H3S10P, Phosphorylation at Ser10 in Mouse Preimplantation Embryos

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H3S10P, PHOSPHORYLATION DE L'HISTONE H3 SUR LA SÉRINE 10 DANS L'EMBRYON PRÉIMPLANTATOIRE DE SOURIS

Résumé

L'hétérochromatine péricentromérique semble jouer un rôle dans la régulation de l'expression génique et par conséquent dans le potentiel de développement des embryons. Nous avons fait l'hypothèse qu'une marque épigénétique, H3S10P, pourrait être un nouveau marqueur permettant le suivi des régions péricentromériques dans l'embryon préimplantatoire de souris.

Par des techniques d'immunofluorescence et d'immuno-FISH couplées à de la microscopie en haute résolution, nous avons montré que la distribution de H3S10P dans les embryons de souris est différente de celle observée dans les cellules somatiques. Durant les stades 1 à 4-cellules, H3S10P est détectée en interphase autour des précurseurs des nucléoles (NPB), où elle colocalise avec les sondes ADN reconnaissant l'hétérochromatine péricentromérique, puis marque les bras chromosomiques sur toute la durée des phases de mitose. Après le stade 4-cellules, la distribution de H3S10P redevient similaire à ce qui est connu dans les cellules somatiques, avec un marquage au niveau des chromocentres seulement en fin d'interphase et sur les chromosomes mitotiques seulement jusqu'à la télophase. Cette cinétique particulière observée semble liée à l'absence de la kinase Aurora B aux stades les plus précoces.

Nous avons également comparé la localisation de H3S10P avec celle d'autres marqueurs associés à l'hétérochromatine péricentromérique comme H3K9me3, HP1 β et la double modification H3K9me3S10P et en avons conclu que H3S10P est un meilleur marqueur pour l'hétérochromatine péricentromérique des deux génomes parentaux.

Enfin, comme les embryons clonés obtenus par transfert nucléaire à partir de cellules somatiques (SCNT) montrent une redistribution anormale de l'hétérochromatine péricentromérique ainsi qu'un développement altéré, nous avons utilisé H3S10P pour détecter les remaniements de l'hétérochromatine après SCNT. Nos résultats montrent que, contrairement aux autres marqueurs, H3S10P n'est présente que sur la portion de l'hétérochromatine qui est correctement remaniée, tandis que l'hétérochromatine incorrectement reprogrammée conserve la signature épigénétique de la cellule donneuse.

Mots-clés: Embryon, Développement, Reprogrammation, Chromatine, Histone, Epigénétique

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Summary

Pericentromeric heterochromatin appears to be involved with gene regulation and therefore with the developmental potential of embryos. We hypothesized that an epigenetic modification, H3S10P, could be a new marker to follow pericentromeric heterochromatin in preimplantation mouse embryos.

Using immunofluorescence, immunoFISH and high resolution microscopy, we observed that H3S10P shows a different distribution pattern in mouse embryos than in somatic cells. It is detected early in interphase around the Nucleolar-Precursor Bodies from 1- to 4-cell, in co-localization with the DNA probes for pericentromeric heterochromatin, and is seen in the chromosome arms throughout mitosis. In fact, H3S10P shows a similar kinetic as seen in somatic cells only after the 4-cell stage: being solely observed in the chromocenters during late interphase and on the mitotic chromosomes until telophase. This distribution seems related to the absence of Aurora B kinase in the earlier stages.

We have also compared H3S10P to other related pericentromeric heterochromatin markers such as H3K9me3, HP1β and the double modification, H3K9me3S10P, and concluded that H3S10P is a better marker for pericentromeric heterochromatin of both parental origins.

Finally, as cloned embryos often show abnormal pericentromeric heterochromatin remodelling and impaired development after Somatic Cell Nuclear Transfer (SCNT), H3S10P was used to track down heterochromatin reprogramming after SCNT. Our results show that H3S10P underlines only the portion of heterochromatin which is remodelled when compared with the other related markers and that the unremodelled portion maintains the epigenetic signature of the donor cell.

Keywords: Embryo, Development, Reprogramming, Chromatin, Histone, Epigenetic

Acknowledgements

It has been eleven years away from home. Eleven whole years of pursuing a dream...

Everything began at high school which is where I had my first contact with Embryology. **Prof Euripides** from the Federal University of Paraiba State came to my school to give us a taste of what Embryology was all about. To be frank, it was love at first sight. I was and still am infatuated by gametes, embryos, embryonic development which to me is like being infatuated with LIFE itself.

After this initial flavour, I chose to study Biological Sciences and was lucky to have the chance to study at the finest university in my city. To my delight, my lecturer in Embryology was Prof Euripides. I felt that was verification that I was following the right path, even though unfortunately I did not have the chance to study or work on Embryology at my university. Instead I studied and worked on Head and Neck cancer looking at the p53. Nevertheless, during 2 ½ years I learnt much about research and I would like to thank my fantastic supervisor, **Dr Arnaldo**, for being such a great example of a scientist.

It was prior to my final semester at university that I met **Prof Henrique Gil**, someone who also encouraged me to follow my dream. It was because of him that I ended up meeting a "angel"; someone who opened the "doors to research and clinical embryology". It was **Dr David Keefe** whom I met in Salvador (Bahia, Brazil) during a Human Reproduction conference; one Prof Henrique Gil strongly suggested that I attended. I quickly finished my last semester at university and travelled all the way to Providence in the USA to be taught Clinical Embryology under Dr Keefe's supervision.

During the year I spent working as a volunteer at the Women and Infant's IVF Clinic, I also had the chance to work in a research lab with a professor from Brown University, **Dr John Pepperell**. Dr John taught me everything I know about mouse embryology. All this experience gave me the opportunity of going further with my studies. After learning all about the practical side, I then wanted to learn more about the theory. I was ready to do a Masters degree.

It was during 2001, while I was still in the USA, that I heard of the Masters degree in Clinical Embryology at Monash University in Melbourne, Australia, the only Masters degree in this field around the world at that time. I therefore applied for a place in this Masters and felt blessed to be accepted.

I arrived in Australia in 2002 and had to do an introductory course about the Australian teaching system. During this period I worked as a volunteer at Monash Institute of Reproduction and Development, the same place where I would eventually do my Masters Degree. For 7 months I worked under the supervision of **Dr Gabriel Sanchez-Partida**, a Prof who also helped me to pursue my dream and would eventually be my lecturer during my masters.

After finishing my Masters degree with "Distinction", I was hired to work as an Embryologist/ Research Assistant at the Monash Institute of Medical Research. I loved working at Monash Institute but I felt that after few years of working, I was ready to go further with my career, I was prepared to do a PhD.

It was in 2007 that I started seeking a PhD position. I decided to come to the ESHRE in Lyon, France, and take the opportunity to visit different labs in Europe. This was also when I decided to visit **Dr Nathalie Beaujean** at INRA Jouy-en-Josas, even though I knew I would not be eligible for the PhD position she was offering due to

European regulations. However, while chatting to me during my quick visit to Jouy-en-Josas, Dr Nathalie asked me if I was still interested in the PhD position. She explained that there was a misunderstanding with the interpretation of the European rules and that I would be suitable to take the PhD position if I wanted. And voilà! I moved to France 1 ½ months later.

It is true that I changed my field of expertise; however I am really glad I did. During these 4 years I learnt a great deal about chromatin and epigenetics. It was difficult during the first two years but with support I managed it. Anyway, it was like discovering a "new world": chromatin biology, nuclear organization, epigenetics, cloning and the techniques which allowed me to visualize the little proteins and DNA sequences, plus the opportunity to learn about microscopy and the various fluorochromes.

These 4 years were very valuable. Through the wonderful European project I was part of I had the chance to visit many labs in Europe and overseas, and had the opportunity to learn different techniques. I have to say that Europe provides some of the best prospects for scientists around the world to pursuit their careers and dreams. I sincerely hope Europe can continue to offer such a wonderful opportunity to young scientists and that European research can get stronger and stronger.

I have to confess I faced many problems in France. Even though life was not easy, my scientific vocation was not disturbed as I was lucky enough to be part of a group containing such caring people. I have to thank everybody from my group for the support, help and especially patience, for making me feel welcomed and for the effort everybody made to communicate with me in English. It was and still is very much appreciated. I owe you these 4 years of learning and progress on my career.

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I would like to dedicate this work to my family in Brazil: **Alvaro, Gisleine, Monika, Netto** and **Lucas**; I would have not been able to pursue this dream without your support and love. Also to my family in Australia, **John, Maryanne and Tim Mason**, for always being so positive and encouraging. Thank you all.

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Merci beaucoup à tous,

Karlla Ribeiro-Mason

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Abbreviations

BSA Bovine serum albumin

CBX1 Chromobox Homolog 1

CENP- A Centromeric protein A

CPC Chromosome passenger complex

CpG shorthand for "C-phosphate-G", i.e. when cytosine and guanine are separated by only one phosphate

DAPI 4'-6-Diamidino-2-phenylindole

DNA Deoxyribonucleic acid

DNMTs DNA methyltransferases

Fab Antigen binding fragment

FISH Fluorescence in situ hybridization

GV Germinal vesicle

GVBD Germinal vesicle breakdown

HATs Histone methyltransferases

ICM Inner cell mass

ICSI Intra cytoplasmic sperm injection

IEGs Immediate early genes

INCENP Inner centromeric protein

IVF In vitro fertilization

MAP Mitogen-activated protein

MS3 Mouse satellite 3

MS4 Mouse satellite 4

MSK1 Mitogen- and stress-activated protein kinase 1

MSK2 Mitogen- and stress-activated protein kinase 2

MI Meiosis I

MII Meiosis II

NIMA Never in Mitosis gene A

NPBs Nucleolar precursor bodies

NSN Non-surrounded nucleolus

N-Tail Amino-terminal tail

PBS Phosphate-buffered saline

p.c. Post-coitum

PCC Premature chromosome condensation

PFA Paraformaldehyde

PMSG Pregnant mare's serum gonadotropin

PTMs Post-translational modifications

PPTs Phosphatases

PP1 Phosphatase 1

PP2A Phosphatase 2A

RNA Ribonucleic acid

RT Room temperature

SCNT Somatic cell nuclear transfer

SDS Sodium dodecyl sulphate

SN Surrounded nucleolus

SUV39H Suppressor of variegation position 3-9 homolog

TCM199 Tissue culture medium-199

TE Trophectoderm

TSA Trichostatin A

VRK1 Vaccinia related kinase 1

ZGA Zygote genome activation

Chapter One : Introduction

1.1 Epigenetics Overview

For many years scientists around the world assumed they knew about everything concerning genetics. They believed that they could understand any existing phenotype based on the knowledge they had acquired about genetics in past decades. However, around the 80's another topic emerged which attracted their attention and led them to realise the sole DNA sequence would not determine a phenotype, as had previously been anticipated. This fascinating new field was more intriguing and mesmerizing than the former one and was named epigenetics.

The term epigenetics – literally "above genetics" – was coined by the developmental biologist Conrad Waddington in the 1940s to describe 'the interactions of genes with their environment which bring the phenotype into being' (Tost, 2008). Nowadays, epigenetics refers to the study of heritable changes in the genome function that occur without alterations to the DNA sequence (Riggs and Porter, 1996). In fact, epigenetics studies the additional layers of information on top of the bare genomic sequence that dramatically extend the information potential of the genetic code (Tost, 2008).

Epigenetics is now a rapidly advancing field of biological and medical research. It is now clear that epigenetic changes play a key role in disease as well as in normal development. Determining what components are affected at a molecular level, and how alterations in these components can cause disease and disturb normal development, is a major challenge for future studies.

1.2 Introduction on Chromatin

The higher-order nucleoprotein complex, the chromatin, is formed by the association of proteins called histones and DNA. Histone proteins directly interact with DNA to form the fundamental unit of the chromatin, the nucleosome, which is in turn an octamer formed by two copies of core histones, H3 and H4, and two dimers of H2A-H2B enclosed in 146 bp of DNA (Kornberg and Thomas, 1974; Luger et al., 1997; Dormann et al., 2006). It is through these entities, the nucleosomes, that the chromatin structure is modulated, leading to either compaction or relaxation of the DNA fibre, achieving a certain organization, leading to either gene expression or silencing. The most accepted idea is that the degree of compaction of the chromatin fibre varies locally according to the need for access: it is less compact in regions undergoing transcription and replication which needs a local and transient decondensation,

whereas it is more compact in transcriptionally silent regions and during mitosis, when dense packing is required for accurate segregation of DNA (Sauvé et al., 1999).

The core histones are highly conserved proteins with respect to molecular weight, structure and amino acid composition (Khorasanizadeh, 2004). Histone H3 is one of the four core histones which with the DNA form the remarkable nucleoprotein complex, the chromatin. The amino-terminal tail of H3 has the highest density of post-translational modifications (PTMs) mapped among histones and thus gives rise to a complex pattern of coexisting or mutually exclusive combinations of markers (Torres-Padilla, 2008; Pérez-Cadahía et la., 2009).

Histone phosphorylation is a well-characterized PTM whereby serine (or threonine) residues on the histone tails are reversibly modified through the covalent addition of a phosphate group (McManus and Hendzel, 2006). Many of the changes in chromatin structure that occur during the cell cycle are mediated by reversible phosphorylation/dephosphorylation reactions (Jeong et al., 2010). Histone phosphorylation has been linked to a variety of cellular processes, such as chromosome condensation and segregation, activation of transcription, gene silencing, apoptosis and DNA damage repair (Prigent and Dimitrov, 2003; Johansen and Johansen, 2006; Houben et al., 2007).

1.2.1 Euchromatin & Heterochromatin

Eukaryotic chromatin is organized in two distinct defined domains known as euchromatin and heterochromatin (Heitz, 1928). The euchromatin referred to as the gene-rich part of the chromatin, has a more “flexible” arrangement, being more accessible to the transcription apparatus because of its “open state” configuration (decompacted). It is “poised” for gene expression, although not necessarily transcriptionally active. On the other hand, the heterochromatin, known as being gene-poor, has a more “closed” chromatin structure, is more compact and hard to access, and has a more regular nucleosomal organization than the euchromatin (Wallrath and Elgin, 1995). These two types of chromatins are even more distinguishable from one another based on chromatin density. By using DAPI (a DNA dye that has high affinity for A-T sequences), species like mouse and humans exhibit high DAPI signal for heterochromatin, while the euchromatin exhibits low DAPI signal intensity (Tanious et al., 1992; Kapuscinski, 1995). Moreover, euchromatin generally keeps genes ready for transcription, whereas heterochromatin contains mainly transcriptionally silent genes (Sims et al., 2003; Santos et al., 2005; Miao and Natarajan, 2005).

Diverse parts of the genome have different types of chromatin configuration depending on their function and importance, which is basically a way of protecting and separating these structures along the genome. For instance, heterochromatin is found on the centromeres and telomeres, two components of the chromosomes formed by repetitive sequences. These elements are thought to have the mission of protecting important structures essential for chromosomal function (Avramova et al., 2002). They basically ensure large and relatively stable heterochromatin domains. The telomeres, which are located at the end of the chromosomes' arms and serve as chromosomal "caps," prevent abnormal fusion and incorrect rearrangements between chromosomes, whereas centromeres are there to ensure appropriate kinetochore formation, the establishment of spindle attachments, chromatid cohesion and segregation (of chromatids and homologous chromosomes) to ensure a faithful inheritance of genetic information. This structure is usually located in the middle or at the end of the chromosomes depending on the chromosome type (Villasante et al., 2007). Consequently, due to their essential roles these structures are well protected from possible genetic recombination, mutations or even invading DNA. It is believed that the presence of repetitive DNA sequences, their chromatin configuration and very specific epigenetic signature are all involved in such protection (Allis et al., 2007). The chromatin which forms these distinct chromosome territories, telomeres and centromeres, is classified as constitutive heterochromatin because it is always condensed and present throughout the entire cell cycle, forms functional structures, has a repetitive nature and replicates late in S phase (Cowell et al., 2002). In contrast, the facultative heterochromatin, which is developmentally regulated, is transient. Being formed only in specific situations to stop gene expression, it is in fact a result of a developmentally regulated condensation of gene-rich euchromatic regions, which are usually decondensed and transcriptionally active (Sabbattini et al., 2007). A good example of this type of heterochromatin is the inactive X, again another way of developmentally regulating the genetic information ensuring that the right dosage of X-linked gene products exists between the sexes (Allis et al., 2007).

1.3 Global Chromatin modifications

Modulation of gene expression occurs through epigenetic modifications, which include DNA methylation and post-translation modifications (PTMs) of histones such as methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP ribosylation (Corry et al., 2009). These epigenetic modifications cause changes in the chromatin structure altering and causing a variation in gene expression. It is through these epigenetic markers that environmental factors such as diet, stress and prenatal nutrition can make an imprint on genes that is passed from one generation to the next (Cloud, 2010). Therefore, these covalent modifications together with DNA methylation are thought to be involved in the inheritance of the genome reprogramming information to the next generation (Lepikhov et al., 2004).

1.3.1 Histone Post-translation Modifications

As mentioned above histones can undergo different post-translational modifications through their N-terminal tails catalyzed by different histone modifiers. There are several chromatin-modifying enzymes such as histone acetylases (HATs) and deacetylases which acetylate/deacetylate specific lysine residues in histone substrates: the histone kinase family and phosphatases (PPTs) which phosphorylate/dephosphorylated specific serine or threonine residues, the histone methyltransferases and demethylases which in turn add/remove methyl groups from specific residues, and the DNA methyltransferases responsible for adding methyl groups to CpG dinucleotides (Allis et al., 2007; Shi and wu, 2009; Delcuve et al., 2009). From these modifications the first to be described and most well-characterized are acetylation and methylation (Bártová et al., 2008). It is generally thought that methylation is related to gene silencing and acetylation to gene activity. In certain cases it can however vary depending on the residue and histones which are being modified (Kwon and Workman, 2008). For example, H3K9me3 and H3K4me, which are both methylation nevertheless present different effects on gene expression: H3K9me3 correlates to gene silencing while H3K4me is a mark associated with initiation of gene expression (Wang et al., 2007). In fact, heterochromatin and euchromatin can also be distinguished by their specific markers, such as post-translational histone modifications and/or associated proteins. Generally, lysine acetylation of histones H3 and H4 is associated to gene expression and found in euchromatin, whereas histones in heterochromatic areas are relatively hypoacetylated (Kwon and Workman, 2008). Moreover, markers like H3K4me2 and H3K4me3, which are markers of transcriptionally active genes, are found on the euchromatin, while transcriptionally

repressive modifications such as H3K9me2 and H3K9me3 are found on the heterochromatin (Ooga et al., 2008).

Histone phosphorylation was first discovered forty years ago and, since then many of the kinases that phosphorylate histones have been identified (Sims et al., 2008). Similarly to other histone modifications, phosphorylation presents contradictory biological functions. In interphase cells, Ser10 (H3S10P) of histone H3 has turned out to be an important phosphorylation site for transcription from yeast to humans, whereas during mitosis and meiosis H3S10P has an opposing role, being correlated to chromosome condensation (Kouzarides and Berger, 2007).

1.3.2 DNA Methylation

The long-term epigenetic marker and the most prominent form of epigenetic modification, DNA methylation, refers to the addition of a methyl group to the fifth carbon of the base C (Wu and Zhang, 2010). DNA methylation at CpG dinucleotides, a major epigenetic modification of the genome, plays an important role in the regulation of gene expression and is essential for mammalian embryogenesis (Liu et al., 2004). In general, DNA methylation is a process involved in genomic imprinting and X inactivation, and plays a central role in silencing specific genes through a transcriptional repression mechanism in both heterochromatic and euchromatic domains (Brenner et al., 2007). The enzymes responsible for this epigenetic modification, DNA methyltransferases (DNMTs), are well characterized and conserved in mammals and plants. The DNMTs are classified into two categories: *de novo* and maintenance DNA methyltransferases, with the DNMT3A and DNMT3B being *de novo* DNMTs and DNMT1 as the maintenance one (Wu and Zhang, 2010). The genomic imprinting by DNA methylation is important for the sex-specific germline "marking" of parental alleles that contributes to appropriate expression of many imprinted genes which should be long-term silenced. This silencing must be sustained throughout the development and lifespan of an organism (Paulsen et al., 2007).

Therefore, DNA methylation and post-translation modifications of histones are processes involved whether, when and how particular genetic information will be read. They are also believed to pass the information on by propagating the epigenetic markers which are required for growth and faithful development (Gilbert et al., 2007).

1.4 Centromeric & Pericentromeric Heterochromatin

Centromeres are structures with very specialized functions important for chromosome alignment and segregation and for the formation of the kinetochores which are essential for normal chromosome attachment and separation during mitosis and meiosis (Vogt et al., 2009). The centromeres are formed by centromeric heterochromatin flanked by large blocks of pericentromeric heterochromatin. These two types of heterochromatin have different characteristics but share similarities: both are formed by repetitive DNA sequences. In mouse the centromeric heterochromatin corresponds to the minor satellite sequences and the pericentromeric one to the major satellite ones which are long stretches of short tandem A-T rich sequences (Guenatri et al., 2004; Martin et al., 2006a; Probst et al., 2007).

Centromeric and pericentromeric heterochromatin present different markers such as histone modifications and/or non-histone chromosomal components. Centromeric heterochromatin is characterized by the presence of centromere-specific histone H3-like proteins (CENP family) which are present at the centromeres throughout the cell cycle. The centromere protein-A (CENP-A) was the first histone variant to be identified as being involved in specification and inheritance of the chromatin state. CENP-A provides the base for the recruitment of kinetochore proteins, for spindle attachment and for normal chromosome segregation in many different organisms (Karpen et al., 2007). The centromeric heterochromatin regions are somehow conserved in many different organisms. In fact, it is the actual epigenetic composition of the centromeric heterochromatin that shows conservation, because CENPs are much more consistent features of the centromeres than the rapidly evolving arrays of repetitive satellite DNA (Henikoff et al., 2004). In mouse and humans the flanking pericentromeric heterochromatin stains strongly with DAPI, a DNA dye that has high affinity for A-T sequences. Remarkably, this type of heterochromatin contains one of the most studied histone modifications, the trimethylation of the histone H3 at Lysine 9 (Banister et al., 2001; Lachner et al., 2001; Torres-Padilla, 2008). This covalent modification together with the heterochromatin protein 1 (HP1) are hallmarks of this type of heterochromatin (Lehnertz et al., 2003). These two markers are seen in the pericentromeric heterochromatin of various types of cells and organisms and are associated with transcriptional repression. In mouse these two markers are involved in heterochromatin formation (heterochromatinization), therefore being expressed in dense heterochromatin compartments called chromocenters which have a totally hostile environment towards transcriptional gene activity. It is believed that these two markers function together to modulate the chromatin in a

way to restrict access of different factors crucial for gene expression (Festenstein et al., 2003). It is likely that the interaction between these two markers in the pericentromeric heterochromatin takes place through the binding site created by the enzyme SUV39H when it trimethylates histone H3 at Lys9 and in which the chromo domain of HP1 binds to (Cowell et al., 2002). HP1 binds to H3K9me3 through its chromodomain and interacts with other proteins through its chromo-shadow domain to accomplish its function. Therefore, by binding to H3K9me3 through its chromo domain, HP1 stabilizes these repressive heterochromatin areas (Lehnertz et al., 2003).

It has been shown that pericentromeric transcripts which are processed to small RNAs, guide heterochromatin formation and establishment of a transcriptionally silent state in fission yeast and plants (Zaratiegui et al., 2007). Even though, many noncoding RNAs are expressed in a developmentally regulated way, it remains to be determined whether a similar mechanism is functional in mammals (Kanellopoulou et al., 2005; Murchison et al., 2005). It has also been recently suggested that transcripts generated by pericentromeric satellite repeats are involved in heterochromatin formation during early embryogenesis (Probst et al., 2010).

These distinct chromosomal structures, the chromocenters which are easily detectable in the interphase nuclei, are the association of centromeres of different chromosomes resulting in these highly condensed clusters (Guenatri et al., 2004). In the mouse nucleus, pericentromeric heterochromatin made up of major satellite repeats tends to cluster into, the already mentioned, chromocenters (Gilbert et al., 2007). The degree of centromere clustering varies depending on the cell type, cell cycle phase or stage of differentiation (Merico et al, 2007). Therefore, it is believed that these chromosomal domains formed by the centromeres may behave as structural centers for chromatin organization in interphase, favouring the creation of functional compartments for essential nuclear processes such as gene expression, DNA replication, and cell division (Alcobia et al., 2000). Moreover, in many cells types these domains are rapidly established after cell division during the G1 phase of the cell cycle and it is exactly on these structures that the pericentromeric markers H3K9m3 and HP1 are first detected (Maison et al., 2002; Monier et al., 2006; Mateos-Langerak et al., 2007).

Another interesting point about the pericentromeric heterochromatin is that there is an increasing body of evidence suggesting a relationship between the repressive marker H3K9m3 and DNA methylation in these regions. It has been reported that histone methyltransferases, such as Suv39h, are required for both H3K9 trimethylation and DNA methyltransferase 3b (DNMT3b)-dependent DNA methylation at pericentromeric repeats

whereas it is independent of it at the centromeric ones (Bártová et al., 2008). Both methylation systems and the presence of the HP1 in the pericentromeric heterochromatin in mouse are likely to be important in reinforcing the stability of heterochromatic subdomains (Lehnertz et al., 2003).

