleus in 8-9 hpf as it is typical for mouse zygotes. The origin of paternal pronucleus was confirmed by Cot1 DNA FISH and by labeling of spermatozoa mid-piece with Mitotracker Green FM. The paternal pronucleus was demethylated absolutely in some zygotes (6/43); however, only partial demethylation with two typical patterns was observed in some of them – a weaker labeling under membrane (20/43) and weak labeling homogenously distributed in the pronucleus (17/43). All these observations confirmed strong remodeling capability of the mouse oocytes. The labeling with other antibodies showed the asymmetrical patterns between pronuclei on H3/K9-me2, H3/K9-me3, H4K20-me3, H3K4-me3, H3K27-me3, and HP1 positions. Histones H3/K9 and H4/K12 were labelled symmetrically in both pronuclei. The epigenetic labeling is generally almost the same to mouse intraspecies zygotes.

The iICSI can be used in related studies to assisted reproduction (Yanagimachi, 2005). For example Heindryckx (2005) used the iICSI with mouse oocytes for the analysis of sperm activation ability. Terada et al. (2004) injected human spermatozoa into rabbit and analyze the sperm centrosomal function. What is more,

iICSI zygotes may be used also for the karyotype analysis (Araki and Yoshizawa, 2005). Besides, the iICSI seems to be really useful for testing of ROSI (testicular biopsy; Tesarik et al., 1999). Our results confirmed that human spermatozoa are able to form the functional paternal pronucleus in oocytes of other species.

3.1.4 Research paper IV

Fulka H, Langerova A, Barnetova I, Novakova Z, Mosko T, Fulka J, Jr.
How to repair the oocyte and zygote?
JOURNAL OF REPRODUCTION AND DEVELOPMENT 2009; 55 (6): 583-587

Specific contribution to the article: IB works on topics discussed in this review

In this review we focused on approaches that can be used to repair oocytes or one-cell embryos. First of all, it is important to point out that defects in cytoplasm (mtDNA) are possible to repair with a micromanipulation technique whereas defects in nucleus (nuclear DNA) are impossible or very difficult to repair (nucleolus). Second, different oocyte stages can be used for manipulation. MII oocytes seem to be more advantageous for micromanipulation than GV, zygote, or 2-cell embryo; nevertheless, the specific stage is dependent on the type of problem which has to be solved. Finally, it is quite clear that the appropriate technical equipment of laboratory is a necessary precondition for micromanipulations (a micromanipulator).

There are different approaches how to repair oocyte and zygote. First attempt is GV transfer. GV could be removed from one oocyte and fused with another cytoplasm from that the original GV was removed. The oocyte then matures and reaches MII. Moreover, GV can be stored in empty ZP and also vitrificated. When GV are stored *in vitro* separately, the GVBD does not occur. It is due to limited volume of cytoplasm which has not enough factors for GVBD induction. As the second attempt, the MI condensing chromosome can be transferred. This technique, however, needs considerable micromanipulation skills. Chromosomes are poorly visible and they cannot be stored for longer time outside of the oocyte. Another attempt is a transfer of pronuclei which is possible in zygote. Similarly to the case of GV transfer, the pronuclei are quite large, therefore a diameter of enucleation pipette must be wide and pronuclei cannot be directly injected into the oocyte. The other attempts are cytoplasmic transfer, destruction of mtDNA, ZP reparation, and nucleoli manipulation. The cytoplasmic transfer means injection of a volume of cytoplasm into the oocyte or zygote as a tool for improvement of developmental potential. It is not commonly used in human medicine because of some abnormalities of born children. The destruction of mutated mtDNA has

to be used before the transfer of donor mitochondria. In this context, it is important to take into consideration the distribution of donor mitochondria which is cell cycle dependent. The ZP reparation is necessary in cases when spermatozoa are not able to penetrate ZP or when the ZP is so hard that the blastocyst does not hatch. Nucleoli are absolutely essential for embryonic development (Ogushi et al., 2008) and the transfer of this structure is very promising. The potential use of all mentioned attempts is wide and quite perspective. As most of these mentioned micromanipulation methods work well in the mouse, some additional experiments must be done to consider their use in another species. All the risks must be compared with benefit before the use in human assisted reproduction.

