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Manipulating the mammalian oocyte and embryo – Biological and epigenetic aspects

PhD Thesis

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I do declare that I have not used this thesis or any part of it to obtain any other academic titles.

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1. Introduction

The epigenetic inheritance is defined as a mitotically and/or meiotically heritable change in gene expression that is not caused by changes in the DNA sequence itself. Among the mechanisms involved in epigenetic regulation there is action of the ATP-dependent remodelling enzymes, DNA methylation, covalent posttranslational modifications of histone proteins, alteration of the non-histone protein content of chromatin and possibly some others.

The field of epigenetics has undergone a remarkable expansion during the last few years. It has become clear that epigenetic regulation and inheritance plays a very important role in many biological processes such as cancer or differentiation. Epigenetic events have also been implicated in regulation of early mammalian development.

For example, one process during which epigenetic changes can be detected is the transition and reprogramming of the meiotic chromatin into the mitotic chromatin that occurs shortly after fertilization. This is quite understandable since both gametes exhibit a very specific chromatin structure and this structure is evidently not permissive for certain cellular functions (e.g. transcription). Yet at a very specific time point the embryo must activate its genome in order to develop successfully. If it fails to do so, the embryonic development is arrested.

Another process that might be linked to an epigenetic reprogramming is the need for parental genomes dedifferentiation and equalisation. Gametes of both sexes are highly specialized and terminally differentiated cells. However, the embryo is toti- or later on pluripotent. This fact might indicate that the program of the gametes needs to be erased and re-established during early phases of embryogenesis. Also both gametes have their own specific program – upon the fertilization these specialised genomes need to function together in one common cytoplasm.

It is beyond the scope of this thesis to discuss in detail all the epigenetic mechanisms involved, thus, we will focus on those aspects that are particularly relevant to the presented work. We will discuss briefly the mechanisms of DNA methylation and covalent histone modifications and then focus on the role of epigenetics in early mammalian development.

1.1. DNA methylation

The primary target where DNA is methylated in mammals is the fifth position of cytosine. Therefore, cytosine is transformed into 5-methylcytosine (5-MeC). Although it has been reported that mammalian DNA also contains methylated adenine, this modification is strictly in minority and the role of this modification is rather unclear. The methylation of cytosine is carried out by a special group of enzymes - the DNA methyltransferases (Dnmts). These enzymes are divided into three groups but all are structurally related to the bacterial DNA methyltransferase. For a review on DNA methylation as well as eukaryotic DNA methyltransferases, see Chen and Li (2004), Goll and Bestor (2005) and Hermann et al (2004).

1.1.1. DNA methyltransferases

Dnmt1

The first group comprises of Dnmt1 and related enzymes. Dnmt1 is known as the "maintenance" DNA methyltransferase because its primary function is to maintain the previously set methylation marks. Correspondingly to its function, Dnmt1 has a very high affinity towards hemimethylated DNA and the processivity is about 5-30x higher when compared to the processivity with an unmethylated substrate (Goll and Bestor, 2005). Apparently, Dnmt1 can also methylate *de novo* an unmethylated DNA. This observation, however, is most probably an artefact obtained in *in vitro* studies (Chen and Li, 2004). The hemimethylated DNA arises most often during the S-phase of the cell cycle when DNA is replicated (Hermann et al, 2004). Indeed, Dnmt1 was localised to replication forks during the S-phase and also many studies document that this enzyme interacts with various proteins of the replication complex (Goll and Bestor, 2005). Another very important way how to generate hemimethylated DNA is via the action of so called *de novo* DNA methyltransferases that will be discussed later.

It should be mentioned that there are several variants of Dnmt1 from which we will mention two most relevant – Dnmt1s that is found in somatic cells and Dnmt1o that is present in oocytes and early mammalian embryos. Dnmt1o exhibits a very interesting behaviour during the early mammalian embryogenesis. As the oocyte grows, the expression of Dnmt1s gradually decreases and is taken over by the expression of Dnmt1o that finally becomes the sole Dnmt1 form in oocytes and early embryos. After fertilization, Dnmt1o is specifically prevented from entering the nuclei and is retained in the cytoplasm until the 8-cell stage when it transiently enters the nuclei and thereafter is excluded from them again (Ratnam et al, 2002; Cardoso and Leonhardt, 1999). This phenomenon is probably linked to a process of passive demethylation and maintenance of imprinting that occur during the preimplantation period of mammalian embryogenesis and will be explained later on.

Dnmt3 family

Another group of DNA methyltransferases is the Dnmt3 family. This family comprises of two enzymes - Dnmt3a and Dnmt3b. Again, there are many different variants of these enzymes generated by alternative splicing as well as by the use of alternative transcription starts. These DNA methyltransferases are called *de novo* Dnmts because they have a high affinity towards unmethylated substrates. These enzymes produce a hemimethylated DNA that can later on act as a substrate for Dnmt1 enzyme. It has been also documented that these two families of Dnmts (Dnmt1 and Dnmt3) can interact and form complexes highly facilitating the process of *de novo* DNA methylation. These proteins have also been found as parts of the ATP dependent remodelling complexes – thus linking DNA methylation, changes in covalent histone modifications and chromatin structure (Geiman et al, 2004). A new member of this family has been identified quite recently – Dnmt3L (Dnmt3 like). This methyltransferase exhibits similarities to the Dnmt3 family methyltransferases, however, it lacks the catalytic activity due to substitutions in the catalytic domain (Goll and Bestor, 2005). In spite of this, Dnmt3L was shown to play an essential role both during the establishment of imprinting marks and meiosis in males. In the male germ line, disruption of this protein causes reactivation of transposable elements and

this in turn ends up in a so-called "meiotic catastrophe" (Bourc´his and Bestor, 2004). Thus, the males with disrupted form of Dnmt3L are infertile due to apoptosis of male germ cells that fail to undergo meiosis properly. In females, this enzyme was shown to be essential for the establishment of correct maternal imprinting. Thus, females lacking this protein are also infertile due to disrupted imprinting and biallelic expression of imprinted genes (Bourc´his et al, 2001).

Dnmt2

The enzyme that remains to be mentioned is Dnmt2. The substrate of this DNA methyltransferase was not known for many years even though there were no indications that this enzyme was inactive (Jeltsch et al, 2006). However, recently one specific tRNA was recognised serving as a substrate for Dnmt2 (Goll et al, 2006). The significance of methylation of this tRNA is unknown.

1.1.2. Genomic imprinting

The experiments that took place in 1984 have clearly demonstrated that both the maternal and paternal genomes are needed for a successful development (McGrath and Solter, 1984; Surani et al, 1984). In these experiments, androgenetic and gynogenetic (containing either two paternal or two maternal genomes, respectively) embryos have been generated. When the development of such embryos was followed, it became clear that unlike the control embryos containing each one of parental genomes these embryos never developed to term. The gynogenetic embryos typically exhibited poor placenta development but the foetus was of normal size. On the other hand, the androgenetic embryos produced large excess of extraembryonic tissues but most of the embryo proper was absent. These initial experiments led to the discovery of so-called genomic imprinting (for review see Reik and Walter, 2001; Smith et al, 2004 and Walter and Paulsen, 2003).

Even though this phenomenon is closely linked to the differential regulation of parental genomes on the whole genome scale which we will discuss in more detail later, the imprinted genes are an example of a differential regulation on the level of individual genes. These genes are expressed in a parent of origin manner. To be more

precise, these genes exhibit a monoallelic expression according to the parental origin – some genes are expressed from the paternal allele exclusively while others are expressed from the maternal allele only.

It has been shown that the differential expression of parental alleles is most often based on DNA methylation. The differential methylation occurs in regions that are called differentially methylated regions (DMRs) or domains (DMDs). The imprinted genes also exhibit one extraordinary characteristic – these genes are often found in clusters and these clusters contain imprinting control regions (ICR) enabling the genes in the cluster to be regulated as one group.

The life cycle of imprinted genes exhibits some specific features. The imprints are established mostly in the germ lineage – the timing, however, is different for males and females. At the time of fertilization when mature germ cells fuse, most of imprinting marks are present. After this, in a majority of cases the methylation marks do not change throughout the whole preimplantation period. The germ line is set aside from somatic lineages very early in mammals (in the mouse around 7.5dpc; McLaren, 2003). As the primordial germ cells migrate into the genital ridges, they multiply and gradually loose DNA methylation including DNA methylation associated with imprinted genes. After the erasure of methylation marks, imprints are again re-established according to the sex of the embryo. In the mouse, the erasure takes place between 10.5-13.5dpc (Hajkova et al, 2002). After the imprinting is established it does not change grossly in somatic lineages and is mostly maintained throughout the whole life of an individual.

1.1.3. Active and Passive demethylation of DNA during early mammalian embryogenesis

During the preimplantation period, large reprogramming steps occur involving both the maternal and paternal genomes of a newly formed embryo. At the time of fertilization, the sperm fuses with the oocyte and the chromatin of both germ cells is remodelled into the paternal and maternal pronucleus, respectively. It is well known that during the process of spermatogenesis histones which help to organise the genome and form a substantial part of chromatin in somatic cells are replaced by

highly basic proteins – the protamines (Meistrich et al, 2003; Lewis et al, 2003). Upon fertilization, these proteins are released from the sperm DNA and are replaced by histones originating from the oocyte cytoplasm. At the same time, massive demethylation of the paternal, but not maternal, DNA is observed (Mayer et al, 2000). This phenomenon has been shown originally by methylation sensitive restriction analysis of DNA in early mouse embryos and more recently by the use of anti-5-methylcytosine (5-MeC) antibody. Because this phase of demethylation occurs before the onset of the first replication, it is termed the "active" demethylation. So far, all attempts to identify the putative active demethylase have failed. Thus, to date the mechanism of active demethylation remains to be elucidated.

