

## RESEARCH ARTICLE

# The maternal nucleolus plays a key role in centromere satellite maintenance during the oocyte to embryo transition

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**ABSTRACT**

The oocyte (maternal) nucleolus is essential for early embryonic development and embryos originating from enucleolated oocytes arrest at the 2-cell stage. The reason for this is unclear. Surprisingly, RNA polymerase I activity in nucleolus-less mouse embryos, as manifested by pre-rRNA synthesis, and pre-rRNA processing are not affected, indicating an unusual role of the nucleolus. We report here that the maternal nucleolus is indispensable for the regulation of major and minor satellite repeats soon after fertilisation. During the first embryonic cell cycle, absence of the nucleolus causes a significant reduction in major and minor satellite DNA by 12% and 18%, respectively. The expression of satellite transcripts is also affected, being reduced by more than half. Moreover, extensive chromosome bridging of the major and minor satellite sequences was observed during the first mitosis. Finally, we show that the absence of the maternal nucleolus alters S-phase dynamics and causes abnormal deposition of the H3.3 histone chaperone DAXX in pronuclei of nucleolus-less zygotes.

**KEY WORDS:** Nucleolus precursor body, Centromere, Chromosome bridging, Satellite DNA, Replication stress, Mice

**INTRODUCTION**

The transition from meiosis to mitosis is a crucial period in animal life. Although extensively studied it is not yet completely understood. Mammalian oocytes and early embryos contain atypical nucleoli [termed nucleolus precursor bodies (NPBs)]. Unlike somatic cell nucleoli, these structures lack the three basic nucleolar compartments (fibrillar centres, granular and dense fibrillar components); instead, they are composed solely of dense fibrillar material of unknown composition (Biggiogera et al., 1994; Pochukalina and Parfenov, 2008). These atypical organelles originate from formerly transcriptionally active nucleoli of growing oocytes. Initially, oocytes increase enormously in size and synthesize large amounts of material (including rRNA), and the oocyte nucleolus structurally resembles the somatic cell-type nucleolus. When oocytes reach full size, transcription ceases and the somatic cell-type nucleolus is transformed into the NPB (Chouinard, 1971).

Interestingly, NPBs can be microsurgically removed (enucleolation) and even transplanted back into oocytes (Fulka et al., 2003; Ogushi and Saitou, 2010). Based on this experimental approach, it was shown that NPBs in zygotes are of maternal (oocyte) origin and are essential for early development: embryos originating from enucleolated oocytes are unable to replace this organelle, form nucleolus-less nuclei and are arrested at the 2-cell stage. When oocytes are enucleolated but the NPB

is transplanted back, healthy offspring can be obtained (Ogushi et al., 2008). The reason for the developmental arrest of nucleolus-less embryos is unclear, although it has been speculated that the problem might lie in the lack of, or aberrant, transcription. Here, we show that whereas rRNA transcription activation and pre-rRNA processing are unaffected by the absence of NPBs, this organelle is essential for the regulation of major and minor satellite sequences and consequently for normal development.

**RESULTS****Early embryos use surprisingly little of the original maternal nucleolar material**

Because the maternal NPB is essential for embryonic development and embryos originating from enucleolated oocytes arrest at the 2-cell stage (Ogushi et al., 2008; Ogushi and Saitou, 2010), the common view of the NPB role is that it serves as the storage site of nucleolar proteins. However, a question remains as to what extent the developing embryo is dependent on such maternal stocks or maternal mRNAs encoding nucleolar proteins. We focused on B23 [nucleophosmin 1 (Npm1)], C23 [nucleolin (Ncl)], fibrillarin (Fbl) and UBF (Ubtf), as these are by far the best-characterised nucleolar components. Interestingly, when the mRNA levels were measured we found no accumulation of these transcripts during the oocyte growth period (Fig. 1A). In fact, in the case of Ubtf and Fbl, their mRNA levels declined markedly. After fertilisation, the transcripts decreased even further, reaching the lowest levels at the 2-cell embryonic stage, when genome activation occurs in the mouse (Fig. 1B). The appearance of paternal transcripts at this stage indicates that the maternal transcripts are actually eliminated prior to this stage (Fig. 1C).

Next we asked whether the nucleolar proteins are carried over to the embryo. With the exception of B23, we found no evidence that even the proteins were accumulated by oocytes. By contrast, a clear decline in the protein levels was observed between the germinal vesicle (GV) and metaphase II (MII) oocyte stages, when fertilisation occurs (Fig. 1D). Therefore, the developing mouse embryo makes very little or no use of the maternal nucleolar proteins and their mRNAs.

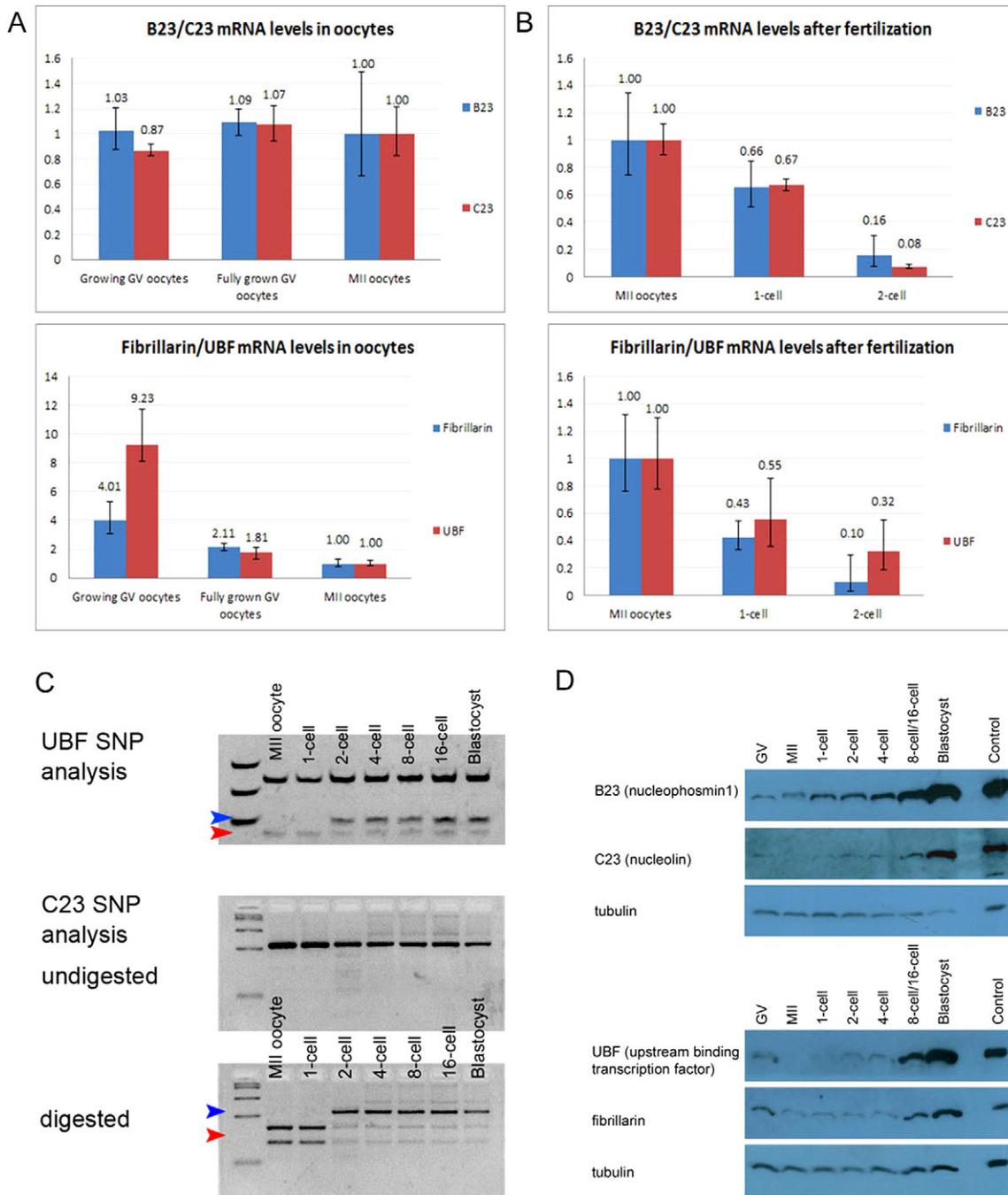
**Embryos successfully activate rRNA transcription and pre-rRNA processing in the absence of NPBs**

To further investigate the possibility that embryos constitute fully functional nucleoli *de novo*, we examined whether rRNA production is initiated in the absence of NPBs.

