THE PRONUCLEI - 20 YEARS LATER

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ABSTRACT
The early mouse embryo is an excellent model to study chromatin changes that underlie zygotic gene activation. Chromatin remodelling in the fertilised mouse egg is intimately linked to protein synthesis and degradation, to protamine by histone replacement and to specific histone modifications. Roumen Tsanev made significant contributions in this field of research. He generated seminal data on pronuclear development and analysed the restoration of transcriptional competence of the inactive gamete chromatin. He identified sperm specific chromatin components, tightly bound to DNA and was one of the first to demonstrate that the histone complement of the male pronucleus appears before the onset of DNA synthesis. In this review we discuss recent genetic data on chromatin remodelling at fertilization. Data from developmentally regulated loci in knock-out mice confirm that first mitosis is associated with a transcriptionally repressive state, gradually relieved by various levels of acetylation and methylation at specific histone motifs. We examine the complexity of the transcriptional revival in the fertilized egg and present results from cutting edge technologies addressing the intricacies of the zygotic genome activation. We illustrate the importance of knowledge about these phenomena for the practical approaches of mammalian cloning and pre-implantation diagnosis.

A next step in the field is to correlate the phenotype with the expression profiles of early markers in preimplantation embryos and open the door to see how regulatory pathways interact to underlie pathology reported after routine in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) protocols.

The flurry of exciting data in this hot field of research speaks even better about the creative intuition in Dr. Tsanev’s projects that traced a pioneering path in pronuclear biology and genetics.

Keywords: pronuclei, zygote, early mouse embryo, chromatin, epigenetics

Introduction
Understanding gene regulation at the beginning of mammalian development is a major challenge in modern biology. Experimental data accumulated with the early mouse embryo have begun to identify cascades of interacting pathways and key factors involved in the control of pre-implantation gene expression. Recent reports confirm that large-scale chromatin remodelling and epigenetic mechanisms would underlie and govern the global patterns of gene expression leading to correct morphogenesis and cell differentiation during development (28, 34).

From the early 80s Rumen Tsanev, a pioneer in the field of epigenetics, designed strategies to look at the chromatin remodelling in sperm and early mouse embryos. By electron microscopy he showed that at two-cell stage all chromatin is organised in nucleosomes and has identified non-ribosomal RNA associated to chromatin fibbers (27). Dr. Tsanev reported seminal data about ultrastructural organization of the sperm nucleus of various mammalian and non-mammalian species. He described different levels of DNA packing in the spermatogenesis and analysed the nucleoprotamine complexes (6, 8, 39). His team identified the presence of a particular category of proteins, tightly bound to the smooth and rough chromatin fibbers. By two-dimensional tryptic peptide mapping they demonstrated that the protein fraction stably linked to DNA was remarkably conserved in mollusces, insects, fishes, amphibians, birds and mammal (7, 9). Rumen Tsanev developed experimental approaches to address zygote genome activation and pronuclear morphology. Using cell microsurgery combined to electron microscopy, he studied the dynamics of protamine to histone replacement and pronuclear chromatin transformations in freshly fertilised mouse eggs and generated pioneering data about the sequence of events in the first zygotic cell cycle (26), (Fig.1). In this review we want to outline the tremendous advances in this field of research generated by the novels tools and cutting edge technology approaches in imaging and bioinformatics. The progress made in pronuclear biology emphasises the fertile intuition and deep insight of Rumen Tsanev’s scientific projects as well as their relevance to major domains of assisted reproduction technologies and pre-implantation diagnosis.