1.5 Histone H3 and its Serine 10 Phosphorylation

The Ser10 of histone H3 is a residue conserved across eukaryotes which becomes highly phosphorylated in mitotic and meiotic cells (Houben et al., 2007). Phosphorylation of histone H3 at Serine 10 (H3S10P) has been specifically identified as being involved in the regulation of gene expression and chromosome condensation/segregation (Cerutti et al., 2009). In fact, the functions of H3S10P are particularly intriguing, because it is associated with two apparently divergent processes. Firstly, H3S10P is associated to chromosome condensation as it peaks at the late G2 phase when the chromatin is condensing to form chromosomes, as well as, to chromatin opening and transcription by the activation of genes in response to certain signals, such as mitogen events or stress (Edmondson et al., 2002). Furthermore, chromosome condensation is of great importance because it is an essential prerequisite to ensure subsequent fidelity of chromosome segregation to avoid for example missegregation events (polyploidy) which are the cause of aneuploidy (Swain et al., 2007).

There are a variety of kinases which can phosphorylate histone H3 at Ser10, such as NIMA in fungi, JIL-1 a possible candidate for phosphorylation of H3 at Ser10 in drosophila, VRK1 in HeLa cells, and a rapid and transient phosphorylation by an MAP kinase signalling cascade resulting in activation of mitogen- and -stress-activated protein kinases MSK1 & MSK2 which phosphorylate H3 at Ser10 have been mentioned (Nowak and Corces, 2004). However, studies in somatic cells have shown that the mitosis-specific phosphorylation of histone H3 at Ser10 occurs via Aurora B kinase, a member of the Aurora kinase family which is also involved in Ser28 phosphorylation (McManus and Hendzel, 2006).

The Aurora kinases are a family of cell-cycle kinases which is to regulate cell proliferation. This family comprises three related isoforms in vertebrates: Aurora A, B and C (Shannon and Slamon, 2002). Furthermore, this family seems highly conserved in different organisms as one or more orthologues can be found in yeasts, flies, worms and other invertebrates (Andrews et al., 2003). These mammalian Aurora kinases display different subcellular

localization and have specialized functions (Sessa et al., 2005). In general, Aurora A is related to centrosome maturation, regulating spindle assembly while Aurora B is involved with monitoring microtubule-kinetochore attachments and spindle mechanism coordination (Ducat and Zheng, 2004). Less is known about Aurora C. It is however believed that this kinase functions exactly like Aurora B and that together they regulate chromosome dynamics (Li et al, 2004). Members of the Aurora family have been found overexpressed in a variety of cancers, emphasizing the pivotal role that these kinases play in governing cell proliferation (Crosio et al., 2002). Due to the association of Aurora kinases overexpression and tumorigenesis, these phosphoproteins have been targeted for cancer therapy, giving rise to the Aurora kinase inhibitors such as hesperadin, ZM447439, VX-680 and others (Carvajal et al., 2006).

Phosphorylation of histone H3 at Ser10 in mammalian mitotic cells, initiates primarily within pericentromeric heterochromatin in interphase during late G2 and then uniformly spreads throughout the chromatin on the chromosome arms as they undergo condensation. Phosphorylation then diminishes or even disappears during late anaphase and early telophase (**Figure 9;** Wang et al., 2006).

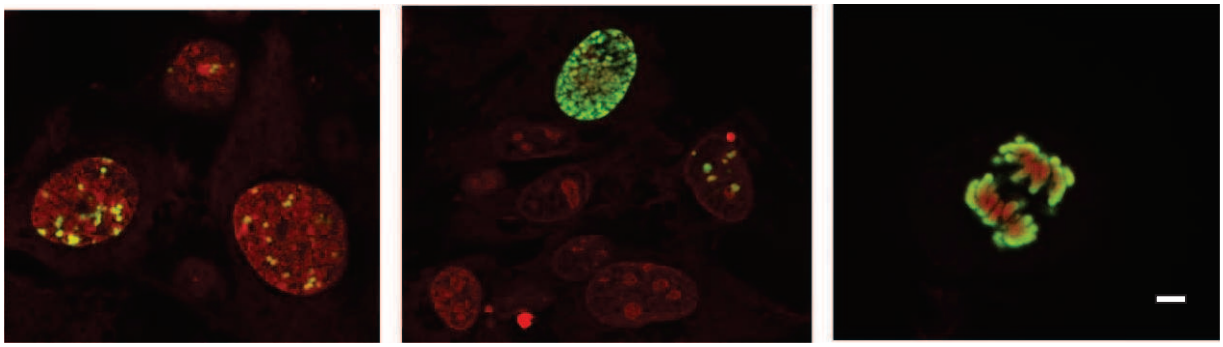

Figure 9. H3S10P staining in mouse fibroblasts, showing the onset of its detection in late G2 and the labeling on the fully condensed chromosomes at metaphase. (H3S10P in green and DNA in read). Scale: 10µm

Indeed, the inhibition of histone H3 phosphorylation prevents the initiation of chromosome condensation and entry into mitosis, while inhibition of its dephosphorylation causes irregularities during mitotic exit (Garcia et al., 2005). The presence of this epigenetic modification during interphase is consistent with a role for H3S10P in regulation of gene expression during this phase of the cell cycle, in addition to its role in higher-order chromatin condensation during mitosis. At interphase, in contrast to what happens at mitosis, the phosphorylation of histone H3 does not affect the whole genome but a subset of genes such

as immediate-early genes (IEGs) which are activated transiently and rapidly in response to a wide variety of cellular stimuli (Clayton and Mahadevan, 2003). H3S10P correlates with the transcriptional activation of these genes after being triggered by different inducer stimuli, such as stress or mitogenic events. It must be emphasized that H3S10P is not a general requirement for transcriptional activation at all promoters but may play a distinct role tailored for specific ones (Johansen and Johansen, 2006).

Different models have been proposed to explain the role of H3S10P during interphase, mitosis and meiosis. In mitosis, H3S10P is viewed as a trigger for chromosome condensation. It is believed that H3S10P is used as a label to indicate to the cell, firstly that chromatin is ready for condensation, then that the chromosomes have passed all the check points required and arrived intact to metaphase and that they can continue to anaphase and go safely through it. Once all these steps have been accomplished "the label" H3S10P is not required anymore and can then be removed (Prigent and Dimitrov, 2003). Another suggestion is that H3S10P can act as a substrate for the binding of chromatin remodelling factors, serving as a docking site for various proteins causing either decondensation (in the promoter regions of the immediate early genes, it would then be an active marker) or condensation in the case of mitosis and meiosis (Pascreau et al, 2003). In fact, this rapid and transient stimulation that occurs on the promoters of these specific genes affects a distinct population of histone H3 which is different from the one detected in dividing cells (Nowak and Corces, 2004). Basically, the general view is that phosphorylation of histone H3 at Ser10 serves to destabilize the chromatin structure in either way (closing or opening it) after interacting with various proteins involved in different processes such as condensation, decondensation, repair and transcription (Pascreau et al, 2003). Histone H3 phosphorylation at Ser10 is sufficient to induce a change in higher-order chromatin structure from a condensed heterochromatin-like state to a more open euchromatic configuration (Deng et al., 2008). It is also believed that the maintenance of the levels of H3S10P at euchromatic regions is necessary to counteract heterochromatinization and gene silencing (Cai et al., 2008).

As can be seen the same epigenetic modification can exert different functions depending on the phase of the cell cycle. It is known that few H3 molecules are phosphorylated in interphase whereas in mitosis all of them become phosphorylated at Ser10. It is not clear how phosphorylation of Ser10, which decreases the overall positive charge of the H3 N-tail, could increase its binding to DNA to shield charge and promote compaction (Sauvé et al., 1999). One of the suggestions to explain H3S10P physical consequences, is that phosphorylation of

H3S10 would neutralize Serine positive charges, increasing the affinity between its N-tail and DNA, leading to compaction (Sauvé et al., 1999). H3S10 phosphorylation could also weaken H3 N-tail binding to DNA thereby allowing association of the "free" DNA with different factors or with other proteins essential for chromatin compaction (Hendzel et al., 1997), in both cases generating a binding site for other proteins (Pérez-Cadahía et al., 2009).

It is known that the addition of negatively charged phosphate groups to histone tails neutralizes their basic charge thereby diminishing the strength of electrostatic interactions between the positively charged histone N-terminus and the phosphate backbone of the DNA, consequently increasing the flexibility of the chromatin fibres (Murnion et al., 2001; Grant, 2001). Basically, phosphorylation might alter the physico-chemical properties of H3, thereby altering nucleosome packing, in one way or another (Pérez-Cadahía et al., 2009).

Therefore, it is evident that more studies need to be carried out to clarify the various biological functions H3S10P has during all these different phases of the cell cycle, as well as to unveil how this epigenetic modification exerts its physical interactions with the DNA and different factors.

1.5.1 H3S10P in somatic cells

In mammalian cells, Aurora B kinase colocalizes with H3S10P, being detected first in the pericentromeric heterochromatin in G2 or even in S phases before the onset of H3S10P, supporting its role as the mitotic H3 kinase (Monier, et al., 2006). Aurora B becomes concentrated in large pericentromeric foci before and during histone H3 phosphorylation initiation in pericentromeric chromatin (Zeitlin et al., 2001). In animals, Aurora B is the enzymatic core of the chromosomal passenger complex (CPC), a group of mitotic regulators that includes the inner centromere protein (INCENP), Survivin and Borealin (Houben et al., 2007). This protein complex moves from the centromeric region of metaphase chromosomes onto the central spindle microtubules during anaphase and functions to modulate chromosome structure and segregation as well as *chromosome* alignment and attachment to the microtubules (Zeitlin et al., 2001). Following the typical pattern of the CPC, Aurora B localizes to centromeres in prophase and remains concentrated at the centromeres until mitosis, when it relocates to the central spindle and subsequently to the spindle midbody (Murnion et al., 2001). For this reason, Aurora B is said to function in both early and late mitotic events, including chromosome segregation and cytokinesis (Yasui et al., 2004).

It is known that regulation of the events related to cell cycle progression is a balance between phosphorylation and dephosphorylation of various proteins. Therefore, an interaction between kinases and phosphatases must be in place. In the case of Aurora B, it has been reported that this kinase might be negatively regulated through interactions with phosphatases such as PP1 or PP2A by preventing accumulation of phosphate in the Aurora B T-loop, causing the inactivation of Aurora B (Yamamoto et al., 2008).

Another interesting biological event related to H3S10P and that many studies are in agreement with is the "binary switch hypothesis". It has been reported that phosphorylation of H3S10 during mitosis in mammalian cells antagonizes the effect of H3K9 trimethylation and leads to the dissociation of HP1 from chromosomes (Karam et al., 2010). Indeed, while most HP1dissociates from chromosomes during mitosis, if phosphorylation of histone H3 at Ser10 is inhibited, HP1 remains chromosome-bound throughout mitosis (Hirota et al., 2005). It is believed that the already trimethylated histone H3 at its Lys9 is further phosphorylated at its Ser10. This extra modification on the same histone N-tail would then cause the ejection of HP1 (Fischle et al., 2005). There are many implications about this dissociation of HP1. One suggestion is that the association of HP1 proteins to chromatin might block the access of condensins to DNA, avoiding chromosome condensation. It was seen that the timing of HP1 release is clearly before the start of condensin loading (Dormann et al., 2006). There are other suggestions about the biological significance of HP1 dissociation, such as the correct resolution and separation of chromatids (if HP1 persisted it could cause segregation defects), and removal of cohesin from chromosome arms aiding in the resolution of sister chromatids. All this is accomplished by Aurora B histone H3 phosphorylation via the ejection of HP1. Therefore, H3 phosphorylation by Aurora B is part of a "methylation-phosphorylation switch" mechanism that displaces HP1 and possibly other proteins from mitotic heterochromatin (Hirota et al., 2005).

This double epigenetic modification, trimethylation of histone H3 at Lysine 9 and phosphorylation at Serine 10 (H3K9me3S10P), is also correlated to heterochromatin. It was stated that both modifications coexist on the same histone tail especially during mitosis based on the results obtained by *in vitro* assays and analysis of the *in vivo* modification pattern of H3 isolated from HeLa cells (Fischle et al., 2003). This double marker was found to strongly stain the centromeric domains of the chromosomes in HeLa cells during mitosis (Fischle et al., 2005). Another research group had revealed the existence of H3K9me3S10P with peptide

competition assays and immunofluorescence experiments. Thereby they described its correlation with pericentromeric heterochromatin in somatic cells (Hirota et al., 2005). In fact, H3K9me3S10P is also detected at later G2 in interphase, however it does not strongly stain the heterochromatin clumps labeled with DAPI. This double marker was found enriched in the centric and pericentric domains with a more spotted appearance on the chromosome arms during mitosis (Fischle et al., 2005).

As can be seen from the above, the epigenetic modification H3S10P presents distinct functions during interphase and mitosis, being modulated in a variety of ways by several kinases during the different phases of the cell cycle and presenting diverse histone interactions.

1.5.2 H3S10P in mammalian gametes

It is now readily accepted that the chromatin structure and nuclear organization are both epigenetically regulated. Different epigenetic signatures are seen in different cell types which are basically the result of specific functions (Alcobia et al., 2000). Gametes are very specialized cells which upon fertilization undergo impressive changes in their chromatin structure and nuclear content. But firstly during gametogenesis, these cells also undergo drastic rearrangements in their chromatin. For instance in the sperm, histones are replaced by protamines in the chromatin in order to highly pack the DNA (Goldberg et al., 1977; Kimmins et al., 2007), whereas the oocyte needs to acquire competence to resume meiosis in an attempt to fully develop and therefore it undergoes a massive chromatin rearrangement (Bui et al., 2004). These two impressive events have raised the interest of many scientists in understanding how spermatogenesis and oogenesis are epigenetically regulated.

From all these events, the maturation process the oocyte goes through is the one which attracts the most attention due to its importance in establishing good quality oocytes that will be capable of originating a viable embryo. Remarkably, as the oocyte approaches prophase I (also known as GV "germinal vesicle" stage) it acquires meiotic competence. In these fully-grown oocytes the chromatin then changes from decondensed (Non-Surrounded-Nucleolus, NSN) to condensed state (Surrounded-Nucleolus, SN) (**Figure 13**; Hirao et al., 1995).

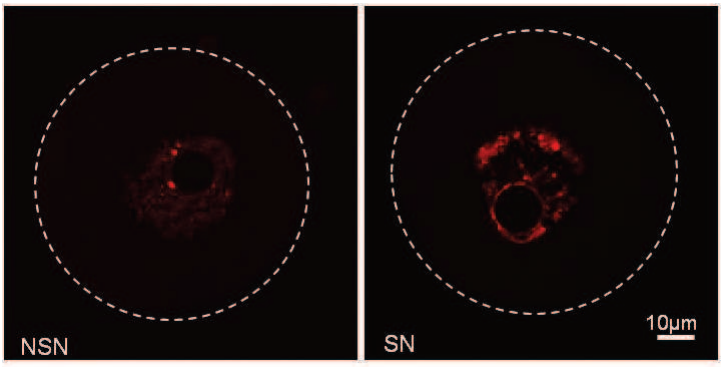

Figure 13. Example of heterochromatin arrangement in NSN and SN GV mouse oocytes. Intense staining showing heterochromatin ring in SN GV oocyte and diffuse DNA staining in the NSN GV one (DNA in red).

Thereafter, many changes occur during the meiotic process in the chromatin and in the nucleus such as chromosome condensation, breakdown of the germinal vesicle (GVBD), arrangement of the microtubule network with extrusion of the first polar body and finally the arrest of the oocytes in meiosis II (Jelínková and Kulbelka, 2006). Upon fertilization the oocytes then resume meiosis completing the second meiotic division with the extrusion of the second polar body.

During the maturation process chromosome condensation is the first visible event that occurs which is crucial for correct chromosome packaging and segregation during PB extrusion. Phosphorylation of histone H3 at Ser10 is linked to chromosome condensation, however, the pattern of histone H3 phosphorylation is mammalian oocyte meiosis is nearly unknown. The first report stating that H3S10P correlates to chromosome condensation during meiotic maturation in pig oocytes was only published in 2004 (Bui et al., 2004).

In pig oocytes, it has been shown that H3S10P is slightly detected in the GV oocyte, however it undergoes gradual dephosphorylation from early to late GV stage, then a transient phosphorylation at the periphery of condensed chromatin is re-established in early GVBD oocytes. Moreover, the H3S10P signal gets stronger, covering all the chromosomes of oocytes from MI to MII (Gu et al., 2008). The authors believe that this gradual dephosphorylation of H3S10P seen from the GV to the GVBD stages in pig oocytes might be involved in regulation of meiotic resumption. Nevertheless, these findings make it clear that H3S10P is related to chromosome condensation in pig oocytes during meiotic maturation.

As for mouse oocytes, it has been shown that H3S10P is clearly detected on the chromatin at the GV stage. As the oocytes enter the first meiosis, H3S10P is seen on all chromosomes. However, when the oocytes proceed through the anaphase of the first meiotic division, H3S10P is barely detected on the chromosomes, being seen only in intense dots indicating association of this modification with pericentromeric domains. Additionally, when the oocytes reach metaphase II the chromosomes are again covered by H3S10P (Wang et al., 2006). The authors believe that this indicates that H3S10P is related to chromosome condensation in mouse oocyte maturation and that it must exert distinct functions during the metaphase of the first and second meiosis, but specifically that H3S10P is involved somehow with chromosome segregation during the transition of anaphase to telophase of meiosis I. Interestingly, another report presents different findings for H3S10P in mouse oocytes even though they seem to have used the same antibodies and protocol. In this study, H3S10P is not detected during the GV stage, but seen only at about the time of GVDB and remaining on the chromosomes during MI and MII. Therefore, this report states that H3S10P must be involved in the later aspects of chromatin condensation (Swain et al, 2007). Controversies can also be found in the studies carried out on pig oocytes. Some say H3S10P is not necessary for chromosome condensation in pig oocytes (Jelínková and Kulbelka, 2006), while others state that the meiotic phosphorylation of histone H3 is essential for oocyte chromosome condensation (Bui et al., 2004).

In regards to spermatogenesis, histone-based chromatin of the sperm is transformed into a protamine-based one, making its chromatin six times more compact than the chromatin found in somatic cells. However, at fertilization, when the two gametes fuse, this protamine-based chromatin is lost, being changed again into a chromatin containing maternal histones (van der Heijden et al., 2005). It has been reported that H3S10 phosphorylation occurs during mammalian spermatogenesis and is closely associated to condensed meiotic chromosomes of mouse spermatocytes, being lost during the post-meiotic differentiation (Govin et al., 2010). Another study has shown that H3S10P is only detected in mouse spermatocytes, showing a dramatic decrease after meiosis (Krishnamoorthy et al., 2006). Moreover, another report states that H3S10P is detected in heterochromatic regions in the nucleus of mitotic spermatogonia and in late meiotic cells from stages IX-XII (Kimmins et al., 2007). Nevertheless, all these studies agree with the fact that H3S10P is somehow related to chromosome condensation during some specific phases of mammalian spermiogenesis.

Due to this conflicting data about H3S10P especially in mammalian gametes and the few studies carried out, more research needs to be performed in order to understand the biological role of this epigenetic modification in these specialized cells, as well as to learn how they are epigenetically regulated. Most importantly, this would consequently bring more insight about the understanding on how mammalian preimplantation embryos are epigenetically regulated.

1.6 The mouse as a model

Mus musculus is the closest model organism to humans that is easy to manipulate and breed and shares about 85% sequence similarity with humans. Its genome is about the same size as the human genome and the order of genes on the chromosomes is very similar in both organisms. The wide area of information about mouse genetics has made it a valuable model system for the study of mammalian development as well as human genetic diseases (The Gale Group, Inc., 2003). The mouse (taxon-*mus*) has always been a good embryological model, easy to generate giving around 8-20 litters and with a quick gestation period of 21 days. Mouse embryology really expanded when molecular biologists started using mice for gene knockouts. Suddenly, the understanding about mouse development was necessary in order to understand the effect of knocking out the genes. There are over 450 different strains of inbred research mice (Hill, 2011). The mouse foetal development significantly matches that of other animals and especially humans. Many research groups study mouse development in order to more fully understand the development of human embryos and foetuses (Steel, 2010).

The role of epigenetics in mammalian development has mostly been elucidated in the mouse, although a number of studies have been translated to diverse human cell lines and primary cultures (Allis et al., 2007). The advantage of gene "knock-out" and "knock-in" technologies has been instrumental for the functional dissection of key epigenetic regulators. For instance, the Dnmt1 DNA methyltransferase mutant mouse provided functional insight for the role of DNA methylation in mammals (Li et al., 1992). Hence, many of the key advances in epigenetic control took advantage of the unique biological features of the mouse.

1.6.1 Preimplantation development

Nuclear remodelling is a crucial process during the early steps of development (Lanctôt et al., 2007). The two very specialized cells, the sperm and the oocyte, undergo dramatic alterations in their nuclear morphology and chromatin structure in order to achieve the appropriate embryonic configuration giving way to events leading to correct development. The incorporated sperm nucleus with its highly compacted chromatin, undergoes nuclear envelope breakdown and nucleoprotein remodelling. The two sets of chromatin decondense and are the origin of the two pronuclei (Mann and Bartolomei, 2002). By going through all this nuclear remodelling, these two unique cells give rise to the zygote (period from 1-cell to blastocyst stage) which has a distinctive nuclear configuration characterized by the already

mentioned pronuclei (nucleus-like structures) which contain Nucleolar-Precursor Bodies (NPBs), an “immature” type of nucleolus.

1.6.2 Mouse preimplantation development

Embryonic development in the mouse begins with fertilization of the oocyte by the sperm. Mouse embryogenesis is much slower than other model organisms such as sea urchin, *Drosophila* and *Xenopus*. By 24 hours, the mouse embryo is still at the 2-cell stage, continues to develop slowly, moving along in the oviduct into the uterus to implant after 4.5 days after fertilization.

It should be mentioned that a problem faced with the production of mouse embryos in laboratories is that the population of embryos recovered from females after spontaneous ovulation and mating will always be heterogeneous. In order to obtain synchronization between fertilization and subsequent development, researchers use schedule hormonal injections of hCG to ensure that mating occurs prior to ovulation and that oocytes are fertilized within one hour of ovulation (Monk, 1987; Nagy et al., 2003). Fertilization is therefore expected to happen at around 12 hours post hormonal stimulation, knowing that ovulation normally occurs between 10 and 13h after hCG injection. For that reason, the hormonal injection of hCG is often used a more accurate starting point to follow embryonic development in mouse.

After fertilization, full maturation of the egg, including completion of the second meiotic division and extrusion of the second polar body, is completed only around within 4 hours (Riggs, 1996). The newly formed embryo then enters the first mitotic cell cycle that starts with a long G1 phase. In the mouse zygote, the cell cycle consists of the usual succession of four phases: G1, S (DNA synthesis), G2 and M (mitosis) as in somatic cells. Around 16h phCG, nuclear membranes form around the separate haploid male and female pronuclei. DNA replication takes place during the migration of the two pronuclei to the center of the oocyte, starting around 21h phCG and being completed by around 29h phCG (Nagy et al., 2003; Bouniol-Baly et al., 1997). The pronuclei do not fuse and it is not before the 2-cell stage that the maternal and paternal genomes are enclosed into a single nucleus and that the activation of the embryonic genome takes place (around 45h phCG) (Flach et al., 1982; Pikó and Clegg, 1982). However, a minor wave of transcription is seen at the late 1-cell stage starting around 26h phCG during the transition from the late S to the G2 phase (Bouniol et

al., 1995; Sonehara et al., 2008). The first two cell cycles each last about 20h *in vivo* while the subsequent cycles each last for about 12h. It is only at the 8-cell stage that the first major change in the morphology of the embryo takes place: the embryo compacts. At the 16-cell stage, for the first time two phenotypically distinct cell populations are found in the embryo: non-polarized inner cells and polarized outer cells. By the 32-cell stage the blastocoelic cavity forms. By the 64- to 128-cell stage a blastocyst with two cell subpopulations has formed: an outer layer of epithelial trophectoderm cells, derived largely from outer cells of earlier stages, surrounds an inner cluster of cells, the inner cell mass (or ICM) located eccentrically within the blastocoelic cavity, and derived largely from the inner cells of earlier stages. In the blastocyst, the trophectoderm will give rise to the extra embryonic tissues and allow the implantation of the embryo in the uterine mucous membrane. The inner cell mass will give rise to the embryo proper (Maro et al., 1990).

1.6.3 Heterochromatin in embryos

As is known from studies carried out on mouse cells, during interphase the centromeres of different chromosomes are organized in clusters called chromocenters (Alcobia et al., 2000). These clusters are tightly formed by the pericentromeric heterochromatin of different chromosomes with the centromeric heterochromatin being just adjacent to it.