To discriminate between the possible impact of the enucleolation procedure and NPB absence on rRNA synthesis, three groups of embryos were prepared. In the first group, the oocyte (maternal) NPB was removed and transplanted back, and these oocytes were allowed to mature to the MII stage and then intracytoplasmic sperm injection (ICSI) was performed. The second group was subjected to the same procedure except that the NPB was completely removed. In the third group, no manipulation was performed prior to ICSI

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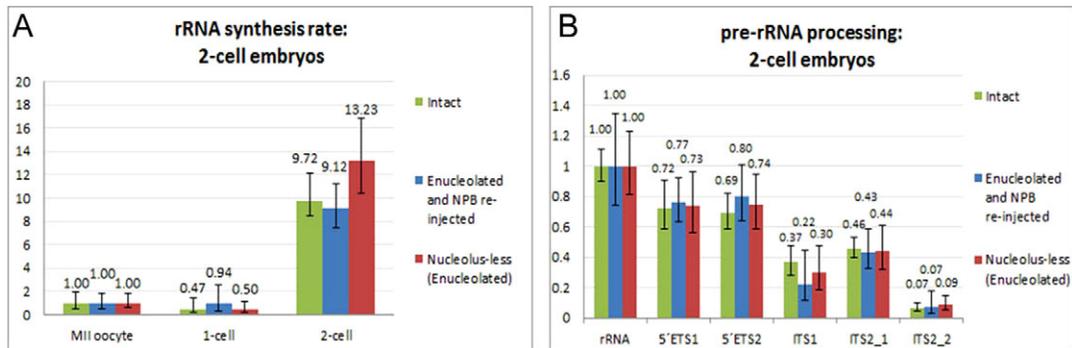
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**Fig. 1. Mouse embryos make surprisingly little use of maternal nucleolar material or nucleolar factor transcripts.** (A) During final phases of growth, oocytes do not accumulate mRNAs encoding typical nucleolar proteins. In fact, as they reach MII, *Ubf* and *Fbl* mRNAs are largely degraded ( $\geq 45$  oocytes analysed in each group). (B) The mRNA levels decline even further after fertilisation (ICSI). This indicates that embryos largely eliminate the maternal transcripts ( $\geq 35$ –45 oocytes/embryos used in each group). Mean ratios are shown, with error bars representing the minimum and maximum ratio values. (C) Single nucleotide polymorphism (SNP) analysis of C57BL/6 $\times$ DBA/2 embryos clearly demonstrates the presence of the paternal transcript at the 2-cell stage (blue arrowheads); maternal transcript, red arrowheads. Three different samples, each consisting of ten oocytes/embryos, were analysed for each stage. (D) The same trend was also observed for the corresponding nucleolar proteins. With the exception of B23, most of the evaluated proteins decline by the MII stage or during the 1-cell embryonic stage (zygote).

(intact *in vitro* matured oocytes). The use of ICSI allows maximum sample synchronisation. Surprisingly, real-time PCR analysis of the rRNA synthesis rate showed that nucleolus-less embryos are able to activate pre-rRNA production at the 2-cell stage at comparable levels to both controls (Fig. 2A). Therefore, the lack of RNA polymerase I activity is not the cause of the developmental arrest of nucleolus-less embryos.

It is well described in mammalian cells, as well as in yeast, that a precise balance of processed/unprocessed pre-rRNA is tightly monitored and the presence of unprocessed pre-rRNA transcripts causes various defects including cell cycle arrest and cell death (Kopp et al., 2007; Ugrinova et al., 2007; Boulon et al., 2010; Holzel et al., 2010; Chakraborty et al., 2011). We tested if aberrant pre-rRNA processing could underlie the developmental arrest.



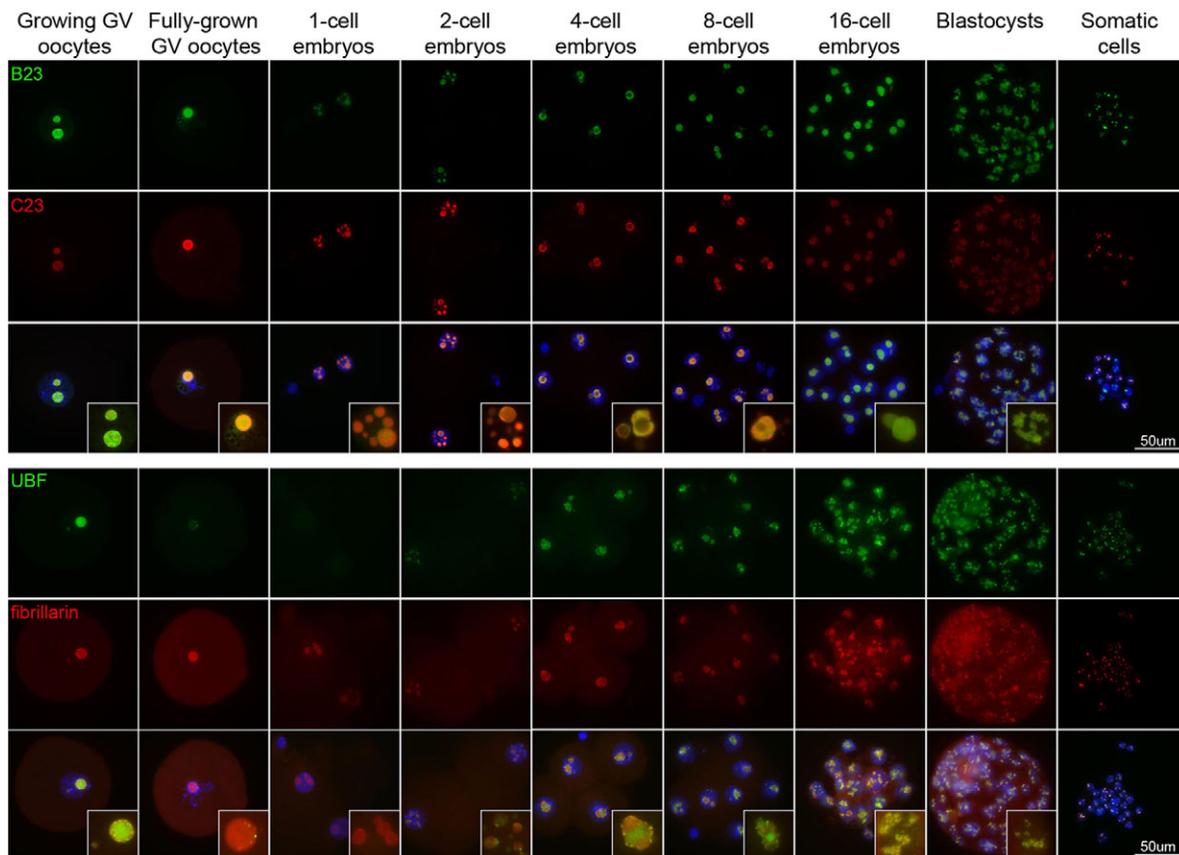
**Fig. 2. Nucleolus-less embryos are able to activate rRNA synthesis.** (A) Nucleolus-less embryos are able to activate rRNA synthesis at levels comparable to control embryos (intact; enucleolated with NPB re-injected). The samples were collected 6 h post-ICSI (1-cell) or 24 h post-ICSI (2-cell). At least 30 oocytes/embryos were evaluated in each group. (B) The levels of different processing products as well as unprocessed pre-rRNA were investigated. Assays targeting different regions of the pre-rRNA (5'ETS, 5' external transcribed spacer; ITS, internal transcribed spacer) produced similar results between controls and nucleolus-less embryos at the 2-cell stage. Mean ratios are shown, with error bars representing the minimum and maximum ratio values; 40 embryos were evaluated in each group. Results of neither the rRNA synthesis rate nor processing experiments were statistically significant ( $P > 0.05$ , Mann-Whitney U-test).

Support for this idea comes from our observation that NPBs contain at least some proteins involved in pre-rRNA processing (B23, C23 and Fbl), but not UBF, which is essential for RNA polymerase I transcription initiation (Fig. 3). However, when the levels of different 47S pre-rRNA processing products and unprocessed transcripts were measured no difference was found between the control and experimental groups (Fig. 2B). Thus, the nucleolus-less

embryos are not only able to activate rRNA transcription but also to process the pre-rRNA transcripts produced.