Nuclear modifications during gametogenesis
Meiosis in the female mammal starts in mid-gestation and is blocked at birth. During postnatal development, oocytes are arrested and maintained at the diplotene stage of meiosis 1 prophase. At puberty the pituitary luteinising hormone (LH) triggers a wave of oocyte growth and differentiation in one or more follicles. In stimulated oocytes, the nucleus, termed germinal vesicle (GV) resumes then meiosis and undergoes dynamic changes in its chromatin structure and function. Initially decondensed GV chromatin progressively condenses forming a heterochromatin rim around the nucleolus (surrounded versus non-surrounded nucleoli) concomitantly...
with gradual global repression of transcriptional activity and establishment of maternally specific genomic imprints by epigenetic marks on individual loci. It is interesting to note that such events in preovulatory oocytes might occur long before full competence to meiosis which consists of GV breakdown and formation of metaphase I chromosomes (Fig. 2). Follows the pairing of homologous chromosomes, the asymmetric cell division of the oocyte I and the rapid succession of metaphases I and II, without intervening DNA replication. Complete egg maturation is achieved in the De Graaf’s antral follicle that releases in the oviducte a metaphase II arrested oocyte competent for fertilisation and able to reprogram the gene expression pattern of somatic nuclei transferred in cloning experiments. The female meiosis phenomena are still quite poorly understood at the level of chromatin. It is established that large scale chromatin restructuring in oocytes (as well as in spermatocytes) is intimately linked to post-translational modifications at specific amino-acids of core histone proteins as well as to versatile use of histone variants in a changing nucleosomal context. It has been shown that in the oocyte global changes in histone H3/H4 acetylation regulated by HDACs can be crucial for the localisation of the heterochromatin binding protein to specific sub-domains of forming metaphase I chromosomes (14). Deacetylation of H4 at lysine 12 was reported at resumption of meiosis in the oocyte, while methylation of histones H3/H4 at arginine residues occurs at chromosome condensation (33). More precisely, the dramatic decrease of H3 and H4 acetylation in female gamete maturation seems to be due to HDAC1 accessibility to the chromosome, as this enzyme co-localises with the chromosome only in meiotic oocytes (18).

In the male, primary spermatocytes entering meiosis are seen only at puberty and they promptly complete both meiotic divisions to generate round spermatids, which differentiate in mature spermatozoa. Throughout meiosis and up to the haploid spermatid stage, DNA is associated to histones and organised in nucleosomes. While spermatids initiate elongation tremendous acetylation of histone H4 and ubiquitination of H2B and H3 shake the stability of the “beaded” chromatin. Subsequently, histones are replaced by transition proteins TP1-4, which are gradually replaced by the small, 50 amino-acids long, and highly basic, arginine rich protamines P1 and P2. Extreme nuclear and DNA condensation is then achieved in the sperm, presumably due to compact alignment of adjacent DNA strands to perform sheet-like arrangement with strong anchoring of the protamines by disulphide bridges (46). This type of crystalline-like structure, sixfold more compact than metaphase chromosomes underlies the heavy transcriptional inertness of mature spermatozoa. Histones and their modification might not be the only cues in gamete differentiation. Recent knock-out experiments indicate that the meiotic cell cycle is governed by specific components of the maturation promoting factor (MPF). Mice lacking the Cdk1 binding protein Cks2 are sterile due to the failure of oocytes and spermatocytes to progress past the first meiosis metaphase (38). Furthermore, conditional knock-out data suggest that subtle regulation by RNA-binding proteins (SLBP) in meiotic cells might be involved in transcripts stabilisation and translational control (5).

**Pronuclei and asymmetry of parental chromatin remodelling**

Cell architecture changes dramatically during the process of fertilization. The oocyte nuclear material terminates another asymmetric division with extrusion of a second polar body, while the sperm nucleus undergoes extensive remodelling in the oocyte cytoplasm (Fig. 2). Classically paternal chromatin reorganisation is divided in three distinct phases (12). First, upon sperm entry in the cytoplasm dispersion and rapid recondensation of the chromatin are observed as the nuclear envelope is removed and various cytoplasmic factors alter the highly compacted sperm nucleus (2). These two phases are concomitant with the replacement of the sperm protamines by histones, synthesised during oocyte growth and stocked in the cytoplasm. The result of this nucleoprotein exchange is that the paternal chromatin now drastically expands as the thick smooth bundles of the sperm nucleus are transformed in thin nucleosome bearing fibbers, able to sustain DNA replication (20, 26). Indeed, the highly relaxed chromatin of both male and female pronuclei is entering the S phase of the first zygotic cell cycle. A striking feature of this phenomenon is that maternal and paternal DNA will replicate in two physically distinct pronuclear compartments. The volume of the male pronucleus enlarges much more importantly presumably by importation of ooplasmic proteins. This process is fundamental for reprogramming and was shown to include import of maternally derived Sp1, TBP, TEAD transcription factors, of kinetochore CENP proteins or MCM minichromosome maintenance proteins (17, 39)
proteins, as well as rapidly accumulating transcripts of the Y-linked genes Zfy and Sry (24).