The described situation is not universal and several species exhibit only partial or no demethylation of the paternal DNA at all. Also, it should be noted that a certain discrepancy between published results exists. Generally, no active demethylation has been observed for sheep, rabbit, goat and sometimes pig. Partial demethylation was described to occur in human, bovine and in some reports goat zygotes. Complete active demethylation was shown to take place in the mouse, rat and in some reports in the pig and human zygotes (readers are referred to the "selected publication" section at the end of this thesis for review of the current literature). These conflicting results are not easy to explain. However, there are several facts that might play a very important role. First, the protocol used as well as antibody concentration plays a pivotal role. The antibody concentration can particularly influence the reported extent of active demethylation so the complete vs. partial or partial vs. no demethylation reported in some species can be primarily caused by the antibody dilution factor. On the other hand, Park et al (2007) have reported that methylation/demethylation can be very dynamic even during the first cell cycle. These authors describe waves of demethylation and remethylation during the first embryonic cell cycle. Thus, the importance of the sample preparation timing might have been slightly underestimated. Moreover, Gioia and colleagues (2005) reported that the extent of active demethylation is highly dependent on the oocyte quality - on the oocyte maturation scheme, respectively. Thus, while the *in vivo* matured oocytes were very efficient in inducing active demethylation of the paternal DNA after fertilization, *in*

vitro matured ones were less efficient in demethylating the male genome. In contrast to results obtained by Beaujean et al (2004) in human zygotes, where these authors reported complete demethylation of the paternal genome, we have found only partial demethylation of the paternal DNA and in about half of the zygotes examined no demethylation at all (Fulka et al, 2004). It is highly probable that since these samples were obtained from patients that underwent assisted reproduction the quality of these embryos was rather compromised. Also, as pointed out by Shi and Haaf (2002) the extent of demethylation can be linked to the developmental potential of embryos and certain reports also show a more frequent occurrence of certain epigenetic diseases in children conceived by assisted reproduction technologies. Thus, the embryo handling can also play an important role in the process of active demethylation. It is also probable that the results can be influenced by the chosen method of DNA methylation analysis. While the anti-5-MeC antibody can be used to investigate the genome wide DNA methylation dynamics, it is not sensitive enough for a single gene analysis. In this case, bisulfite sequencing or methylation sensitive restriction analysis are more appropriate. However, these methods also have their limitation as only a limited number of sequences or CpG sites can be analysed and this does not give us the picture about the whole genome. As pointed out earlier, the reason for sometimes conflicting results is not easy to explain (this covers also the bisulfite sequencing vs. immunofluorescence data) and the above mentioned explanations are rather hypothetical.

DNA demethylation continues beyond the first cell cycle. It has been published that this second phase of demethylation exhibits more gradual decline that can be observed with every replication. The probable mechanism has been outlined above. The Dnmt1o that is the major form of Dnmt1 in early mammalian embryos is specifically retained in the cytoplasm and therefore cannot reach its target DNA in the nucleus. The only exception is the 8-cell stage. At this stage, Dnmt1o is released from its block by an unknown mechanism and is translocated into the nucleus. Thereafter, it is again sequestered in the cytoplasm (Ratnam et al, 2002; Cardoso and Leonhardt, 1999). The significance of this step is largely unknown but it is possible that this translocation is essential for the maintenance of methylation of imprinted genes.

1.1.4. Remethylation and differentiation at the blastocyst stage

The anti-5-MeC antibody has also allowed the investigation of dynamic changes of DNA methylation in early mammalian embryos without the need of large samples. This can be highly beneficial in those cases, where for example complicated micromanipulation schemes must be employed (e.g. somatic cell nuclear transfer, SCNT). It is now clear that demethylation of parental genomes extends well beyond the first mitotic cell cycle of early embryonic development. In fact, the level of methylation gradually declines and the lowest level is in the mouse observed at the morula stage (Dean et al, 2001). After that, the first somatic lineages differentiate and remethylation of the cells of the ICM (inner cell mass) occurs. However, it must be mentioned that the situation described is species specific – a completely opposite situation has been observed in human and monkey blastocysts, where it is the trophectoderm that becomes hypermethylated (Fulka et al, 2004; Yang et al, 2007), and an intermediate situation was described in bovine embryos where remethylation takes place earlier (8-16 cell stage) and both the TE and ICM exhibit substantial methylation (Santos et al, 2003). Initially, we have analysed the methylation/demethylation pattern in human embryos and later on in the pig (Fulka et al, 2004; Fulka et al, 2006). The pig is particularly interesting since this species does not seem to exhibit passive demethylation throughout the whole preimplantation period. A detailed analysis has shown that while the pattern of methylation at the blastocyst stage is similar to the mouse (hypermethylated ICM and hypomethylated TE), the mechanism leading to this result is different for both species. Whilst in the mouse, the lowest level of methylation is at the morula stage and both TE and ICM undergo *de novo* methylation, in the pig the level of DNA methylation stays approximately the same in TE cells (when compared to previous stages) and the cells of the ICM gradually remethylate their DNA.

From the above described phenomena, it appears that the maternal and paternal genomes are differentially regulated after fertilization. We have briefly discussed the dynamics of DNA methylation during early mammalian embryogenesis. In accordance to differences between methylation levels of the parental genomes,

presence of distinct covalent histone modifications was also observed and will be discussed in the next section.

1.2. Posttranslational covalent histone modifications

It is well known that DNA is not loosely located in the nucleus but is organised at several levels by various proteins. The first level of organisation is the nucleosome. This chromatin unit comprises of about 146 (148) bp of DNA and an octamere of histones composed of two copies of each histone H2A, H2B, H3 and H4. These protein molecules are often the target of many covalent histone modifications that take place posttranslationally (e.g. methylation, acetylation, phosphorylation or ubiquitination). Between these units so called linked DNA (20-80bp of DNA) can be found. This "spacer" DNA is associated with linker histones – H1 and its variants. It should be noted that the above mentioned histones forming the nucleosome core are known as the canonical histones, however, there are also histone variants that can replace the classical histone molecules giving very often different properties to the nucleosome. The histone variants are also important from the point of various posttranslational covalent histone modifications – often having different target sites that can be modified or lacking these sites altogether (Henikoff et al, 2004; Mellor, 2006). The significance of histone modifications is such that these can be recognised by various proteins that can influence either the structure of chromatin (e.g. heterochromatinisation) or transcription. It is beyond the scope of this thesis to describe in detail all the modifications that can be found on histone molecules and the enzymes responsible for both introducing and removing these modifications. In this section, we will focus only on histone methylation and acetylation as being by far the most studied and especially relevant to the presented publications.

1.2.1. Histone methylation and acetylation

Generally, the covalent histone modifications can be divided into two groups – repressive and activating in the sense of transcription. However, the situation is not so simple and there are also many modifications playing role in more specialised cellular processes, for example DNA repair or recombination (Tamburini and Tyler, 2005).

We will now focus on the repressive modifications – histone lysine methylation (Martin and Zhang, 2005). The most studied are the methylation on H3/K9, H3/K27 and H4/K20. The H3/K9 methylation is a classical repressive modification found in heterochromatin and non-transcribed genes. However, recent results also found this modification in the body of actively transcribing genes slightly opposing the previously set role (Vakoc et al, 2005). Methylated H3/K27 is another example of a repressive modification. The location of this modification is more restricted being preferentially found on the inactive X chromosome in females. The last mentioned modification is restricted to the centromeric regions – to the pericentric heterochromatin to be more precise (Kourmouli et al, 2004). It is thus clear, that histone modifications can either serve as general purpose modifications (as in the case of H3/K9 methylation) or have very specific roles (the latter two).

On the other hand, histone acetylation is generally associated with active or transcriptionally permissive environment (Struhl, 1998). Both the histone H3 and H4 are the target of acetylation at various sites. The exact mechanism by which the histone acetylation causes higher transcription rate is unknown. It was supposed that highly acetylated histones are unable to bind DNA as strongly when compared to unmodified ones, thus loosening the nucleosome and leaving the DNA more accessible to transcription factors. However, this is most probably not the case and recent results contradict this hypothesis (Brower-Toland et al, 2005).

The above mentioned facts might give the impression that histone methylation is generally a repressive mark. However, this is not the case. Methylation of H3/K4, H3/K36 and H3/K79 is associated with active genes. Thus, in the case of histone methylation approximately the same number of activating as well as repressive marks does exist (Sims III et al, 2003).

The importance of covalent histone modifications lies in the fact that these can be recognised and bound by different proteins that can carry out executive functions or serve as docking sites for other factors affecting chromatin structure and activity. An example of such recognising protein is HP1. This protein recognises the methylated H3/K9 (trimethylated) by its chromodomain. Moreover, this protein is able to oligomerise by its chromoshadow domain, thus helping to form the

heterochromatin structure (Maison and Almouzni, 2004). A similar mechanism can be implicated also for other histone modification.

1.2.2. Enzymes affecting histone methylation and acetylation

There are a number of enzymes that methylate histones at different sites. These include G9a, Suv39h or Dot1 and others. These enzymes act in a site specific manner and are often a part of some chromatin modifying complexes. All of these proteins, except for the Dot1 family, exhibit the presence of a so-called SET domain (Dillon et al, 2005). This domain was originally identified in Drosophila Melanogaster proteins (Suppressor of variegation - Su(var)3-9; Enhancer of zeste - E(z) and Trithorax - Trx). For a long time, the histone methylation was considered to be irreversible (Bannister et al, 2002). The proposed mechanisms of methylation removal were for example either releasing the whole histone molecule from the nucleosome or histone tail clipping. However, recently a number of proteins capable of demethylating histones at different sites have been identified and the number is still growing. The readers are referred to the article by Shi (2007) for review. These proteins often contain JmjC domain. The first identified demethylase was the LSD1 – this protein apparently does not contain the above mentioned domain but instead requires Co-REST (chromatin-associated transcriptional repressor) for its function *in vivo* (for review see Klose and Zhang, 2007).

As for histone methylation there is a number of histone acetyltransferases (HATs) that are capable of transferring the acetyl groups to histones and other target molecules. An opposing group of enzymes are the histone deacetylases (HDACs) which in turn remove this mark. These are divided into several groups.

Histone acetylation can be found on lysine residues, thus the lysine residue can be either methylated or acetylated. The enzymes that carry out histone acetylation are histone acetyltransferases (HATs) and the reverse reaction is carried out by histone deacetylases (HDACs). Nuclear HATs can be divided into several groups (Gcn5/PCAF family, MYST – MOS, Ybf2/Sas3, Sas2 and Tip60 - family and p300/CBP family) but all use Acetyl-CoA as a source of acetyl groups (Roth et al, 2001).

HDACs are also divided into several groups – class I (HDAC1-3 and -8), class IIa (HDAC-4, -5, -7 and -9), class IIb (HDAC-6 and -10) and class III (also called Sirtuins) and class IV (HDAC-11). Classes I, II and IV show similar structural properties and require Zn ion as a cofactor (de Ruijter et al, 2003). Generally, both HATs and HDACs are parts of the ATP dependent remodelling complexes again facilitating the whole process. As mentioned earlier, histone acetylation is closely associated with activation of gene expression. On the other hand, deacetylated histones are associated with silenced genes. As in the case of other histone modifying enzymes, the dysregulation of these enzymes is linked to cancer and thus these proteins are considered as possible targets of the anticancer therapy.