#### Impact of enucleolation on chromosome morphology

Because this result was unexpected, we verified that we are indeed microsurgically removing the whole NPB. The protocols used to label nucleoli in somatic cells are unsuitable for labelling NPBs



**Fig. 3. The presence and localisation of nucleolar proteins in NPBs of mouse oocytes and preimplantation embryos.** Antigen retrieval allows the localisation of typical nucleolar proteins involved in pre-rRNA processing (B23, C23, fibrillarin) and rRNA transcription (UBF) to NPBs in oocytes as well as in preimplantation embryos prior to genome activation. Note the decline and absence of UBF in fully grown GV oocytes and 1-cell embryos, respectively. Exposure times were kept constant, except for B23: GV oocytes, 1-cell and 2-cell embryos, 25 ms; thereafter, 10 ms. The bottom row in each case is a merge. Insets show the detail of the NPB/nucleolus.

because of their compact structure. We modified a classical antigen retrieval protocol that, combined with immunofluorescence, can be used to monitor the presence of various nucleolar proteins (Fig. 3; supplementary material Fig. S1), and we confirmed that we are indeed removing most of the maternal nucleolus, leaving on average less than 1% of the NPB material in the oocyte nucleus (Fig. 4A).

It is well known that centromeres and pericentric heterochromatin associate closely with NPBs in oocytes. In theory, these sequences might be subjected to DNA damage during enucleation. However, at 1-2 h post-enucleation, most of the GV oocytes did not exhibit higher levels of phosphorylated H2A.X (S139), which is a marker of DNA damage (47/56 oocytes; 84%). The percentage of phospho-H2A.X-positive oocytes (16%) matches the typical ratio of these oocytes that fails to mature to the MII stage (10-20%). The overall maturation rate is comparable between control and enucleated oocytes. Also, no differences were found between intact and enucleated MII oocyte chromosomes. Both groups exhibited normal chromosome morphology with prominent pericentric heterochromatin and easily detectable centromeres as well as telomeres (Fig. 4A). Typically, the chromosomal spreads contained undamaged chromosomes, although occasionally a single DNA lesion, mostly located distally, could be found [present in 16% and 17% of intact and enucleated oocyte spreads (89 and 82 oocytes analysed), respectively]. Thus, DNA damage induced by enucleation is unlikely to be the cause of the developmental arrest at the 2-cell stage.

### Genome activation and replication during the first embryonic S phase in nucleolus-less embryos

In somatic cells, the nucleolus is involved in many cellular processes, including cell cycle progression. For this reason we used the mouse cell cycle array, which allows the simultaneous profiling of 89 selected target genes involved in cell cycle control. However, it was first necessary to verify that the nucleolus-less embryos do actually activate their genome. This was confirmed by BrUTP incorporation assay (not shown).

The expression of most of the genes investigated was not altered in nucleolus-less 2-cell embryos (including DNA damage genes such as *Atm*, *Atr*, *Brca1* and *Brca2*). The only difference was a significant downregulation of *Cdc7* and *Cdk1* [5.11-fold and 2.3-fold difference, respectively;  $P < 0.05$ ].

This prompted us to investigate whether DNA replication might be affected by NPB absence. Control and nucleolus-less zygotes were incubated with BrdU [*in vitro* fertilisation (IVF) or ICSI]. Although both groups entered S phase at approximately the same time (~6-7 h post-ICSI) and completed replication, nucleolus-less embryos (both IVF and ICSI) exhibited delayed cleavage to the 2-cell stage, as described previously (Ogushi and Saitou, 2010). The reason for this delay might be the altered chromosome morphology, especially at the pericentric chromatin region, that was detected in nucleolus-less zygotes (Fig. 4B).

### Major and minor satellite sequences are affected by NPB removal

To investigate the altered chromosome morphology in more detail, we measured the content of the major and minor satellite sequences (pericentric and centric chromatin, respectively) by real-time PCR. Whereas no difference was observed between intact and enucleated MII oocytes (Fig. 4C), 2-cell embryos originating from enucleated oocytes exhibited decreased content of both major and minor satellite DNA (to 88% and 82%, respectively;  $P < 0.05$ ) when compared with controls (Fig. 4C). Thus, a significant

amount of satellite DNA is lost during the first embryonic cell cycle if NPBs are absent.

Because dysregulation of satellite sequence transcription leads to abnormal centromere function and, in embryos, to developmental arrest (Probst et al., 2010; Bouzinba-Segard et al., 2006), we measured the level of the satellite transcripts. Downregulation of both major and minor satellite transcripts was found in nucleolus-less embryos (Fig. 4C). This decrease was even higher than for the DNA (~2.4-fold decrease;  $P < 0.05$ ).

### Parental-specific effect of NPB removal on satellite DNA and transcript levels

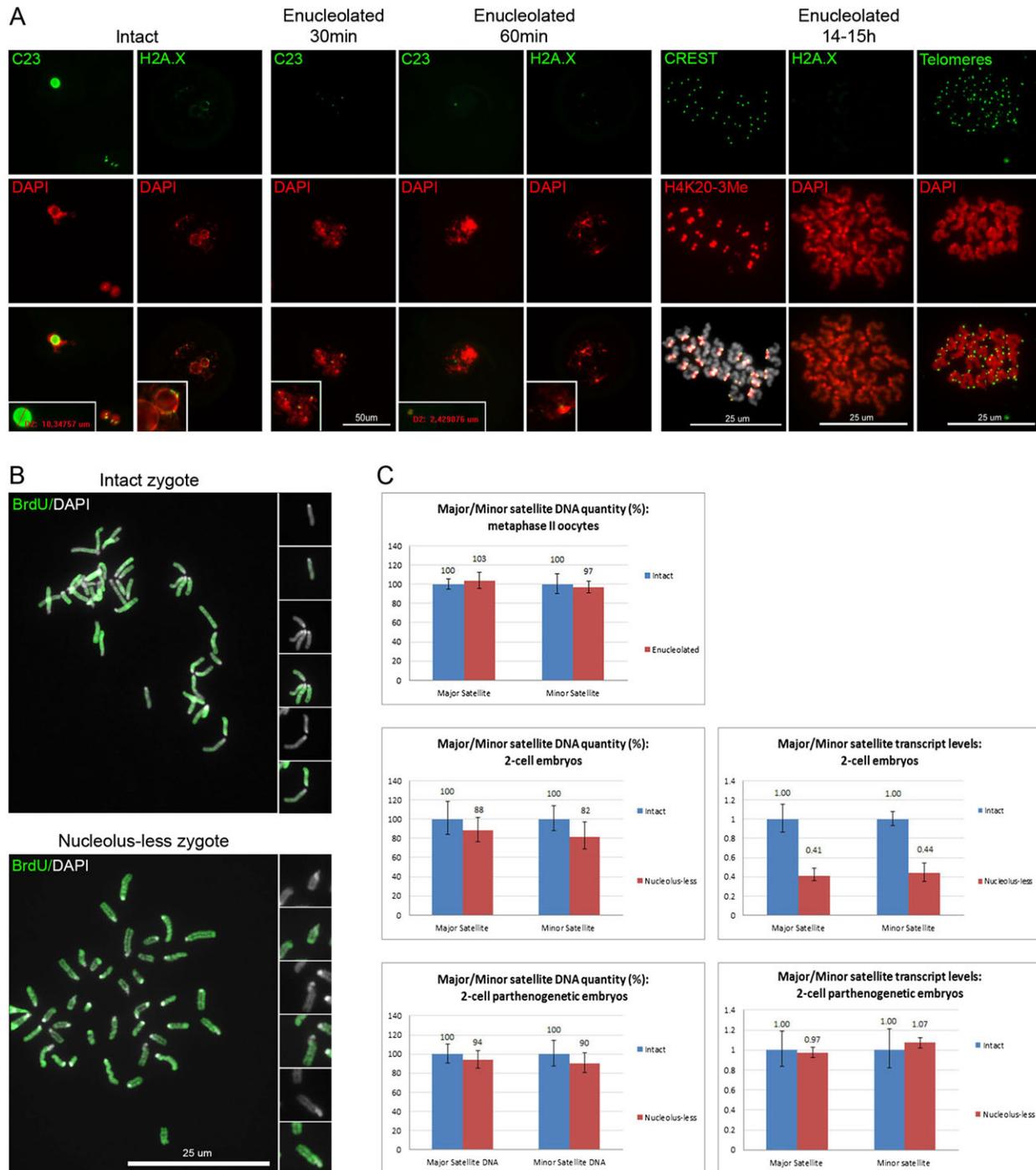
To dissect the roles of the parental genomes and to examine the effect of NPB removal more precisely, haploid parthenogenetic embryos were analysed. Again, a significant reduction of both major and minor satellite DNA was found in nucleolus-less parthenogenetic embryos (to 94% and 90%, respectively;  $P < 0.05$ ; Fig. 4C). Because this reduction is approximately half of that observed in biparental embryos (88% for major satellite DNA and 82% for minor satellite DNA), we conclude that the satellite DNA reduction affects both parental genomes. However, when the satellite transcript levels were measured, no marked difference between the control and the nucleolus-less group was found (Fig. 4C). Therefore, whereas the satellite DNA reduction affects both parental genomes after fertilisation, only the paternal satellite transcripts are downregulated in nucleolus-less embryos. This is in agreement with a previous report (Probst et al., 2010) that showed that it is the paternal transcript that is expressed during early embryogenesis.