The clearly detectable pronuclear asymmetry is accompanied by a number of molecular differences. Distinct characteristics of the male chromatin are the transient hyperacetylation of histone H4 as well as the replication independent demethylation of DNA. Paternal pronucleus hyperacetylation disappears just before DNA replication and is likely to be associated with assembly of H3 and H4 on DNA at the onset of zygotic S-phase (1). As far as both pronuclei are concerned, a particular localisation of selected acetylated forms of histones H3 and H2A was detected at the nuclear periphery of one and two-cell stage embryos (39). Establishment of regulated gene expression in both pronuclei and blastomeres is also associated to the presence of a particular complement of histone H1 subtypes (15). The dynamics of histone modifications is likely to be controlled at the mRNA processing level as a special category of stem-loop binding proteins (SLBP) protecting transcripts from degradation display abundant accumulation in pronuclei and cytoplasm during the first cell cycle (3).

The chromatin architectural non-histone proteins HMG14 and HMG17 were detected at all stages of cleavage and have been shown to participate in cell cycle regulation. Transient displacement of these proteins slows the progression of pre-implantation development and causes reduction of RNA and protein synthesis (25). Unlike histone modifications that have not been reported to persist after genomes fusion, maternal DNA remains methylated in the early cleavage stages; while the male pronucleus will lose its DNA-methylation marks 6 hours after fertilisation. This window of time follows histone assembly, precedes DNA synthesis and is regulated by specific unidentified ooplasmic factors unable to affect maternal DNA (32). Very recently specific interest was focused on pronuclear heterochromatin remodelling. In fact, the asymmetry described above, extends to the pericentric heterochromatin repeats. It was shown that paternal pericentric heterochromatin lacks H3K9 tri-methylation and other downstream marks, while the maternal counterpart is enriched in H3K9me3 and H4K20me3 (29). Gene targeting of maternally provided polycomb repressive complex1 (PRC1) components to paternal heterochromatin has shown that levels of pericentric major satellite transcripts are increased at the paternal but not the maternal genome. In maternally deficient zygotes, PRC1 also associates with maternal heterochromatin lacking H3K9me3, thereby revealing hierarchy between repressive pathways. Therefore, in early embryos, H3K9me3 constitutes the dominant maternal transgenerational signal for pericentric heterochromatin formation. In absence of this signal, PRC1 functions as the default repressive back-up mechanism (30).

(vg - germinal vesicle; MI – metaphase I; MII – metaphase II, spz – sperm nucleus; gp – polar body; pn – pronucleus; ZM- zygote metaphase plate; B – blastomeres).
Parental epigenetic asymmetry is also observed along cleavage chromosomes. It is not resolved before 8-cell stage and marks the end of the maternal-to-embryonic transition.

**Zygotic genome activation**

In mice and human zygotic genome activation (ZGA) occurs in few waves of gradually increasing transcriptional competence. The mouse late syngamic zygote displays the capacity of endogenous and ectopic transcription that increases even more at the two cells stage. ZGA is initiated by maternally derived proteins and RNAs and relies on chromatin remodelling complexes and covalent histone modifications. The first DNA replication might be the critical factor for ZGA as it disrupts nucleosomes and allow access of transcription factors to cis-regulatory sequences. In a very intriguing way, the activation of the embryonic genome is coupled to the formation of a transient chromatin mediated transcriptionally repressive state at the end of the two-cell stage. The complexity of this reprogramming is certainly dictated by the need to transform the extremely differentiated oocyte and sperm in totipotent blastomeres of the early preimplantation embryo. Activation and repression states are required to impose the right pattern of gene expression necessary for a correct and successful embryonic and adult development.