1.2.3. Covalent histone modification during early mammalian embryogenesis

As mentioned above, the fertilizing sperm is soon after fusion with the oocyte transformed into a male pronucleus. The protamines are rapidly replaced by histones originating from the oocyte cytoplasm. On the contrary, the maternal chromatin is organised with the aid of histones throughout meiosis and these proteins are not released and replaced by any special forms of structural proteins. However, during the course of meiosis, these histones are differentially covalently modified. It has been shown that the GV (germinal vesicle) oocyte exhibits a high degree of histone acetylation on various residues as well as H3/K4 trimethylation – typical marks of active chromatin (or chromatin which is actively transcribed). However, several articles have shown that the transcriptional activity in GV stage oocytes is negligible and the residual activity can be ascribed to RNA Polymerase I that is responsible for rRNA production (Bouniol-Baly et al, 1999; Miyara et al, 2003). These modifications are most certainly not localized only to the perinucleolar ring – the site where the residual transcriptional activity can be found. Upon the re-entry into the cell cycle, the chromatin condenses and individual chromosomes are formed. Together with this condensation, the chromatin looses the acetylation marks and on the other hand gains repressive methylation marks such as methylation of H3/K9 (Fulka, 2007; Kim et al, 2003). After fertilization when the pronuclei form, the specific histone modifications are retained by the maternal chromatin. The paternal chromatin, on the other hand,

shows very dramatic changes. First of all, the paternal pronucleus exhibits the presence of highly acetylated histones (in comparison, the maternal chromatin shows a more gradual rise in histone acetylation; Adenot et al, 1997). Some authors speculate that this phenomenon can be linked to the fact that the paternal pronucleus is more transcriptionally active than the maternal one. This, of course, seems to be true. However, the higher transcriptional activity might be the effect and not the cause of the presence of highly acetylated histones. In the nineties, articles describing the mechanism of histone incorporation into nucleosomes have indicated that histones (especially H4) are incorporated as already acetylated (Sobel et al, 1995). Since histones are mostly absent from the paternal chromatin and need to be re-introduced into it, we can conclude that acetylated histones are readily incorporated into the paternal pronucleus. The situation is more complex for other modifications investigated and for example the methylation of H3/K4 shows a gradual and successive appearance with the monomethylated form being the first one that can be detected, followed by di- and still later on tri-methylated forms (Lepikhov and Walter, 2004). On the other hand, the maternal chromatin contains methylated histones and these must be either removed or demethylated prior to acetylating histones at the same residues. This fact could be the reason, why the maternal chromatin exhibits a much slower rate of histone acetylation.

1.3. Somatic Cell Nuclear Transfer

The first mammal generated by somatic cell nuclear transfer was born in 1996 – this was Dolly, the sheep (Wilmut et al, 1997). Since then, little progress has been made in this rather inefficient procedure, albeit many species have been successfully cloned to date. The overall efficiency generally ranges from 0-5%, even though in some species (such as cattle) the successful rate has been reported to reach about 20% (Meissner and Jaenisch, 2006). It is well known that the genome of a somatic cell does not change (with some exceptions) throughout the life of an organism. Thus, we can speculate that another level at which the regulation of gene expression takes place is the level of epigenetic regulation. Indeed, it has been documented that the somatic cell gains tissue specific epigenetic marks as differentiation steps occur (Shiota et al,

2002; Song et al, 2005). We can conclude that when a somatic cell is transferred into an enucleated oocyte (cytoplast) the epigenetic marks that have accumulated throughout the differentiation steps must be erased and new epigenetic marks must be introduced into the genome in order to achieve a successful development. Therefore we can expect a gross epigenetic remodelling after somatic cell nuclear transfer. This has been indeed described. However, we can expect many abnormalities and these were also observed during this process. For example, in SCNT embryos only a limited demethylation as well as precocious remethylation has been observed (Dean et al, 2001). Moreover, the overall chromatin distribution seems to resemble more the nuclear architecture of the donor cell and not the one of an early embryo. Especially large blocks of heterochromatin were reported to maintain the distribution that is typical for a somatic cell – in somatic cells the pericentric heterochromatin is organised into so called chromocenters that are not typically observed in early embryos in the form that is equivalent to the somatic ones (Dean et al, 2001; Probst et al, 2007).

Surprisingly, when the expression of imprinted genes was investigated, both normal and abnormal expression patterns were reported (Humpherys et al, 2001; Wakayama et al, 2006). Moreover, when embryonic stem cells derived from SCNT blastocysts were used for an analysis they were mostly comparable to *in vivo* derived control lines in terms of gene expression (Wakayama et al, 2006). This points to the possibility that most cloned embryos die after their transfer into recipient females and that the most probable cause is an abnormal function of the placenta that is critical in terms of the nutrition supply.

This fact is very important for the possible application of the SCNT technique in human medicine. It is assumed that this procedure might be used to generate so called patient specific embryonic stem cells. These cells could be later on used to treat many devastating diseases such as Parkinson disease without the danger of being rejected. In this scheme a somatic cell from a patient would be transferred into an enucleated oocyte and the resulting embryo would be allowed to develop up to the blastocyst stage. From this stage, embryonic stem cells could be potentially derived and transplanted back to the patient. As pointed out earlier, experiments comparing

SCNT embryonic stem cells with their control counterparts have shown that these cells are highly comparable (Wakayama et al, 2006). However, many problems still remain to be solved. For example, it has been shown that humans are highly refractory to the technique of SCNT and to date no SCNT produced embryos were generated. The only ESC line reported that came from South Korea was, in fact, shown to be a human parthenogenetic embryonic stem cell line (Kim et al, 2007).

Thus, it is clear that many problems remain associated with this technique. In our experiments, we have investigated mostly the epigenetic changes in nuclei after somatic cell nuclear transfer. The information about epigenetic events in early embryos is, however, still rather limited. In order to judge the reprogramming success or failure a good reference system must be used. From these reasons we have investigated epigenetic changes in normal as well as SCNT generated embryos. Therefore, we have first focused on DNA methylation as well as various histone modifications in normal early mammalian embryos.

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Yang JF, Yang SH, Beaujean N, Niu YY, He XC, Xie YH, Tang XH, Wang L, Zhou Q, Ji WZ, Epigenetic marks in cloned Rhesus monkey embryos: comparison with counterparts produced in vitro. Biol Reprod, 2007, 76: 36-42

2. Aims of the study

The general aim of this study was to characterize the remodelling and reprogramming activity of the oocytes and cytoplasts prepared from oocytes at different stages of maturation. Special attention was paid to epigenetic factors such as DNA and histone methylation, histone acetylation as well as localisation of specific protein factors involved in chromatin remodelling.

Specific aims were:

1. To establish a relevant reference system of covalent histone modifications that would allow us to follow the epigenetic processes associated with oocyte maturation.
2. Characterize, at least partially, the oocyte reprogramming activities (namely changes in covalent histone modifications, DNA methylation and other chromatin features) in nuclear transfer experiments.
3. To evaluate the effects of selected experimental procedures that are currently used for the creation of embryos on chromatin remodelling of parental genomes.
4. To evaluate the epigenetic processes during early embryonic development in different species (e.g. pig).
5. To develop new micromanipulation approaches and new techniques for the cryopreservation of mammalian oocyte nuclear (GV) material.

3. Comments and discussion on specific publications and unpublished results

3.1. Comments and discussion on specific publications

The below listed publications are not arranged chronologically but rather according to their relevance and are grouped as belonging to a given topic. Publications can be located in public databases under the author name Helena Fulka.

Fulka J, Fulka H, Slavik T, et al.
DNA methylation pattern in pig in vivo produced embryos
HISTOCHEMISTRY AND CELL BIOLOGY 126 (2): 213-217, 2006

Specific contribution to the article: pig embryo culture, embryo labelling, image analysis

The pattern as well as dynamics of DNA methylation was shown to be species specific, as mentioned. The first detailed reports about this epigenetic mark were published in the mouse. In this species, a rapid and very intensive demethylation was observed in the case of the paternal pronucleus. Thereafter, a more gradual demethylation is observed up to the morula stage and at the blastocyst stage, concomitant with the first differentiation events, two distinct cell populations can be identified. These are the trophectodermal (TE) lineage and cells of the ICM (inner cell mass) – both these lineages exhibit differential levels of DNA methylation with the cells of the ICM being hypermethylated compared to the TE cells. We have performed a similar analysis in *in vivo* produced pig embryos. We have taken the advantage of the low sample numbers when an antibody against 5-MeC is used. In this species, the paternal pronucleus becomes rapidly demethylated as in the mouse. On the other hand, we were unable to detect any gross changes in the level of DNA methylation thereafter. However, dynamic changes of DNA methylation were detected in blastocysts in association with the cell number – the ICM lineage becomes gradually hypermethylated while the TE lineage retains basically the same levels of DNA methylation when compared to the morula stage.

These results become important when judging for example the culture conditions or procedures used to generate embryos. Gioia et al (Reproduction, 2005,

130: 29-39) have shown for example that the demethylation of the paternal pronucleus is highly influenced by the oocyte quality (oocyte maturation conditions, respectively). While the *in vivo* matured oocytes were able to demethylate the paternal genome in most cases, this ability became compromised when *in vitro* matured oocytes were used. This fact implicates that a compromised gamete quality as well as inappropriate gamete handling can have devastating impact on the embryo development.

Fulka H, Fulka J, Jr.
No differences in the DNA methylation pattern in mouse zygotes produced in vivo, in vitro, or by intracytoplasmic sperm injection
FERTILITY AND STERILITY 86 (5): 1534-1536, 2006

Specific contribution to the article: in vitro fertilization, intracytoplasmic sperm injection, immunolabelling

The study of impact of gamete or embryo handling and culture conditions on the epigenetic dysregulation has become very important. As pointed out by several authors, the epigenetic dysregulation can dramatically influence the developmental potential of embryos. The dysregulation was shown to occur both at the level of imprinted genes and on the whole genome scale (Khosla et al, Biol Reprod, 2001, 64: 918-926; Shi and Haaf, Mol Reprod Dev, 2002, 63: 329-334). For example, Shi and Haaf (Mol Reprod Dev, 2002, 63: 329-334) have shown that an abnormal level of global methylation can be linked to the ability of embryos to reach the blastocyst stage. Maybe not so surprising was that certain epigenetic disorders occur with a higher frequency in children conceived by human assisted reproduction techniques (Allen and Reardon, BJOG, 2005, 112:1589-1594). In this case, it is evident that gametes with a compromised quality have to be used and this problem can not be surpassed easily. However, what can be influenced is the choice of techniques that will be used. For example in the case of ICSI (intracytoplasmic sperm injection) the sperm is directly injected into the oocyte cytoplasm thus surpassing many naturally occurring steps. Ramalho-Santos et al (Hum Reprod, 2000, 15: 2610-2620) have shown that in Rhesus monkey, the sperm head components are not removed as

rapidly when compared to IVF (in vitro fertilization). Thus, the remaining head structures can potentially interfere with proper protamine removal and possibly epigenetic remodelling. From these reasons we have analysed the impact of techniques widely used in human assisted reproduction on remodelling of the paternal genome in the mouse and found there were no differences between the individual methods. It should be noted that a direct extrapolation of these results to human medicine might be difficult to make. Even though, this field has undergone a marked expansion the mouse in vitro embryo production and culture are far more standardized.