3.2 Unpublished results

3.2.1 Research paper V - submitted manuscript

Barnetova I, Vackova I, Firla P
Dynamics of epigenetic remodeling in interspecies porcine zygotes
CZECH JOURNAL OF ANIMAL SCIENCE

Specific contribution to the article: oocytes isolation, intracytoplasmic sperm injection, immunolabeling, image analysis, manuscript preparation

The quality of oocytes may be reflected in oocyte remodeling capabilities. The ovulated oocytes seem to be much better quality then the oocytes matured *in vitro* (Gioia et al., 2005). In our previous work we have used iICSI for mouse interspecies embryo production (Barnetova et al., 2010) and we observed different capabilities of ovulated and *in vitro* matured oocytes to form paternal pronucleus of interspecies origin (porcine).

In this work we used a reversed approach of iICSI - mouse sperm head injection into porcine oocytes. Two types of porcine oocytes were used for injection and epigenetic remodeling evaluation - ovulated oocytes and *in vitro* matured oocytes. The presumptive zygotes were labeled with antibodies against Pan Histone, 5-MeC, HP1, H3/K9-me2 and H3/K4-me3. The labeling patterns were compared with control zygotes, produced by porcine spermatozoa injection into ovulated oocytes (intraspecies ICSI). First, we injected mouse sperm head into ovulated oocytes. In this part of the study, the paternal pronucleus was formed in 37.6 % of zygotes. The labeling was symmetrical for all the mentioned antibodies except of H3/K9-me2, where the labeling was asymmetrical. Second, we injected mouse sperm head into *in vitro* matured oocytes. These oocytes were not able to form paternal pronucleus of mouse origin. We therefore activate the oocytes additionally (electric pulses) and then the paternal pronucleus was formed. The rate of pronuclei formation after additional activation was 30.6 %. The labeling with the mentioned antibodies was essentially the same as in the first part of the study. Third, as a control the intraspecies zygotes were produced by porcine sperm head injection into ovulated oocytes. The antibody labeling of control

zygotes was the same as the labeling of interspecies zygotes from both parts of the study. From these results it is evident that the oocytes of different origin differ in their activation capabilities; however, their remodeling capabilites are comparable. Moreover, we do not observed the DNA demethylation in porcine inter- and also intraspecies zygotes. The paternal pronucleus was labeled in the same intensity as the maternal pronucleus in both types of interspecies zygotes (from ovulated and *in vitro* matured oocytes). What is more, the symmetrical labeling was observed even in progressed stages of porcine intraspecies zygotes. These results are in agreement with the study of Deshmukh et al. (2011) who analyzed naturally fertilized zygotes and even there he did not observed DNA demethylation.

It seems that the final remodeling is mainly affected by the oocyte cytoplasm. The pronuclei size in porcine interspecies zygotes was the similar, as it is typical for porcine zygotes. In contrast, according our previous work the pronuclei differ in size in mouse intraspecies zygotes and also interspecies zygotes. The epigenetic remodeling is also similar to the pattern typical for the species from which originate the oocytes. For example, the paternal pronucleus or porcine origin was partly demethylated on 5-MeC and it was not labeled after H3/K4-me3, H3/K9-me2 and HP1. Similar pattern is typical for mouse zygotes. In contrast, the paternal pronucleus of mouse origin was not demethylated in porcine oocytes and it was labeled with HP1 and H3/K4-me3. Similar patterns were observed also in porcine control zygotes.

From our results it seems that the ovulated and *in vitro* matured oocytes differ in some capabilities (activation) but their remodeling potential is comparable. These results are in contrast to the results of Gioia et al. (2005) who observed significant difference in active DNA demethylation between ovulated and *in vitro* matured oocytes. This discrepancy is difficult to explain. Although Gioia et al. (2005) used another method for embryo production (IVF) we do not suppose that the method affects the final remodeling.

It seems that the inconsistent data of DNA demethylation observed in the pig are affected mainly by some unknown factors and lesser by the quality of oocytes. More studies must be done to evaluate the factors affecting the epigenetic remodeling.

4. CONCLUSIONS

According to the aims of the study, main results are summarized as follows:

1. We compared two techniques commonly used for embryo production - conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). We did not observed statistically significant differences in epigenetic remodeling of zygotes produced by these two techniques. Both pronuclei remain highly methylated (no marks of DNA demethylation) on 5-MeC in zygotes produced by IVF or ICSI. More than half of embryos had also symmetrically labeled pronuclei on H3/K9-me2. From these results we concluded that the method for embryo production does not affect the epigenetic remodeling of zygotes.