### Heterochromatin recombination frequency is not altered in nucleolus-less embryos

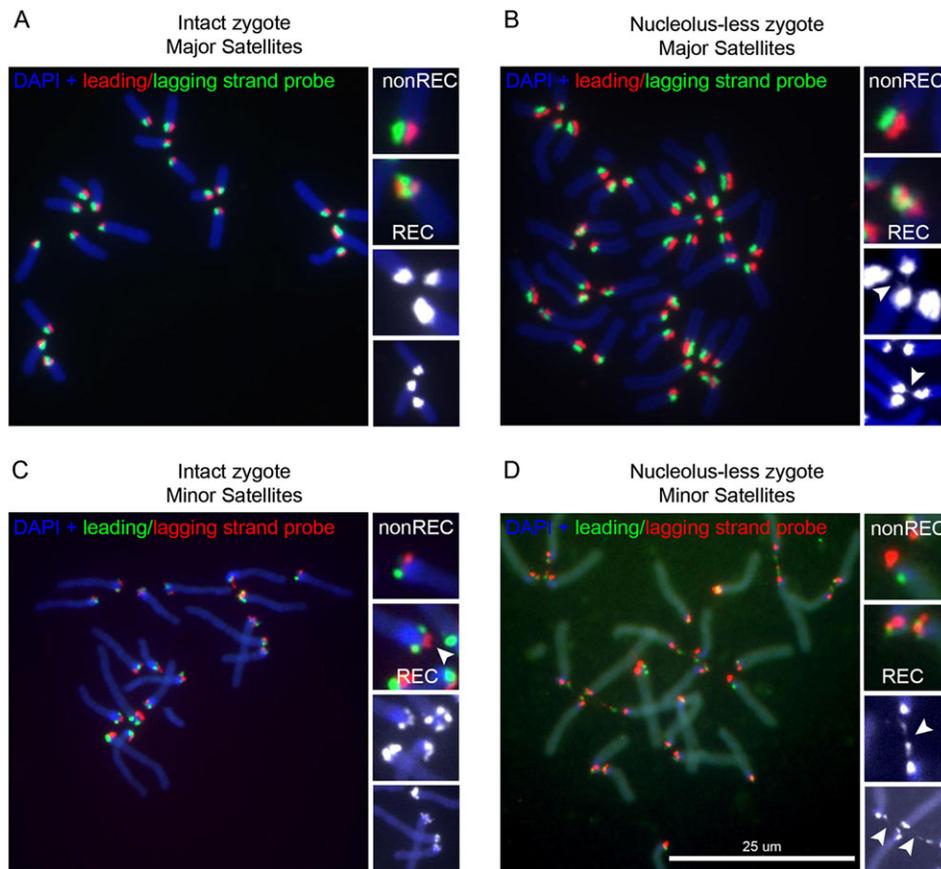
There are two possible mechanisms that could lead to the satellite DNA decrease: replication-associated loss or extensive mitotic recombination of satellite domains (Talbert and Henikoff, 2010; Jaco et al., 2008). Previously, CO-FISH (chromosome orientation fluorescence *in situ* hybridisation) was successfully applied to investigate the recombination frequencies of satellite DNA. Both control and nucleolus-less IVF embryos showed comparable frequencies of major satellite recombination events (16% and 13%, respectively; chi-squared test,  $P > 0.05$ ; Fig. 5A,B). Furthermore, no differences were found in the recombination frequency between *in vivo* produced and IVF embryos (17% and 16%, respectively; chi-squared test,  $P > 0.05$ ). However, nucleolus-less embryos exhibited distorted and irregular pericentric heterochromatin morphology, often accompanied by chromosome bridging (Fig. 5B, insets). A similar analysis was performed for the minor satellites (Fig. 5C,D). Again, comparable frequencies were found between nucleolus-less, control IVF and *in vivo* produced embryos (all 87%; chi-squared test,  $P > 0.05$ ) but again extensive chromosome bridging was observed (Fig. 5D, insets). Thus, nucleolus-less embryos fail to effectively separate chromosome bridges that form from the centric/pericentric chromatin.

### Nucleolus-less embryos exhibit signs of replication stress during the first S phase and aberrant chromatin remodelling

In somatic cells and yeast, failure to separate chromosomes effectively during mitosis was observed under replication stress conditions (Sofueva et al., 2011; Chan et al., 2007, 2009). To determine whether the same applies to the nucleolus-less embryos, EdU pulse labelling followed by staining for phosphorylated histone H2A.X (S139) was used. This histone modification has been shown to be associated not only with double-strand DNA



**Fig. 4. The effect of NPB removal (enucleolation) on oocytes and embryos.** (A) Typically, GV oocytes contain a single nucleolus, here detected by the presence of C23, and exhibit only low levels of phosphorylated H2A.X (intact). From 30 min post-enucleolation, several small C23-positive foci can be found (enucleolated 30 min). These typically fuse later on (enucleolated 60 min). The levels of phospho-H2A.X remain unchanged. Based on the diameter (insets) of the C23-positive foci the residual NPB material volume following enucleolation was estimated to be ~1% (in this case corresponding to 1.3%). Despite the close association of centromeres with nucleoli, once matured to MII, enucleolated oocytes (enucleolated 14-15 h) contain intact chromosomes (telomeres) with easily detectable pericentric chromatin (H4K20-3Me) and centromeres (CREST) and show no signs of DNA damage (H2A.X). Bottom row, merged images. In total, over 250 good spreads were analysed. (B) The first embryonic S phase occurs irrespective of nucleolus presence. However, nucleolus-less zygotes contain decompacted chromosomes with an asymmetric distribution of pericentric heterochromatin. Insets show chromosomes at higher magnification. (C) The absence of the nucleolus affects major and minor satellite DNA content and the abundance of major and minor satellite transcripts. The satellite DNA content is not influenced by the enucleolation procedure, as enucleolated and intact oocytes contain comparable levels of satellite DNA. However, this changes after the first embryonic cycle. The reduction in the satellite DNA content of parthenogenetic embryos was approximately half that of biparental embryos, whereas the transcript levels remained unchanged. Therefore, the satellite DNA loss affects both parental genomes but it is only the paternal transcript that is downregulated. The decrease of both major and minor satellite DNA/transcripts in nucleolus-less embryos (biparental/parthenogenetic) is statistically significant ( $P < 0.05$ , Mann-Whitney U-test). Mean ratios are shown, with error bars representing the minimum and maximum ratio values; in each group 55 embryos were analysed.

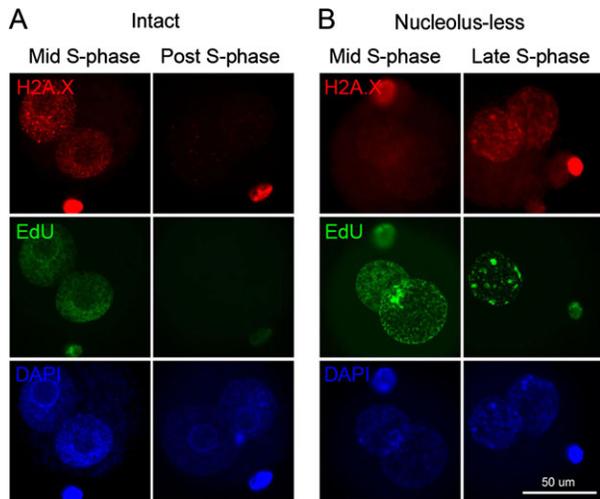


**Fig. 5. NPB absence does not alter satellite recombination frequency but causes extensive chromosome bridging.** (A) Major satellite sequences (pericentric chromatin) only rarely recombine in control and nucleolus-less embryos as shown by CO-FISH. Typically, separate non-interrupted signals are observed when no recombination occurs (nonREC). However, when recombination takes place the signals become overlapping or discontinuous (REC). Signals are pseudocoloured white for a better visualisation (insets). (B) A comparable recombination frequency was observed in nucleolus-less embryos. However, when closely evaluated, chromosome bridges (stretched chromatin) were found to emanate from the pericentric part of the chromosomes (insets, arrowheads). (C) In contrast to somatic cells, minor satellites (centric chromatin) were found to be highly recombinant in zygotes. Again, non-recombined satellite domains are marked by separate uninterrupted signals. Chromosome bridges were found only rarely in controls (insets). (D) Also, nucleolus-less embryos show a high degree of centric chromatin recombination (discontinuous or overlapping signals) but many chromosomes remain connected together by centric chromatin bridges (insets, arrowheads). The centric chromatin bridges (highly stretched chromatin) were even more distinguishable than those of the pericentric region. Altogether, 250-700 chromosomes were evaluated in each group and experiment. Statistical analysis was performed by chi-squared test. Figure shows merged images of leading and lagging strand probe hybridization (red, green).