Kren R, Fulka J, Fulka H
Cryopreservation of isolated mouse germinal vesicles
JOURNAL OF REPRODUCTION AND DEVELOPMENT 51 (2): 289-292, 2005

Specific contribution to the article: oocyte dissection, evacuated Zonae Pellucidae preparation, transfer of karyoplasts into evacuated Zona Pellucida

Fulka J, Jr., Fulka H, St John JC
Transmission of mitochondrial DNA disorders: Possibilities for the elimination of mutated mitochondria
CLONING AND STEM CELLS 9 (1): 47-50, 2007. Review

Fulka H, Fulka J, Jr.
The use of micromanipulation methods as a tool to prevention of transmission of mutated mitochondrial DNA
CURRENT TOPICS IN DEVELOPMENTAL BIOLOGY 77: 187-211, 2007. Review

The above listed publications are engaged in development of new techniques and their possible use in human medicine, endangered species or valuable livestock preservation. The use of micromanipulation techniques has become widely discussed method how to improve the oocyte or embryo quality or to eliminate mutated mtDNA. As in all cells, the oocyte is composed of a nuclear and a cytoplasmic component. Both these parts bare a certain amount of genetic material. It is clear that most of the genetic material is stored in the nucleus, however, in the cytoplasm

mitochondria contain their own genome. This DNA (mtDNA) is especially vulnerable to mutations and malfunction of mitochondria caused by these mutations can be clearly linked to several devastating diseases (Spinazzola and Zeviani, Biosci Rep, 2007, 27: 39-51). It is particularly interesting that the mtDNA is transferred to the offspring only through the maternal lineage (Shoubridge and Wai, Curr Top Dev Biol, 2007, 77: 87-111). At present, there is no method that could be potentially used to prevent the maternal transmission of mutated mtDNA. However, the micromanipulation techniques offer this possibility. It is, however, clear that the micromanipulation techniques are rather invasive and in turn can influence the epigenetic state of the transferred nucleus. Since the correct function of mitochondria requires absolutely the precise cooperation between the mitochondrial and nuclear genetic information, the epigenetic dysregulation of the nuclear content can negatively influence the mitochondrial function.

These facts are relevant not only to mitochondrial diseases transmission but also to the field of endangered species preservation. It is often speculated that interspecies nuclear transfer could be used for this task. However, here we are facing many problems with mitochondrial dysfunction and epigenetic dysregulation – these facts are considered as the major obstacles.

Fulka J, Jr., Fulka H

Somatic cell nuclear transfer (SCNT) in mammals: The cytoplast and its reprogramming activities
ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY 591: 93-102, 2007. Review

Fulka H
Changes in global histone acetylation pattern in somatic cell nuclei after their transfer into oocytes at different stages of maturation
MOLECULAR REPRODUCTION AND DEVELOPMENT,
DOI: 10.1002/mrd.20840

It is clear that DNA methylation and covalent histone modification are in close relationship. As pointed out, after fertilization the paternal pronucleus becomes

rapidly demethylated while the maternal one retains high levels of both DNA and histone methylation. As the sperm fuses with the oocyte, the protamines are replaced by histones originating from the oocyte cytoplasm. It has been reported that the paternal pronucleus exhibits high histone acetylation that appears rapidly after the protamine replacement and most repressive histone modifications are missing. On the other hand, the maternal pronucleus shows the presence of many repressive modification as well as some modifications associated with active chromatin.

When a somatic cell is transferred in to a cytoplast, some proteins associated with its chromatin are most probably rapidly replaced by proteins originating from the oocyte cytoplasm. One example is the linker histone – H1. It has been documented that the oocyte contains an oocyte specific form of this protein –H1foo (Tanaka et al, Biol Reprod, 2005, 72: 135-142). When a somatic cell is introduced in to the cytoplast, the somatic H1 is rapidly released and exchanged for H1foo (Teranishi et al, Dev Biol, 2004, 266: 76-86). In our work, we have focused on changes of covalent histone modification upon transfer of a somatic cell into cytoplasts generated from oocytes at different stages of maturation. We have used GV (germinal vesicle), MI (metaphase I), MII (metaphase II) and activated oocytes as the source of cytoplasts. As the somatic cell is transferred into a GV stage cytoplast, its nucleus swells but the nuclear membrane remains intact. The intact GV stage oocyte contains high levels of acetylated histones in its chromatin and also the trimethylated H3/K4 is present. The same pattern can be observed is interphase somatic cells. When a somatic cell is transferred to such cytoplast, there are no gross changes in the labelling pattern against these modifications. However, when a somatic cell is transferred into a metaphase (irrespectively whether MI or MII) cytoplast, the nuclear membrane is disassembled and the chromosomes condense. As the oocyte resumes the meiotic cell cycle, the chromosomes condense and the histones are rapidly deacetylated. The same situation is observed in the case of introduced somatic cell nucleus. The deacetylation of somatic cell chromatin is very rapid and occurs within 1h after transfer. A complete deacetylation can be detected after a prolonged culture. We have also investigated the changes in H3/K4 trimethylation after the transfer of a somatic cell into a metaphase cytoplast. The metaphase oocyte contains high levels of H3/K4

methylation in its chromatin even as there is no transcription and the chromosomes are highly condensed. Upon the transfer of a somatic cell into a metaphase cytoplast, no changes in the level of H3/K4 trimethylation can be observed in the somatic chromatin. This observation is not very surprising. However, after a prolonged culture interval, the situation changes and the somatic cell chromatin is, unlike the oocyte chromatin, demethylated. This fact was rather unexpected, since there are no changes in the level of this modification in the oocyte chromatin even after a prolonged interval. Thus, these results point to the fact that the chromatin originating from the somatic cell is different in certain respect when compared to the oocyte chromatin. Generally, it can be concluded that the somatic cell chromatin behaves after transfer neither like the maternal nor the paternal chromatin.

Fulka H, St John, JC, Fulka J, Hozak, P
Chromatin in early mammalian embryos: achieving the pluripotent state
DIFFERENTIATION, 2007. Review accepted for publication

In this review, we have focused mainly on epigenetic changes occurring during normal embryogenesis in different species. We have described namely the species-specific differences in patterns of both active and passive demethylation as well as remethylation at the blastocyst stage. Since the methylation changes are often linked with changes in covalent histone modifications, we have also discussed this topic. According to currently available literature, we have defined the normal epigenetic state in different species and compared this with abnormal development that occurs namely in somatic cell nuclear transfer embryos. These embryos exhibit often, in contrast to normal embryos, epigenetic dysregulation both at the level of DNA methylation, covalent histone modifications, as well as chromatin structure in general. For example, it has been shown that the somatic cell nuclear transfer generated embryos show the presence of large heterochromatin blocks that are typical for donor cells but not to their normal counterparts (Dean et al, PNAS, 2001, 98: 13734-13738). Also, imprinted genes are often dysregulated in these embryos. However, an interesting phenomenon was noted in the case of embryonic stem cells derived from these embryos. These cells, once stably in culture, are comparable to

lineages obtained from control embryos. Several types of analyses were performed including general gene expression, imprinted genes expression and methylation of these genes and no significant differences were noted (Wakayama et al, Stem Cells, 2006, 24: 2023-2033). Thus, these results indicate that the reprogramming failure occurs mostly in later periods of embryogenesis and the dysregulation might mostly affect the extraembryonic tissues. These results are highly promising from the point of view of so called therapeutic cloning as a mean to generate patient specific embryonic stem cells that could be used in human medicine to treat several devastating human diseases.

3.2. Unpublished results

Inability of the fully grown GV stage oocyte to transcriptionally reprogram the transferred transcribing nucleus

Authors: Helena Fulka, Josef Fulka, Jr. and Pavel Hozak

Abstract

During the process of somatic cell nuclear transfer cytoplasts from metaphase II oocytes are exclusively used. However, it is evident that certain reprogramming activities are present in oocytes even at earlier stages. The mammalian oocyte is a highly specialized cell that harbours many specific properties. One of these properties is a particularly large size when compared to somatic cells. As the oocyte enters the growth phase, its volume as well as the amount of material enlarges considerably. It is clear that the mammalian oocyte must possess the machinery to accomplish this incredible material accumulation. However, when the growth phase is completed the transcription ceases and the oocyte becomes transcriptionally inactive. In this study, we have used the model system of oocyte fusion (transcribing x non-transcribing GV stage oocytes) as a substitute for a somatic cell nuclear transfer schemes where the somatic cell is introduced into a cytoplast obtained from a GV stage oocyte. We wanted to determine if the fully grown GV stage oocyte could induce silencing in a transcriptionally active nucleus. In order to evaluate possible changes in the transcriptional properties after nuclear transfer, we investigated the mechanisms of the transcriptional silencing taking place when the oocyte reaches its full size as well as the fate of the components of the RNA Polymerase II (Pol II) transcriptional and splicing machinery. Here we show that while the RNA Polymerase II is degraded in fully grown GV stage oocytes and splicing proteins undergo significant rearrangement, the oocyte is unable to induce similar changes in transcriptionally active nuclei even after a prolonged culture interval.