2. The remodeling capabilities of paternal genome were characterized by interspecies intracytoplasmic sperm injection (iICSI). We injected mouse spermatozoa into porcine oocytes, boar spermatozoa into mouse oocytes, and human spermatozoa into mouse oocytes. All the used spermatozoa formed the paternal pronucleus and were remodeled in oocytes of mentioned species. We clearly demonstrated that boar spermatozoa, which were not demethylated in porcine oocytes (Aim no. 1), are capable of demethylation in cytoplasm of mouse oocyte. In contrast, mouse spermatozoa, which are commonly demethylated in mouse oocytes, were not demethylated in porcine oocytes. According to these results it seems that the cytoplasm of oocyte has a major impact on the paternal genome reprogramming. We also showed that human spermatozoa are able to undergo epigenetic remodeling in oocytes of other species.

3. The maturation conditions of oocytes are reflected in the quality of oocytes. Naturally ovulated oocytes are supposed to have better quality than oocytes matured *in vitro*. We have used ovulated and *in vitro* matured oocytes of mouse and pig for ICSI and iICSI. A difference between ovulated and *in vitro* matured oocytes was observed in both species. The major difference was in activation capabilities of oocytes. Mouse *in vitro* matured oocytes were not able to form the paternal pronucleus of porcine origin in spite of that the ovulated oocytes formed the paternal pronucleus frequently. Porcine ovulated oocytes also formed the paternal pronucleus of mouse

origin; however, *in vitro* matured oocytes were able to form the paternal pronucleus only after additional activation. In contrast, epigenetic remodeling capabilities of ovulated and *in vitro* matured oocytes seem to be similar. Mouse ovulated and *in vitro* matured oocytes differ only in the rate of active demethylation in 12 hpf. No difference in epigenetic remodeling was observed between ovulated and *in vitro* matured porcine oocytes.

5. DISCUSSION

The real significance of active DNA demethylation in mammalian zygotes remains unknown. It is supposed that the remodeling of gametes is important for further ability of cell to differentiate into all lineages of the embryo. Aberrant remodeling is connected with low efficiency of SCNT and it is the reason of the "large offspring syndrome". Several genetic diseases are associated with defects in gene methylation in humans (De Rycke et al., 2002). Therefore it seems that a remodeling is essential for successful development.

The contrasting data of active DNA demethylation observed in porcine zygotes are not explained so far. In our study we excluded some factors as technique, sperm factors, or the quality of oocytes. However, there are still some other factors which must be evaluated. Such factors are, for example, the age of oocytes (and also of animals), the media composition, or the animal breed (strain).

One of the mentioned factors is the age of oocytes and eventually, the age of animals. It is known that oocytes after maturation undergo aging. The aging means decrease of MPF activity which may be connected with decrease in activity of some other factors. Nevertheless, the age of ovulated oocytes is difficult to estimate because of different reactivity of animals on stimulation. Another important factor is the age of animals. In our system we used prepubertal gilts which are known to have a lower quality oocytes compared to sows (Lechniak et al., 2007). However, prepubertal gilts were used also by Gioia et al. (2005) who observed the active demethylation. Moreover, the study of Deshmukh et al. (2011), where no demethylation was observed, evaluated oocytes and zygotes from sows.

Another factor, the possible effect of media composition on final epigenetic remodeling was published by several authors. Alternations in methylation and expression levels were observed for some imprinted genes in the mouse (Doherty et al., 2000; Khosla et al., 2001). Prolonged culture *in vitro* may deregulate epigenetic mechanism. However, this aspect does not explain the difference in zygotes produced without cultivation *in vitro* (naturally fertilized).

It is unexplained why both the active demethylation and no demethylation were found in naturally produced zygotes by different research groups (Fulka et al., 2006a; Deshmukh et al., 2011). Also in our laboratory the demethylation of paternal genome

in naturally produced zygotes were observed in miniature pig (Fulka et al., 2006a) in contrast to the absence of active demethylation observed in zygotes from hybrid pig (Barnetova, submitted). The procedure and the source of antibodies used for evaluation were simply the same. In spite of that the zygotes of breeding pig were produced differently (*in vitro*) we do not suppose that the final remodeling would be affected by the *in vitro* production technique. We suppose that the difference would be explained rather by various remodeling strategies between miniature and breeding pig. Nevertheless, this notion must be tested furthermore.