breaks but also with collapsing replication forks and replication stress in general (Sirbu et al., 2011; Chanoux et al., 2009; Ward and Chen, 2001). An EdU pulse was applied at different time points and zygotes were staged according to Bouniol-Baly et al. (Bouniol-Baly et al., 1997). Surprisingly, early S-phase and mid-S-phase nucleolus-less zygotes showed a weaker phospho-H2A.X signal than controls. However, as control embryos reached the end of S phase, most of the phospho-H2A.X signal was lost (Fig. 6). By contrast, a strong diffuse phospho-H2A.X signal persisted in pronuclei of nucleolus-less zygotes even after they ceased replicating. It should be noted that the nucleolus-less zygotes often exhibited replication asynchrony between pronuclei, as described previously (Bouniol-Baly et al., 1997), whereas very little replication asynchrony was observed in controls (both *in vivo* and IVF produced).

Based on the phenotype and published results, we investigated the presence and localisation of ATRX and DAXX proteins. ATRX is a maternal chromatin remodelling factor that is essential for development (Baumann et al., 2010). It contains an ATPase/helicase domain, interacts with the H3.3 histone chaperone DAXX and is necessary for recruiting it to pericentric heterochromatin in oocytes

and telomeres in somatic cells (Baumann et al., 2010; Lewis et al., 2010; Drane et al., 2010). In both enucleolated and intact GV oocytes, ATRX and DAXX were localised to DAPI-dense pericentric heterochromatin regions as described by Baumann et al. (Baumann et al., 2010). Moreover, no differences were found in chromosomes from enucleolated and control MII oocytes. After fertilisation, ATRX and DAXX localised to both pronuclei in control IVF embryos at relatively high levels. By contrast, whereas ATRX levels remained largely unchanged, markedly less DAXX could be detected in pronuclei of the nucleolus-less zygotes (Fig. 7A). When Triton X-100 permeabilisation was applied together with the fixation, the difference became even more evident (Fig. 7B). Under these conditions, in control embryos DAXX localised to the vicinity of NPBs (especially in female pronuclei), where centric/pericentric chromatin is localised, whereas no DAXX was detected in nucleolus-less zygotes. Thus, even though DAXX is transported to pronuclei in nucleolus-less zygotes it is not stably associated with DNA. This strongly suggests abnormal remodelling of the centric/pericentric chromatin in both parental pronuclei and is in agreement with the observation that satellite DNA loss affected chromosomes irrespective of their parental origin.



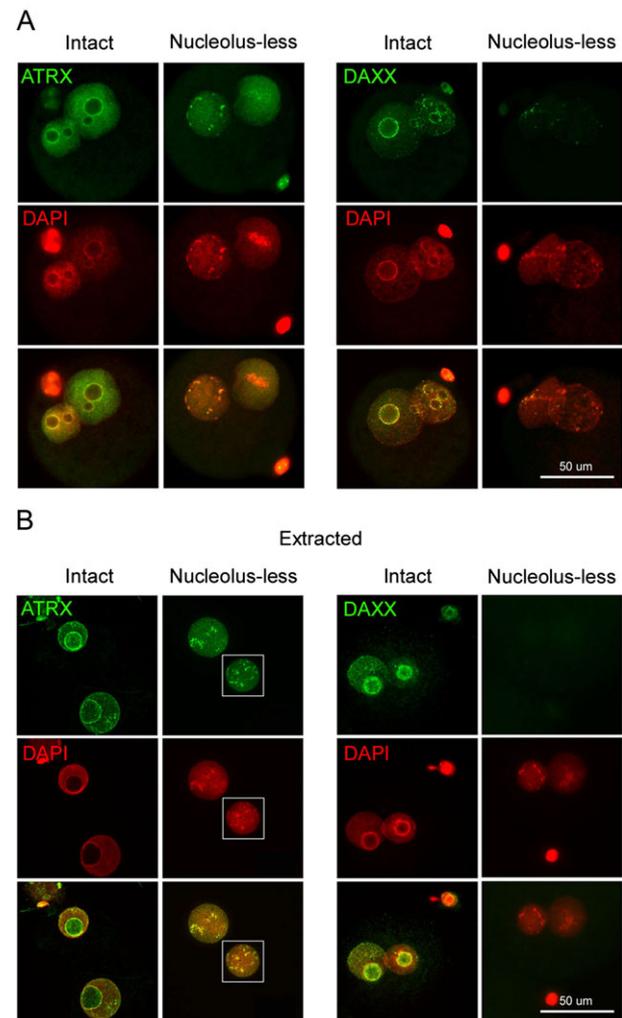
**Fig. 6. Nucleolus-less embryos show a persistent phospho-H2A.X signal even after termination of DNA replication.** (A) Control zygotes (intact) show a strong phospho-H2A.X signal from very early S-phase stages (here mid-S phase is shown). However, when replication is finished (post-S phase, G2) the phospho-H2A.X signal is largely lost from the pronuclei (note the absence of EdU incorporation). Overall, 126 *in vivo* and 74 *in vitro* produced embryos were evaluated at different S-phase time points. (B) Although nucleolus-less embryos initially show a lower phospho-H2A.X signal (mid-S phase) than controls, this signal persists even after the pronuclei have ceased replicating DNA (late S phase). Note that the pronuclei in nucleolus-less embryos tend to be slightly smaller than controls. Moreover, a pronounced replication asynchrony can often be observed (late S phase); here, the maternal pronucleus is finishing replication (heterochromatin) whereas the paternal pronucleus has already ceased replicating.

In summary, abnormal chromatin remodelling, replication and expression of the centric and pericentric satellite DNA accompany the developmental arrest of nucleolus-less 2-cell mouse embryos.

## DISCUSSION

Altogether, our results show an unusual but essential role of the maternal nucleolus (NPB) in very early mammalian development. Contrary to our expectations, this structure does not seem to be involved in RNA polymerase I transcription activation; instead, removal of this nuclear organelle has a profound effect on the regulation of centric and pericentric DNA sequences and results in alterations in the expression profile at the time of embryonic genome activation.

Initially, we aimed to clarify the extent to which maternal nucleolar proteins and/or maternal mRNAs for nucleolar factors are used during early embryogenesis. We focused on four of the best-characterised nucleolar factors: UBF, B23, C23 and Fbl. Surprisingly, oocytes do not show a tendency to stockpile these mRNAs and proteins and their abundance is substantially reduced by the time of fertilisation or soon after. This challenges the classical view that, during their growth, oocytes make large stocks of material and that NPBs serve as the storage site of nucleolar proteins that are subsequently used by the embryo (Chouinard, 1971; and references therein). That NPBs are dispensable for rRNA synthesis is further documented by the fact that even nucleolus-less embryos are able to activate pre-rRNA production and processing at levels comparable to controls; thus, the function of NPBs during early embryogenesis is unrelated to ribosome production. To dissect the impact of NPB absence and the possible negative effect of enucleation on rRNA production, embryos originating from intact *in vitro* matured oocytes were included in the study, but essentially no difference



**Fig. 7. NPB absence causes the loss of DAXX labelling in pronuclei.** (A) Whereas ATRX was found to be present in both pronuclei irrespective of NPB presence (left), substantially less DAXX was detected in the pronuclei of nucleolus-less zygotes (right). Note the residual DAXX signal on heterochromatin. Bottom row, merged images. In total, ~70 embryos in mid-S phase were analysed in each group. (B) When detergent treatment (extracted) is applied during fixation, the DAXX labelling in nucleolus-less zygotes is completely lost (left), indicating that DAXX is not stably bound to DNA. ATRX signals remain largely unchanged even with detergent treatment (right). Boxes show the corresponding female pronucleus. Overall, at least 20 embryos in mid-S phase were analysed in each group. Bottom rows, merged images.

was detected. Therefore, enucleation does not negatively affect these parameters. In fact, embryos lacking the nucleolus show a slightly higher pre-rRNA synthesis rate (although this difference was not statistically significant).