Introduction

The mammalian oocyte is a highly specialized cell. First, soon after the primordial germ cells enter the genital ridge the oogonia enter meiosis and the oocytes are arrested in the prophase of the first meiotic division (Mehlmann, 2005). After the follicular recruitment, the oocytes enter a growth phase (Picton et al, 1998). During this time, the oocytes accumulate all the necessary material that will be used during early embryogenesis before the major embryonic genome activation takes place and the embryo becomes self-sufficient in basic cellular processes. Only when the oocyte reaches its full size it becomes competent to resume meiosis and thus reach the metaphase II at which the oocyte awaits the fertilizing sperm (Sorensen and Wassarman, 1976; Motlik et al, 1984). It has been shown that small oocytes are unable to re-enter meiosis, as indicated by absence of GVBD (germinal vesicle breakdown), when released from follicles (Motlik and Kubelka, 1990). Thus, it is evident that the accumulation of material during the oocyte growth phase is important for subsequent resumption of meiosis and its completion. It is well documented that the oocyte accumulates both RNA Polymerase I and RNA Polymerase II products (Picton et al, 1998). However, when the oocyte reaches its final size, the transcription ceases (Miyara et al, 2003; Bouniol-Baly et al, 1999). Although, the transcriptional shut down is almost absolute, residual transcriptional activity can still be detected mainly in the perinucleolar ring. Bouniol-Baly and colleagues (1999) used the inhibitor amanitin to dissect the activity of RNA Polymerase I and II and showed that this residual transcription can be attributed to RNA Polymerase I. The exact mechanism of the transcriptional shut down is, however, unclear. For these reasons, we have investigated the fate of the RNA Polymerase II in mouse oocytes before and after the oocytes reach its full size.

The transition to the fully grown state is also accompanied by changes in the oocyte chromatin distribution (De La Fuente, 2006). These states are termed as either "non-surrounding nucleolus" (NSN) or "surrounding nucleolus" (SN) distribution of DNA. This refers to either DNA being decondensed and not tightly associated with the nucleolar precursor body (NSN type) or condensed and clustered to the perinucleolar ring with few larger chromatin threads protruding towards the periphery

of the germinal vesicle (SN type; Debey et al, 1993). The chromatin redistribution is tightly associated with the intensity of transcription and changes of these two processes go hand in hand. While the NSN oocytes are highly transcriptionally active, as the chromatin condensation occurs and the oocytes transit to the SN type, the transcription gradually declines. In this respect, growing oocytes (NSN) exhibit the same properties as somatic cells, however, they are naturally much larger. In this study, we have taken the advantage of this fact and used the growing oocyte instead of a somatic cell facilitating the evaluation of results.

In our experiments, we have used mainly immunofluorescence with antibodies against RNA Polymerase II and in vitro transcription assay using BrUTP incorporation into nascent RNA. The anti-RNA Polymerase II antibodies available are specific for various epitopes of RNA Polymerase II and are highly dependent on the phosphorylation status. It is well known that the RNA Polymerase II CTD (C-terminal domain) exhibits different phosphorylation states according to the transcriptional cycle (Meinhart et al, 2005; Komarnitsky et al, 2000). The H14 antibody was described to recognise the initiation state of RNA Polymerase II, whilst the H5 antibody recognises mainly the elongating form of RNA Polymerase II. The CTD 4H8 antibody has been reported to recognise both the hyperphosphorylated as well as hypophosphorylated RNA Polymerase II according to the manufacturer (Covance), however, a certain doubt about the specificity of this antibody exists. Finally, the 8WG16 antibody recognises the unphosphorylated form only (Cho et al, 2001).

From our results, we conclude that the transcriptional silencing is caused mainly by degradation of the RNA Polymerase II in fully grown GV stage oocytes. This degradation is not, however, induced in the transferred nucleus concomitantly with the ongoing transcription in this nucleus. We also show that the splicing factors undergo a marked rearrangement during these processes.

Materials and methods

Unless stated otherwise, all chemicals have been purchased from Sigma, Czech Republic.

Mouse oocyte collection and fusion

ICR mice were kept under standard housing conditions with the 12h light cycle (light on 7:00-19:00, 22°C, 55% relative air humidity). The females were injected intraperitoneally 48h prior to oocyte collection with 5 I.U. PMSG – Pregnant Mare Serum Gonadotropin (Intervet International, Boxmeer, Holland, www.intervet.com). The females were sacrificed by cervical dislocation and ovaries were collected into the pre-warmed HTF-HEPES medium (Cambrex, Verviers, Belgium, www.cambrex.com) supplemented with BSA (bovine serum albumin, 4mg/ml). The oocytes were then cultured in MEM (minimal essential medium) supplemented with BSA (4 mg/ml), Na-pyruvate (0,2mM), gentamicin (50μg/ml) and dbcAMP (dibutyryl cyclic adenosine monophosphate, 150 μg/ml) in order to prevent the germinal vesicle breakdown (GVBD). For the oocyte fusion, we have sorted the oocytes according to their size. The zona pellucida was removed from individual oocytes by incubating them in Acid Tyrode´s solution. Thereafter, the oocytes were agglutinated in phytohemmagglutinin solution (200 μg/ml in PBS) and the fusion was induced by a double pulse (50 μs/ 50V, distance between electrodes 500 μm) in the isoosmolar:hypoosmolar buffer mixture (1:1; Eppendorf, Hamburg, Germany, www.eppendorf.com) using the Eppendorf Multiporator. After the fusion, the oocyte pairs were returned to the culture medium and incubated for either 3 or 20h and processed for immunofluorescence or in vitro transcription assay. The unfused oocyte pairs served as controls.

Immunofluorescence

Prior to immunofluorescence, the zona pellucida was removed also from some GV stage control oocytes as described. In the case of fusion pairs, this step was performed before the induction of fusion. After this, the samples were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. After fixation, the

samples were washed in PBS and permeabilized for 10 minutes in 0.2% Triton X-100 in PBS. Then, the samples were blocked in 1% BSA in PBS and incubated with one or two of the above mentioned antibodies. The antibodies used were: clone H5 recognising RNA Polymerase II phosphorylated on Ser2, H14 recognising the Ser5 phosphorylated form, 8WG16 recognising the unphosphorylated for of RNA Polymerase II and CTD4H8 recognising both hyper and hypophosphorylated form of RNA Polymerase II - all were purchased from Covance (www.crpinc.com, www.covance.com), the FK1 and FK2 clones recognising polyubiquitinylated and mono- and polyubiquitinylated proteins and anti-core of 20S proteasome were purchased from Biomol International (Exeter, UK, www.biomol.com), anti-SC35 were purchased from Sigma, anti-Sm (clone Y12) was obtained from Lab Vision Corporation (Fremont, CA, USA, www.labvision.com), anti-BrdU antibody was obtained from Roche (Prague, Czech Rep., www.roche-applied-science.com). After an overnight incubation, the antibodies were washed off and the samples were incubated with secondary goat antibodies conjugated with FITC and/or Texas Red for 2h at room temperature (Santa Cruz Biotechnology, Santa Cruz, CA, USA, www.scbt.com). After this incubation, the secondary antibodies were washed off and the samples were mounted in Vectashield mounting medium supplemented with DAPI (Vector Laboratories, Burlingame, CA, USA, www.vectorlabs.com) and evaluated under the Olympus IX71 fluorescence microscope. The images were captured with the use of ImagePro software (MediaCybernetics, Bethesda, MD, USA, www.mediacy.com).

In vitro transcription assay

The assay was essentially performed as described in Aoki et al (1997). Briefly, the oocytes were permeabilized in physiological buffer supplemented with 0.03% Triton X-100 and transferred to the transcription buffer (physiological buffer + nucleotides and BrUTP) for 15 minutes. After this incubation, the samples were washed in physiological buffer and fixed in 4% paraformaldehyde in physiological buffer). After fixation, the samples were subjected to immunofluorescence with anti-BrdU antibody.

SDS PAGE and Western Blotting

The oocytes were sorted according to their size into three groups – small (<40μm in diameter), intermediate (~60-70μm) and large (>80μm). Briefly, samples of equal number of oocytes were lysed in a Laemmli sample buffer (Bio-Rad Laboratories, Prague, Czech Republic, www.bio-rad.com) containing inhibitors of phosphatases (1mM NaF and Na Orthovanadate) as well as inhibitors of proteases (Complete Mini, Roche). The samples were boiled and loaded onto a 7.5% SDS PAGE gel. To test the properties of the CTD 4H8 antibody, the following commercial lysates were used: unstimulated A431 cells, Jurkat cells (Upstate, Lake Placid, NY, USA, www.millipore.com) – both 10μg of protein per lane. The proteins were transferred to a PVDF membrane (Bio-Rad Laboratories) and RNA Polymerase II was detected using the CTD 4H8 antibody (Covance). Secondary goat anti-mouse IgG conjugated to peroxidase (Santa Cruz Biotechnology) with ECL Advance substrates (Amersham Biosciences, www.amersham.com) was used to detect the primary antibody recognizing RNA Polymerase II.

Results

Localization of RNA Polymerase II and BrUTP incorporation in GV stage oocytes after fusion

In the first experiment, we wanted to investigate whether the fully grown oocyte can potentially induce transcriptional silencing in somatic cells nuclei when transferred to a GV stage oocyte. For these purposes, we have performed model fusion experiments between the fully grown GV and growing GV oocytes. As pointed out earlier, the growing oocyte is highly transcriptionally active and in this respect exhibits characteristics similar to somatic cells. The larger size of the nucleus when compared to somatic cells highly facilitates the analysis. After 3h post fusion, the nucleus of the small GV oocyte was stained strongly by the H14, CTD 4H8 and the 8WG16 antibody while the nucleus of the large GV oocyte was negative for the H14 labelling (Fig1A) and mostly negative for the latter two antibodies – however, the nucleus from fully grown GV oocyte was still positive when labelled by the CTD

4H8 and 8WG16 antibody. This labelling was, however, significantly weaker when compared to the nucleus of the small GV oocyte (Fig1B). The unfused oocyte pairs were used as a control - no differences in labelling pattern or intensity were observed between the fused and unfused samples. To address if this situation corresponds really to differential transcriptional activity of these two nuclei, BrUTP incorporation was assessed in the fused as well as unfused GV oocyte pairs. As expected, the nucleus originating from the growing GV stage oocyte incorporated BrUTP with strong intensity while its counterpart originating from the fully grown GV stage oocyte was essentially negative (Fig1C). These results showed that the 3h interval is not sufficient to induce silencing of an introduced transcriptionally active nucleus. As the next step, we have performed the same experiments, however, this time with the incubation interval of 20h. After this incubation period, essentially same results were obtained again indicating that even the 20h incubation of nuclei in the common cytoplasm is not sufficient for transcriptional silencing of the active nucleus. Again, the unfused oocyte pairs were taken as a control. No difference in the intensity of labelling between fused and unfused oocyte pairs was observed. The presence of the strong signal in the nucleus of a growing oocyte when stained by the H14 antibody might also indicates that dephosphorylation of the RNA Polymerase II CTD is not the mechanism that leads to transcriptional silencing in fully grown GV oocytes. Based on these results, we next wanted to investigate the fate of RNA Polymerase II and related proteins during oocyte growth.