Differently from somatic cells, the mammalian embryos present a unique organization for this type of heterochromatin. It is known that the same pericentromeric heterochromatin which forms clumps in somatic cells, is organized in spherical structures around the Nucleolar Precursor Bodies (NPBs), forming rings of heterochromatin instead of clusters in the interphasic nuclei of the preimplantation embryos, at least until the 4-cell stage in mouse embryos. However, these major heterochromatic domains, the chromocenters, will be formed the during late 2-cell stage in mouse embryos, characterizing the transition from a zygotic to a somatic cell-like chromatin organization (Martin et al., 2006a).

As pericentromeric heterochromatin is characterized by the histone marker H3K9me3 and the protein HP1, several research groups have used them as markers to study pericentromeric heterochromatin in preimplantation embryos. It should be mentioned that HP1 is highly conserved from yeast to humans (Wang et al., 2008). In mammals, three subtypes of HP1 have been identified: HP1α, HP1β and HP1γ. The three isoforms have different localization in the nucleus and exert different functions (Meglicki et al., 2008). In mammalian cells, HP1α and HP1β are mainly heterochromatic, whereas HP1γ is observed in both heterochromatin and euchromatin (Lomberk et al., 2006). It was believed that the HP1α and HP1β are constantly present at pericentromeric. However, several studies showed that the majority of HP1α diffuses into the cytoplasm before prometaphase (Sugimoto et al., 2001; Fischle et al., 2005; Hirota et al., 2005; Terada, 2006) and re-associates with pericentromeres at anaphase (Terada, 2006). Similarly, HP1β also seems to dissociate from the pericentromeres during mitosis (Fischle et al., 2005). Arney et al 2002, were the first ones to investigate HP1β and HP1γ in mouse embryos; they concluded that HP1β is the isoform involved with epigenetic asymmetry between the parental genomes because they failed to detect HP1γ in mouse zygotes. Van der Heidjen et al. (2005), also looked at two of the isoforms HP1β and HP1α in embryos. They did not detect HP1α in any of the parental pronuclei, however HP1β was observed shortly after PN formation. Meglicki et al. (2008) with their study comparing HP1α and HP1β in mouse oocytes, have concluded that HP1β is the isoform with the strongest affinity for heterochromatin, because they found that HP1β was always co-localized with areas of condensed chromatin in growing and fully-grown mouse oocytes. Moreover, they observed that HP1β was still visible around the chromatin even 10 minutes after GVBD, while HP1α was never detected at this time. For all these reasons, HP1β has been more extensively studied in preimplantation embryos than the other isoforms and is the one which we have focused on.

It has thereby been showed that an asymmetry is clearly evident in the pericentromeric heterochromatin at the beginning of development. At the beginning of development, only the maternal constitutive heterochromatin is labeled by HP1β and H3K9me2/3 marks inherited from the oocyte, whereas the paternal one presents other repressive histone modifications and/or proteins such as H3K27me2/3 and PRC1 (Polycomb Repressive Complex 1), respectively (Liu et al., 2004; Puschendorf et al., 2007). It is known that methylated H3K9 recruits heterochromatin protein 1 (HP1), an essential component of constitutive heterochromatin which is then seen accumulated only in the maternal pronucleus. This higher

level of H3K9me3 and HP1 in the maternal pronucleus promotes the formation of late replicating heterochromatin (Liu et al., 2004).

There are however conflicting reports and different speculations about HP1β. There are reports showing that the heterochromatin in the paternal pronucleus is devoid of HP1β, acquiring it only later at around the 4-cell stage (Merico et al., 2007; Puschendorf et al., 2007), whereas others say that HP1β can already be seen in the paternal complement around 18hp hCG (Santos et al., 2005; Yeo et al., 2005; Probst et al., 2007).

Different studies try to explain the reasons for the asymmetry seen for H3K9me3 and HP1β in mammalian embryos. It is speculated that HP1β protects the maternal pronucleus from its interaction with DNA methylases in the maternal complement. It might be that the lack of these heterochromatic factors in the paternal pronucleus gives way to the DNA demethylation seen in this haploid part of the genome (Mayer et al., 2000 ; Santos et al., 2002) and that the presence of these factors in the maternal pronucleus protect it against demethylation (Arney et al., 2002). Indeed, HP1β creates a compact chromatin structure without transcription or any other processes (Stewart et al., 2005). Moreover, is has been suggested that the H3K9me3/HP1 interactions observed in early embryos could be linked to the preferential de novo DNA methylation of the maternal genome (Arney et al., 2002). As for the H3K9me3 asymmetry seen between the two parental complements, a plausible explanation is that HP1β binds to the already existing H3K9me in the paternal pronucleus, occluding it and preventing further methylation of the histone H3 N-tail (Corry et al., 2009). Moreover, the absence of H3K9me3 in the male part of the genome correlates with the DNA demethylation process (Hou et al, 2008). Basically, it is believed that the lack of this epigenetic modification in this part of the genome allows for DNA demethylation (Santos et al., 2005). It has been shown that absence of the two histone lysine methyltransferases specific to the H3K9 residues reduces CpG methylation within heterochromatic repeat sequences (Lehnertz et al., 2003). All these differences seen between the two parts of the genome can also be a reflection of the paternal pronucleus initiating transcriptional activity before the female one (Bouniol-Baly et al., 1997; Aoki and M Schultz, 1999).

The double modification H3K9me3S10P which is said to correlate to pericentromeric heterochromatin in cell has also been shown in preimplantation mouse embryos. Hayashi-Takanaka and colleagues, have microinjected Fab fragment antibodies (which do not impair normal development) to check the double modification in live mouse embryos. They state

that the H3K9me3S10P labels maternal chromatin, however it is clear from the video made after they injected Fab313 which preferentially recognizes H3S10P adjacent to di- and trimethylated Lys9, that the paternal chromatin also shows signal for this epigenetic modification to a certain extent (Hayashi-Takanaka et al., 2009). Therefore, it would be interesting to further investigate this double modification in preimplantation mouse embryos to check if this double marker is also a pericentromeric heterochromatin marker and if so, if it can be used to "read" heterochromatin reprogramming during preimplantation development.

It is very important for developmental biologists and epigeneticists to know if these epigenetic modifications represent constraints to the acquisition of a specific nuclear organization, since it is now clear that epigenetic changes are the "organizer" of the genome. For this reason it is extremely important to understand how gametes carrying special epigenetic signatures and unique nuclear organization, attain such specific higher-order chromatin arrangements seen in the embryos which is crucial for normal development. Moreover, once the embryo is formed, it is also especially essential to learn how the embryo sustains the particular nuclear and chromatin configurations which are central for further development.

Amongst the chromatin categories, the one which is always remarkable is the heterochromatin. This type of chromatin basically regulates gene activity by creating repressive gene environments. It is also the one which shows the most intriguing changes on its arrangement. Due to all the differences seen between both parental genomes, especially in relation to pericentromeric heterochromatin, it is fundamental to further investigate this heterochromatin domain in regards to its epigenetic signature and the influence it has on nuclear organization and gene expression. Consequently, the investigation of this kind of chromatin and its centromeric and pericentromeric domains are thought to be keys leading to the understanding of the epigenetic regulation from gametes to embryos.

With such diverse forms and examples of nuclear organization and therefore different systems for gene regulation seen from gametogenesis to embryogenesis, further studies need to be carried out in order to better understand how the epigenetic modifications affect the chromatin structure and consequently the nuclear organization and gene expression.

1.7 H3S10P in mammalian embryos

Despite all this information on these two pericentromeric heterochromatin markers, H3K9me3 and HP1β, in mammalian preimplantation embryos, nothing much is known about H3S10P. It is said that this epigenetic modification is a marker of pericentromeric heterochromatin in somatic cells and different organisms, nevertheless it is an open question as to whether this histone modification is also a marker of this type of heterochromatin in preimplantation embryos. So far only three reports have shown association of H3S10P and pericentromeric heterochromatin in preimplantation mouse embryos. They however present conflicting data.

In the work done by Wang and colleagues, they have showed for the first time that H3S10P is present during interphase of 1- and 2-cell embryos, as well as on condensed chromosomes during prophase and anaphase of the first mitosis (Wang et al., 2006). Moreover, they state that this distribution pattern for H3S10P is completely different from the one they observed in mouse oocytes. In the first oocyte meiosis, dephosphorylation of histone H3 at Ser10 is seen in the transition of anaphase to telophase, an event not observed in mouse embryos. They also suggested that H3S10P overlaps with pericentromeric heterochromatin around the NPBs in 1-cell embryos. Thereafter Huang et al. (2007), with their study on the comparison of some epigenetic modifications between *in vivo* and IVF embryos, found that H3S10P is detected at the pronuclear stage, with strong labelling in the perinucleolar region (around the NPBs) and diffusely detected throughout the nucleoplasm. As for the first mitosis, they observed strong H3S10P staining on the chromosomes which they say gradually decreases at anaphase. Furthermore, during the following divisions, H3S10P is obviously detected in the interphasic blastomeres from the 2-cell to morula stage, but undetectable at the blastocyst stage, except for the strong staining co-localized with the chromosomes.

As for the study carried out by Teperek-Tkacz et al. (2010), they have shown that H3S10P is detected in the condensed chromatin regions surrounding the NPBs very early during the pronuclear stage (19-21h phCG), showing a very short window of dephosphorylation of the Ser10 at the early-middle S phase (22-25h phCG) and *de novo* phosphorylation around 26h phCG seen only on the heterochromatin of the paternal pronucleus. H3S10P is then detected in the whole chromatin of both pronuclei at the G2 phase and all chromosomes from prophase to telophase of the first mitosis. Moreover, they say that a similar pattern of phosphorylation/dephosphorylation of H3S10 was observed in the interphase of the second

cell cycle and that H3S10 remains highly phosphorylated on the chromosomes on the subsequent mitosis from prophase to telophase.

These three reports have shown contradicting results for the same histone modification even though they have used the same antibody for the detection of H3S10P. In addition Wang et al. (2006) and Huang et al. (2007) seem to adopt the same approach for detection of H3S10P. Nevertheless they obtained different results. On the other hand Teperek-Tkacz and colleagues used a slightly different protocol which included pronase treatment for zona pellucid removal, fixation with a higher concentration of PFA and the use of M2 as culture medium (Teperek-Tkacz et al., 2010). These different steps could have caused some disturbance of the H3S10P distribution, leading to different conclusions. Consequently, more investigation needs to be carried out on H3S10P in preimplantation embryos in order to clarify the discrepancies found in these reports.

As it can be seen, the epigenetic regulation scenario in mammalian embryos is puzzling and the studies done so far cannot explain the biological significance and differences seen for many of these epigenetic markers. Even though it is known that there are species-specific contributions to the epigenetic regulation which could cause the differences seen for epigenetic markers in various types of mammalian embryos, it is important to further investigate to understand these divergences in an attempt to clarify even better how preimplantation embryos are epigenetically regulated.

1.8 Somatic Cell Nuclear Transfer (SCNT)

1.8.1 SCNT and its issues

During normal development the genomes of the two very specialized cells, the sperm and the oocyte, undergo profound changes. They lose their characteristic nuclear and chromatin configuration giving way to the establishment of a zygotic nuclear and chromatin organization. It is exactly this distinctive embryonic configuration that should be achieved by the somatic nucleus after Somatic Cell Nuclear Transfer, which basically consists in injecting a somatic mature donor nucleus into an enucleated oocyte. This conversion of a more differentiated nuclear condition into an embryonic one is one of the ways of carrying out nuclear reprogramming. In this case, the mature cell is forced to setback its differentiated state to an undifferentiated immature embryonic one. It is the faithful reprogramming of the somatic genome by the complete elimination of the epigenetic memory of the donor nucleus (Hochedlinger and Jaenisch, 2006).

Nuclear reprogramming by Somatic Cell Nuclear Transfer has caught the interest of many research groups due to the opportunity of testing nuclear potency, distinguishing between genetic and epigenetic state and alteration of various donor cells, as well as the possibility of generating embryonic stem cells for the treatment of animal model disease and the prospect of generating patient-specific human ES cells (Hochedlinger and Jaenisch, 2006). Moreover, the opportunity to clone by Somatic Cell Nuclear Transfer has been achieved in a variety of mammalian species and has potential applications for human health, improvement of agriculture species, protection of exotic and endangered species and advancement of basic biological research (Murphey et al., 2008).

Despite the great achievement in cloning mammals like mice, sheep, cattle and pigs using nuclear transfer, the overall efficiency of producing live cloned offspring is quite low.

It is thought that the causes for the low efficiency of this technique could be: mechanical damage of the samples during nuclear transfer, incomplete nuclear reprogramming or inappropriate *in vitro* culture systems (Kawasumi et al., 2007). It is known that three aspects are crucial for development: nuclear architecture, genome organization and gene expression. Therefore, if one of these factors is found irregular or faulty in embryos, the development will be compromised and this is usually true for cloned embryos.

1.8.2 Epigenetic reprogramming after SCNT

It is also believed that incomplete epigenetic reprogramming of the somatic nucleus, in other words, the incomplete re-establishment of the embryonic epigenetic patterns may be one of the causes of development failure of cloned animals (Mann and Bartolomei, 2002). The nuclear-transferred donor genome should be reprogrammed to activate the appropriate embryonic genes at the appropriate time, just as occurs in normal fertilized embryos (Kawasumi et al., 2007). It is conceivable that the normal activation of these genes associated with early embryonic development and/or the inactivation of somatically expressed genes may not occur readily in reconstructed embryos (Chung et al., 2003). Therefore, nuclear reprogramming is often abnormal or incomplete in cloned embryos.

There are many studies pointing out the different constraints seen in such type of embryos which disturb their development, such as aberrant DNA methylation patterns, incomplete chromatin reorganization and irregular nuclear compartmentalization, as well as abnormal histone modification patterns. Several studies have been published about irregular reprogramming of epigenetic markers, such as DNA methylation and histone acetylation and methylation (Wang et al., 2007; Zhang et al., 2009; Kang et al., 2011).

For instance, the signals for H3K4me2 which are supposed to increase at the 2-cell stage concomitant to the genome activation in mouse embryos, have been observed in lower levels during the same stage in cloned mouse embryos (Shao et al., 2008). Another epigenetic marker found abnormal in cloned embryos is H3K9 acetylation. This modification is detected in lower levels in cloned embryos when compared to their *in vivo* counterparts (Wang et al., 2007). As mentioned before the DNA methylation pattern is also disturbed in cloned embryos. One of the enzymes related to DNA methylation, the methyltransferase Dnmt1, is precociously detected in cloned mouse embryos at the 8-cell stage, whereas in normal embryos this protein should only be expressed in later stages (Chung et al., 2003). Moreover, studies carried out by Yamagata et al. (2007) have shown altered DNA methylation in reconstructed embryos by SCNT as well. They have found by live-cell imaging experiments that the centromeric and pericentromeric heterochromatin domains of cloned embryos were highly methylated when compared to their IVF counterparts. These results clearly illustrate the heterogeneity of epigenetic alterations found in cloned embryos. Taken all together, further research needs to be done to unveil the basic mechanisms regulating the development

of SCNT embryos, as well as to acquire a better understanding of these mechanisms to deeply analyse nuclear reprogramming in order to improve this fabulous technique.

1.8.3 TSA and the improvement of reprogramming after SCNT

Different methods have been employed by several research groups in attempts to improve the SCNT technique, such as inhibiting cytokinesis, changing the time of enucleation and injection of the donor nucleus, using various types of donor cells and the use of drugs like trichostatin A (TSA), an inhibitor of histone deacetylases. Histone acetylation has the greatest potential for unfolding chromatin, making it more accessible to different transcriptional factors which will lead to gene transcription (Wang et al., 2007). In fact, the treatment of the reconstructed embryos with TSA has shown a significant improvement in cloning efficiency in mouse, pig, bovine and rabbit (Maalouf et al., 2009; Bui et al., 2010). It is said that TSA improves nuclear reprogramming as a consequence of the modulation of histone modifications. As TSA is an inhibitor of deacetylases, this drug causes hyperacetylation, therefore enhancing the acetylation state of the cloned embryos which do then reach almost normal levels of acetylation as observed in normal embryos. In fact, the TSA treatment brought many benefits to the SNCT technique. It increased the time taken for chromosome condensation, enhanced the levels of H3K4me2 (a marker of transcriptionally active chromatin) and reduced the levels of a marker of inactive chromatin, H3K9me3 (Bui et al., 2010). Improved levels of H3K9 acetylation was also seen when the mouse-cloned embryos were incubated with TSA (Wang et al., 2007).

1.8.4 Pericentromeric Heterochromatin remodelling after SCNT

As mentioned before, the epigenetic status of the reconstructed embryo is a crucial step in normal embryonic development after nuclear transfer. As a matter of fact, different epigenetic modifications are found misregulated after this procedure including DNA methylation and histone modifications, as mentioned above. Additionally, as these epigenetic modifications are in close relation to chromatin remodelling and nuclear higher-order organization such misregulation can be one of the causes of the abnormal nuclear reprogramming seen in cloned embryos. Therefore, providing the means for the donor-cell genome to find its way to the correct reorganization after nuclear transfer is primordial for the improvement of cloning procedures.

It is clearly evident that the different nuclear arrangements seen in various types of cells and embryos result from the interaction between epigenetic markers and chromatin. In mouse, the somatic cells possess a characteristic way of organizing their chromatin which consists of an agglomeration of pericentromeric domains of different chromosomes forming blocks of constitutive heterochromatin which are called chromocenters. In embryos for example, the parental genomes display a unique distribution of centromeres and pericentromeric heterochromatin organized around the nucleolar precursor bodies (NPBs), with the chromocenters being formed later by the end of the 2-cell stage in mouse (Martin et al., 2006a) and around the 8-cell stage in bovine embryos (Santos et al., 2002). It is exactly these epigenetic markers together with chromatin that dictate the way the nucleus should be organized. Therefore, it is very interesting to look at how these heterochromatin domains behave after SCNT because the donor cell injected in an enucleated oocyte is thought to overcome its epigenetically imposed nuclear organization and higher-order chromatin structure, undergoing profound changes in its global nuclear structure in order to achieve the same epigenetic markers and embryonic nuclear configuration as a normal embryo.

Even though there is a great chromatin reshape imposed by the oocyte reprogramming factors in the donor nucleus, this switch from a somatic configuration to an embryonic one is not well achieved. It is known that with nuclear transfer, the donor cell nucleus which has blocks of heterochromatin (chromocenters) can be remodeled into a zygotic-like heterochromatin which is characterized by the arrangement of the centromeres around the NPBs (Martin et al., 2006a; Merico et al., 2007). However, it has been shown that a great number of cloned mouse embryos present a high number of centromeres not associated to the NPBs compared to normal embryos (Martin et al., 2006b). Moreover, the percentage of cloned embryos showing this abnormal heterochromatin redistribution correlates to the proportion of the cloned embryos which failed to develop to blastocyst stage (Maalouf et al., 2009).

It is evident that the somatic heterochromatin is often not well remodelled by nuclear reprogramming after SCNT. It is therefore important to further investigate how these heterochromatin domains are “reshuffled” through nuclear reprogramming so that new tools and approaches can be found to improve reorganization of these regions after SCNT.

1.9 Project aims

Even though the phosphorylation of histone H3 at Ser10 is one of the most characterized post-translational modifications of core histones (Garcia et al., 2005; Kouzarides and Berger, 2007), data concerning this epigenetic modification in mouse embryos and even in mouse oocytes are limited and controversial. As a matter of fact, only few reports have been published so far concerning H3S10P and no information is available regarding this epigenetic modification during the whole preimplantation development of mammalian embryo. Due to the lack of information and the fact that this post-translational modification can be classified as a marker of pericentromeric heterochromatin in somatic cells, we were prompted to investigate how this epigenetic modification would behave in preimplantation mouse embryos. As our interest also lays in nuclear organization and specially chromatin structure, we have included in our investigation nuclear reprogramming of embryos generated by SCNT.

Chapter Two: H3S10 phosphorylation marks constitutive heterochromatin during interphase in early mouse embryos, until the 4-cell stage

2.1 Introduction

So far H3S10P findings are controversial and no clear information about this epigenetic modification has been provided. Considering this, our main objective with this work was to investigate the spatial distribution pattern of H3S10P throughout the whole preimplantation development using a specific antibody against this epigenetic modification having the mouse as our model, in an attempt to give a clear picture of its distribution pattern, to examine its correlation to pericentromeric heterochromatin, as well as to try to determine if this epigenetic modification was a new marker of pericentromeric heterochromatin, as suggested by the three previous reports in this epigenetic modification in mouse preimplantation embryos (Wang et al., 2006; Huang et al., 2007; Teperek-Tkacz et al., 2010). All these studies have concluded that H3S10P is somehow linked to pericentromeric heterochromatin due to its obvious detection in the heterochromatin domains and chromosomes.

For this purpose we have used indirect immunostaining with antibodies against different proteins and immunoFISH (immunostaining plus 3-D FISH). Indirect immunostaining is a conventional method which can be employed in diverse scientific scenarios. It is basically used for the detection of specific proteins through antibodies containing colourful particles called fluorochromes. ImmunoFISH is one of the best approaches found so far to observe DNA sequences and their related proteins. This procedure has been found to circumvent some issues brought about by the immunostaining technique, such as the presence or absence of antigens. With this method it is possible to accurately locate the repetitive DNA sequences and to investigate their co-localization with different proteins. For this part of the work, we used specific probes to the pericentromeric sequences together with specific antibodies against histone (H3S10P) and non-histone proteins (HP1β).

2.1 Original Paper #1

This paper is presently submitted to the “Journal of Reproduction and Development”.

Supplementary figures related to this paper are shown next (§ 2.2).

H3S10 PHOSPHORYLATION MARKS CONSTITUTIVE HETEROCHROMATIN DURING INTERPHASE IN EARLY MOUSE EMBRYOS, UNTIL THE 4-CELL STAGE

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ABSTRACT

Phosphorylation of histone H3 at Ser10 (H3S10P) has been linked to a variety of cellular processes, such as chromosome condensation and gene activation/silencing. Remarkably, in mammalian somatic cells, H3S10P initiates in the pericentromeric heterochromatin during late G2 phase and phosphorylation spreads throughout the chromosomes arms in prophase, being maintained until the transition from anaphase to telophase when it gets dephosphorylated. Considerable studies have been carried out about H3S10P in different organisms, however there is few information about this histone modification in mammalian embryos. We hypothesized that this epigenetic modification could also be a marker of pericentromeric heterochromatin in preimplantation embryos.

We therefore followed the H3S10P distribution pattern in G1/S and G2 phases through the entire preimplantation development in *in vivo* mouse embryos. We paid special attention to its localization relative to another pericentromeric heterochromatin marker HP1β and performed immunoFISH using specific pericentromeric heterochromatin probes. Our results indicate that H3S10P presents a remarkable distribution pattern in preimplantation mouse embryos until the 4-cell stage and is a better marker of pericentromeric heterochromatin than HP1β. After 8-cell, H3S10P kinetic is more similar to the somatic one, initiating during G2 in chromocenters and disappearing upon telophase.

Based on these findings, we believe that H3S10P is a good marker of pericentromeric heterochromatin, especially in late 1- and 2-cell stages as it labels both parental genomes and that it can be used to further investigate epigenetic regulation and heterochromatin mechanisms in early preimplantation embryos.

INTRODUCTION

Nowadays, it is known that the way in which the chromatin is positioned in the nucleus inside the cell, can direct all the nuclear and chromatin functions essential for cell cycle and development [1-3].

It is believed that chromatin organisation and nuclear architecture are governed by epigenetic mechanisms which are not random. Therefore, chromatin and nucleus are controlled by these epigenetic modifications to achieve such a spatial organization and structure leading to the creation of functional nuclear compartments during cell cycle progression and development [4, 5].

Epigenetic modifications have been the focus of intense investigation. It includes DNA methylation and post-translational histone modifications such as methylation, acetylation, phosphorylation, ubiquitination and ADP ribosylation. It is through these processes that chromatin and nuclear organization can be modulated to change gene expression. Many epigenetic modifications have been studied in different organisms and it is believed that some of them are conserved in a variety of metazoan, fungi, plants and protozoa [6].

It is known that a same histone modification can have different functions depending on the samples studied, implying that there is some specific factors orchestrating the event. Histone phosphorylation of H3 at Ser10 for instance, has been linked to a variety of cellular processes, such as chromosome condensation and segregation, regulation of gene expression (activation of transcription or gene silencing), apoptosis and DNA damage repair [7]. This modification is clearly involved in cell cycle progression [8]. In mammalian cells this modification initiates in the late-replicating/early condensing heterochromatin surrounding the centromeres during G2 phase and is coincident with the initiation of chromosome condensation of centromeres [9]. At the beginning of mitosis in prophase this phosphorylation spreads throughout the euchromatin in the chromosomes arms [10]. This distribution pattern is maintained until the transition from anaphase to telophase when histone H3 gets dephosphorylated. Because of this distribution pattern and timing, phosphorylation of histone H3 at Ser10 (H3S10P) is often referred as a marker of mitosis. On the other hand, it is believe that H3S10P can also function as a “chromatin-opening factor” in interphase for a subset of genes, allowing many different elements to access the chromatin, keeping it in a more open state enabling transcription [11-13]. Remarkably, H3S10P seems to be highly dependent on post-translational modification of neighboring residues amino acids. This is the case of H3 (tri) methylation at Lys9 (H3K9me3) that gave rise to the binary switch

hypothesis. It is for example known that constitutive heterochromatin is enriched with H3K9me3 and that the heterochromatin protein HP1β is recruited to these sites, thus mediating heterochromatin organization and gene silencing [14]. Conversely, it was shown that when cells start mitosis, the same histone H3 is phosphorylated at Ser10 thereby promoting the ejection of HP1β [15]. This probably facilitates the release of tightly bound factors from the chromatin, inducing its rearrangement to higher-order structures which are required for chromosome condensation [16].