With respect to a possible negative impact of enucleation, DNA damage was also examined. The majority of enucleated oocytes showed no signs of excessive DNA damage, as only 16% exhibited a higher phospho-H2A.X signal. Because this number is comparable to the average ratio of oocytes that fail to reach the MII stage, the phospho-H2A.X-positive oocytes might simply become arrested and never reach the MII. When MII chromosomal spreads were analysed, DNA lesions were only rarely observed and were typically located distal to the centromeric part that is known to associate closely with NPBs in both oocytes and early embryos (Longo et al., 2003; Aguirre-Lavin et al., 2012). Thus, no indications of DNA damage were found

in oocytes following enucleation. The reason for the absence of DNA damage might lie in the fact that when enucleation is performed the oocytes are transcriptionally inactive (transcriptionally active oocytes cannot be simply enucleated) and rDNA is probably no longer located inside NPBs (Mirre and Stahl, 1978; Vagner-Capodano et al., 1987; Zatsepina et al., 2000). In agreement with this, Kyogoku et al. (Kyogoku et al., 2011) showed that, if actinomycin D is used to block RNA polymerase I activity in transcriptionally active oocytes, such oocytes can also be enucleated.

Overall, very few differences between control and nucleolus-less embryos were found. The only clear variation was the altered chromosome morphology that was apparent when zygotes were analysed. Specifically, we noted changes in chromosome condensation and asymmetric pericentric heterochromatin distribution. We found reduced satellite DNA in both IVF and ICSI biparental and parthenogenetic nucleolus-less embryos. However, the reduction in satellite transcripts was observed only in embryos containing the paternal genome and not in parthenotes. Because parthenogenetic embryos also fail to develop beyond the 2-cell stage, the reduction in satellite transcripts does not appear to be the only cause of developmental arrest. Altered satellite transcript levels have, however, been reported to be important for restructuring male centromeres (Probst et al., 2010). The role of the maternal nucleolus in this process has not been described previously.

We then focused on the potential mechanism of the satellite DNA reduction. Generally, there are two possible explanations: the first is extensive mitotic recombination of the satellites; the second, aberrant replication and/or remodelling (Chan et al., 2009; Lukas et al., 2011; Talbert and Henikoff, 2010).

The overall recombination frequencies were similar between control and experimental embryos. Therefore, extensive mitotic recombination is unlikely to be the reason for the developmental arrest. Interestingly, the recombination frequency of minor satellites seems to be much higher in zygotes (on average ~87% of these sequences were found to be recombinant) compared with somatic cells, where the frequency ranges from 15% to 20% (Jaco et al., 2008). This is unlikely to be caused by any differences in the methodology and/or probes used because we followed the published protocol in detail and our preliminary experiments in parthenogenetic embryos showed a much lower recombination frequency. Whether this high recombination frequency is the result of combining two different parental genomes after fertilisation remains to be elucidated. Although the major satellite recombination rate in somatic cells is not known precisely, in our system it was ~16-17% and was roughly comparable to the minor satellite recombination frequency in somatic cells. Again, no difference in the frequency was observed between experimental and control groups.

The second potential mechanism of satellite DNA loss might include abnormal first embryonic S-phase progression. Indeed, an altered dynamics of phospho-H2A.X deposition during this phase was observed in nucleolus-less embryos. Although there is some discrepancy in the literature with respect to the presence of phospho-H2A.X in mouse zygotes, our results in control embryos are in agreement with several published articles in which this histone modification was closely monitored (e.g. Wossidlo et al., 2010; Ziegler-Birling et al., 2009).

Bouniol-Baly et al. (Bouniol-Baly et al., 1997) described that the paternal pronucleus initiates and finishes DNA replication earlier than the maternal pronucleus. Interestingly, when we followed the patterns of EdU incorporation at different time points the described replication asynchrony between pronuclei was observed in nucleolus-less zygotes only. By contrast, such a marked replication asynchrony

was never observed in control zygotes (both *in vivo* and IVF produced) under our conditions. Indeed, several articles questioning the replication asynchrony have been published (Yamauchi et al., 2009; and references therein). For example, Yamauchi et al. (Yamauchi et al., 2009) reported that, even though mouse zygotes can tolerate asynchronous DNA replication in their pronuclei, this does not normally occur after IVF or ICSI.

An interesting phenomenon has been described in yeast *cdc14* mutants. Cdc14 is a cell cycle regulatory protein that localises to nucleoli throughout most of the cell cycle. These yeast mutants exhibit heterochromatin under-replication but this does not trigger cell cycle checkpoints and does not alter replication-associated gene expression immediately; rather, this defect is manifested during the next cell cycle (Dulev et al., 2009). Essentially the same situation was observed in our system, including the changes in replication-associated transcripts at the 2-cell stage. However, the alteration in gene expression at this stage might be primarily related to the fact that the major genome activation occurs at this time, and the lag in gene expression change might be simply explained by the presence of maternal transcripts and/or proteins that are involved in sustaining the first embryonic cell cycle. Currently, we cannot exclude this possibility.

In addition to the replication-associated changes, the alteration in gene expression and the satellite DNA loss in nucleolus-less embryos, we show that the absence of the NPB causes extensive chromosome bridging during the first embryonic mitosis. The reason why we initially did not observe these fine satellite bridges in our primary replication/karyotype analysis might be related to the use of HCl in the BrdU assay. It is well known that HCl causes DNA hydrolysis and thus might have caused the loss of these DNA bridges. However, the use of CO-FISH with peptide nucleic acid (PNA) probes that require minimal or no denaturation of target DNA allowed their detection. Interestingly, both minor and major (centric and pericentric) satellite sequences were involved in this bridging. This contrasts with results obtained in somatic cells, as only centric chromatin was indicated to be involved in ultrafine bridge formation (Baumann et al., 2007; Chan et al., 2007). Recent results in somatic cells show that the formation of chromosome bridges is relatively common, even under optimal conditions, but is enhanced significantly with replication stress. Indeed, when we analysed closely chromosomal preparations from zygotes these bridges were present even in control embryos. Typically, 0-3 bridged pairs were found per 40 chromosomes (on average ~8% of all chromosomes evaluated were involved in bridging). By contrast, in nucleolus-less embryos the vast majority of chromosomes were affected (sometimes as many as 38 out of 40 chromosomes were bridged) and the overall frequency was 78%. Chan et al. (Chan et al., 2007) proposed two possible models to explain DNA bridge formation: the sequences are either fully replicated but fail to separate (catenate) or they are only partially replicated and thus the DNA remains hemicatenated. If we apply the first model to our system, then chromosome bridging would have to be followed by massive DNA degradation because nearly 20% of the minor satellites is lost between the 1- and 2-cell embryonic stages (plus the concomitant major satellite loss). However, recent results in somatic cells do not support the degradation model, as the DNA lesions were shown to be protected by a protein sheltering mechanism and are effectively repaired in the subsequent G1 phase (Lukas et al., 2011).

Concomitantly, we show that the H3.3 histone chaperone DAXX fails to stably bind to DNA in nucleolus-less zygotes. This strongly implies abnormal or absent remodelling of the satellite sequences. In contrast to somatic cells, where H3.3 is incorporated into DNA in a replication-independent manner, recent results in embryos show that H3.3 incorporation occurs even during S phase (Akiyama et al.,

2011; Santenard et al., 2010). This is understandable given the necessity of extensive remodelling of both parental genomes after fertilisation. Unfortunately, no anti-H3.3-specific antibodies are currently available and the direct test cannot be performed readily. Our results are in agreement with those of Santenard et al. (Santenard et al., 2010), who injected mRNA for different histone H3 variants into embryos. They showed that H3.3 is specifically necessary for the remodelling of centromeres and the introduction of a mutant version causes developmental arrest.

DAXX forms a complex with ATRX and this is necessary for its correct targeting (Drane et al., 2010; Lewis et al., 2010; Baumann et al., 2010). In nucleolus-less embryos, however, ATRX localisation remains unaffected. Therefore, it is highly probable that there are additional – presumably nucleolar – proteins involved and engaged in another level of ATRX/DAXX regulation. This, however, remains to be investigated.

Taken together, our data show an unusual and previously unappreciated role of the maternal nucleolus in early mammalian embryogenesis.

## MATERIALS AND METHODS

### Oocyte and embryo collection

Mice (C57BL/6 and B6D2F1; Charles River Laboratories) were kept under standard housing conditions with 12 h light/dark cycle (light on at 8:00 a.m.). Females were injected intraperitoneally with 7.5 I.U. PMSG (Calbiochem). After 44 h, mice were sacrificed by cervical dislocation and GV stage oocytes were released from follicles into HTF-HEPES medium (Zenith Biotech). The oocytes were then cultured in MEM medium supplemented with gentamicin (50 µg/ml), sodium pyruvate (0.22 mM), dibutyl cyclic adenosine monophosphate (dbcAMP; 150 µg/ml) and bovine serum albumin (BSA; 4 mg/ml) at 37°C in an atmosphere of 5% CO<sub>2</sub> and used for further manipulations. Alternatively, after a brief culture, oocytes were either washed in phosphate buffered saline (PBS)/0.1% polyvinyl alcohol (PVA), collected in Laemmli sample buffer and used for western blotting or fixed and used for immunofluorescence.