Recent advances in technology shed new lights on the process of ZGA in the tiny mammalian embryo.

The quantification of BrU incorporation by laser-scanning confocal microscopy allows monitoring transcription in one and two-cell embryos and assessing the role of DNA replication and chromatin structure in the process. It was shown that transcription of the total pool of endogenous genes starts during the zygote S-phase, the total amount of incorporation being about 20% of that at the two cells stage. In the same time the amount of transcription over the male pronucleus was about five fold greater than the one ensured by the female pronucleus.

In two blastomeres embryos the rate and the overall amount of transcription keeps increasing and does not depend on inhibition of DNA replication. Recruitment of maternal mRNA is crucial for the acquisition of transcriptional competence. By inhibiting its polyadenylation, gene expression was markedly decreased, demonstrating that newly synthesised proteins, and not post-translation modified maternal products are operational. Inhibiting its polyadenylation, gene expression was markedly decreased, demonstrating that newly synthesised proteins, and not post-translation modified maternal products are operational. Inhibiting its polyadenylation, gene expression was markedly decreased, demonstrating that newly synthesised proteins, and not post-translation modified maternal products are operational. This was also tackled by direct injection of HDAC6 in germinal vesicles and pronuclei. Intriguingly, in both cases a drastic premature compaction of chromatin was observed and this effect is dependent on the integrity of HDAC6 motifs involved in proteasome degradation pathways (42), (Fig. 3). The role of maternal factors was challenged by recently developed approaches of small interfering RNA (siRNA) and double strand RNA (dsRNA) targeting. It was shown that maternal cyclin A2 mRNA and recruitment of CDK2 are essential for genome activation and progress through the two-cell stage (16). On the front of histone modifications, RNAi – mediated reduction of HDAC1 in two-blastomeres embryos induces hyperacetylation of histone H4K5 despite up-regulation of HDAC2 and HDAC3. Development of HDAC1 depleted embryos at specific histone-DNA concentrations was shown to restore promoter repression and subsequent relief of this repression by functional enhancers or by histone deacetylase inhibitors. The extent of enhancer-mediated stimulation seems to depend on the acetylation status of the injected histones as well as on developmentally regulated enhancer-specific coactivators. Supercoiling assays indicate that co-injected plasmids rapidly assemble in transcriptionally competent chromatin structures (31). If injection of histones in zygotes is able to induce chromatin mediated transcription normally observed in two-cell embryos, then the main prerequisite for ZGA appears to be the correct chromatin organization of the parental genomes. The role of histone modifying enzymes was also tackled by direct injection of HDAC6 in germinal vesicles and pronuclei. Intriguingly, in both cases a drastic premature compaction of chromatin was observed and this effect is dependent on the integrity of HDAC6 motifs involved in proteasome degradation pathways (42), (Fig. 3). The role of maternal factors was challenged by recently developed approaches of small interfering RNA (siRNA) and double strand RNA (dsRNA) targeting. It was shown that maternal cyclin A2 mRNA and recruitment of CDK2 are essential for genome activation and progress through the two-cell stage (16). On the front of histone modifications, RNAi – mediated reduction of HDAC1 in two-blastomeres embryos induces hyperacetylation of histone H4K5 despite up-regulation of HDAC2 and HDAC3. Development of HDAC1 depleted
embryos is retarded and a high number of genes display enhanced expression, while normally they are repressed at this stage. Inactivation of other HDACs had no effect on protein levels, acetylation status of H4K5 or embryo development to implantation (22). Results obtained by the technology of conditional targeting suggest that the zygote transcriptional machinery could directly access gene promoters. The identification of RNA polymerase II holoenzyme components like the ATP-dependant chromatin remodelling SWI/SNF complex indicated that gene activation could operate by nucleosome disruption and facilitation of transcription factor binding to regulatory DNA sequences. Indeed, knockout data show that mutants for the SWI/SNF subunits BRGs and BAFs die at the blastocyst stage. Furthermore, Brg1 inactivation targeted by Cre-loxP technology in the oocytes leads to ZGA defects. The mutant embryos are arrested at the two-four cells stages, transcriptional activity is highly reduced and the genes involved in transcription, RNA processing and cell cycle regulation are particularly affected (10).