It has been shown that mitosis specific H3S10 phosphorylation occurs via Aurora B kinase [17]. The Aurora kinases family consists of evolutionary conserved serine/threonine kinases that are important for centrosome duplication, mitotic spindle assembly, chromosome condensation, alignment and segregation [18]. The mammalian Aurora family includes three protein kinases referred as Aurora A, B and C [19]. Among these kinases, Aurora B is said to function in both early and late mitotic events, including chromosomes segregation and cytokinesis [20]. Immunofluorescence studies have shown a temporal and spatial relationship between Aurora B, chromosome condensation and phosphorylation of H3S10 [21]. It has also been shown that in opposition to the kinases, mitotic histone H3 phosphorylation is also regulated by at least one phosphatase protein [22]. Work carried out with *Xenopus* egg extract has shown that the phosphatase PP1C can directly dephosphorylated histone H3 at Ser10 and that its inhibition activates Aurora B, increasing the levels of H3S10P [23]. Thus, a balance between both kinases and phosphatases activity appears to be in place to regulate histone H3 phosphorylation [24].

Considerable studies have been carried out about phosphorylation of histone H3 at Ser10 in different organisms, however there is little information about this histone modification in mammalian embryos [25-27]. These studies showed that H3S10P behaviour in early mouse embryos differs from somatic cells, with genome-wide phosphorylation in interphase. In fact, preimplantation embryos are very interesting to investigate when it comes to chromatin organization, simply because of their unique chromatin architecture [28, 29]. To form an embryo, two specialized cells, the gametes, need to undergo genome reprogramming, meaning that their chromatin structure needs to be reshaped in order to create a totipotent zygote. Among the reprogrammed nuclear compartments, pericentromeric heterochromatin tends to form chromocenters in somatic cells [30]. However, in mammalian preimplantation embryos pericentromeric heterochromatin is organized in rings around the Nucleolar Precursor Bodies (NPBs) in the pronuclei of the 1-cell embryos. It is only at 2-cell stage that

the pericentromeric heterochromatin of different chromosomes clusters together forming the characteristic heterochromatin domains called chromocenters [31, 32]. Two very well established markers of pericentromeric heterochromatin are the epigenetic modification H3K9me3 and the protein HP1β that have been widely used to study chromatin arrangement and nuclear organization in preimplantation embryos [33-38]. However, there is a clear parental asymmetry in early preimplantation embryos until the 4-cell stage and H3K9me3 as well as HP1β, constitute the major markers for maternal pericentric heterochromatin only. Other epigenetic markers characterize pericentromeric heterochromatin (H4K20me3 or H3K27me3) but none of them have been observed in both parental inherited genomes [36].

Previous studies suggested that perinucleolar heterochromatin in 1-cell embryos could contain H3S10P marker but did not investigate this hypothesis in details [25, 26, 27, 39]. As H3S10P correlates to heterochromatin organization/condensation in different cells and organisms, we decided to further investigate H3S10P in preimplantation mouse embryos with a specific focus on pericentromeric heterochromatin. Therefore, we followed the spatial and temporal pattern of phosphorylation of histone H3 at Ser 10 in *in vivo* mouse embryos from fertilization to the blastocyst stage. We compared its nuclear localization with HP1β distribution and performed immunoFISH as well, using specific pericentromeric heterochromatin DNA probes. Our results clearly indicate that H3S10P staining partially co-localizes with HP1β in 1- and 2-cell embryos, even in interphase, and fully overlaps with the pericentromeric DNA probes. It labels the heterochromatin rings around NPBs in both parental pronuclei of 1-cell embryos and later on also co-localizes on the chromocenters appearing during the 2-cell stage. Remarkably, we also observed that interphasic H3S10P staining on heterochromatin regions disappears after the 4-cell stage, concomitantly with interphasic Aurora B staining.

Based on these findings, we believe that H3S10P is a good marker of pericentromeric heterochromatin and that it can be used to further investigate epigenetic regulation and heterochromatin mechanisms in early preimplantation embryos.

MATERIALS AND METHODS

Animal care and handling were carried out according to European regulations on animal welfare.

Embryo production

C57/CBA F1 female mice, 6-8 weeks of age, were superovulated with 5 IU of PMSG (pregnant mare serum gonadotropin) followed by injection with 5 IU of hCG (human chorionic gonadotropin) 48 hours later. For *in vivo* embryo production, females were placed together with males (one by one) after hCG administration. Embryos were collected (at either at 1- or 2-cell stage) in M2 medium and then cultured in M16 at 37C in a humidified atmosphere containing 5% CO2 until fixation for immuno-fluorescent staining. Fertilization occurred at about 12 h after hCG injection which was used as reference point for embryonic development (hours post-hCG, i.e., hphCG).

Embryo immuno-fluorescent staining

The following antibodies were purchased from the indicated companies: rabbit polyclonal antibody against H3S10P (Abcam #5176); mouse monoclonal antibody against HP1β (Euromedex #MOD-1A9-AS) and rabbit polyclonal antibody against Aurora B (Santa Cruz Biotechnology #ARK-2 H-75); FITC-conjugated anti-rabbit & TRITC-conjugated anti-mouse secondary antibodies (Immunoresearch, Jackson laboratories).

Embryos at different developmental stages were fixed with 4% PFA (paraformaldehyde) in PBS at 4C overnight and permeabilized with 0.5% Triton X-100 (15 min, room temperature). The fixed embryos were blocked in PBS containing 2% BSA (1hour at room temperature) and incubated overnight at 4C with the specific first antibody diluted in 2% PBS-BSA (H3S10P 1:300; HP1β 1:200; Aurora B 1:100). The embryos were then washed twice in PBS to remove any first antibody excess. After this step the embryos were incubated with fluorescent labeled secondary antibody for 1 hour at room temperature (1:200). DNA counterstaining was performed with ethidium homodimer 2 (Invitrogen). Embryos were then post-fixed with 2% PFA for 15 min at room temperature, washed and mounted on slides with an antifading agent Citifluor under coverslips.

All the staining experiments were repeated at least three times with not less than 15 embryos analysed per stage.

ImmunoFISH

FISH pericentromeric DNA probes (major satellites probes) were prepared by PCR on genomic mouse DNA using the two following primers followed by Cy5-labeling through random priming (Invitogen Kit, Ref 18095-011): 5'-CATATTCCAGGTCCTTCAGTGTGC-3' and 5'-CACTTTAGGACGTGAAATATGGCG-3'.

For this procedure H3S10P immunostaining was performed first and the immunolabeled embryos were then further processed for 3D-FISH. All steps were performed at room temperature unless otherwise specified. Zona pellucida was removed with Tyrode's acid (Sigma). The embryos were then rinsed in PBS and gently plated with a minimum amount of PBS on glass slides to allow adherence. They were then post-fixed in 4% PFA for 30 min, permeabilized for 30 min in 0.5 % Triton X-100, and rinsed once for 5 min in 2x saline-sodium citrate (SSC) pH 6.3. RNA digestion was performed by incubation with 200µg/ml RNase (Sigma) in 2xSSC for 30 min at 37C. After rinsing twice for 5 min in 2xSSC at room temperature, the slide was equilibrated in the hybridization buffer (50% formamide, SCC 2X, Denhardt 1X, 40 mM NaH2PO4, 10% dextran sulfate) for 1 to 2h. Denaturation was performed separately for probes and embryos during 10 min at 85C in the hybridization buffer. After deposition of the probes onto the slide, embryos were then placed in an humidified chamber at 37C for 24 h. After rinsing twice in 2x SSC at 42C, samples were permeabilized in 0.5 Triton X-100 for 10 min, rinsed with PBS for 5 min and DNA counterstained with propidium iodide for 15 min at 37C (Sigma, 1 µg/ml). The embryos were then post-fixed in 2% PFA for 15min and a last wash was done in PBS for 5 min. The immunoFISH experiments were repeated two times with not less than 15 embryos analysed per time point.

High resolution microscopy

3D-preserved embryos were observed either with an "Apotome" fluorescent microscope or a confocal Laser-Scanning Microcospe (Zeiss LSM 510). Immunofluorescent stainings were observed using a Carl Zeiss AxioObserver fluorescence microscope equipped with the Apotome slider (MIMA2 Platform, INRA). The samples were observed with a 63x Plan-Neofluar oil objective (NA 1.3) and digital optical sections were collected every 1µm using a Z-series acquisition feature. immunoFISH experiments were visualized with a Zeiss LSM 510 confocal laser scanning microscope equipped with an oil-immersion objective (Plan Apochromatic 63X n.a.1.4) and imaging was performed with the 488-, 535- and 633-nm wavelengths of the lasers. Entire embryos were scanned with 0.37 µm distance between light optical sections.

RESULTS

Distribution pattern of H3S10P from fertilization to implantation

To begin with, we followed the spatial and temporal pattern of H3S10P by immunostaining in *in vivo* fertilized mouse embryos from the 1-cell stage (fertilization) to the blastocyst stage (implantation). Where possible, embryos were fixed at two different time-points during the embryonic cell cycle, i.e. in G1/S-phase (G1 is often very short during the first cell cycles and difficult to distinguish from the S-phase) or in G2 and then during mitosis [40]. As shown in Figure 1, we observed H3S10P signal in the nucleoplasm of interphasic and mitotic blastomeres until the 4-cell stage. During the first embryonic cycle, histone H3 phosphorylation at Ser10 was present in both male and female pronuclei. H3S10P displayed homogeneous staining in the whole nucleoplasm and intensely marked NPBs periphery (Nucleolar Precursor Bodies) during both the S-phase (~18 hphCG) and G2-phase (~28 hphCG) (Figure 1A and 1B). During the second cell cycle, the staining for H3S10P signal was also maintained in the nucleoplasm and an intense signal was still seen around NPBs of early 2-cell embryos in G1/S-phase (~36 hphCG) (Figure 1D). Later on, during the G2-phase (~48 hphCG), pronounced H3S10P staining appeared in foci (Figure 1E) not associated with any nucleoli, which become then transcriptionally active [41, 42]. During the first and second mitosis, phosphorylation of histone H3 at Ser10 was detected on the whole chromosomes following a similar pattern to that of somatic cells (Figure 1C and 1F).

During the 4-cell stage, H3S10P was clearly detected in the interphasic blastomeres uniformly labeling the nucleoplasm and staining more strongly specific regions, i.e. the nucleolar periphery in G1/S-phase (~50 hphCG) and isolated foci in G2 (~58 hphCG) (Figure 1G and 1H). However, H3S10P labeling began to differ from the earlier embryonic stages. The diffuse nucleoplasm staining and the perinucleolar rings intensity started to weaken when compared to previous stages, especially during the G1/S-phase of the cell cycle.

From the 8-cell to the blastocyst stage, H3S10 showed exactly the same phosphorylation/ dephosphorylation kinetics seen in somatic cells. Early interphasic H3S10P staining did not exist anymore and intense foci only appeared in G2-phase spreading throughout the euchromatin in the chromosomes arms upon mitosis (Figure 1I until 1O). At 8-cell embryos for instance, when blastomeres are still synchronized, no labeling of H3S10P was seen at all in early interphase (~63 hphCG) (Figure 1J) and foci were only noticed at the end of the cell cycle, during the G2 phase (~77 hphCG) (Figure 1K). Upon the transition from the 8- to 16-cell stage, labeling was clearly observed in prophase/ metaphase/anaphase but not in

telophase (Figure 1L) as already described in mitotic somatic cells. In the more advanced stages like morula and blastocyst, blastomeres are no longer synchronized and various H3S10P staining patterns could be observed simultaneously: no staining, foci or whole-chromosome staining that most probably illustrate Ser10 phosphorylation during cell cycle progression as in somatic cells (Figure 1N and 1O).

H3S10P peculiar pattern in 1-cell and 2-cell stage embryos

Since the nuclear distribution pattern of H3S10P in early preimplantation embryos seemed very peculiar and different from the one observed in somatic cells, we pursued our observations and analyzed more developmental time-points at the 1-cell and 2-cell stages (every two hours approximately from 16 to 50 hphCG). Because we used *in vivo* fertilized embryos, some asynchrony can be observed between several mice and describing 1-cell stage becomes difficult. We therefore used the nomenclature that we previously set-up to describe the morphological changes of the pronuclei occurring during the first cell-cycle [43] and classified embryos from PN0 (fertilization) to PN5 (end of the 1-cell stage).

As shown on Figure 2, H3S10P signal was present shortly after fertilization in both sets of parental inherited genomes, i.e. the decondensing sperm head and the maternal chromatin going through the last steps of meiosis II (Figure 2A). H3S10P staining was quite homogeneous although some dense foci could be observed in the maternal genome. This staining pattern was maintained upon formation of the pronuclei (PN1, Figure 2B). At PN2, diffuse nucleoplasm H3S10P labeling was still present but some accumulations appeared at NPBs periphery (Nucleolar Precursor Bodies) (Figure 2C). This perinucleolar staining became more intense in PN3 stage (that corresponds to the beginning of the S-phase) forming complete rings around the NPBs (Figure 2D). The same signal was observed in PN4 and PN5 embryos, i.e. upon exit of the S-phase and during the G2-phase (Figure 2D, 2E and 2F). During mitosis of the first embryonic cycle, H3S10P staining covered the entire length of all chromosomes arms (Figure 2G and 2H).

After formation of the nuclei in early 2-cell stage (~32hphCG, Figure 2I), a diffuse H3S10P staining was observed within the nucleoplasm as well as intense perinucleolar rings, as in late 1-cell embryos. This staining was maintained upon the S-phase (~36hphCG, Figure 2J). It is only at the beginning of the G2-phase (~42hphCG, Figure 2K) that isolated foci of H3S10P, not attached to any NPBs, appeared. Finally, by the end of the 2-cell stage, H3S10P perinucleolar rings had almost completely disappeared; conversely at that time the number of H3S10P nucleoplasmic foci was much more numerous (~48hphCG, Figure 2L).

Co-localization with the pericentromeric heterochromatin marker, HP1β

We observed that the immunodetection of H3S10P in early preimplantation embryos is quite different from somatic cells and that its most intriguing features are the perinucleolar staining detected in G1 phase at the 1- and 2-cell stages and the transition to intense foci, often not related to any nucleoli, during the G2-phase of the second cell cycle. This correlates with the distribution of pericentromeric heterochromatin around NPBs at 1-cell and the formation of the so-called chromocenters, when the pericentromeric regions of several chromosomes assembled together, as previously described by ourselves and others [31, 32, 35]. Here we used the well-established pericentromeric marker HP1β to further characterize H3S10P distribution in relation to this type of heterochromatin and performed double immunostaining. In pronuclear stage embryos (20 hphCG), H3S10P and HP1β uniformly label the nucleoplasm of both pronuclei. Interestingly, co-localization of both markers within the heterochromatin rings around the NPBs is only seen in the maternal pronucleus (Figure 3A). We were indeed unable to detect the same perinucleolar signal for HP1β in the paternal PN as previously described [31-33]. The striking observation during this stage is that some heterochromatin left in the periphery of the nucleus is stained by HP1β but not with H3S10P (Figure 3A, arrowhead). As for early 2-cell embryos (36 hphCG), HP1β was partially detected around the NPBs (Figure 3B) with the exception of rare single foci accumulating at the nuclear periphery (Figure 3B, arrowheads). However H3S10P was only concentrated around the NPBs forming rings and excluded from the nucleoplasmic isolated foci (Figure 3B, arrowheads). In late 2-cell embryos (48 hphCG), HP1β is localized within the newly formed chromocenters as well as H3S10P; only few HP1β foci did not contain H3S10P at the same time (Figure 3C). Conversely, H3S10P staining, not colocalized with HP1β, could be observed on some nucleoli and also within small nucleoplasmic aggregates (Figure 3C, arrowheads). This suggests that H3S10P indeed labels pericentromeric heterochromatin in G1/S-phase of early embryos and that supplementary H3S10P labeled domains appear in late G2, underlying the entrance into mitosis.

In later stages such as blastocysts, H3S10P and HP1β completely co-localize on the chromocenters of some blastomeres only, most probably those going through the G2 phase (Figure 3D). In early interphasic blastomeres only HP1β could be observed within pericentromeric heterochromatin, as no H3S10P was ever detected. Conversely, HP1β staining was diffuse in the nucleoplasm of mitotic blastomeres whereas H3S10P was concentrated on chromosomes.

H3S10P co-localization with pericentromeric DNA repeats using immunoFISH

ImmunoFISH is a refined technique that makes it possible to specifically check co-localization of proteins and DNA sequences. We therefore used it to check co-localization of H3S10P with the DNA repeats that constitute pericentromeric heterochromatin using probes for major satellites. This technique was performed under conditions which preserved the 3D nuclear structures of the embryos [35, 44].

In 1-cell embryos, H3S10P staining clearly co-localized with the pericentromeric repeats forming rings around the NPBs of both pronuclei, except for some pericentromeric heterochromatin foci seen in the periphery of the pronucleus (Figure 4A). As for the 2-cell stage, in earlier examples, the perinucleolar rings labeled with the major satellite probes also stained for H3S10P, showing faithful co-localization (Figure 4B). At that stage, the very few chromocenters already present were labeled with the major satellite probes and not H3S10P (Figure 4B, arrowhead). In contrast, for late 2-cell embryos, co-localization of H3S10P with major satellite sequences was obvious on the chromocenters. Scarcely, few nucleoli show some pericentromeric heterochromatin signal. However it always co-localized with H3S10P (Figure 4C). Some aggregates of H3S10P that do not correspond to pericentromeric heterochromatin are seen in the nucleoplasm in late G2-phase, as already suggested by the double immunostaining of H3S10P and HP1β (Figure 4C).

Altogether, these observations show that H3S10P almost perfectly labels the pericentromeric heterochromatin of both parental origins at the 1- and 2-cell stages, except for some isolated foci at the nuclear periphery.

Presence of Aurora B staining is coincident with interphasic H3S10P signal

In somatic cells, the kinase responsible for the mitotic phosphorylation of histone H3 at Ser10 is the Aurora B kinase. This kinase phosphorylates the histone H3 at Ser10 starting from late S/G2 phase maintaining this phosphorylation until anaphase. We therefore decided to perform immunodetection of Aurora B kinase in preimplantation mouse embryos to check whether maintenance of interphasic H3S10P signal was related to the presence of this kinase. Interestingly, Aurora B staining was detected during the G1/ S-phases of 1-, 2- and 4-cell embryos, concomitantly to histone H3 phosphorylation (Figure 5A, 5B and 5C). On the other hand, in 8-cell embryos Aurora B staining was no longer observed during S-phase (Figure 5D) which coincides with the lack of H3S10P signal at this stage. Aurora B signal was only detected in 8-cell embryos going through late G2-phase (Figure 5E), when H3S10P signal was also present. It therefore seems that Aurora B might well be involved with phosphorylation of histone H3 at Ser10 during interphase in early embryos, as well as with the mitotic phosphorylation of histone H3 at Ser10 during mitosis in late ones.

DISCUSSION

In this study, we have used indirect immunofluorescence, immunoFISH and high resolution microscopy to investigate the spatial distribution of an epigenetic modification, phosphorylation of histone H3 at serine 10, in preimplantation mouse embryos. In somatic cells, histone H3 phosphorylation initiates during G2 in pericentric foci prior and is lost during anaphase. However, this epigenetic modification clearly presents unusual kinetics in mammalian embryos as it is present from early interphase through mitosis over several embryonic cycles. In the interphase of these early developmental stages, H3S10P stains not only the entire nucleoplasm but also the pericentromeric heterochromatin, i.e. the perinucleolar regions or the chromocenters. In this study, we performed for the first time that a precise observation during the whole preimplantation period, trying to distinguish G1/S versus G2 phases of each embryonic cycle. These results are in agreement with previous global observations made by Huang and collaborators who compared *in vivo* versus *in vitro* fertilized preimplantation mouse embryos [27]. By looking more precisely at pericentromeric heterochromatin, we clearly observed a shift during the 2- and 4-cell stages when this heterochromatin moves away from the nucleoli and clusters together assembling the chromocenters [31, 32]. Remarkably, at 4-cell, the interphasic staining of H3S10P was

weaker when compared to earlier embryonic stages and disappeared completely at the 8-cell stage. This probably reflects the shift from the H3S10P embryonic phosphorylation pattern to the somatic one. Indeed, at 8-cell embryos, H3S10P was solely noticed on the chromocenters at the end of G2 phase and as expected on the chromosomes of mitotic blastomeres. The same spatial distribution was observed in morula and blastocyst stages with H3S10P labeling the chromocenters in G2 and the chromosomes during mitosis.

It has been shown that the mitotic phosphorylation of histone H3 at Ser10 is mediated by one of the kinases of the Aurora family, named Aurora B [45]. In mammalian cells, co-localization of Aurora B and H3S10 phosphorylation was detected from late G2 phase until metaphase [46]. The few studies done so far in oocytes pointed out a role for Aurora B as the kinase behind the phosphorylation of histone H3 at Ser 10. It is believed that this kinase is responsible for the chromatin remodeling and spindle formation during meiosis I [24]. In our study, we observed for the first time Aurora B staining during the G1/ S-phases of 1-, 2- and 4-cell embryos, concomitantly to the maintenance of interphasic histone H3 phosphorylation. Similarly, after the 8-cell stage, Aurora B staining follows H3S10P kinetics and can be detected only upon mitosis. It therefore seems that Aurora B and H3S10P are closely related in preimplantation embryos. This is confirmed by Aurora kinase inhibition with a specific drug, Zm447439, which impairs phosphorylation of histone H3 at Ser10 and causes aberrant chromosome condensation during mitosis in 1-cell stage embryos [25].

However, mitotic histone H3 phosphorylation is the result of a balance of competing kinase and phosphatase activities [23] and Aurora B kinase may indeed be negatively regulated through interactions with phosphatases like PP1 or PP2A [16, 17]. When we attempted to perform immunostaining to detect PP1 in preimplantation embryos, we detected a diffuse spotted signal in anaphase/telophase both during the first and second cell cleavages. Quantification of the signal intensity however suggested that PP1 concentration is much lower in these embryos than in later stages (data not shown).

Interestingly, it was recently shown that another member of the Aurora kinase family, Aurora C, might also be involved in meiotic progression and phosphorylation of histone H3 at Ser10 in mouse oocytes [47]. Aurora C could therefore also be a good candidate for H3S10P regulation during interphase of the first four cell cycles in preimplantation mouse embryos. Further study is clearly required to elucidate all the mechanisms behind this peculiar kinetic of H3S10 phosphorylation.

Several authors have already suggested that H3S10P interphasic staining observed in early embryos likely corresponds to pericentromeric heterochromatin domains, however they did

not demonstrate it [25, 26, 27, 39]. Here we demonstrate for the first time a clear correlation between H3S10P and pericentromeric heterochromatin in 1- and 2-cell embryos by double immuno-stainings and immunoFISH. Indeed, immunoFISH with major satellite DNA probes clearly shows that H3S10P is labeling the major satellite sequences that form these pericentromeric heterochromatin rings around the NPBs in 1- and 2-cell embryos. When we compared H3S10P marker with HP1β, we clearly saw a better co-localization of H3S10P and pericentromeric probes in the heterochromatin rings around the NPBs, especially as it labels the paternal PN when HP1β does not. Remarkably, some HP1β/ major satellites foci isolated at the nuclear periphery at 1-cell embryos did not contain any H3S10P labeling. These foci had already been previously described both by immunostaining and immunoFISH [31, 35]. At this stage, it has been suggested that chromosomes are forming "cartwheels" around the NPBs [31]. It would be interesting to see whether the absence of H3S10P from some pericentromeric domains would explain why some chromosomes are not associated to NPBs.

When looking at early 2-cell embryos, it appears that HP1β is partially detected in the pericentromeric heterochromatin rings around the NPBs, however in lower amounts than H3S10P. At the end of second cell cycle, when the heterochromatin undergoes impressive rearrangement, H3S10P and the major probes completely overlap on the newly formed chromocenters that also contain HP1β as previously shown [35]. It seems possible that this heterogeneity of pericentromeric labeling by the two markers during the second cell cycle in fact corresponds to differences between the two parental genomes. It has also been previously suggested that in 2-cell embryos the HP1β-positive chromocenters contain maternal inherited pericentromeric heterochromatin already labeled in 1-cell stage whereas HP1β-negative chromocenters correspond to the paternal part [32]. Altogether this suggests that H3S10P is faithfully following all the pericentromeric heterochromatin movements in early embryos, better than HP1β.