The fate of RNA Polymerase II during the oocyte growth

The small oocytes show a decondensed chromatin (the "non-surrounding" type of chromatin distribution) as judged by DAPI labelling. In accordance with previously published results the transcriptional activity is very high in these oocytes as shown by high rate of BrUTP incorporation throughout the nucleus. This is also documented by the presence of a very strong signal after labelling with all the antibodies against RNA Polymerase II. Moreover, the antibodies against both initiating and elongating RNA Polymerase II - H5 and H14 antibodies - show a

homogeneous pattern of labelling with equal distribution throughout the whole germinal vesicle of growing oocytes (Fig2A).

However, as the oocytes undergo further growth the chromatin starts to condense and in the final phases forms a main perinucleolar ring with a few chromatin protrusions which eradiate from this ring towards the periphery. As this process takes place, the transcription is gradually being shut down. This can be shown by a decrease in the BrUTP incorporation and thus by the decrease in the intensity of labelling with anti-BrdU antibodies. This process is also characterized by gradual decline of the H14 antibody signal (Fig2A). The H5 antibody does no longer exhibit homogenous labelling pattern but instead the signal is aggregated into a large number of foci (Fig2B). The CTD 4H8 antibody as well as the 8WG16 antibody exhibits a homogenous pattern of labelling although somewhat weaker when compared to small oocytes (Fig2C). We assume that the decrease of the signal from anti-RNA Polymerase II antibodies might be caused by either an enlargement of the volume of germinal vesicle or by degradation of Polymerase II.

As the oocytes finally reach the "surrounding" type of chromatin distribution, the pattern of labelling with antibodies against RNA Polymerase II changes again. The H14 signal is totally absent, the 8WG16 and CTD 4H8 antibodies still show homogenous pattern of labelling although very weak. The biggest change occurs in the labelling pattern of the H5 antibody. The H5 antibody at this phase labels large proteins structures or bodies in the nucleoplasm. The number of these bodies typically ranger from 3 to 6 per germinal vesicle. These bodies are also typically found in the close vicinity of the nucleolar precursor body. Moreover, the BrUTP incorporation becomes restricted to the vicinity of the nucleolar precursor body and is very weak.

To investigate whether the decrease in the RNA Polymerase II signal is caused by the germinal vesicle volume enlargement or degradation of this protein we have performed a western blot. We decided to use the CTD4H8 antibody that, as indicated by the manufacturer (Covance), recognises both the phosphorylated and unphosphorylated form of RNA Polymerase II. However, as pointed out earlier, some doubts about the specificity of this antibody do exist. Thus, we have decided first to verify, if this antibody truly recognises both the hyper- and hypophosphorylated

forms of RNA Polymerase II. This analysis confirmed that this antibody recognises all phosphorylation forms of RNA Polymerase II as indicated in the datasheet for this antibody (Fig2D). Then we have decided to follow the protein levels of RNA Polymerase II in oocytes. The results show that the amount of RNA Polymerase II gradually decreases as the oocytes grow. In spite of evident decrease in the intensity of transcription and RNA Polymerase II degradation, the RNA Polymerase II is mostly found in the hyperphosphorylated form, as indicated by the upper position of the band on western blots, and thus most probably engaged in transcription (Fig2D).

To further evaluate the effects of incubation of the nucleus originating from the growing GV oocyte, we have detected the levels of RNA Polymerase II in fusion pairs after 3 and 20h interval. Unfused growing oocytes were used as standard to witch the fused oocytes samples were compared. The levels of this protein are comparable between all the probed groups. Thus, these results again strengthen the view that the fully grown oocyte can induce neither degradation nor dephosphorylation of the growing oocyte nucleus.

Localization and redistribution of nuclear speckles during the oocyte growth

As mentioned above, the H5 antibody stains large domains in fully grown mouse oocytes. However, the H5 antibody has been also reported to cross-react with phosphorylated factors of the splicing machinery. This fact might implicate that the observed pattern is due to the cross-reactivity of this antibody. On the other hand, in GV stage Xenopus oocytes various nuclear bodies have been isolated and indeed some of these bodies have been shown to contain RNA Polymerase II (Doyle et al, 2002). This in turn might implicate that the RNA Polymerase II might be sequestered and unable to reach the DNA where it could engage in transcription. Even though, our previous results speak strongly against this assumption we have decided to further verify the nature of the H5 domains. To assess the content of the bodies observed in mouse GV stage oocytes, we have labelled the GV stage oocytes with the 8WG16 antibody. Previously published results have shown that this antibody recognises the unphosphorylated RNA Polymerase II. Moreover, binding of this antibody is inhibited by phosphorylation of Ser2 (an epitope recognised by the H5 antibody

clone; Cho et al, 2001). Thus, if the observed bodies contained RNA Polymerase II phosphorylated on Ser2, the 8WG16 antibody signal should be excluded from these domains. When the samples were evaluated, a completely homogenous labelling was observed by 8WG16 antibody albeit this labelling was rather weak (Fig2C). No accumulation in or exclusion from any bodies was observed. This indicates that the phosphorylated RNA Polymerase II is most probably not present in the H5 positive bodies and the observed signal is indeed most probably caused by a cross-reactivity of the H5 antibody that was previously suggested.

Next, we wanted to know if these bodies might be the site of RNA Polymerase II degradation. For these experiments, we have used the antibodies against poly- and mono- and polyubiquitinylated proteins as well as against the core subunit of the 20S proteasome. The antibody against mono- and polyubiquitinylated proteins showed a homogenous signal throughout the germinal vesicle with no visible enrichment. The antibody detecting polyubiquitinylated proteins also showed a homogenous pattern throughout the germinal vesicle and even a slight exclusion in certain sites. These sites again markedly resemble the H5 domains. The anti-20S proteasome antibody showed a clear enrichment of the proteasome complex in the nucleus. The labelling pattern was homogenous without any enrichment in any part of the germinal vesicle (not shown). Thus, the H5 stained domains are not the site of RNA Polymerase II degradation.

Because of the suspected cross-reactivity of the H5 antibody to phosphorylated splicing factors we have performed double labelling of the H5 antibody with either anti-SC35 or anti-Sm antibodies. Both these antibodies are often used to localize the speckles, domains where splicing of mRNA takes place. The signal from the H5 antibody seems to closely correspond to the SC35 signal (Fig3A), confirming that this antibody exhibits high cross-reactivity to the phosphorylated splicing factors. The signal from H5 antibody does not, however, correspond absolutely with the signal when labelling by the anti-Sm antibody is used (Fig3B). While the SC35 is localised to few larger domains that appear homogenously granular, the anti-Sm antibody stains typically the perinucleolar ring as well as 1-2 round domains in the nucleoplasm and these domains clearly show the presence of

various vacuoles. Unfortunately, the anti-SC35 and anti-Sm double labelling cannot be performed due to the nature of both antibodies (mouse IgG). Thus we can not confirm that the bodies stained by these two antibodies are either identical or distinct.

HDAC1 localization in mouse GV stage oocytes

Several experiments using Trichostatin A, an inhibitor of histone deacetylases – HDACs, have shown that this drug can induce chromatin decondensation in fully grown mouse GV stage oocytes. De La Fuente and colleagues (2004) investigated the relationship between this drug, chromatin decondensation and transcription and reported that while the chromatin transits back to a highly decondensed form upon Trichostatin A treatment, no sign of BrUTP incorporation can be observed. Thus, the treatment of fully grown mouse oocytes by Trichostatin A causes the chromatin decondensation but is unable to restore transcription. Borsuk and Milik (2005) have proposed that the HDACs are responsible for transcriptional silencing when 8-cell stage nuclei are transferred into a GV stage cytoplasts. These authors have again used Trichostatin A to inhibit HDACs in the nuclear transfer produced cells. Therefore, we have decided to follow the localization of HDAC1 in mouse GV stage oocytes and in fusion pairs after 3 and 20h post incubation.

In the growing mouse oocytes, the HDAC1 is distributed homogenously throughout the whole germinal vesicle without any visible clustering or enrichment in any parts of the nucleus (Fig4A). However, as the oocyte chromatin starts to condense, the HDAC1 becomes localised to the regions of condensing chromatin. At the final phases of this process, the HDAC1 is localised in relatively large foci located on or in close vicinity of the oocyte chromatin. As expected, most of these HDAC1 sites are distributed around the nucleolar precursor body where most of the oocyte chromatin is sequestered (Fig4A). Again, respecting the typical chromatin distribution the HDAC1 foci are also located on the protruding chromatin fibres reaching the nuclear periphery.

After 3h post fusion, no marked changes in the HDAC1 distribution were observed. The nuclei of the small GV oocytes still exhibit fully homogenous labelling while the nuclei from the large GV stage oocytes show the focal type of HDAC1

distribution (Fig4B). Therefore, at this time no signs of chromatin condensation in the nuclei of small oocytes can be detected.

At 20h post incubation, no signs of abnormal HDAC1 activity were observed in the nuclei of transcribing oocytes (Fig4B). Thus, as in controls HDAC1 showed a diffused labelling pattern in the small GV oocyte nuclei without any indication of foci forming. On the other hand, in nuclei originating from fully grown oocytes HDAC1 exhibited focal type of labelling. These results indicate that HDAC activity is unlikely the primary mechanism of transcriptional silencing during oocyte growth.

Discussion

In our experiments we have investigated the transcriptional status of fully grown GV stage mouse oocytes as well as of the growing ones. It is well established that the GV stage oocyte must undergo an intensive growth phase during which the cell accumulates all the necessary material that will be subsequently used during the early development and is necessary for the resumption of meiosis (Sorensen and Wassarman, 1976; Mehlmann, 2005). This is accompanied by a very strong RNA Polymerase I and Polymerase II transcription that is indispensable for the production of ribosomes as well as specific mRNAs. These components will be utilised during the very early embryogenesis before the embryonic genome is activated and the embryo becomes self-sufficient.

It is now known that during the growth phase the GV oocyte undergoes substantial changes. One of these changes encompasses the altered distribution of chromatin and transcriptional silencing also occurs (Miyara et al, 2003; Bouniol-Baly et al, 1999). The chromatin is originally decondensed and occupying the whole nuclear volume, later on it transits to a highly condensed state and relocates to the close vicinity of the nucleolar precursor body with only a few protrusions towards the nuclear periphery. This process is also accompanied by a gradual transcriptional silencing.