MII oocytes were obtained from stimulated females [7.5 I.U. PMSG, followed by 7.5 I.U. hCG (Intervet) 48 h later]. Ovulated oocytes were collected from ampulae of sacrificed mice.

*In vivo* produced embryos were obtained from stimulated females as above, except that after hCG injection females were caged with males. Next morning, females were sacrificed and 1-cell stage embryos were released from ampulae into HTF-HEPES medium and further cultured in KSOM medium (Zenith Biotech).

### Enucleation and intracytoplasmic sperm injection (ICSI)

Enucleation of GV stage oocytes and nucleolus re-injection were performed essentially as described (Fulka et al., 2003). Enucleated and control (nucleolus re-injected) oocytes were allowed to recover in MEM supplemented with dbcAMP (to prevent GV breakdown) for 30 min or 1 h and used for immunofluorescence. Alternatively, manipulated oocytes were washed free of dbcAMP and cultured in MEM medium to MII. The MII oocytes were used for ICSI as described (Yoshida and Perry, 2007) and then cultured in KSOM medium. ICSI was used especially in experiments in which maximum embryo synchronisation was required (e.g. rRNA production and processing, gene expression profiling).

### *In vitro* fertilisation (IVF)

IVF of *in vitro* matured MII oocytes (manipulated and control) was carried out as described (Takeo and Nakagata, 2011). Fresh sperm from cauda epididymides of DBA/2 male mice (minimum 8 weeks old) were used.

### Parthenogenetic activation

Parthenogenetic activation of MII oocytes was performed as described (Kishigami and Wakayama, 2007) except that the activation was carried out in KSOM medium and cytochalasin B was omitted.

### BrdU incorporation

Embryos were incubated in medium supplemented with 5-bromo-2'-deoxyuridine (BrdU; 50 µM). Embryos were fixed in 4% paraformaldehyde (PFA) for 10 min and processed for immunofluorescence. Alternatively, demecolcine was added (0.1 µg/ml; Sigma-Aldrich) in order to arrest embryos at the first embryonic mitosis. Chromosomal spreads were prepared as described (Kamiguchi and Mikano, 1986). A short incubation in 4 M HCl (15 min) followed by immunofluorescence was used to detect BrdU. YOYO-1 (10 nM; Life Technologies) labelling for 10 min was used to counterstain the chromosomes.

### Conventional immunofluorescence

Zona pellucida-free oocytes/embryos were fixed in 4% PFA in PBS for 15 min and permeabilised by 0.2% Triton X-100 in PBS. After blocking in 1% BSA in PBS, the samples were incubated with selected primary antibodies overnight at 4°C, washed, incubated with secondary antibodies, mounted in ProLong Gold supplemented with DAPI (Life Technologies) and evaluated under an Olympus IX71 microscope. The images were captured by ImagePro software (Media Cybernetics) and processed by Adobe Photoshop. Antibodies used: anti-BrdU (Roche, 11170376001; 1:100; also used for BrUTP detection), anti-ATRX and anti-DAXX (Santa Cruz Biotechnology, sc-15408 and sc-7152; both 1:500), anti-γH2A.X (Millipore, 05-636-I; 1:2000). All secondary antibodies were purchased from Jackson ImmunoResearch; a 1 h incubation at 37°C or room temperature was used.

### Chromosome spreading and antigen retrieval (AR)

Oocytes and embryos were gently spread on SuperFrost slides as described (Hodges and Hunt, 2002). The slides were either used directly for immunofluorescence or AR was first performed. For AR, the slides were boiled in 10 mM sodium citrate containing 0.05% Tween 20 (pH 6.0) for 15 min, briefly washed in PBS and blocked/permeabilised as above. Primary antibody incubation was performed for 1 h at 37°C. The samples were examined under an Olympus BX61 microscope. Antibodies used: anti-ATRX, anti-DAXX and anti-γH2A.X (see above), anti-C23 (Abcam, ab70493; 1:400), anti-B23 (Sigma-Aldrich, B0556; 1:200), anti-Fibrillarin (Cell Signaling, mAB#2639; 1:400), anti-UBF (Abnova, H00007343-M01; 1:200), anti-H4/K20-3Me (Abcam, ab9053; 1:2000), CREST antiserum (Fitzgerald Industries International, 90C-CS1058; 1:1000), anti-SC35 (Sigma-Aldrich, S4045; 1:200), anti-nuclear pore complex (NPC; Covance, MAb414; 1:100). Secondary antibodies were as above.

### BrUTP injection

For the assessment of general transcription, embryos were injected with 5-bromouridine-5'-triphosphate (BrUTP) as described (Bouniol-Baly et al., 1997). After 1 h incubation, the embryos were fixed in 4% PFA and nascent transcripts were detected by immunofluorescence.

### Telomere peptide nucleic acid (PNA) fluorescence *in situ* hybridisation (FISH)

Chromosome spreads were prepared as described above. FISH was performed according to the PNA probe manufacturer (Panagene). TelC-Cy3 probe was used.

### Chromosome orientation FISH (CO-FISH)

CO-FISH was performed as described (Jaco et al., 2008; Falconer et al., 2010). In brief, this method involves the incorporation of BrdU into DNA followed by UV exposure and enzymatic degradation of the newly synthesized strand. When such chromosomes are hybridised to oppositely oriented probes (coupled to different dyes) separate uninterrupted non-overlapping signals are obtained if no recombination has occurred.

### EdU labelling

5-ethynyl-2'-deoxyuridine (EdU) labelling was performed as recommended by the manufacturer (Life Technologies) except that 100 µM EdU was added to the KSOM medium for 1 h. Subsequently, conventional immunofluorescence was performed as described above.

### Western blotting

Samples of 100 mouse oocytes or embryos were separated by 10% SDS-PAGE. A control sample of 1 µg total 3T3A31 cell lysate was included. Proteins were transferred onto a PVDF membrane (GE Healthcare), which was blocked in 5% nonfat dried milk (Bio-Rad) in Tris-buffered saline supplemented with 0.1% Tween 20 (TBST). Antibodies used were: anti-UBF (1:5000), anti-B23 (1:2000), anti-fibrillarin (1:1000), anti-C23 (1:2000) and anti- $\alpha$ -tubulin (Sigma-Aldrich, T6074, 1:2000). HRP-conjugated secondary antibody was obtained from Bio-Rad. Proteins were visualised using the ECL Advance Western Blotting Detection Kit (GE Healthcare) as recommended by the manufacturer.

### RT-PCR and real-time PCR

Samples of five oocytes/embryos were processed using the FastLane Cell RT-PCR Kit (Qiagen) as recommended by the manufacturer, snap-frozen in liquid nitrogen and stored  $-80^{\circ}\text{C}$  until use. A genomic DNA elimination step was performed followed by reverse transcription (RT) with random hexamers and SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Synthetic EGFP mRNA was added to the sample prior to RT. No-RT controls were also evaluated. The reaction was diluted and 2 µl used for PCR with Power SYBR Green Master Mix (Life Technologies). Total PCR volume was 20 µl. Serial dilution of the template was used to determine the PCR efficiencies for data evaluation according to Pfaffl (Pfaffl, 2001). Primers are listed in supplementary material Table S1. PCR was performed using the 7900HT Fast Real-Time PCR System (Life Technologies) with cycling conditions:  $95^{\circ}\text{C}$  10 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 s and  $59^{\circ}\text{C}$  for 1 min. Expression values are given as relative ratios=minimum and maximum relative ratios. All PCR products were cloned using the TOPO-TA Cloning Kit For Sequencing (Life Technologies) and sequenced.

### SNP analysis

cDNA was amplified with PerfectTag polymerase (5 PRIME). The PCR product was digested by *Van911* (C23) or *HhaI* (UBF) (Fermentas) and analysed on either 4% TBE agarose (C23) or on standard PAGE (UBF, Ubtg) gels. In both cases, SybrSafe (Life Technologies) was used to visualise the fragments.