It is also worth noticing that H3S10P is part of the switch involving H3K9me3 and HP1β. It is know that when histone H3 is phosphorylated at serine 10, HP1β is ejected from the complex in cells in culture [48]. However, this is not the case in early stage embryos since our immunostaining results showed that H3S10P co-localizes with HP1β even in G1/S-phase. This is especially true for the maternal inherited genome as the paternal pronucleus does not stain for HP1β on the enriched intense heterochromatin areas [31, 32], or only very weakly [34]. The work done by Mateescu and collaborators states that phosphorylation of histone H3 at serine 10 is not enough by itself to eject HP1β from the complex formed with H3K9me3

and that H3K14 acetylation is also required [49]. Consequently, further studies need to be carried out to understand the mechanism behind HP1β association/ejection.

Some other epigenetic marks also have an asymmetric distribution between the two parental genomes, e.g. H3K27me3 or H3K9me3 [36]. Altogether it appears that the paternal pronucleus is positive for H3k27me3 and H3S10P but negative for H3K9me3 and HP1β. On the contrary, the maternal pronucleus is positive for H3K9me3 and H3S10P and HP1β. It is tempting to speculate that in the paternal pronucleus the lack of H3K9me3 and HP1β and presence of H3S10P, could explain why the paternal genome is more accessible to transcription factors being replicated and transcribed earlier than the maternal one. Conversely, the presence of H3K9me3, $HP1_{\beta}$ and H3S10P in the maternal pronucleus, would account for the more repressive state of the maternal heterochromatin and could explain its very late replication timing [50, 51].

Despite of all these findings about phosphorylation of histone H3 at Ser10, there is still need for some elucidation about this epigenetic modification during embryonic development. The maintenance of H3S10P from fertilization until the 4-cell stage suggests that H3S10P is involved with gene expression whereas in mitosis it most likely recruits factors that act on chromatin condensation. In interphase of the later stages, when H3S10P is only present on the heterochromatin clumps at the end of the G2 phase, this epigenetic modification must function as a signal, indicating to the cell that replication is complete and that mitosis can proceed (as in somatic cells). It is tempting to speculate that H3S10P purpose during interphase of 1- and 2-cell embryos is to function as a factor holding the open-state of the pericentromeric heterochromatin in order to allow transcription to happen. It has indeed been recently shown that pericentromeric transcripts are involved in the recruitment of HP1β and formation of chromocenters [52, 53]. Interestingly, these transcripts are present in 1-cell/2-cell embryos whereas a sharp downregulation is observed at 4-cell/8-cell ones. This kinetic correlates with the peculiar H3S10P dynamic within pericentromeric heterochromatin. It would therefore be very interesting to use H3S10P as a marker to unveil the fundamental mechanisms behind pericentromeric heterochromatin movements in other species with later formation of chromocenters, e.g. bovine [37].

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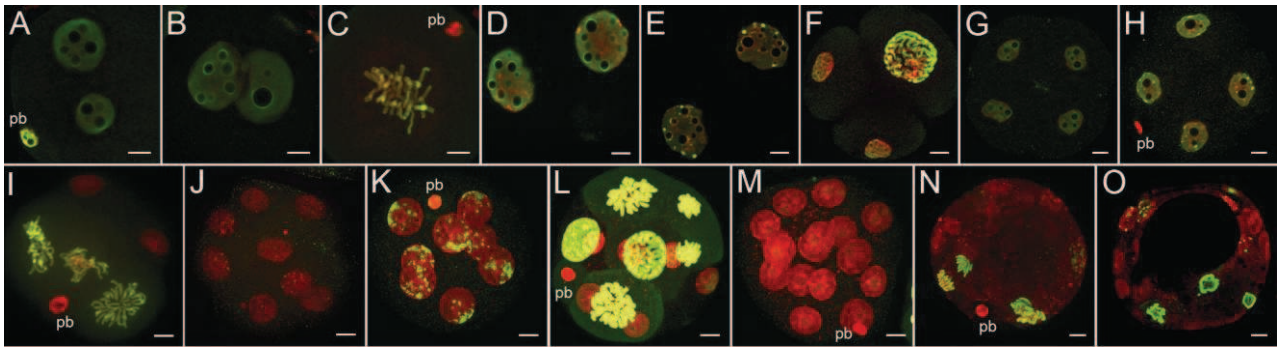


Figure 1: Distribution of H3S10P during the entire mouse preimplantation development. Immuno-fluorescent staining of H3S10P (green) and DNA (red) was performed on preimplantation embryos at different time points: at 18hphCG (**A**) and 28hphCG (B) for 1-cell, 30hphCG for metaphase of first mitosis (C), 36hphCG (D) and 48hphCG (E) for 2-cell, 50hphCG for 3-cell (F) and early 4-cell (G), 58hphCG for late 4-cell (H), 63hphCG for transition for 5-cell (I) and early 8-cell (J), 77hphCG for late 8-cell (K), 79hphCG for 12-cell (L), 79hphCG for 16-cell (M), 89hphCG for morula (N) and 96hphCG for blastocyst(O).

All the embryos were observed on an inverted Apotome microscope and z-series were performed for later analysis. Representative single z-sections are shown here for 1-cell to 4-cell embryos as well as for morula and blastocyst. Z-series projections are shown for 5-cell to 16-cell embryos. Note that remains of the second polar body are often present (pb). Scale bars: 10µm.

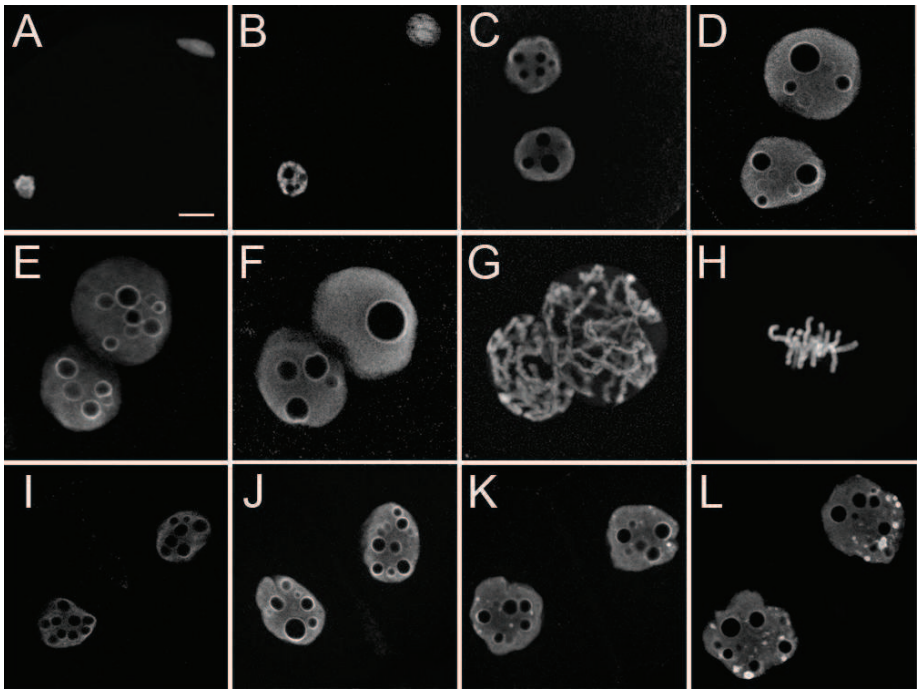


Figure 2: Distribution of H3S10P during the first and second cell cycle in mouse embryos. Immuno-fluorescent staining of H3S10P was performed on 1- and 2-cell stage embryos. At the 1-cell stage (from 16hphCG until 29hphCG), we classified the embryos into PN0 (A, just after fertilization), PN1 (B, formation of the pronuclei), PN2 (C, pronuclei increase in size but remain at the nuclear periphery), PN3 (D, large pronuclei now in the center of the cytoplasm), PN4 (E, large pronuclei close to each other) and PN5 (F, pronuclei are then apposed). For all these PN stages we chose to turn the images in order to have the paternal pronuclei in the upper part. Two representative embryos in prophase and anaphase of the first mitosis (29/30hphCG) are also shown (G and H, respectively). On the last row are representative images of 2-cell embryos at 32hphC (I), 36hphCG (**J**), 42hphCG (**K**) and 48hphCG (**L**). All these images are single light-optical sections taken on the Apotome microscope, except for the two embryos in mitosis for which Z-series projections are shown. Scale bar: 10µm

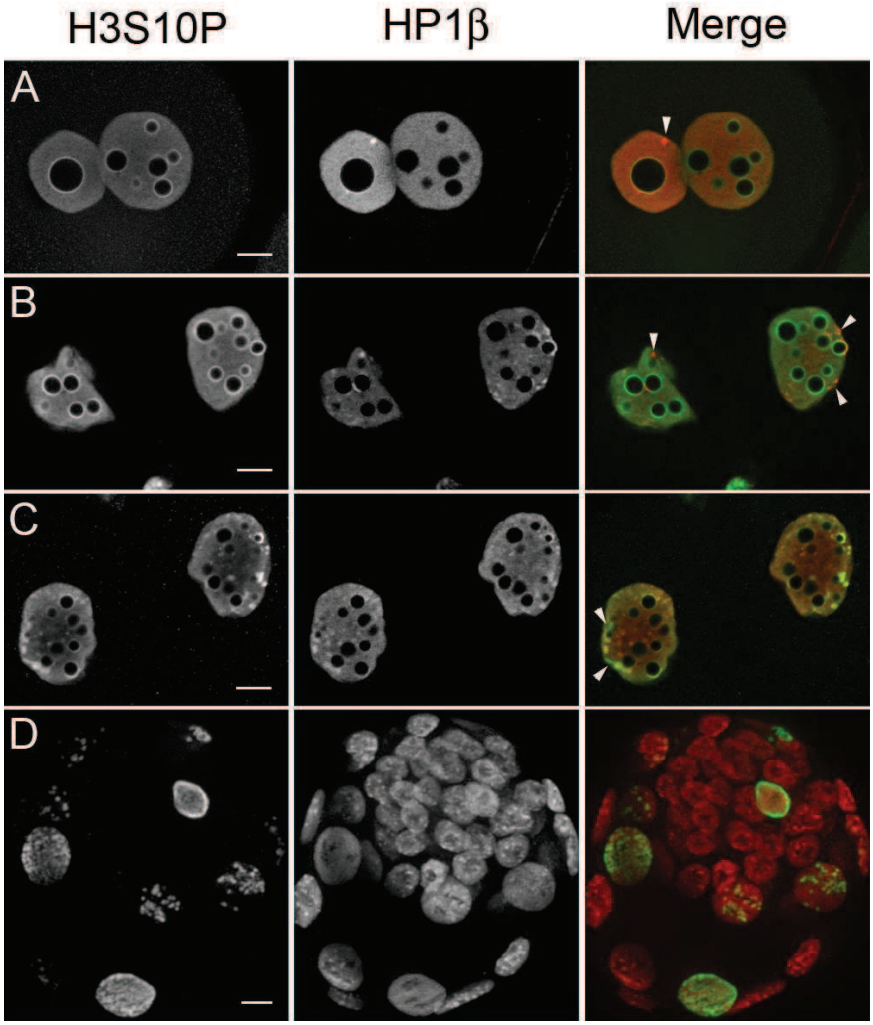


Figure 3: Distribution of H3S10P in relation to pericentromeric heterochromatin protein HP1β. Distribution of H3S10P (green) and HP1β (red) was analyzed in 1-cell (**A**, 20hphCG), early (B, 36hCG) and late 2-cell (C, 48hphCG) and blastocyst stage (D) on the Apotome microscope. Representative light-optical sections are shown here. Obvious co-localization of H3S10P and HP1β in the nucleoplasm is observed in 1-cell and early 2-cell stage embryos except for some HP1β foci, often located at the nuclear periphery (arrowheads in A and B). Perfect co-localization of both markers was also observed in late 2-cell embryos especially within the newly formed chromocenters. However some H3S10P staining not colocalized with HP1β could also be observed (arrowheads in C). In blastocysts (D), H3S10P and HP1β completely co-localize on some blastomeres only, within the chromocenters. Scale bars: 10μm

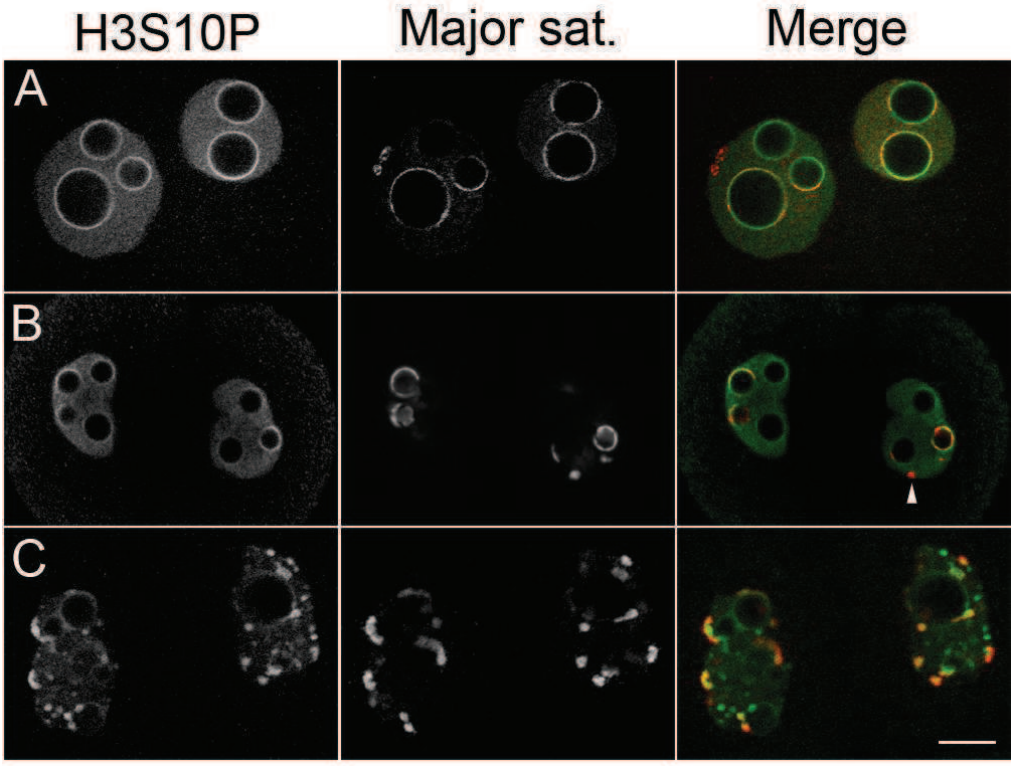


Figure 4: Co-localization of H3S10P and pericentromeric heterochromatin

3D immunoFISH with H3S10P antibody (green) and major satellite probes for pericentromeric DNA repeats (red) was performed on 1-cell (A, 24hphCG), early (B, 36hphCG) and late 2-cell embryos (C, 48hphCG). Z-series were taken for each embryo on the LSM510 confocal microscope. Single z-sections are shown on this figure. A: note the co-localization of H3S10P and pericentromeric repeats around the NPBs and presence of some isolated pericentromeric foci at the nuclear periphery. B: complete co-localization between H3S10P and pericentromeric repeats is observed in early 2-cell, except for isolated chromocenters (arrowhead). C: in late 2-cell embryos, H3S10P perfectly overlaps with the pericentromeric probes in the newly formed chromocenters but supplementary H3S10P foci appeared. Scale bar: 10µm

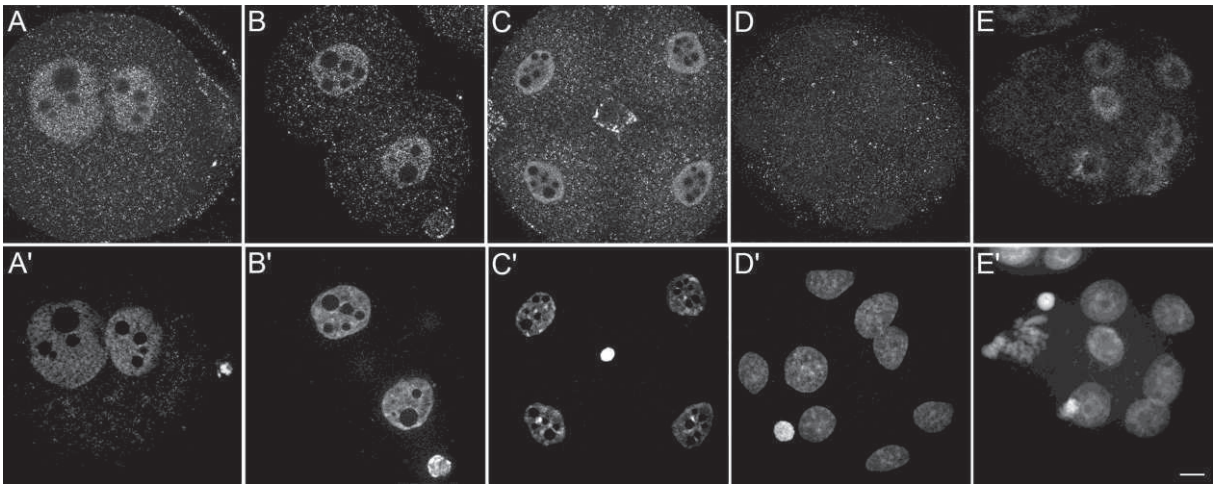
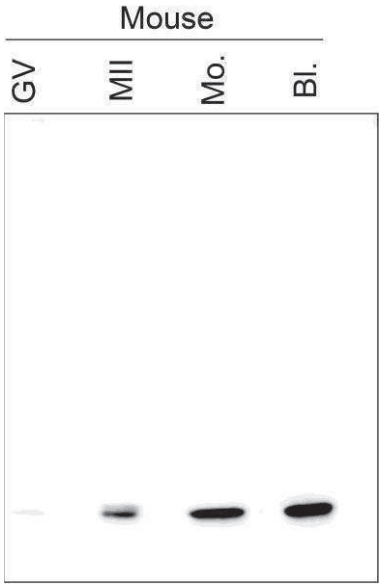


Figure 5: Detection of Aurora B kinase in preimplantation mouse embryos

Aurora B immuno-fluorescent staining (upper panel) with DNA counterstaining (lower panel) was performed during S-phase in 1-cell (A, 24hphCG), 2-cell (B, 36hphCG) and 4-cell embryos (C, 50hphCG) and samples were observed on an inverted Apotome microscope. A diffuse staining was observed in all these embryos (single light-optical sections from z-series). On the other hand, 8-cell embryos in S-phase did not show any signal (D, 63hphCG, projection of z-series); it appeared only in late 8-cell (E, 74/78hphCG, projection of z-series). Scale bar: 10µm

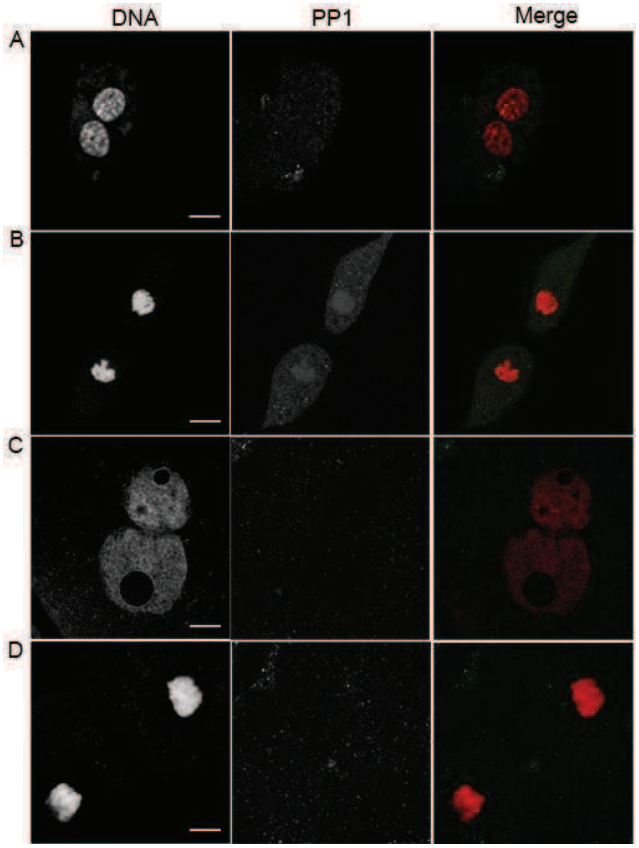
2.2 Supplementary figures



Supplementary Figure 1: Detection of H3S10P by Western blot.

Total proteins were extracted from oocytes and embryos and then subjected to SDS-PAGE as previously described (Gall et al., 2008). Immunoblot was performed using a commercially available rabbit polyclonal anti-H3S10P antibody (Abcam #5176).

Four samples were compared: mouse immature oocytes (GV stage), metaphase II as well as morula and blastocyst stages. The antibody recognized a 17-kDa protein in MII oocytes (150 oocytes were loaded). The same antibody failed to detect this 17-kDa protein in GV oocytes (n=148) as it was expected from the literature (Swain et al., 2007). Conversely, a single band at ~20kD could be detected in morula (n=248) and blastocysts (n=205). This result indicates that the antibody used is specific for histone H3 phosphorylation at serine 10 in mouse embryos.



Supplementary Figure 2: PP1 detection in mouse fibroblasts and early embryos.

Immunostaining was performed using the same protocol as described in the first paper with slight differences: the antibody used was a mouse monoclonal antibody against PP1 (Millipore #07-1217, dilution 1:300). Images were acquired on the Apotome microscope as previously described. Representative images are shown here (single z-sections, bar: 10µm).

A striking difference in the levels of PP1 is observed in mouse fibroblast between interphase (**A**) and telophase during mitosis (**B**). However, in early mouse embryos this difference in PP1 levels was not as obvious between 1-cell embryo in interphase (**C**) and telophase of the first cell cycle (**D**). Note that PP1 staining seems nearly absent in the preimplantation mouse embryos. However, after some image quantification tests we concluded that PP1 was present in early mouse embryo but in very low quantity. We indeed evaluated that the staining intensity was 2 times higher in telophase than in interphase for fibroblasts (89,7 vs. 43,9; arbitrary units of intensity) and not statistically different in embryos (6,1 in telophase vs. 3,9 in interphase). Therefore, we cannot state that PP1 is completely absent from preimplantation mouse embryos and supplementary experiments (especially western blots) are required. However this very low level could explain why we do not see histone H3 Ser10 dephosphorylation during the early embryonic stages in mouse embryos.

Chapter Three: Nuclear dynamics of histone H3 trimethylated on Lys9 and/or phosphorylated on Ser10 in mouse cloned embryos as new markers of reprogramming?

3.1 Introduction

The fact that H3S10P can be classified as a marker of pericentromeric heterochromatin in preimplantation embryos, prompted us to examine this epigenetic modification in cloned embryos. The main idea was to use this epigenetic modification as a tool to investigate how the donor nucleus was being reprogrammed, if it was acquiring a similar nuclear pattern as seen in a normal embryo, especially in regards to the pericentromeric heterochromatin rearrangement. It is known that the heterochromatin is presented in blocks (chromocenters) in the mouse somatic cell nucleus and that these blocks of heterochromatin are not present in its 1-cell embryos. Instead the heterochromatin of the two gametes rearranges itself forming rings of heterochromatin around the NPBs at the pronuclear stage. As is known nuclear organization and chromatin conformation are all epigenetically regulated. The donor nucleus injected in the enucleated oocyte should therefore undergo epigenetic changes losing its somatic characteristics and restructuring its heterochromatin conformation to acquire the embryonic one. According to research carried out by Martin et al. (2006b), cloned mouse embryos display an irregular distribution of constitutive heterochromatin. It was found that there were up to six blocks of heterochromatin not associated to the rings in the NPBs in clones, whereas in normal embryos usually only one isolated heterochromatin block is detected which is not associated to the NPBs. As can be seen, cloned mouse embryos usually fail to achieve the characteristic zygote organization of constitutive heterochromatin. Therefore, as our main hypothesis was to check if H3S10P was also a marker of pericentromeric heterochromatin in early embryos, the aim of this part of the work was to investigate if this epigenetic modification could be used as indicative of the quality of nuclear reprogramming after SCNT in terms of heterochromatin reorganization. In other words, we sought to apply this epigenetic marker to track down the rearrangements of this type of heterochromatin through its spatial distribution, in order to characterize the state of chromatin reorganization in reconstructed embryos. For this purpose we compared the distribution pattern of H3S10P to that of H3K9m3 and HP1β which are well-established pericentromeric heterochromatin markers, as well as to the double modification, H3K9me3S10P which is also said to be related to this type of heterochromatin in somatic cells. Therefore, H3S10P could be a tool to further evaluate the nuclear reprogramming status of the donor nucleus to try to understand even better the fundamental mechanisms behind the chromatin reorganization and nuclear reprogramming by SCNT.

3.2 Original Paper #2

This manuscript has just been submitted to “Cellular Reprogramming”.

A supplementary figure about H3S10P in *in vitro* fertilized embryos is shown next (§ 3.3).

NUCLEAR DYNAMICS OF HISTONE H3 TRIMETHYLATED ON LYS9 AND/OR PHOSPHORYLATED ON SER10 IN MOUSE CLONED EMBRYOS AS NEW MARKERS OF REPROGRAMMING?