Chromatin remodelling and global changes of gene expression in the early embryo - implications for ART, pre-implantation diagnosis and cloning

Chromatin remodelling establishes epigenetic marks on large domains of the zygotic genome by nucleosome disruption and histone modifications, imposing global and specific patterns of gene expression. Transcript profiling of one-cell and cleavage embryos became increasingly crucial in order to monitor molecular processes that accompany normal development, pathology and assisted reproduction technologies. Microarrays data initially confirmed that expression profiles of oocytes and zygotes were quite similar and that bursts of gene expression noted in two-cell embryos differ considerably from the profiles at morula and blastocyst stages. In the same time it appeared that global gene activation is much more selective than anticipated, with genes involved in transcription and RNA processing being preferentially expressed at the two-blastomeres stage (47). As expression analysis was combined to identification of molecular pathways as well as interactions among them, it was demonstrated that over-representation of genes controlling ribosome assembly and protein synthesis hinges on the regulatory role of the c-Myc gene (48). Such body of data is extremely important not only to explain the arrest of cleavage in Myc-ablated embryos, but above all to help improving a variety of assisted reproductive technologies applied to address human infertility and embryonic pathology.

In the early 90s normal fertilization was obtained in mice and humans following oocyte injection with testicular spermatozoa that activated the eggs and produced large pronuclei. Since the global use of ICSI to overcome male fertility has been quite successful for couples for whom traditional IVF has failed. As chromatin and cytoskeletal events that occur during ICSI are quite different from normal fertilization, abnormal remodelling and gene expression are likely to have an impact on the safety of this manipulation. Indeed, in the best clinics only 3 out of 10 couples bring babies home after ICSI. As remodelling of pronuclear chromatin seems to be much more asynchronous after ICSI, compared to IVF, a series of improvements of this technology were tested in non-human primates including removal of the acrosome with specific detergents or introduction of mature centrosomal material. This type of interventions synchronise reprogramming and increase the overall rate of successful development. In addition, ICSI assays of chromatin remodelling under various experimental conditions may lead to discovery and treatment of new types of male deficiencies, until now accounted for as “unexplained" infertility (35, 40). Returning to the safer frames of the mouse embryo experimentation, a recent study tested by transgenesis approach of reprogramming and global changes of gene expression in the early embryo - implications for ART, pre-implantation diagnosis and cloning

In the field of embryo culture and pre-implantation genetic diagnosis (PGD) the morphological criteria are not the best strategy to design a robust embryo. Culturing morulae is a delicate procedure likely to perturb the expression of imprinted genes, crucial for correct development. As expression analysis was combined to identification of molecular pathways as well as interactions among them, it was demonstrated that over-representation of genes controlling ribosome assembly and protein synthesis hinges on the regulatory role of the c-Myc gene (48). Such body of data is extremely important not only to explain the arrest of cleavage in Myc-ablated embryos, but above all to help improving a variety of assisted reproductive technologies applied to address human infertility and embryonic pathology.

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knowing exactly how remodelling complexes move and how histone code functions is essential for the success of early embryo manipulations and cloning procedures. Cloning by
somatic cell nuclear transfer is based on the capacity of oocyte cytoplasm to reprogram the somatic nucleus and make it able to sustain embryonic development. The poor efficiency of the cloning process is mainly due to the difficulty to “revive” the proper number of key transcription factors and the “domino effect” from abnormal expression of their target pathways. Indeed, transcriptome analysis of clones at one and two-cell stages reveals aberrant gene transcription and disruption in the regulation of maternally encoded mRNAs. About 1000 genes involved in mRNA processing, protein biosynthesis and degradation, oxidative phosphorylation and membrane trafficking kept their somatic profile of expression what could have profound consequences on long term development (43). A comparative microarray study of about 8 000 cDNA from cloned and normally fertilised bovine embryos suggest that low success rate of nuclear transfer procedures might be potentially caused by abnormal gene reprogramming during fetal and placental development (36, 37). It remains to be determined if and how early remodelling errors are magnified during embryonic development.

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