The chromatin of the GV stage oocyte represents from the epigenetic point of view a certain paradox. Several investigators have assessed the presence of different histone modifications in GV stage oocyte chromatin. These results have shown that

the chromatin is highly acetylated at both H3 and H4 and also shows the presence of trimethylated H3/K4 (Fulka, 2007). Both acetylation and methylation at this specific residue have in somatic cells been linked to the transcriptionally active chromatin (Struhl, 1998). As pointed out, this is not the case in GV stage oocytes. De La Fuente and colleagues (2004) have investigated the effects of Trichostatin A, a widely used histone deacetylases (HDACs) inhibitor, on chromatin structure and transcription. In this study, the authors have shown that Trichostatin A can induce chromatin decondensation but it cannot induce the renewal of transcription. In our study, we have shown that most of the RNA Polymerase II is degraded by the time the oocytes reach their full size – in this respect the result described by De La Fuente and colleagues are not so surprising. Moreover, since the cytoplasm of a transcriptionally inactive oocyte cannot induce the same condition in a small oocyte, we can speculate that the transcriptional silencing is not an intrinsic property of the oocyte and that cumulus cells might play an important role in this process. This assumption is in agreement with the previously published results (De La Fuente and Eppig, 2001).

We have confirmed by BrUTP incorporation assay the previously published results. As the oocytes reach their final growth phase BrUTP is mostly not incorporated implicating that most transcription has ceased by this time. However, weak signal can be detected in the area around the nucleolar precursor body. This residual transcriptional activity was attributed to RNA Polymerase I (Bouniol-Baly et al., 1999). In certain cases, we have observed not only this type of labelling but also a very weak homogenous labelling by the anti-BrUTP antibody that obviously excluded certain structures in the nucleoplasm. Again, these structures strongly resembled SC35 positive speckles. However, again double labelling could not be performed due to the nature of the two antibodies. Even though, we can not confirm this, it is our opinion that even the fully grown GV stage oocytes are not as transcriptionally inactive as previously thought. Moreover, the weak but still clear labelling of the germinal vesicles by both 8WG16 and CTD 4H8 antibodies indicates that residual RNA Polymerase II is present in oocytes.

We have also investigated the relationship between nuclear speckles, RNA Polymerase II and the site of its degradation. Initially, our hypothesis was that the

speckles might represent a storage sites for RNA Polymerase II during the time of transcriptional repression. In this case, the RNA Polymerase II would be sequestered and prevented from reaching the target DNA. This, however, is most probably not the case since most of the RNA Polymerase II is degraded during transcriptional repression and the remaining RNA Polymerase II is not enriched in these structures. Our results are in agreement with previous studies, even though we do not agree, at least in our model, that the speckles contain a Ser2 phosphorylated form of RNA Polymerase II (Xie et al, 2006). However, the speckles still might serve as the storage site for other proteins involved in transcription. Sun et al. (2007) have localized many of transcription factors as well as RNA Polymerase II in mouse GV stage oocytes and not an insignificant number of factors of the transcriptional machinery exhibited the same pattern as the anti-SC35 antibody. These authors particularly highlight the role of physical separation of chromatin and general transcription factors as well as transcriptional regulators. Another protein that was found in speckles was STAT1 (Truchet et al, 2004). Moreover, when Swiech et al. (2007) localised the MCM proteins in mouse oocytes the described pattern closely resembles the localisation of speckles. If this fact is just a coincidence or the precise localization of these proteins represents a regulatory mechanism remains to be determined. Generally, these data altogether indicate that probably multiple modes of regulation exist to ensure the transcriptional silencing in the fully grown GV stage oocyte.

From our experiments it is quite clear that the H5 antibody cannot be taken as a reliable indicator of transcriptional activity in GV stage oocytes. Labelling by this antibody becomes relevant in this respect again after fertilization when the nuclei are negative up to the late 2-cell stage when the signal suddenly appears (although, it should be noted that a very weak signal can be observed in the male pronucleus in zygotes, unpublished observations). We suggest that in this system, the H14 antibody is a better choice when investigating the activity of RNA Polymerase II.

The original idea behind our experiments was to evaluate the properties of GV stage oocytes as the source of cytoplasts in nuclear transfer experiments. In these experiments, cytoplasts from metaphase II oocytes are almost exclusively used. During the period of incubation of the somatic cell nucleus in the oocyte cytoplasm,

large reprogramming steps must occur in order to achieve successful development of such reconstructed embryos. Our experiments indicate that GV stage oocytes might not be a good source of cytoplasm for somatic cell nuclear transfer experiments. The fact that the GV cytoplasm is not able to terminate the transcriptional program of the newly introduces nucleus strongly speaks against the use of such cytoplast. On the other hand, when metaphase II cytoplasts are used, the donor chromosomes undergo premature chromosome condensation and the transcription terminates rather abruptly. It is highly possible that this condensation imposes repressive marks on the introduced chromatin which might lead not only to reprogramming of the somatic cell nucleus but also to a compromised developmental potential. In our previous work, we propose that the oocyte reprogramming activities can have both beneficial as well as negative effects on the somatic cell chromatin (Fulka, 2007). Moreover, the use of certain drugs (such as Trichostatin A) has shown to be beneficial for the outcome of somatic cell nuclear transfer. It is possible that a sequential scheme of somatic nuclear transfer where the transfer to the GV stage cytoplasm is an intermediate step might improve the outcome of the nuclear transfer technique.

The role of epigenetic mechanisms in transcriptional silencing was proposed by Borsuk and Milik (2005). These authors observe the silencing of transcription before approximately 30h post fusion in transferred 8-cell stage blastomere. Based on their results these authors conclude that HDAC activity is responsible for this process. We have followed the localization of HDAC1 in oocytes during their growth as well as after fusion. At 3h post fusion, no abnormal activity of HDAC1 in nuclei originating from small and transcriptionally active nuclei could be detected. Moreover, the localization of HDAC1 even after 20h post fusion showed again no abnormal activity of this enzyme in the nuclei originating from small transcribing oocytes. This indicates that the activity of this protein is unlikely as the primary cause of transcriptional silencing in this system.

Finally, our results indicate that even after a very careful selection based on the oocyte size, approximately 10-20% of oocytes exhibit a less advanced localization pattern of components of the transcriptional machinery. This implies a certain asynchrony in the isolated oocyte population. This might in turn be linked to different

properties of oocytes and this fact might be crucial not only to the technique of SCNT in respect to the cytoplast quality but also in human medicine, especially in human assisted reproduction. Thus, even after a very careful selection of oocytes, the cytoplasts prepared from them might not be equivalent in their properties and ability to support further development.

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Figures

Localisation of RNA Polymerase II and BrUTP incorporation in fusion pairs:

Fig1A: Left – Labelling of oocyte fusion pairs with the H14 antibody recognising the phosphorylated form of RNA Polymerase II. Notice the presence of the elongating form of RNA Polymerase II in both fused (top) and unfused (control, bottom) oocyte pairs. The labelling pattern was similar at 3h post fusion as well as 20h post fusion.
Right – Parallel labelling by DAPI.

Fig1B: Left – The presence of unphosphorylated RNA Polymerase II in fusion pairs. This labelling shows the presence of RNA Polymerase II in all nuclei of both fused as well as unfused oocyte pairs. Again, the labelling pattern was similar for both 3h and 20h intervals post fusion.
Right – DAPI staining.

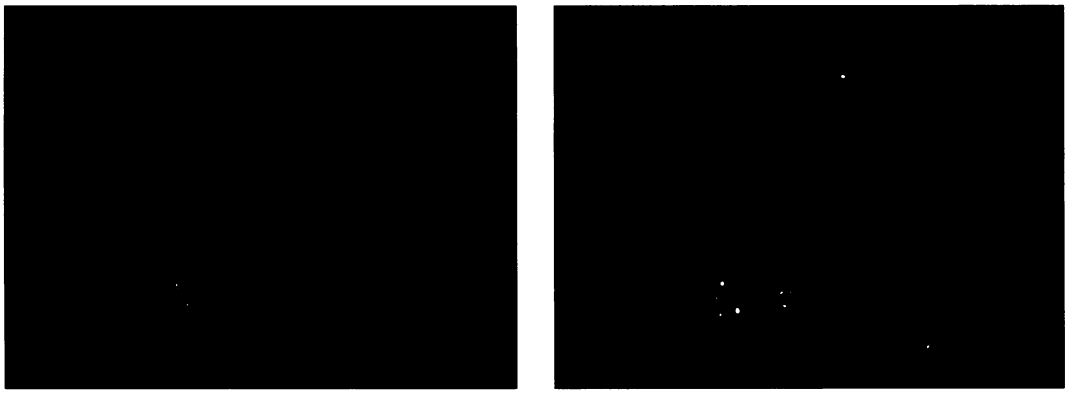
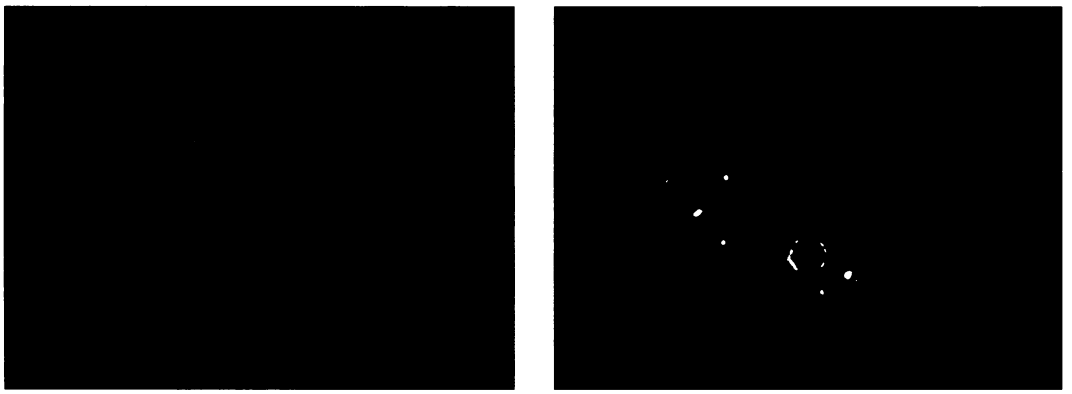


Fig1C: Left – Labelling of nascent transcripts by BrUTP incorporation in oocyte fusion pairs. Notice the relatively high transcription activity in the nucleus of the growing oocyte and absence of this activity in the nucleus of the fully grown oocyte at 3h post fusion. In the case of the 20h interval, the pattern of nascent transcript labelling was rather similar.
Right – Parallel DAPI staining.