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Running Title: H3S10P and H3K9me3S10P in mouse cloned embryos

ABSTRACT

Cell Nuclear Transfer refers to the injection of a donor nucleus into an enucleated egg. However, this technique is not quite efficient and it is believed that one of the causes for this is incorrect genome reprogramming. Embryos produced by nuclear transfer (cloned embryos) very often present abnormal epigenetic signatures and irregular chromatin reorganization and of these two issues, chromatin rearrangements within the nuclei after transfer is the least studied.

It is known that cloned embryos often present pericentromeric heterochromatin clumps very similar to the chromocenters structures present in the donor nuclei. It is therefore believed that the somatic nuclear configuration of donor nuclei, especially that of the chromocenters, is not completely lost after nuclear transfer, i.e. not well reprogrammed.

To further investigate pericentromeric heterochromatin reorganization after nuclear transfer, we decided to study its rearrangements in cumulus-derived clones using several related epigenetic markers such as H3S10P, H3K9me3 and the double marker H3K9me3S10P.

We observed that from all these markers H3S10P and H3K9me3S10P are the ones found on the part of the pericentromeric heterochromatin which is well remodelled, resembling exactly the normal embryonic heterochromatin configuration. Conversely, H3K9me3 and HP1β associated protein were also detected in the perinuclear clumps of heterochromatin, making obvious the maintenance of the somatic epigenetic signature within these nuclear regions. Our results thereby demonstrate that H3S10P and H3K9me3S10P can be applied to evaluate heterochromatin reorganization following nuclear reprogramming.

INTRODUCTION

Nuclear transfer (or cloning) is a technique with broad applications such as the production of patient ES cells (regenerative medicine), the creation of models for the study of human diseases, improvement of animal production in agriculture, the protection of endangered species and especially the opportunity to deeply investigate how epigenetic and structural changes occur in the somatic nucleus during nuclear reprogramming (Yang et al., 2007). After nuclear transfer it is known that the donor nucleus injected into the enucleated egg must undergo biochemical changes to reverse the established constraints on the genetic potential imposed by the process of differentiation (Kikyo and Wolffe 2000). After fertilization the genetic material of the two specialized cells, sperm and oocyte, undergoes deep remodelling to acquire the embryonic genome configuration in order to start the process of development. Similarly, the donor cell nucleus must lose its original genome conformation and be fully reorganized to acquire the embryonic genome configuration and as a result proper gene regulation essential for normal development. However, the efficiency of this reversal will determine the subsequent developmental success of the reconstructed embryo and despite the successful production of clones of different species by nuclear transfer, this technique still shows extremely low success rates with high abortion and fetal death rates as well as a variety of abnormalities including obesity, large placenta and abnormal expression of genes important for development (Kang and Roh, 2010).

It is thought that these undesirable results are due to inappropriate genome reprogramming in this type of embryo which in turn leads to aberrant epigenetic status' such as abnormal DNA methylation and irregular histone modification patterns (Kang & Roh, 2010). However, most of the reports focused on the presence/absence of epigenetic modifications in reconstructed embryos and few analyzed the impact of the inadequate epigenetic status on chromatin reorganization within the resulting nuclei. In previous studies we demonstrated such chromatin rearrangement abnormalities in cloned embryos after ES and cumulus cell nuclear transfer using a marker of pericentromeric heterochromatin, HP1β (Martin et al 2006a; Maalouf et al., 2009).

Undeniably, after fertilization, both parental genomes reorganize to form pronuclei, which display a peculiar distribution of pericentromeric heterochromatin with the centromeres distributed mostly around the Nucleolar Precursor Bodies (NPBs) forming a ring-like structure, while the rest of the chromosomes most probably stretch out to the periphery of the nucleus (Martin et al., 2006b). This 'cartwheel' organization exclusive to that stage has been suggested to maintain transcriptional silencing during parental genome maturation.

In embryos obtained by nuclear transfer it has been shown that pericentromeric heterochromatin is rapidly reorganized as in naturally fertilized embryos (Martin et al., 2006a; Merico et al., 2007; Maalouf et al., 2009). However, reprogramming after nuclear transfer is not perfect and aberrations are quite frequent: remains of somatic-like heterochromatin clumps are often observed in late 1-cell and early 2-cell clones (Martin et al., 2006b; Maalouf et al., 2009). These heterochromatin clumps are very similar to the chromocenters seen in the nucleus of somatic cells from G1. Chromocenters are formed by the clustering of various centromeres of different chromosomes being basically constituted by pericentromeric and the bordering centromeric heterochromatin (Alcobia et al., 2000; Alcobia et al., 2003).

Interestingly, we have evidenced a link between the developmental inefficiency of cloned embryos and aberrant chromatin reprogramming. We indeed observed that ES cell nuclei, that give a higher rate of survival to term after cloning, undergo better remodelling after nuclear transfer than cumulus cell nuclei (Maalouf et al., 2009). Importantly, incubation of cloned mouse embryos in the very early hours after transfer with an histone deacetylase (HDAC) inhibitor, improved the structural remodelling of pericentric heterochromatin at 1-cell and dramatically increased the rate of full term development (a 10 fold increase) (Maalouf et al., 2009).

Altogether it indicated that the nuclear configuration of donor nuclei, and especially pericentromeric heterochromatin clustering into chromocenters, was not completely lost after nuclear transfer and that it impaired development. To further investigate this hypothesis we decided to follow pericentromeric heterochromatin rearrangements in cumulus-derived clones using various epigenetic markers. Therefore, we performed immunostainings on the two most obvious and important stages after meiosis resumption, i.e. the 1-cell stage with formation of the pronuclei and the 2-cell stage when embryonic genome activation normally occurs.

Firstly we analyzed phosphorylation of H3 at serine 10 (H3S10P). In mammalian cells, phosphorylation of histone H3 at serine 10 is first evident in pericentromeric heterochromatin in late G2-interphase cells, spreading throughout the chromosomes arms during prophase and only getting dephosphorylated around late anaphase (Perez-Cadahia et al., 2009). The fact that H3S10P is only detected at the end of interphase and observed in the entire chromosomes lengths led to the conclusion that this epigenetic modification could be related to chromosome condensation (Garcia et al., 2005). However, this post-translational modification has also been observed outside mitosis and it is believed that it is involved with

gene activation (Lim et al., 2004; Drobic et al., 2010). Some research groups have investigated this epigenetic modification during early mouse embryogenesis. Their reports all suggest that H3S10P is linked to pericentromeric heterochromatin (WangQ et al 2006, Huang et al 2007 and Teperek-Tkacz et al 2010).

As it is known, the chromocenters are also characterized by trimethylation of histone H3 at Lysine9 (H3K9me3) that provides a binding site for HP1β; this complex then induces transcriptional repression and heterochromatinization in the pericentromeric heterochromatin domains (Lachner et al., 2001). In fertilized embryos, these two markers are distributed asymmetrically between maternal and paternal pronucleus: at the pronuclear stage only the maternal pronucleus contains H3K9me3 and HP1β (Cowell et al., 2002; Santos et al., 2005).

Likewise, H3K9me3, HP1β and H3S10P, the double modification H3K9me3S10P (trimethylation of histone H3 at Lysine 9 phosphorylated at Serine 10) is also detected in the pericentromeric heterochromatin domains in somatic cells. It was first stated that both modifications coexist on the same histone tail especially during mitosis based on the results obtained by *in vitro* assays and analysis of the *in vivo* modification pattern of H3 isolated from HeLa cells (Fischle et al., 2003). However, its correlation with pericentromeric heterochromatin was later described in somatic cells by peptide competition assays and immunofluorescence experiments (Hirota et al., 2005). In fact, H3K9me3S10P can be detected at late G2 in interphase; it was found enriched in the centric and pericentric domains with a more spotted appearance on the chromosome arms during mitosis (Fischle et al., 2005; Monier et al., 2007). Only one study mentioned this epigenetic modification in fertilized mouse embryos, showing that upon the first mitosis it was preferentially associated with maternal chromosomes (Hayashi-Takanaka et al. 2009).

In this study, we verified the status of three epigenetic markers, related to pericentromeric heterochromatin (H3K9me3, H3S10P, and H3K9me3S10P), in reconstructed mouse embryos at the 1- and 2-cell stages to find out if one of them could be used as another tool to monitor chromatin reorganization after nuclear transfer.

MATERIAL & METHODS

Animal care and handling were carried out according to European regulations on animal welfare.

Oocytes and embryos production

C57/CBA F1 female mice, 6-8 weeks of age, were superovulated with 5 IU of PMSG (pregnant mare serum gonadotropin) followed by injection with 5 IU of hCG (human chorionic gonadotropin) 48 hours later. For *in vivo* embryo production, females were placed together with males (one by one) after hCG administration. Embryos were collected in M2 medium containing 1 mg/ml hyaluronidase and then cultured in M16 at 37°C in a humidified atmosphere containing 5% CO2 until fixation for immunofluorescent staining. Fertilization occurred at about 12 h after hCG injection which was used as reference point for embryonic development (hours post-hCG, i.e., hphCG). To overcome asynchrony problems, we used the nomenclature that we previously set-up to describe the morphological changes of the pronuclei occurring during the first cell-cycle (Adenot et al., 1997) and classified embryos from PN0 (fertilization) to PN5 (end of the 1-cell stage).

Cumulus Cell Nuclear Transfer

Oocytes were prepared by superovulating C57/CBA mice. Superovulation was induced by injecting pregnant mare serum gonadotropin (PMSG, Intervet, 5 UI) and human chorionic gonadotropin (hCG, Intervet, 5 UI) at intervals of 48 hours. Oocytes were collected from oviducts 14 hphCG and washed in M2 medium containing 1 mg/ml hyaluronidase. Subsequently, they were incubated in M2 containing 5 μg/ml cytochalasin B and placed in a chamber on the stage of an inverted microscope (Nikon) equipped with micromanipulators (Nikon-Narishige MO-188). The chromatin spindle (visualized under differential interference contrast) was aspirated into the pipette as previously described (Zhou et al., 2000). For nuclear transfer, donor chromosomes were derived from cumulus cells that previously surrounded the oocytes, gently aspirating them in and out of the injection pipette (inner diameter 7–8 μm) followed by microinjection into the cytoplasm of the enucleated oocytes. The nuclear transfer embryos were activated by incubation for 6 h in Ca^{2+}-free medium containing 10 mM Sr^{2+}, 5 μg/ml cytochalasin B. Embryos with visible nuclei were then considered as activated, transferred into fresh M16 medium and cultured at 37°C in a humidified atmosphere containing 5% CO_2. Embryos were fixed during the first cell cycle (at 4, 5, 8 and 10 hours post-activation, hpa), and early and late 2-cell stages (21 hpa and 33 hpa respectively).

Immunofluorescent staining

The following antibodies were purchased from the indicated companies: rabbit polyclonal antibody against H3S10P (Abcam #5176); mouse monoclonal antibody against HP1β (Euromoedex #MOD-1A9-AS); rabbit polyclonal antibody against H3K9me3 (Upstate #07-523); rabbit polyclonal antibody against H3K9me3S10P (Abcam #5819); FITC-conjugated secondary antibody and Cy5-conjugated secondary antibody from donkey (Immunoresearch, Jackson laboratories).

Embryos in different developmental stages were fixed with 4% PFA (paraformaldehyde) in PBS at 4°C overnight and permeabilized with 0.5% Triton X-100 (15 min, room temperature: RT). The fixed embryos were blocked in PBS containing 2% BSA (1hour at RT) and incubated overnight at 4°C with the specific first antibody diluted in 2% PBS-BSA (at 1:300 for H3S10P; 1:400 for HP1β; 1:400 for H3K9me3 ; 1:300 for H3K9me3S10P). The embryos were then washed twice in PBS to remove any first antibody excess. After this step the embryos were incubated with FITC or Cy5 labeled secondary antibody for 1 hour at RT (dilution 1:200). DNA counterstaining was performed with ethidium homodimer 2 or propidium iodide (Invitrogen). Embryos were then post-fixed with 2% PFA for 15 min at RT, washed and mounted on slides with an antifading agent (Citifluor) under coverslips.

High resolution microscopy

3D-preserved embryos were observed with either a Carl Zeiss AxioObserver Zl fluorescence microscope equipped with the ApoTome slider or a Zeiss LSM 510 confocal laser scanning microscope (MIMA2 Platform, INRA). On the Apotome**,** embryos were observed using a 63x Plan-Neofluar oil objective (NA 1.3) and single wavelength LEDs at 470 nm, 530 nm and 625 nm (Colibri illumination). Digital optical sections were collected using a Z-series acquisition feature every 0.24 μm. As for the confocal system, embryos were visualized with an oil-immersion objective (Plan Apochromatic 63X NA 1.4) and imaging was performed with lasers at 488-, 535- and 633-nm wavelengths. Entire embryos were scanned with a distance of 0.37 μm between light optical sections.

RESULTS

H3S10P distribution pattern in fertilized and cumulus cloned embryos

As previously described (Huang et al., 2007), histone H3 was phosphorylated at serine 10 shortly after fertilization: the decondensing sperm head was already labelled for H3S10P as well as the maternal chromosomes (n=27 PN0 stages at 18hphCG; **Fig 1**). Upon formation of the pronuclei, a diffuse nucleoplasm H3S10P labeling was present in all embryos with some accumulations appearing at NPBs periphery (Nucleolar Precursor Bodies) (n=35 PN1 stages at 18 hphCG; **Fig 1**). This perinucleolar staining then formed full heterochromatin rings around the NPBs (n=29 PN2 stages at 20hphCG) that became more intense in late 1-cell embryos (n=33 PN4 stages at 26hphCG; **Fig 1**). When entering mitosis of the first cell cycle, H3S10P staining had spread out throughout the whole chromosomes length (n=25 at 28-30hphCG; **Fig 2**). For early 2-cell stage embryos, the staining was again present uniformly in the nucleoplasm and more intensely on the heterochromatin rings surrounding the NPBs (n=18 at 36hphCG; **Fig 1**). As the second cell cycle progresses, the pericentromeric regions of different chromosomes are assembled together to form these unique structures called chromocenters that are strongly labeled with H3S10P (n=48 at 48hphCG; **Fig1**). Regarding the second cell cycle, phosphorylation of histone H3 at serine 10 was also detected on the whole chromosomes following the same pattern as seen during the first mitosis (data not shown).

We also performed immunodetection of H3S10P in *in vitro* fertilized embryos to identify any abnormality that could have been caused by the embryo culture conditions. Based on our observations from fertilization to late 2-cell stage, we can state that the same distribution pattern for H3S10P is observed in *in vivo* fertilized and *in vitro* fertilized embryos (data not shown).

We then checked constitutive heterochromatin rearrangements by nuclear reprogramming in cloned mouse embryos produced by somatic cell nuclear transfer with cumulus cells. In these embryos, phosphorylation of histone H3 at Ser10 was detected as early as 2hpa (post activation) on the chromatin of the mouse cumulus cell which is undergoing PCC (Premature Chromosome Condensation) inside the enucleated mouse oocytes (n=22; **Fig 1**). At 4hpa when pseudo-pronuclei are formed, H3S10P recapitulates exactly the same pattern as observed in early fertilized embryos with uniform staining in the nucleoplasm (n=19 PN1 stages, **Fig 1**). Only 3 of these embryos showed a slight labeling of the heterochromatin rings

surrounding the NPBs. One hour later, 100% of the cloned embryos analyzes had these perinucleolar rings labeled with H3S10P (n=17 PN2 stages, 5hpa, **Fig 1**). At the end of the 1-cell stage, all the cloned embryos showed a strong H3S10P labelling corresponding to the one observed in fertilized embryos (n=16 at 7hpa; n=17 at 8hpa and n=29 PN4 stages at 10hpa, **Fig 1**).

Regarding the 2-cell stages, all the cloned embryos had the same H3S10P pattern as seen in fertilized ones (**Fig 1**). At 21hpa (corresponding to early 2-cell), H3S10P was observed uniformly in the nucleoplasm and a strong signal was still seen on the heterochromatin rings at NPBs periphery. This staining was then replaced by labeling in the chromocenters forming at late 2-cell (33hpa).

Remarkably, cumulus cells had a typical somatic cell pattern for H3S10P and only very few showed a positive signal, i.e. in late G2 and mitosis (**Fig 3**). However, 100% of the cloned embryos we observed were positive for this marker over the two first embryonic cycles suggesting that reprogramming had happened.

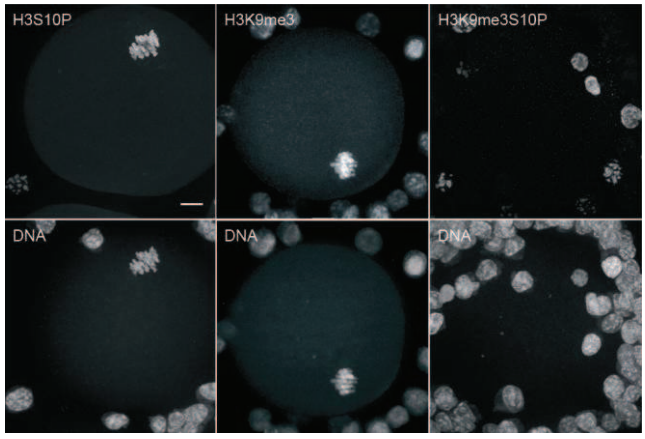

Figure 3. H3S10P / H3K9me3 / H3K9me3S10P detection in cumulus cells. Metaphase II embryos with remaining cumulus cells were processed to detect H3S10P / H3K9me3 / H3K9me3S10P. Representative images (confocal single z-sections) are shown on the upper panel, with DNA counterstaining on the lower panel. Note the difference in cell numbers positively stained between the three markers; H3K9me3 is the marker which presents the highest number of positive cumulus cells. The fact that H3K9me3 is commonly observed in somatic cells at G1, whilst H3S10P and H3K9me3S10P are only observed at the G2 phase, certainly explains this difference. Scale bar: 10µm.

H3S10P and HP1β label distinct types of heterochromatin in early cloned embryos

In order to evaluate whether H3S10P reprogramming after cloning was concomitant with nuclear remodelling and especially heterochromatin rearrangements, we then analyzed the co-localization of H3S10P and HP1β. As mentioned before, HP1β has been extensively used as a marker to check pericentromeric heterochromatin distribution and it was demonstrated that some portion of pericentromeric heterochromatin was accumulating at the nuclear periphery in embryos after nuclear transfer (Martin et al., 2006; Merico et al, 2007; Maalouf et al., 2009).

We observed a high number of HP1β perinuclear accumulations already at 4hpa, as well as a uniform euchromatic staining, but none of these HP1β foci were labelled with H3S10P (n=11, **Fig 4**). These foci are similar to the ones observed in the cumulus donor cells (**Fig 3**). Remarkably, HP1β and H3S10P showed co-localization only when heterochromatin rings appeared around the NPBs. At 10hpa HP1β was still observed in numerous perinuclear foci but also around all the NPBs. However H3S10P did not show any co-localization with HP1β in the clumps of heterochromatin found at the nuclear periphery, but only in the ones surrounding the NPBs (n=21; **Fig 4**).

At the 2-cell stage, HP1β followed the same distribution pattern as previously described. In early 2-cell (21hpa) HP1β was detected in the nucleoplasm, around the NPBs and in isolated foci. As in 1-cell cloned embryos, H3S10P showed almost no co-localization with HP1β in isolated foci but only on NPBs periphery (n=15, **Fig 4**). This difference disappeared by the late 2-cell stage (36hpa) when both markers mostly co-localized within the newly formed chromocenters (n=10, **Fig 4**).

Based on these findings, we suspected that cloned mouse embryos have two types of heterochromatin after nuclear transfer: one that is being remodeled, showing H3S10P and HP1β, around the NPBs and another one, with only HP1β, at the nuclear periphery. In order to confirm whether this perinuclear heterochromatin indeed corresponds to non-reprogrammed somatic heterochromatin we then focused on another typical marker of pericentromeric heterochromatin: H3K9me3.

Comparison with H3K9me3

As expected, only the maternal genome stained for this marker in fertilized embryos while the paternal one showed no labeling (n=18 PN0/PN1 at 18hphCG; **Fig 5 upper panel**). This staining was then distributed all over the nucleoplasm and formed partial rings around NPBs (n=21 PN2 stages at 20/21phCG). At 26hphCG we observed full staining of the NPBs periphery and, remarkably, we regularly noticed one clump of H3K9me3 at the nuclear periphery similar to the clumps previously observed with HP1β (n=32 PN4 stages; Martin et al., 2006). We later confirmed that H3K9me3 and HP1β are co-localized within these perinuclear clumps (n=33, data not shown). Finally, we observed that the paternal genome became faintly stained upon entry in mitosis (n=33, **Fig 2**) whereas H3K9me3 strongly labelled almost the whole maternal genome as expected (Puschendorf et al., 2008)

In comparison, both pseudo-pronuclei formed in early cloned mouse embryos showed H3K9me3 staining and no asymmetry (**Fig 5 upper panel**). At 4hpa and 6hpa (n=16 PN1 stages and n=14 PN2 stages, respectively), we observed heterogeneous nucleoplasmic staining and partial perinucleolar rings (as in fertilized embryos) but also a high number of H3K9me3 perinuclear foci similar to the ones observed in the cumulus donor cells (**Fig 3 and 5**). At 10hpa, H3K9me3 was still present in numerous perinuclear foci but was then clearly accumulated all around the NPBs (n=25 PN4 stages, **Fig 5 upper panel**). Again, this suggested that cumulus inherited heterochromatin was not fully remodeled after nuclear transfer.

As previously described, H3K9me3 followed the same distribution pattern as HP1β in 2-cell stage fertilized and cloned embryos (**Fig 4** and **Fig 5**; Merico et al., 2007, Maalouf et al., 2009): asymmetric diffuse staining in the nucleoplasm with few perinuclear accumulations and strong perinucleolar stainings were observed at 36hphCG (n=16 early 2-cell, **Fig 5 upper panel**) whereas the nucleoplasm was homogeneously stained at 48hphCG with intense labeling appearing on the chromocenter-like structures (n=18 late 2-cell). Cloned embryos showed a similar dynamic with the exception of supplementary isolated foci in early 2-cell embryos (n=9 at 21hpa and n=16 at 36hpa, **Fig 5 upper panel**). Altogether it clearly appeared that H3K9me3 staining was very similar to the one observed for HP1β, both in 1-cell and 2-cell cloned embryos, and that it partially differed from H3S10P after nuclear transfer.

H3K9me3S10P as a new marker of nuclear reprogramming

It is well documented that H3K9me3 is a marker of heterochromatin. Conversely, some studies stated that the double modification H3K9me3S10P could also be a marker of heterochromatin staining chromocenters in G2-phase of somatic cells. As this double modification had never been carefully investigated in mouse embryos, we analyzed its redistribution after fertilization and nuclear transfer.

In fertilized embryos, H3K9me3S10P could only be detected in the maternal genome upon fertilization (n=14 PN0 at 18hphCG, **Fig 5 lower panel**). Upon formation of the pronuclei, heterochromatin accumulations then appeared around the NPBs (n= 14 PN1 stages and 16 PN2 stages at 20/21hphCG, **Fig 5 lower panel**). We noticed that H3K9me3S10P intensity was decreasing from 20hphCG onwards (n= 14 at 27hphCG, **Fig 5 lower panel**). However, in late 1-cell embryos nice heterochromatin rings could be seen in all the female pronuclei (PN5 stages, n=15 at 29hphCG and n= 14 at 30hpCG, **Fig 2**). Remarkably, both parental genomes were labelled during mitosis, although the paternal remained more weakly stained and could still be distinguished (n=22 at 29-30hphCG, **Fig 2**).

Before nuclear transfer, cumulus cells had a similar somatic cell pattern for H3K9me3S10P: only very few showed a positive signal, i.e. in late G2 and mitosis (**Fig 3**). However, H3K9me3S10P was present within all the cloned embryos undergoing PCC (n=12 at 2hpa, **Fig 5 lower panel**) and both pseudo-pronuclei showed strong staining of heterochromatin clumps just after (n=20 PN1 stages at 4hpa, **Fig 5 lower panel**). We then observed a clear remodelling of these regions during the first cell cycle (n=20 PN2 stages at 7hpa, **Fig 5 lower panel**) although the overall intensity seemed to decrease as in fertilized embryos. Finally, H3K9me3S10P was concentrated only on the NPBs periphery in late 1-cell cloned (n=20 PN4 stages at 10hpa, **Fig 5 lower panel**) and not on any perinuclear heterochromatin accumulations, as already observed for H3S10P.

In early and late 2-cell stage embryos H3K9me3S10P pattern was very similar in fertilized and cloned embryos. Just after cleavage, H3K9me3S10P accumulations were observed mostly around the NPBs and in some isolated foci (n= 21 early 2-cell at 36hphCG and n=20 late 2-cell at 21hpa, **Fig 5 lower panel**). This staining was then replaced by clumps on chromocenters in late 2-cell embryos (n=14 at 48hphCG and n=15 at 33hpa, **Fig 5 lower panel**). The only difference between the two groups was that H3K9me3S10P staining accumulated in one pole of the nuclei (most probably the maternal inherited one) whereas this asymmetry was completely lost in clones.