A question arising from the study is concerned to a possibility of various strategies of epigenetic remodeling between mammals. The embryonic genomes must be remodeled before EGA (embryonic genome activation). The EGA occurs in different stages of embryos in mammals. In the mouse, where the EGA occurs at 2-cells stage, it is necessary to remodel the genome shortly after the fertilization. In livestock, where the EGA occurs much later (4-8 cell stage), remain plenty of time for genome remodeling. For example in the goat, there is an assumption published that the active demethylation occurs at 2-cell stage embryo (Park et al., 2010). Moreover, the DNA is demethylated passively during the later development. As the porcine blastocyst contains much more cells (200-300 cells) compared to the mouse blastocyst (70-80 cells), the more extensive methylation decrease may be expected in porcine blastocyst during the passive demethylation. According to the work of Deshmukh et al. (2011), the methylation level decreases from the 2- to the 8-cell stage of embryonic development. Another decrease in methylation level was observed between early and late blastocysts (Deshmukh et al. 2011). Fulka et al. (2006a) also observed the decrease in methylation rate in blastocyst; however, the methylation level from the 2-cell to the morula stage was unchanged. Is the difference in the active demethylation between zygotes connected with the difference of passive demethylation during early development? We do not know whether the passive demethylation during early development can substitute the active demethylation in zygotes. Nevertheless, even this possibility must be further evaluated. Finally, the other epigenetic remodeling occurs with the first differentiation event in blastocyst (ICM *vs.* TE).

There are many factors which may participate in the remodeling. In our work we excluded some factors; however, there are still many of them to be tested. Therefore it is necessary to continue in the research to explain the reason of inconsistent data observed in DNA demethylation and to understand more the process of epigenetic

remodeling. The knowledge will be used for production of high quality embryos which are proper for biotechnologies and human medicine.

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7. LIST OF PRESENTED PUBLICATIONS

Research paper I

Barnetova I, Okada K. Genome reprogramming during the first cell cycle in in vitro produced porcine embryos. Czech J. Anim. Sci. 2010; 55 (2): 49-57

Czech Journal of Animal Science - IF 2009: 1,008

Research paper II

Barnetova I, Fulka H, Fulka J, Jr. Epigenetic characteristics of paternal chromatin in interspecies zygotes. J Reprod Develop 2010; 56 (6): 601-606

Journal of Reproduction and Development - IF 2009: 1,697

Research paper III

Fulka H, Barnetova I, Mosko T, Fulka J, Jr. Epigenetic analysis of human spermatozoa after their injection into ovulated mouse oocytes. Hum Reprod 2008; 23 (3): 627-634

Human Reproduction - IF 2008: 3,773

Research paper IV

Fulka H, Langerova A, Barnetova I, Novakova Z, Mosko T, Fulka J, Jr. How to repair the oocyte and zygote? J Reprod Develop 2009; 55 (6): 583-587

Journal of Reproduction and Development - IF 2009: 1,697

Research paper V - Submitted manuscript

Barnetova I, Vackova I, Firla P. Dynamics of epigenetic remodeling in interspecies porcine zygotes. Czech J Anim Sci

Declaration of participation of the author on presented publication:

*Research paper I (*Czech J. Anim. Sci. 2010; 55 (2): 49-57) - IB as the first author is responsible for major parts of experiments and participated on manuscript preparation and submission

*Research paper II (*J Reprod Develop 2010; 56 (6): 601-606) - IB as the first author is responsible for some experiments and participated on manuscript preparation and submission

*Research paper III (*Hum Reprod 2008; 23 (3): 627-634) - IB as co-author participated on additional experiments

*Research paper IV (*J Reprod Develop 2009; 55 (6): 583-587) - IB as co-author works on topics discussed in this review

*Research paper V, submitted manuscript (*Czech Journal of Animal Science) - IB as the first author is responsible for major parts of experiments and participated on manuscript preparation and submission

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Ing. Josef Fulka, DrSc., Jr.
Representative of IB co-authors

8. APPENDIX