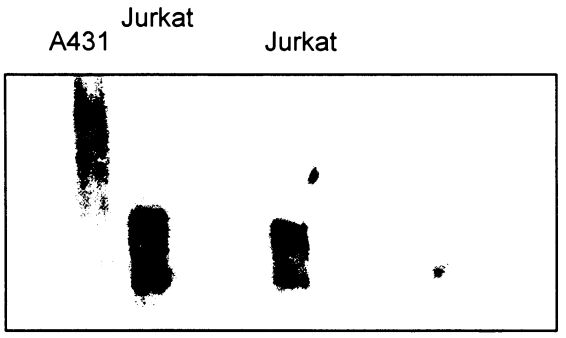
The fate of RNA Polymerase II during the oocyte growth:

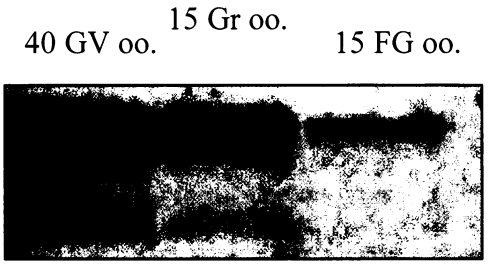
Fig2A: Left – The localization of the elongating form of RNA Polymerase II during oocyte growth. Notice the decline in labelling in the fully grown oocyte nucleus (left).
Right – DAPI staining.

Fig2B: Left – The labelling pattern of the antibody recognising an initiating form of RNA Polymerase II. Notice the change in the labelling pattern that accompanies the chromatin condensation occurring during oocyte growth. Here, we are showing an intermediate and advanced labelling pattern. The sharply localised signal is in this case caused by a cross-reactivity of this antibody - most probably to phosphorylated splicing proteins. For these reasons, we have decided not to use this antibody in further studies.
Right - DAPI labelling.

Fig2C: Left – The localization of unphosphorylated RNA Polymerase II during oocyte growth. During the process of oocyte growth, the signal decreases. Notice the decrease in the labelling intensity together with a highly condensed chromatin in the lower oocyte.
Right – DAPI labelling.

Fig2D: Left – The characterisation of the labelling pattern of the CTD4H8 antibody. As indicated by the manufacturer, this antibody recognises both the hyper- as well as hypophosphorylated form of RNA Polymerase II. The samples loaded were unstimulated A431 cells and Jurkat cells – both available as commercial lysates from Upstate.
Right - A western blot analysis of the protein levels of RNA Polymerase II during oocyte growth. The loaded samples were: 40 growing GV stage oocytes, 15 growing GV stage oocytes and 15 fully grown GV oocytes. Notice especially the decrease in the amount of RNA Polymerase II in fully grown GV stage oocytes. Also, the position of the bands in the first sample indicates that this protein is mostly present in the hyperphosphorylated form and thus, with a high probability, actively engaged in transcription.





Localisation and redistribution of nuclear speckles during oocyte growth:

Fig3A: Left – Oocytes labelled by the anti-SC35 antibody. Notice the remarkable similarity between this labelling and labelling by the H5 antibody. These results confirm the cross-reactivity of the H5 antibody to phosphorylated splicing proteins, thus making this antibody inappropriate for the study of RNA Polymerase II activity in this system.
Right – DAPI labelling.

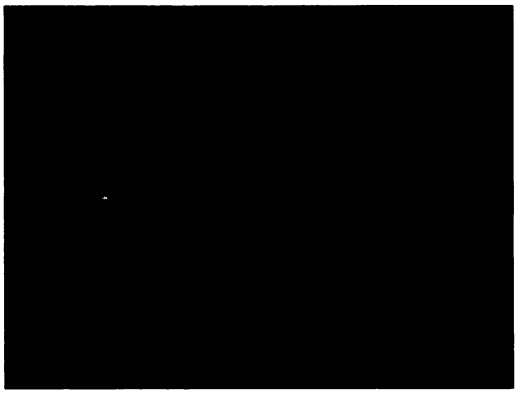
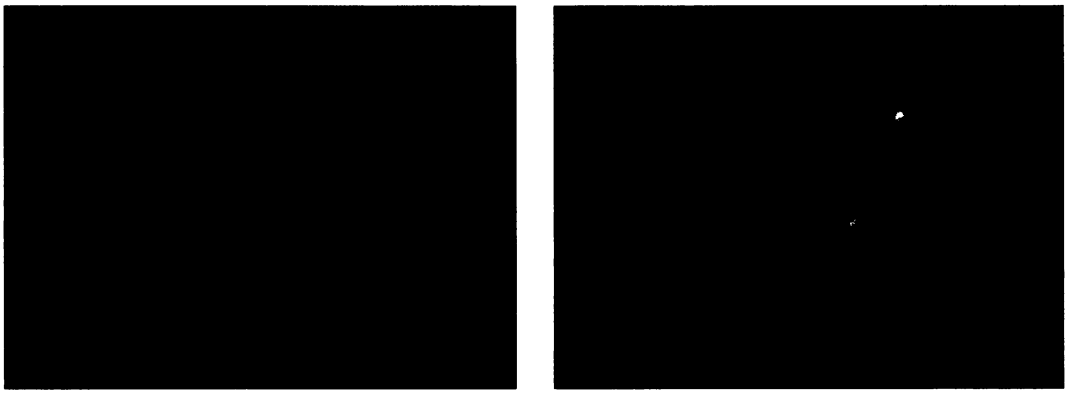


Fig3B: Left – Labelling of oocytes with the anti-Sm antibody. Although both the anti-SC35 and anti-Sm antibodies are often used as a marker of the speckles domain interchangeably, these antibodies show a different pattern in mouse oocytes.
Right – Parallel DAPI labelling.



HDAC1 localization in mouse GV stage oocytes:

Fig4A: Left – Localization of HDAC1 (Histone deacetylase 1) in mouse oocytes. While in the growing oocyte nucleus, the HDAC1 is distributed homogenously (left), in the nucleus of a fully grown oocyte (right) the distribution changes and most of HDAC1 is found in the close vicinity of the nucleolar precursor body (NPB).
Right – Parallel DAPI labelling.

Fig4B: Left – The localization of HDAC1 in fused oocytes pairs. No marked difference in the labelling pattern can be observed between control samples, fused oocytes 3h post fusion and 20h post fusion. This indicates that the HDAC1 activity is most probably not the cause of transcriptional silencing in oocytes.
Right – DAPI labelling.

4. Conclusions and perspectives

During the last two decades, human assisted reproductive technologies (ART) as well as some new experimental procedures (e.g. somatic cell nuclear transfer – SCNT or so called therapeutic cloning) have become widely used and gained much attention. These techniques hold a great promise in treating and preventing many devastating diseases, treating of certain forms of infertility in humans, as well as preserving of valuable livestock or endangered species. We have focused mainly on the field of reprogramming failure that is manifested very often in SCNT (somatic cell nuclear transfer) embryos and also on development of new approaches that could be potentially used in human medicine as well as in other fields (oocyte nuclear material storage and new micromanipulation approaches). In order to at least partly characterise the eventual negative impacts of these techniques we have specifically focused on epigenetic regulation or dysregulation during the early mammalian embryogenesis.

Epigenetics play a very important role during normal preimplantation development and aberrant epigenetic regulation has a negative impact on embryo development and even later on in adult life. It has been shown that this naturally represents a serious potential risk in human assisted reproduction. Thus, we have demonstrated, with the use of new experimental approaches, that many problems persist – and these are both technical as well as biological (e.g. reprogramming failure during SCNT); moreover certain ethical aspects cannot be ruled out (e.g. oocyte donation and embryo destruction in therapeutic cloning). These issues are not easy to deal with and no clear answers can be given. However, the growing interest in these techniques is a clear signal that the technical and ethical difficulties are fully outweighed by the possible beneficial outcomes.

It was not the intention of this thesis to either definitely answer the ethical issues or give an exhausting description of possible technical obstacles that can be encountered during the use of some specialised techniques of embryo production. That is not possible. In the specific publications, we have touched both the above-

mentioned topics. Generally, the presented publications can be divided into two main groups: characterisation of epigenetic oocyte reprogramming activities and development of new approaches that could be potentially used in endangered species or valuable livestock preservation. The second topic is also very close to human medicine.

During the somatic cell nuclear transfer, the somatic cell chromatin is introduced into a foreign cytoplasmic environment (so called cytoplast). The cytoplasts can be potentially prepared from oocytes at different stages of maturation. We have explored the reprogramming activity of different cytoplasts.

We have shown that the germinal vesicle cytoplasts or cytoplasts prepared from zygotes are unable to reprogram efficiently the introduced somatic cell chromatin. This includes reprogramming both epigenetic as well as transcriptional. The absence of epigenetic reprogramming was assessed by immunofluorescent detection of different covalently modified histones. On the other hand, the metaphase cytoplasts (that are currently almost exclusively used in somatic cell nuclear transfer) are able to induce the reprogramming changes in the chromatin of a somatic cell, however, these changes are both beneficial as well as negative. Thus, these results indicate that the outcome of somatic cell nuclear transfer can be viewed as rather stochastic.

Generally, the reprogramming activities and events occurring after the transfer of the somatic cell nucleus are poorly characterised and not well understood. Therefore, we have also investigated the possible transcriptional reprogramming potential of GV stage oocyte. It is well documented, that the fully grown GV stage oocyte is transcriptionally inactive, and thus we wanted to know whether it will be able to induce similar changes in an introduced nucleus that is transcriptionally active. Theoretically, a slow and sequential reprogramming might be beneficial for the nuclear transfer outcome. Moreover, the properties of the GV stage cytoplast are important for the characterisation of the reprogramming activities of the sequential nuclear transfer schemes.

The use of micromanipulation and other techniques opens a very promising new era of embryo production. This includes not only the already mentioned somatic cell nuclear transfer but also for example exchange of nuclear material between oocytes,

nuclear material cryopreservation or cytoplasm donation. Apart from somatic cell nuclear transfer, we have also adapted and modified the method of oocyte nuclear material cryopreservation thus, surpassing the need of whole oocyte freezing that can be highly problematic. As mentioned above, we can expect the use of these techniques mainly in endangered species preservation but certain methods are experimentally used also in human medicine. However, the risks are not yet fully characterised and this is beginning to prove to be more important than previously thought. The precise coordination of events between the nuclear and cytoplasmic compartment of the cell is absolutely essential and we can speculate that this interaction might also influence some epigenetic events. The oocytes and embryos are no exception.

Thus, the new biotechniques are on one hand very promising but on the other, great care should be taken to eliminate the possible risks before these techniques are introduced into for example human medicine and are routinely used. Moreover, some recent studies have also indicated a higher incidence of certain epigenetic syndromes in children conceived by intracytoplasmic sperm injection. Therefore, the safety issues should under no circumstances be underestimated.