In conclusion, H3K9me3S10P staining did not correspond to the combination of H3K9me3 and H3S10P staining demonstrating that these two epigenetic modifications are not always adjacent within the same histone H3 tail in mouse early embryos. It also clearly appears that H3K9me3S10P double modification is a good marker to follow pericentromeric heterochromatin remodeling after nuclear transfer.

DISCUSSION

One of the main issues with the nuclear transfer technique is genome reorganization of the somatic donor nucleus, a process triggered by nuclear reprogramming which is controlled by the enucleated oocyte. For that reason, in this study we have investigated the dynamics of genome restructuring in early mouse embryos derived from cumulus nuclear transfer with an emphasis on pericentromeric heterochromatin related markers such as H3S10P, H3K9m3, HP1β and the double modification H3K9m3S10P.

Our results show that indeed the cumulus donor nucleus is remodelled to a certain extent in cloned mouse embryos as evidenced by the chromatin configuration seen as early as 4hpa (~ 18hp hCG). At this time point H3S10P showed the same distribution pattern as observed in fertilized embryos. The pseudo-pronuclei showed uniform staining for H3S10P in the nucleoplasm and strong labelling in the heterochromatin rings around the NPBs from 5hpa to 10hpa. However, at 10hpa we observed that both H3K9me3 and HP1β accumulated around the NPBs but also in the nuclear periphery, an aberration known to correlate with poor development (Martin et al., 2006b; Maalouf et al., 2009). Differently from these two markers, H3S10P only co-localized with the heterochromatin located around the NPBs. Altogether, this indicates that H3S10P, as opposed to H3K9me3 and HP1β, only labels remodelled pericentromeric heterochromatin located around the NPBs, resembling exactly the normal embryonic heterochromatin arrangement. This also proves that heterochromatin clumps located at the nuclear periphery are unremodelled, maintaining the epigenetic signature of the cumulus cells (with H3K9me3 and HP1β staining on chromocenters).
As for the other epigenetic modification studied, H3K9me3S10P, a strong staining was also detected in heterochromatin accumulations in the nuclear periphery but only at very early stages (4hpa). Later on (7hpa/10hpa), the spatial distribution of H3K9me3S10P shifted and the double modification only co-localized with the heterochromatin rings around the NPBs.

We can hypothesize that this double modification was being progressively reprogrammed during the first cell cycle and finally overlapped with the portion of the remodelled heterochromatin. In light of these results, we are presently convinced that both epigenetic modifications H3S10P and H3K9me3S10P can be applied to chase reprogrammed heterochromatin during nuclear remodelling.

The perinuclear accumulations seen in 1-cell stage cloned embryos with H3K9me3 and HP1β staining are probably due to irregular chromatin rearrangement. The reason for this preferential positioning is unknown, however we can infer that oocytes proteins responsible for nuclear and chromatin organization are involved. It is known that the somatic chromatin configuration must be reshuffled by the reprogramming factors present in the cytoplasm of the enucleated oocyte. Among them are the nuclear lamin filaments that lie on the interface of the nuclear envelope and chromatin playing a major role in nucleoskeleton support, chromatin remodeling, as well as protein recruitment to the inner nucleolus (Hall et al., 2005). It has also been suggested that additional "motor proteins" are in place to assist with chromatin organization, such as nuclear actin in mouse embryos (Nguyen et al., 1998). It is not known how and what makes chromatin move inside the nucleus and if this process happens in a coordinated or random way. However, it might be that during the nuclear transfer procedure lamins and motor proteins are disrupted, and that as a result chromatin is misplaced. It would be of great relevance to further investigate this hypothesis, in order to better understand the role of these proteins in nuclear and chromatin organization within the early stages of reprogramming.

In fertilized embryos, it unmistakably appears that H3S10P is a better marker of pericentromeric heterochromatin as compared to other epigenetic markers such as H3K9me3 and H3K9me3S10P, since it is always correlated to this type of heterochromatin in both inherited parental genomes, from the very beginning of development. Indeed, both H3K9me3 and H3K9me3S10P epigenetic modifications showed parental asymmetry over the whole first cell stage. Similarly to H3K9me3 (Cowell et al., 2002; Santos et al., 2005; this study), only the maternal PN is labelled for H3K9me3S10P epigenetic modification in the rings around the NPBs and a diffuse staining is observed in the nucleoplasm at PN1. However, a slight decrease in H3K9meS10P intensity is seen starting from 20h phCG (~PN2). This decrease most probably corresponds to chromatin decondensation and incorporation of new histones

H3 upon the first replication phase as already described for H3K9me3 (Liu et al., 2004; Wang et al., 2007).

Regarding entry in the first mitosis, we observed that all three epigenetic markers were present: 1) H3S10P covered equally both parental genomes, 2) H3K9me3 strongly labelled the maternal genome and faintly stained the paternal one only at the very end of the 1-cell stage and 3) The intensity of H3K9me3S10P clearly increased covering both parental genomes. Conversely, it is known that HP1β proteins preferentially localize within condensed inactive heterochromatin and that it dissociates from chromatin during mitosis (Minc et al., 1999; Hayakawa et al., 2003; Puschendorf et al., 2008). In fact, H3K9me3 and HP1 are working together to propagate heterochromatin and cause gene silencing (Lachner et al., 2001) but the binding of HP1β to the methylated H3-tail is fully reversible and highly dynamic, thereby supporting the rapid exchange of HP1β from heterochromatin (Fischle et al., 2005). It has been proposed in somatic cells that H3S10 phosphorylation prevents the binding of HP1 to the adjacent tri-methylated Lys 9 residue of histone H3 (Fischle et al., 2003; Hirota et al., 2005).
Also separate observations of H3S10P staining versus H3K9me3 staining first gave the impression that the scenario in mouse embryos was in contradiction with the hypothesis made in somatic cells, we then observed that both parental genomes present a strong signal H3K9me3S10P only at the end of G2 phase, exactly when HP1 starts to disappear. We, however, do not know whether additional phosphorylation at the Ser10 occurs on the already tri-methylated Lys9 histone H3 or vice-versa. Experiments using ZM447439 (ZM), an inhibitor of Aurora kinases activity, showed that embryos lacking almost completely H3S10 phosphorylation did not cleave properly (Teperek-Tkacz et al., 2010 and our own unpublished results). Similarly, disruption of the two mouse Suv39h HMTases (Histone Methyl Transferase) that abolishes H3-Lys9 methylation of constitutive heterochromatin induces gestation death or postnatal growth delay (Peters et al., 2001; Peters et al., 2002). Both phosphorylation at the Ser10 and tri-methylation on Lys9 might therefore be involved in embryonic chromosome condensation.

At 2-cell stage, heterochromatin undergoes massive distribution changes, moving from the rings surrounding the NPBs towards the nuclear periphery, to form new heterochromatin domains, the chromocenters (Martin et al., 2006a; Merico et al. 2007). However, in cloned embryos, it appears that the heterochromatin markers H3K9me3 and HP1β have a distinct

behavior. In early 2-cell cloned embryos, the two markers were observed in rings around the NPBs, a characteristic of normal early 2-cell stage embryo, but also in numerous foci within the nucleoplasm (Martin et al., 2006a; Merico et al. 2007; this study). These foci most probably correspond to the remains of unremodelled heterochromatin clumps inherited from the donor cells. On the other hand, we observed that H3S10P and H3K9me3S10P are both labelling only pericentromeric heterochromatin rings around the NPBs in early 2-cell, following the same nuclear movement in fertilized and cloned embryos. It therefore seems that the most important restructuring events occur during the first cell stage, with relocalization of remodelled pericentric heterochromatin towards the NPBs and that cloned embryos do not undergo further reprogramming at the 2-cell stage.

There have been few studies addressing the importance of genome reorganization after nuclear transfer. In these studies, inhibitors of specific epigenetic modifications like DNA methylation and histone deacetylation were applied in an attempt to improve cloning efficiency by improving chromatin remodelling (Yamagata et al., 2007; Wang et al., 2007; Maalouf et al., 2009; Bui et al., 2010). It has been shown that the use of trichostatin A (TSA), an inhibitor of deacetylases, which is known to increase the acetylation levels in somatic cells and in embryos improves cloned mouse development (Wang et al., 2007). Moreover, culturing reconstructed embryos in the presence of this drug improved chromatin reorganization. The centromeric and pericentromeric heterochromatin pattern from the TSA-treated cloned embryos resembled more the spatial distribution seen in fertilized ones and a lower amount of embryos displayed irregular heterochromatin clusters not associated to the NPBs (Maalouf et al., 2009). Therefore, rectifying reprogramming of these epigenetic modifications at an early stage may be a strategy to improve cloning efficiency (Shao et al., 2009). As H3S10P and H3K9me3S10P seem to be good markers to trace remodelled pericentromeric heterochromatin after nuclear transfer, we speculate that, by the use of specific drugs, we could increase the levels of this histone H3 phosphorylation and somehow heterochromatin remodelling could be enhanced. Caffeine, a protein phosphatase inhibitor has for example been used to treat oocytes before nuclear transfer. This treatment increased the frequency of PCC as well as the development of cloned sheep embryos (Lee and Campbell, 2008). These authors believe that this increases the removal of chromatin bound proteins, thus allowing the access of oocyte derived factors involved in the reprogramming of the somatic DNA. The same principle could also be applied in regards to histone H3 phosphorylation. Treating cloned embryos with caffeine would in all probability raise the

levels of the Aurora kinase responsible for H3S10 phosphorylation, making the chromatin more accessible to remodelling factors thus facilitating even more heterochromatin remodeling.

When taken all together, it is clear that genome rearrangement after nuclear transfer is compromised. Therefore, further studies need to be carried out in order to understand more the mechanisms behind nuclear reprogramming, so that new tools can be found and consequently be applied to assist with genome rearrangement and improve the technique. Following this thread of thought, our main intention with this work was to find a new tool to evaluate heterochromatin reorganization upon nuclear reprogramming and both H3S10P / H3K9me3S10P match perfectly with these requisites. However, this only represents one modest step in the vast world of nuclear reprogramming, therefore more research needs to be carried out to find other coadjuvants that can be applied.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that no conflicting financial interests exist.

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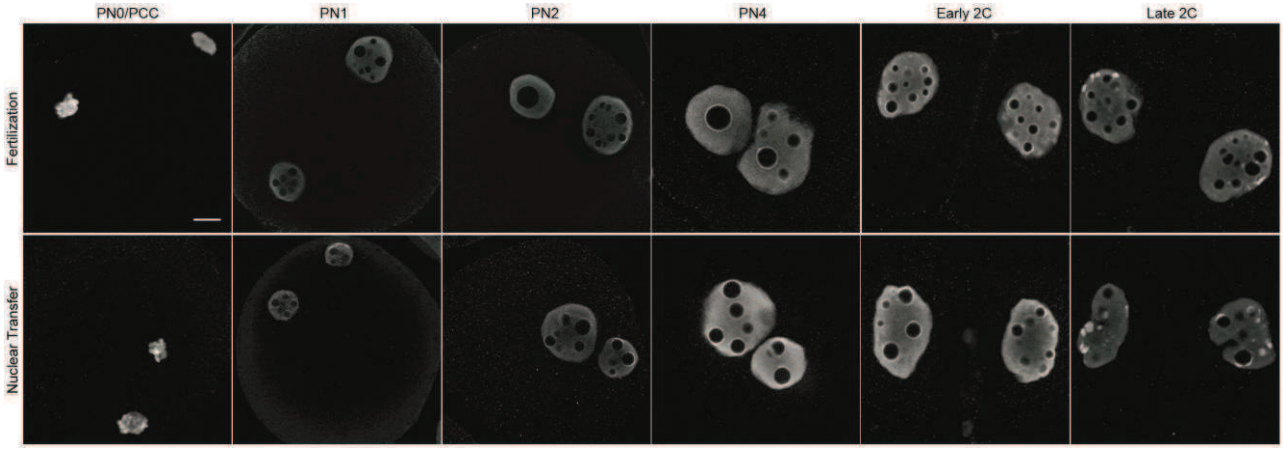


Figure 1. H3S10P detection in fertilized and cloned embryos

Representative images (Apotome single z-sections) of embryos produced by natural fertilization (upper panel) or nuclear transfer (lower panel), and stained for H3S10P are shown here. The same signal which is seen on the parental genomes (decondensing sperm head and oocyte metaphase) at the beginning of development (PN0) in fertilized embryos, is also accurately observed on the PCC in cloned embryos. During pronuclei formation (PN1), both types of embryos present the same diffuse nucleoplasm H3S10P labeling. At PN2, signals for H3S10P in the heterochromatin rings around the NPBs start appearing. Finally, at PN4 in late 1-cell, the heterochromatin rings are completely formed and a strong staining for H3S10P is seen around the NPBs for both types of embryos. At the 2-cell stage, H3S10P was first detected in the heterochromatin rings around the NPBs (Early 2C) and then on the newly formed chromocenters (Late 2C). We again observed a similar distribution pattern in fertilized and cloned embryos. Scale bar: 10µm.

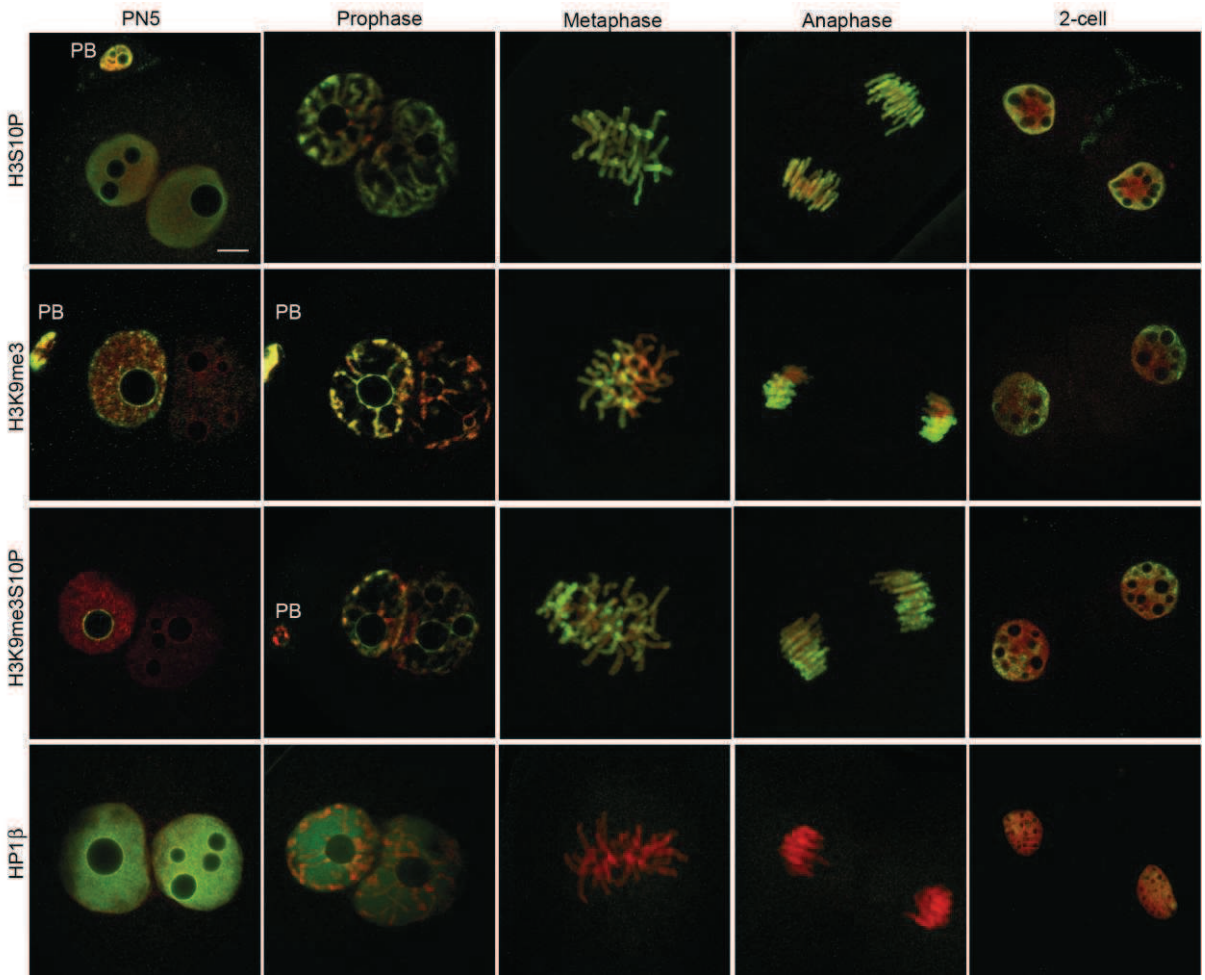


Figure 2. Histone H3 epigenetic modifications and HP1β detection upon the first mitosis

Naturally fertilized embryos were stained for various histone H3 post-translational modifications (H3S10P, H3K9me3 and H3K9meS10P) as well as for the related associated protein HP1β. Representative images from the late 1-cell stage (PN5) until the formation of 2-cell stage nuclei (~1hour post cleavage) are shown here in green with DNA counterstaining in red. All images were taken on the confocal microscope (H3K9me3, H3K9meS10P and HP1β) or on the Apotome (H3S10P): PN5 and 2-cell are single z-sections; Prophase / Metaphase / Anaphase correspond to z-stack projections. In one-cell embryos the two parental pronuclei can clearly be distinguished. To make comparisons easier we rotated the images, when required, to show the paternal one on the right hand side. PB: Polar Body; scale bar: 10μm.

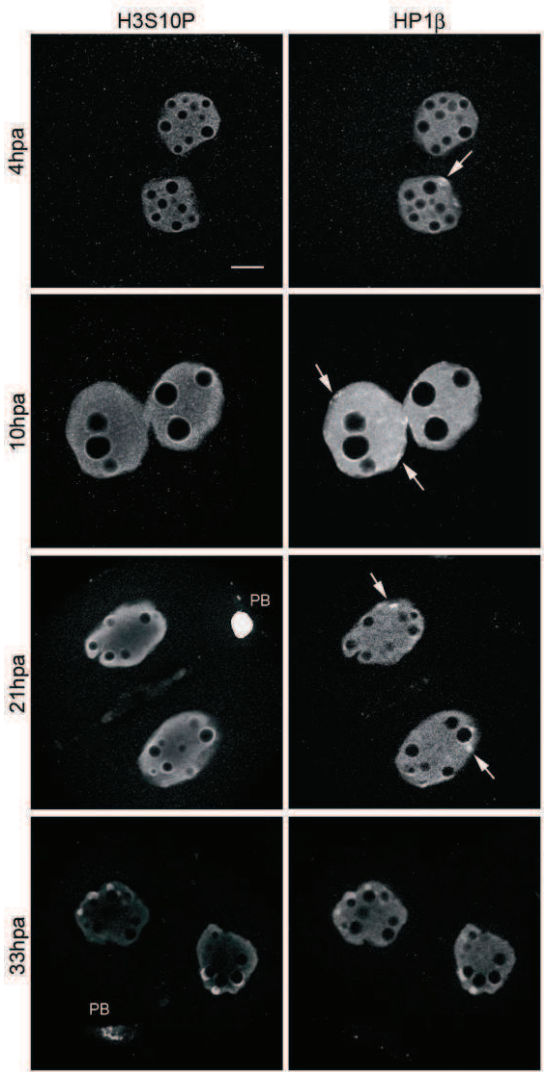


Figure 4. Double immunostaining with H3S10P and HP1β in early cloned embryos.

Cloned embryos were stained for H3S10P and HP1β protein at various time points after activation and observed on the Apotome microscope (only single z-sections are shown here). At the 1-cell stage, it appears that HP1β shows accumulation in the nuclear periphery (arrows) while a stronger signal for H3S10P is visualized around the NPBs, both at 4hpa and 10hpa. At early 2-cell (21hpa) H3S10P and HP1β only show co-localization on the rings of heterochromatin; again the HP1β foci lack a signal for H3S10P. Nearly full co-localization of both markers is seen by the end of the 2-cell stage (33hpa). Scale bar: 10μm.

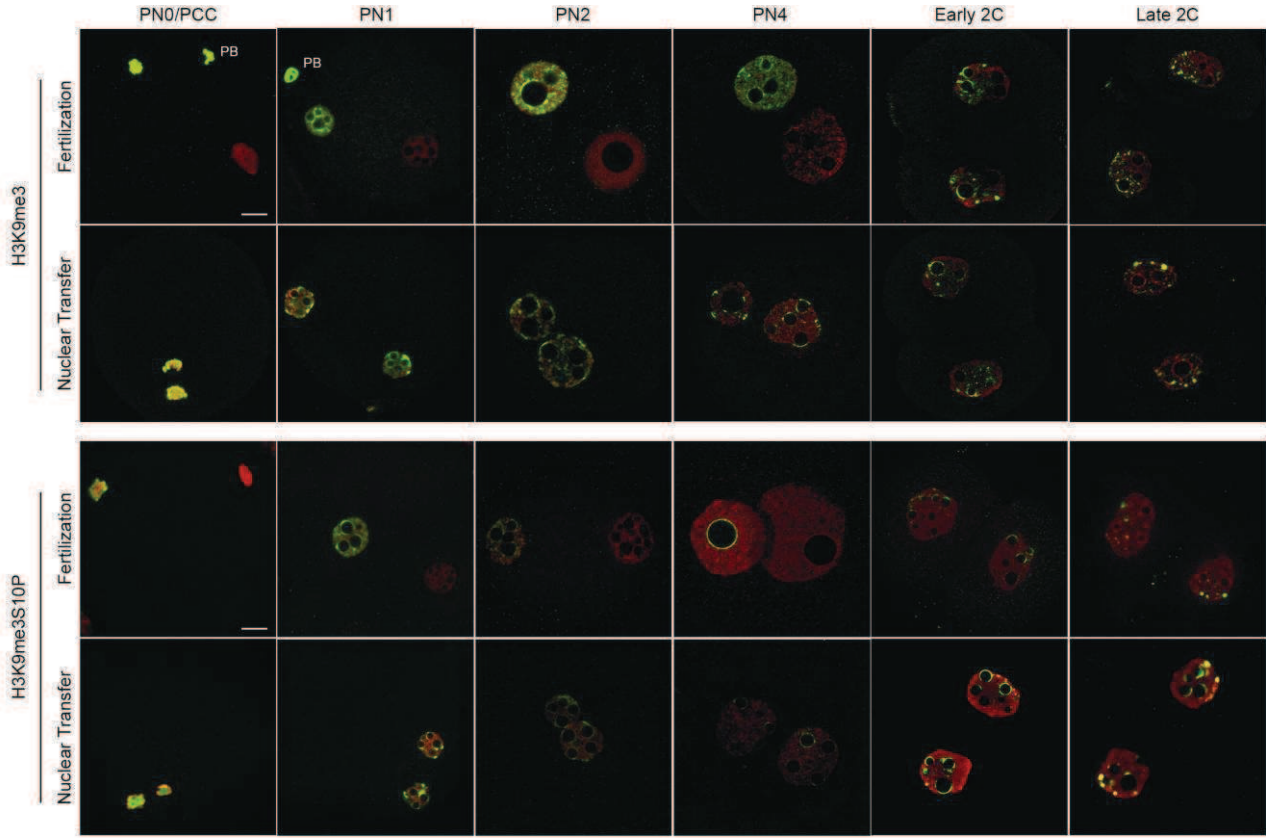
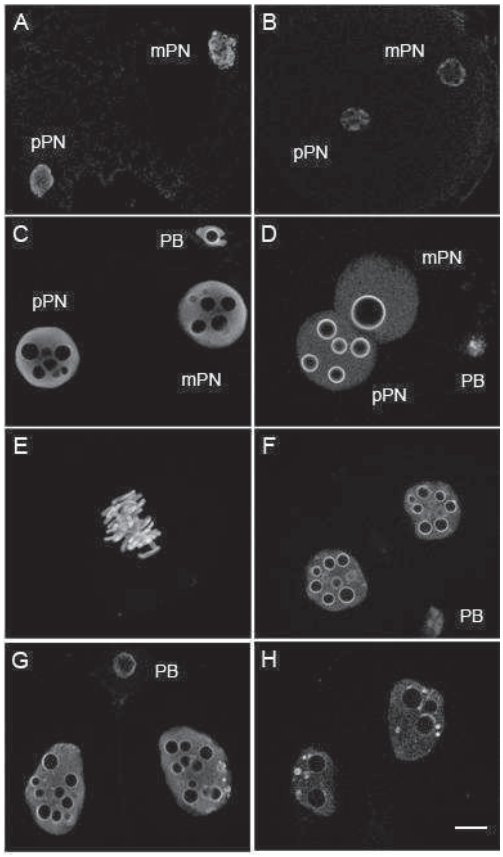


Figure 5. Comparison of H3K9me3 and H3K9me3S10P distribution in fertilized and cloned embryos.

Naturally fertilized and cloned embryos were stained for H3K9me3 and H3K9me3S10P (green) and counterstained (DNA staining in red). Representative images from early 1-cell stage (PN0/PCC) until late 2-cell stage (Late 2C) shown here are single-sections from z-stacks taken on the confocal microscope (scale bar: 10µm). In one-cell embryos the two parental pronuclei can clearly be distinguished. To make comparisons easier we rotated the images, when required, to show the paternal one on the right hand side (PB: Polar Body). Remarkably, we observed that: 1) both H3K9me3 and H3K9me3S10P epigenetic modifications showed parental asymmetry over the whole first cell stage and even within the 2-cell stage nuclei; 2) H3K9me3S10P intensity decreases and the staining remains only on the NPBs periphery by PN4; 3) in contrast to fertilized embryos, clones often present perinuclear blocks of heterochromatin at 1-cell with no asymmetric distribution of either marks.

3.3 Supplementary Figure: Immunodetection of H3S10P in IVF embryos



We followed the same immunostaining protocol to investigate the spatial and temporal pattern of H3S10P in IVF (*In Vitro* Fertilized) embryos as in *in vivo* fertilized ones (see Paper #1). Representative images (single z-sections, except for E that is a z-projection) are shown here. In one-cell embryos the two parental pronuclei can clearly be distinguished (maternal: mPN and paternal pPN) as well as the remaining second polar body (PB).

Very early during the first embryonic cycle (A), H3S10P was already present in the decondensed sperm head and maternal chromatin (PN0). During pronuclei formation (PN1), both parental complements had a strong signal (B). Both pronuclei started showing signals for H3S10P in the heterochromatin rings around the NPBs at PN2 (C). In late 1-cell, at PN4, the heterochromatin rings showed strong H3S10P staining (D). During mitosis chromosomes were strongly stained for H3S10P (E). At the 2-cell stage, H3S10P was first detected in the heterochromatin rings around the NPBs (F) whereas from middle (G) to late 2-cell (H), H3S10P could be detected on the newly formed chromocenters. Scale bar: 10µm. Therefore, as IVF embryos showed the same distribution pattern for H3S10P as their *in vivo* counterparts, we concluded that our culture system did not affect this epigenetic mark in developing embryos.

Chapter Four: General discussion & Perspectives

It has been shown that phosphorylation of histone H3 at Ser10 initiates in pericentromeric heterochromatin in late G2 phase, spreading throughout the chromatin as it undergoes condensation and staying phosphorylated up to the end of mitosis (Houben et al., 2007). This post-translational modification has also been observed outside mitosis and it is believed that it is involved with gene activation.

Scant few research groups have investigated this epigenetic modification during early mammalian embryogenesis and the three articles that have been published so far about H3S10P in preimplantation mouse embryos all suggest that H3S10P is linked to pericentromeric heterochromatin. Wang et al. (2006), Huang et al. (2007) and Teperek-Tkacz et al. (2010) have hypothesized that H3S10P may be involved with pericentromeric heterochromatin. Wang et al 2006 and Teperek-Tkacz et al. (2010) have indicated that H3S10P was found on compact heterochromatin areas in 1- and 2-cell mouse embryos; and Huang et al 2007 have mentioned an intense H3S10P labelling in the perinucleolar domains until morula stage. It was evident that further studies needed to be carried out in order to verify the involvement of H3S10P with this type of heterochromatin.

With this purpose in mind, we have studied H3S10P throughout preimplantation development, using techniques such as double immunostaining or immunoFISH, looking at its spatial distribution, with emphasis on pericentromeric heterochromatin, and related heterochromatin markers such as H3K9m3, HP1β and the double modification H3K9m3S10P.

We have shown that H3S10P truly co-localizes with the pericentromeric heterochromatin rings around the NPBs (Nucleolar-Precursor Bodies) during interphase from the pronuclear stage. Interestingly, H3S10P labels both parental genomes just after pronuclear formation and until the end of the first cell cycle.

As expected from previous studies focusing on the heterochromatin protein 1 β (HP1β) (Martin et al., 2006a; Santos et al., 2005), co-localization of HP1β and H3S10P in the pericentromeric heterochromatin was only seen in the maternal pronucleus at 1-cell and the paternal pericentromeric heterochromatin domains contained only H3S10P. Moreover, frequently during the end of that stage, one perinuclear clump of heterochromatin positively labelled by HP1β but not for H3S10P was observed in the maternal pronucleus. We have also carried out immunoFISH using specific probes to the pericentromeric DNA in order to specifically visualize the major satellite sequences and to confirm the co-localization with

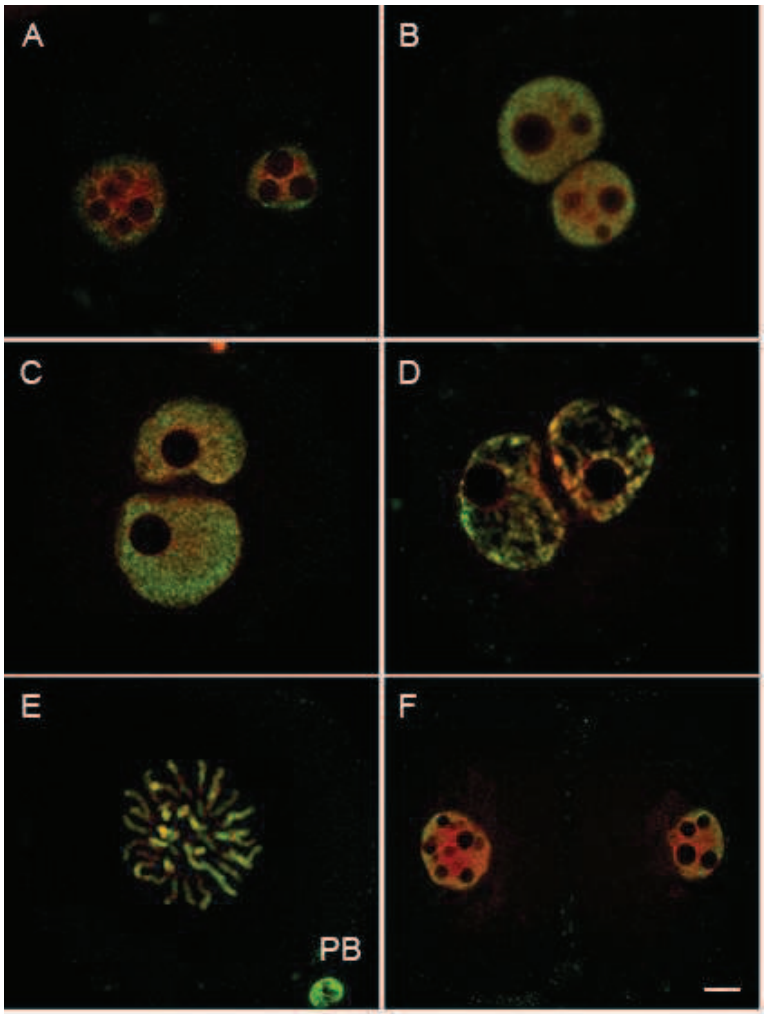
H3S10P. With this technique we observed that H3S10P faithfully overlaps with the probes corresponding to the major satellite sequences: full co-localization is seen in the NPBs in the two pronuclei of 1-cell stage. However, some isolated pericentromeric heterochromatin foci were again seen at the nuclear periphery which did not stain for H3S10P, suggesting that H3S10P does not label all pericentromeric heterochromatin regions.
However, in comparison to other epigenetic markers such as H3K9me3 and H3K9me3S10P, it unmistakably appears that H3S10P is a better marker of pericentromeric heterochromatin since it is always correlated to this type of heterochromatin in both inherited parental genomes, from the very beginning of development. Indeed, both H3K9me3 and H3K9me3S10P epigenetic modifications showed parental asymmetry over the whole first cell stage. Regarding H3K9me3, we confirmed that only the maternal pronucleus presents a strong signal around the NPBs and a diffuse staining in the nucleoplasm (Cowell et al., 2002; Santos et al., 2005). Similarly, at PN1, only the maternal PN is labeled for H3K9me3S10P epigenetic modification in the rings around the NPBs and a diffuse staining is observed in the nucleoplasm. However, a slight decrease in H3K9meS10P intensity is seen starting from 20h phCG (~PN2). This decrease most probably corresponds to chromatin decondensation and incorporation of new histones H3 upon the first replication phase as already described for H3K9me3 (Liu et al., 2004; Wang et al., 2007).

Upon entry in the first mitosis, we observed that all three epigenetic markers were present: 1) H3S10P covered equally both parental genomes, 2) H3K9me3 strongly labelled the maternal genome whereas the paternal one became faintly stained only at the very end of the 1-cell stage and 3) H3K9meS10P intensity clearly increased covering both parental genomes.
Conversely, it is known that HP1β proteins preferentially localize within condensed inactive heterochromatin and that it dissociates from chromatin during mitosis (Minc et al., 1999; Hayakawa et al., 2003; Puschendorf et al., 2008). In fact, H3K9me3 and HP1 are working together to propagate heterochromatin and cause gene silencing (Lachner et al., 2001) but the binding of HP1β to the methylated H3-tail is fully reversible and highly dynamic, thereby supporting the rapid exchange of HP1β from heterochromatin (Fischle et al., 2005). It has been proposed in somatic cells that H3S10 phosphorylation prevents the binding of HP1 to the adjacent tri-methylated Lys 9 residue of histone H3 (Fischle et al., 2003; Hirota et al., 2005). However, it has also been proposed that the additional phosphorylation at the Ser10 on the already tri-methylated Lys9 histone H3 does not completely prevent the binding of HP1

to heterochromatin and that a third modification, namely acetylation of histone H3 at Lsy14, needs to occur in order to eject HP1 from chromatin (Mateescu et al., 2004).

In mouse embryos this scenario is completely different. Looking at H3K14ac in 1-cell embryos (see supplementary figure on next page), we have seen that the paternal heterochromatin presents H3S10P and H3K14ac, while the maternal one has H3K9me3, HP1β, H3S10P plus H3K14ac all together. This seemed to be in contradiction with the hypothesis made in somatic cells. We therefore came up with the hypothesis that dissociation of HP1 from chromatin only happens upon tri-methylation on Lys9 and phosphorylation of S10P within the same histone H3 tail. Based on the immunostaining experiments with a specific antibody targeting H3K9me3S10P we confirmed that both parental genomes present a strong signal for this modification upon prophase of the first mitosis, exactly when HP1 starts to dissociate from chromatin. The presence of the third modification H3K14ac on the maternal chromatin did not have any effect on the ejection of HP1. It will be of interest to further investigate this unique HP1 dissociation scenario in embryos to unveil the factors controlling HP1 ejection from the embryonic heterochromatin. We, however, do not know whether additional phosphorylation at the Ser10 occurs on the already tri-methylated Lys9 histone H3 or vice-versa. Experiments using ZM447439 (ZM), an inhibitor of Aurora kinases activity, showed that embryos lacking almost completely H3S10 phosphorylation did not cleave properly (Teperek-Tkacz et al., 2010 and our own results). Similarly, disruption of the two mouse Suv39h HMTases (Histone Methyl Transferase) that abolishes H3-Lys9 methylation of constitutive heterochromatin induces gestation death or postnatal growth delay (Peters et al., 2001; Peters et al., 2002). Both phosphorylation at the Ser10 and tri-methylation on Lys9 might therefore be involved in embryonic chromosomes condensation.

Supplementary Figure: Immunodetection of H3K14ac in fertilized embryos



We followed the same immunostaining protocol and used the same parameters to investigate the spatial and temporal pattern of H3K14ac in *in vivo* fertilized embryos previously described (see Paper #1). H3K14ac antibody used was purchased from Millipore (#06-911, diluted at 1:250). Representative images are shown here (A-C and F are single z-sections; D-E correspond to z-stack projections).

During the whole first embryonic cycle, histone H3 acetylation on lysine 14 was present on both parental pronuclei (A: 19hphCG, B: 26hphCG). In late 1-cell and upon prophase condensation, H3K14ac clearly stained the whole genome with the exception of the heterochromatin rings around NPBs (C: 29hphCG, D: 30hphCG). During mitosis staining for H3K14ac covered the whole chromosomes arms but not the pericentric heterochromatic regions (E: 30hphCG). At the 2-cell stage, H3K14ac was again first detected in the nucleoplasm and not within peri-NPBs heterochromatin rings (F: 30hphCG). PB: polar body; bar: 10µm.

During interphase in 2-cell embryos, H3S10P is detected labelling pericentromeric heterochromatin rings around the NPBs and later on, during chromocenter formation (G2 phase), H3S10P stained newly formed chromocenters. During early 2-cell stage, co-localization of HP1β and H3S10P was observed partially on some of the perinucleolar rings whereas the few HP1β foci seen at this stage were not stained for H3S10P, as already observed in late 1-cell embryos. We could then speculate that in this stage, the heterochromatin areas positive for HP1β and H3S10P came from the female genome, whereas the ones with only H3S10P were from the male set. This is in agreement with Merico et al. (2007) who suggested that the pericentromeric heterochromatin domains positively labelled by HP1β in the 2-cell stage were of maternal origin, while the negative ones were from the paternal complement which is this case contains only H3S10P.

As for the immunoFISH in early 2-cell embryos, faithful co-localization between the major satellites DNA probes and H3S10P is seen in the heterochromatin rings around the NPBs. However the few chromocenters already seen at this stage only showed labelling for the probes. At late 2-cell stage, the probes and H3S10P co-localized in the chromocenters and in the remainders of pericentromeric heterochromatin detected around the NPBs. It is only at the 4-cell stage that H3S10P changes its distribution pattern. At this stage H3S10P was weaker in the heterochromatin rings around NPBs and strong labelling was seen in the chromocenters.
It should be mentioned here that the reinitiation of rDNA transcription occurs only in late 2-cell embryos (44–45 hphCG) at the surface of the Nucleolar-Precursor Bodies (NPBs), upon recruitment of the RNA polymerase I (pol I) transcription complex. However, the NPBs are not equal in their ability to support recruitment of pol I and rDNA transcription and it is believed that at some of the NPBs are still not transcriptionally active even in 4-cell stage embryos (Zatsepina et al., 2003; Romanova et al., 2006).
It is not known if there is a link between heterochromatin withdrawal from the NPBs and their transcriptional activation but this would explain why H3S10P gradually disappears from these regions with development and why heterochromatin rings can still be seen surrounding NPBs at 4-cell. It might certainly well be that the NPBs positively labelled by H3S10P are still inactive through recruitment of unknown factors that maintain heterochromatin rings and that heterochromatin eventually leaves these structures when they become transcriptionally activated. It would also fit with the fact that embryos acquire the somatic cell pattern for H3S10P after this stage, once the NPBs are “mature” and fully active: staining is then observed in the interphasic blastomeres labelling only the chromocenters during G2 phase

and obviously staining the mitotic chromosomes in the blastomeres going through mitosis. Further studies are required to understand what actual relation heterochromatin and NPBs have, in regards to NPBs transcriptional activation.

In regards to H3K9m3 at the 2-cell stage, a partitioned distribution is observed in the nuclei: Only half of both nuclei present labelling for this modification. It is now widely accepted that this positive labelling must correspond to the maternal part of the genome, previously labelled by this marker at 1-cell (Cowell et al., 2002; Merico et al., 2007; Puschendorf et al., 2008). A tendency to concentrate on one pole of the nuclei was also observed for the double modification H3K9me3S10P. Moreover, both epigenetic marks showed partial rings labelled and few foci in the nucleoplasm in early 2-cell embryos. At 48h phCG strong staining is seen in the chromocenters and nearly no heterochromatin rings are seen around the NPBs anymore. This is in agreement with the view that the heterochromatin during this stage is undergoing massive changes in its structure, moving from the rings surrounding the NPBs towards the nuclear periphery, to form new heterochromatin domains, the chromocenters (Martin et al., 2006a).

The mechanisms involved in heterochromatin formation are still subject to discussion. It has been suggested that small non coding RNAs are involved with heterochromatin formation (Chu and Rana, 2007; Prasanth & Spector, 2007). In fission yeast and plants, pericentromeric transcripts are processed to small RNAs that in turn guide heterochromatin formation and establishment of a transcriptionally silent state (Zaratiegui et al., 2007). Similarly, transcripts generated by pericentromeric repeats represent possible candidates for chromocenters formation in mouse embryos (Probst et al., 2010; Santenard et al., 2010). It has indeed been suggested that the presence of histone variant H3.3 and low levels or absence of H3K27 methylation, would provide a chromatin environment for transcription of pericentromeric chromatin, leading to heterochromatin formation at pericentromeric repeats (Santenard et al., 2010). Both the transcription of these domains and the gradual accumulation of H3K27 methylation would then lead to heterochromatinization and correct spatial positioning of these sequences around NPBs. Moreover, these authors have also shown that HP1β co-localizes with the newly formed chromocenters after injection of major satellites double-strand RNA in mutant embryos devoided of chromocenters, suggesting that HP1β must function as an architectural component for the propagation of heterochromatin.

The involvement of pericentromeric transcripts with heterochromatin organization and chromocenter formation during the 2-cell stage has also been recently shown by RNA-FISH (Probst et al 2010). Moreover, these authors demonstrate the paternal-specific major satellite is preferentially expressed, in particular the Forward transcripts, and that HP1β shows a preference for major forward satellite transcripts. All this suggests that somehow epigenetic modifications and non-histone proteins are implicated with the establishment of heterochromatin.

Regarding H3S10P, which is detected in both pronuclei very early in development, we can hypothesize that it might be assisting the establishment of heterochromatin by maintaining the chromatin in an open state giving the opportunity for transcription to take place in the paternal pronucleus and to some extent in the maternal one. Undeniably, the repressive heterochromatin markers H3K9me3 and HP1β, which are initially only detected in the maternal pronucleus, might be helping with the establishment of the stage-specific organization of the pericentromeric domains depending on the parental origin. Likewise the double modification H3K9me3S10P, might also be involved with this mechanism since it shows the same epigenetic asymmetry between the parental genomes. It would be interesting to further explore the relation between these epigenetic markers, H3S10P and H3K9me3S10P, and the transcription of pericentromeric satellite sequences upon formation of heterochromatin in 1- and 2-cell mouse embryos.

Due to its evident involvement with pericentromeric heterochromatin and correlation with chromatin structural changes during development, we hypothesized that H3S10P would be a good candidate to assist in the study of nuclear reprogramming after Somatic Cell Nuclear Transfer (SCNT). It has certainly been shown that reconstructed embryos by SCNT are usually not properly reprogrammed and previous data from the group suggested that the low efficiency of reprogramming is in part due to donor cell heterochromatin which is unable to lose its somatic heterochromatin configuration in order to acquire the embryonic one. Consequently, blocks of condensed heterochromatin are observed in the nuclear periphery: nearly half of the cloned embryos at 10hpa shows up to six clumps of heterochromatin not associated to the NPBs in the formed pseudo-pronuclei (Martin et al., 2006b; Maalouf et al., 2009).

In order to test whether these clumps of heterochromatin correspond to chromocenters which had not lost their somatic configuration, we decided to check pericentromeric

heterochromatin remodelling during SCNT using H3S10P epigenetic marker and related heterochromatin markers such as, HP1β, H3K9me3 and H3K9me3S10P.

Our results showed that H3S10P behaves the same in reconstructed embryos as it does in naturally fertilized ones. H3S10P staining is seen in the pericentromeric heterochromatin around the NPBs in the pseudo-pronuclei of cloned embryos, resembling exactly the same distribution pattern seen in *in vivo* fertilized ones. No pericentromeric heterochromatin containing H3S10P was ever detected dispersed in the nuclear periphery in late 1-cell embryos (10hpa). On the other hand with H3K9me3, perinucleolar and perinuclear blocks of heterochromatin were seen at 10 hpa in the reconstructed embryos, in both pseudo-pronuclei, as observed for HP1β (Martin et al., 2006b; this study). Looking at the double H3K9me3S10P modification, we observed that this epigenetic marker which is only present in the heterochromatin rings around the NPBs in the maternal pronucleus, is following the same distribution pattern in cloned embryos.. Altogether, this indicates that H3S10P, as opposed to H3K9me3 and HP1β, only labels remodelled pericentromeric heterochromatin located around the NPBs, resembling exactly the normal embryonic heterochromatin arrangement. This also proves that heterochromatin clumps located at the nuclear periphery are unremodelled, maintaining their somatic epigenetic signature (with H3K9me3 and HP1β stainings).

In light of these results, we believe that H3S10P is a good marker to trace remodelled pericentromeric heterochromatin after SCNT. It is therefore plausible to speculate that by the use of specific drugs, we could increase the levels of this histone H3 phosphorylation and somehow heterochromatin remodelling could be enhanced. Caffeine, a protein phosphatase inhibitor has been used to treat oocytes before SCNT. Lee & Campbell have shown that the treatment of sheep oocytes with caffeine increased the quality of embryos by increasing the levels of MPF and MAPK, two kinases essential for oocyte maturation (Terret et al., 2001), and consequently increasing the frequency of PCC (Premature Chromosome Condensation) as well as the development of cloned sheep embryos (Lee & Campbell, 2008). These authors believe that this increases the removal of chromatin bound proteins, thus allowing the access of oocyte derived factors involved in the reprogramming of the somatic DNA. The same principle could also be applied in regards to histone H3 phosphorylation. Treating cloned embryos with caffeine would in all probability raise the levels of the Aurora kinase responsible for H3S10 phosphorylation, making the chromatin more accessible to remodeling factors thus facilitating even more heterochromatin remodeling.

HDACi (Histone DeACetylase inhibitors) have also been shown to increase nuclear reprogramming. It is known, for example, that TSA (trichostatin A) has the potential to open chromatin by elevating the levels of acetylation, thereby affecting development of cloned embryos even to term (Kishigami et al., 2006 ; Rybouchkin et al., 2006). In a previous study from the group, we demonstrated that by treating the cloned mouse embryos with TSA, less clumps of unremodelled heterochromatin is observed at the nuclear periphery, indicating that the TSA facilitated the remodelling of somatic heterochromatin (Maalouf et al., 2009). One idea that could be tested now is to combine these two drugs, caffeine and TSA: we could treat the cloned embryos with caffeine first to start opening the chromatin and then apply TSA to unfold the chromatin even more, to observe the effect of these two drugs on nuclear reprogramming after SCNT.

As mentioned above, cloned mouse embryos often show more clumps of heterochromatin not associated to the NPBs than normal embryos (Martin et al., 2006b). Moreover, these heterochromatin clumps are usually located in the nuclear periphery. The reason for this preferential positioning is unknown, however we could infer that it must be related to the proteins responsible for nuclear and chromatin organization. Mammals have three related proteins called lamins A, B and C (Houliston et al., 1988). These proteins are part of a complex meshwork of nuclear lamin filaments that lies on the interface of the nuclear envelope and chromatin and are important for cell maintenance, nucleoskeleton support, chromatin remodeling, and protein recruitment to the inner nucleolus (Hall et al., 2005). It is not known how and what makes chromatin move inside the nucleus and if this process happens in a coordinated or random way. It has been suggested that additional “motor proteins” are in place to assist with chromatin organization, such as nuclear actin in mouse embryos (Nguyen et al., 1998). It might be that during enucleation of the oocyte and injection of the donor nucleus these proteins (lamins and motor proteins) are disrupted, and that as a result chromatin is misplaced. Furthermore, the protocol of nuclear transfer requires the use of drugs such as cytochalasin B which causes the disruption of actin filaments and inhibits actin polymerization; this might also contribute to incomplete nuclear remodelling. Therefore, it would be of great relevance to further investigate nucleoskeleton proteins plus the “motor” ones, in order to better understand their role in nuclear and chromatin organization within the early stages of reprogramming.

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Appendix

Appendix 1: In Vitro Fertilization Protocol

For IVF (In Vitro Fertilization), C57/CBA F1 female mice, 6-8 weeks of age, were superovulated with 5 IU of PMSG (pregnant mare serum gonadotropin) followed by an injection 48 hours later with 5 IU of hCG (human chorionic gonadotropin). Cumulus-Oocytes complexes were collected at around 13-14h post hCG and denuded in a 100µl drop of M2 medium + hyaluronidase. The denuded MII oocytes were washed in 20µl drops of M2, transferred into pre-equilibrated 20µl drops of M16 culture medium and finally placed in the incubator until the time of insemination. Fresh sperm was obtained from the caudae epididymides and vasa deferentia which were placed in an eppendorf tube containing 1ml of pre-equilibrated M16 and incubated for 10 minutes at 37^{o}C, 5% CO_2, 95% air. Subsequently, the tissue was removed and the released sperm were further incubated (50 minutes) for capacitation. After the capacitation period, the sperm preparation was checked for sperm morphology and motility. If these two criteria were satisfied, 10µl of this suspension was added to pre-equilibrated 90µl drops of M16. For insemination, the MII oocytes were transferred to the drops containing fresh capacitated sperm and then incubated for 4 hours at 37^{o}C, 5% CO_2, 95% air. Four hours post insemination, the fertilized oocytes (with a second polar body and 2 pronuclei) were washed through several drops of M2 to remove any sperm excess and then placed into pre-equilibrated 20µl drops of M16 and cultured at 37^{o}C, 5% CO_2, 95% air. Embryos were left in culture until the embryonic stage needed for immunostaining.