### Major/minor satellite real-time PCR

To investigate the content of major and minor satellite sequences, embryos were allowed to pass through the first embryonic S phase (zygotes) and were collected at the very early 2-cell stage (after the first replication but prior to the second round,  $\sim 22$  h after ICSI or IVF). Genomic DNA was isolated as described (Zuccotti and Monk, 1995) and the lysate was amplified by real-time PCR as described (Zhu et al., 2011). Alternatively, cDNA from 2-cell embryos was used as a template. To monitor replication *36B4* (*Rplp0*) was used, a well-characterised single-copy gene that is often used in quantitative telomeric assays (Callicott and Womack, 2006). The major and minor satellite primer sets were validated for both DNA and RNA. The results were subjected to statistical analysis (Mann-Whitney test). For real-time PCR analysis at least nine different measurements were evaluated in each group.

### Gene expression profiling

Samples of five embryos were collected as for real-time PCR analysis. Reverse transcription was performed with the RT<sup>2</sup> PreAMP cDNA Synthesis Kit and RT<sup>2</sup> PreAMP Pathway Primer Mix (SABiosciences, now Qiagen) as described by the manufacturer. The Mouse Cell Cycle Array (PAMM-020ZC) was used for gene expression analysis (SABiosciences). Results were evaluated with the help of the SABiosciences Excel application designed for this array.

### Acknowledgements

We thank Josef Fulka, Jr, Atsuo Ogura and Stanislava Martinkova for suggestions and help with some experiments.

### Competing interests

The authors declare no competing financial interests

### Author contributions

H.F. conceived the study, designed and performed experiments and wrote the manuscript. A.L. performed the oocyte manipulations and immunofluorescence.

### Funding

H.F. is supported by the Czech Science Foundation [P302/11/P069].

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105940/-/DC1>

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# Somatic Cell Nuclear Transfer–Derived Embryonic Stem Cell Lines in Humans: Pros and Cons

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## Abstract

The recent paper, published by Mitalipov's group in *Cell* (Tachibana et al., 2013), reporting the production of human somatic cell nuclear transfer (SCNT) embryonic stem cells (ESCs), opens again the debate if, in the era of induced pluripotent stem cells (iPSCs), the production of these cells is indeed necessary and, if so, whether they are different from ESCs produced from spare embryos and iPSCs. It is our opinion that these questions are very difficult to answer because it is still unclear whether and how normal ESCs differ from iPSCs.

## Dedifferentiation of Somatic Cells

THERE ARE SEVERAL METHODS of dedifferentiating the somatic cell nucleus (Halley-Stott et al., 2013), but it is also evident that somatic cell nuclear transfer (SCNT) represents the most efficient artificial approach, although it has not yet fully explained why. We suggest that an important point of SCNT reprogramming efficiency is that the transferred nucleus, in the course of reprogramming, uses some oocyte components that are incorporated into the remodeled nucleus. The other reprogramming approaches—induced pluripotent stem cells (iPSCs) production, heterokaryons—are in fact “closed” systems, *i.e.*, reprogramming is induced in a given cell with an intact nucleus. On the other hand, SCNT is an “open” system in which the nuclear membrane breakdown occurs within a very short time interval after introduction of the nucleus into the cytoplasm and then the chromosomes condense. Following a subsequent artificial activation, which is usually applied approximately 1 h after nuclear transfer (NT), the pseudopronuclei start to form and, in the mouse, for example, they are typically very visible after about 6 h. Morphologically they are much larger when compared to the original transferred nucleus and contain very visible nucleoli (nucleolus precursor bodies, NPBs) that are morphologically indistinguishable from similar structures that can be found in pronuclei of normally fertilized or parthenogenetic one-cell-stage embryos. These dramatic changes cannot be detected for example in iPSCs or heterokaryons.

## The Role of Oocyte Organelles in Reprogramming

The invention of the enucleation method, in which NPBs can be microsurgically removed either from oocytes (or even from one-cell-stage embryos; Fulka Jr et al., 2003; Fulka and

Fulka Jr., 2010), clearly demonstrated that if enucleolated oocytes are fertilized or parthenogenetically activated, normal-sized pronuclei are formed in one-cell stage embryos; however, these pronuclei or pseudopronuclei do not contain typical nucleoli (NPBs) and consequently embryos fail to develop (Fulka et al., 2004; Ogushi et al., 2008). In SCNT experiments, where enucleolated and subsequently matured oocytes were used for the preparation of cytoplasts, the nucleus that is transferred into the cytoplasm contains its own nucleolus(i) but this nucleolus(i) cannot substitute for the original oocyte nucleolar material that is dissolved in the oocyte cytoplasm. Thus, pseudopronuclei do not contain nucleoli.

These observations clearly explain, of course to a certain extent, why early NT experiments in mammals where nuclei were transferred into enucleated zygotes were unsuccessful (McGrath and Solter, 1984). The nucleoli were removed along with the entire pronuclear material. Typically, only two-cell-stage mouse embryo nuclei supported development, but no development was observed when more advanced nuclei were used. It must be noted here that in the mouse the transformation of nucleoli in embryos begins at the late two-cell stage. Moreover, this also explains why mitotic one-cell-stage embryos can be used as cytoplasts (Noggle et al., 2011). Here the zygote nucleolar material is also dissolved in the cytoplasm. Nucleoli are released into the cytoplasm when zygotes are enucleated selectively, and this also results in successful development of reconstructed embryos (Greda et al., 2006).

The role of nucleolar material in the process of normal and SCNT embryo development is not yet understood completely. The developmentally competent oocytes and zygotes

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contain atypical nucleoli (NPBs) that are composed from dense fibrillar material. On the other hand, somatic and advanced embryonic cells contain nucleoli composed from fibrillar, dense fibrillar, and granular material. It has been commonly accepted that the original oocyte nucleolar material is, as the embryo develops, gradually transformed into the somatic cell nucleoli and that the oocyte nucleolus (NPB) serves as a depot or storage site of material that is, after fertilization, used by the early embryo. Some recent results, however, have forced us to reconsider the whole concept of the NPB as a passive storage site of nucleolar proteins because they indicate that this, in fact, might be very far from the primary role of this structure. Currently, it is believed that the NPB actually plays an active role in supporting early development unrelated to ribosomal RNA production and is probably involved in the regulation of certain cell cycle processes during very early embryogenesis. However, some very recent results also demonstrate that NPBs might also be essential for certain structural and chromatin modification processes that occur within a very short time interval after fertilization, when both the maternal and paternal chromatin are remodeled extensively. For further successful development, heterochromatin at pericentric satellites must be in a very close contact with NPBs (Probst and Almouzni, 2011).

All of these hints of the actual role of NPB are supported by experiments performed by Ogushi and Saitou (2010), who transferred NPBs into metaphase II oocytes originating from germinal vesicle (GV)-stage enucleolated oocytes. These oocytes were subsequently fertilized *in vitro*. Eventually, NPBs were transferred into early and late one-cell-stage embryos that were produced by fertilization of previously enucleolated oocytes. When NPBs were transferred into mature oocytes, the development of embryos was rescued. However, this was not the case after transfer of NPBs into zygotes without nucleoli. The involvement of NPBs in structural and chromatin modifications seems to be true also for SCNT embryos. The proper nuclear genome organization in pseudopronuclei, which is also characterized by a close association of heterochromatin with NPBs, is necessary for genome reprogramming and probably for correct gene expression during further development (Martin et al., 2006a, b). Which other of the oocyte cellular components are used in the process of transferred nucleus remodeling remains to be determined.

Taken together, the above results can explain why NT is more efficient than other reprogramming approaches, but they do not answer the question whether SCNT embryonic stem cells (ESCs) are better than iPSCs. The ESC lines can be established efficiently from SCNT embryos (Wakayama et al., 2006), even though the production of cloned offspring is still disappointingly very low (Ogura et al., 2013). It is, however, commonly accepted that the main problems in cloning is the abnormal function of the placenta. This, however, is not relevant when the aim is ESC line derivation.

## Conclusion

It is our opinion that at present it is difficult to say which is the best way that will lead to an efficient approach of somatic cell reprogramming of and the production of

patient-compatible stem cell lines. The production of a patient's iPSC lines may take at least few months (Takahashi and Yamanaka, 2013). The SCNT approach would be, in theory, much shorter. Thus, at present the evident advantage of SCNT ESC derivation is the speed of this approach. On the other hand, we must keep in mind that the production of SCNT ESC lines requires the supply of very high-quality human oocytes as a source of cytoplasts. This represents the first ethical problem. The second ethical problem is in the opening the gate to the production of cloned humans.

## Acknowledgments

H.F. is supported from GACR P302/11/P069 and J.F. Jr. from GACR 13-03269S.

## Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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# Pluripotent Stem Cells from Maturing Oocytes

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## Abstract

Embryonic stem cells are mostly derived from mature oocytes that were either fertilized or activated parthenogenetically and then reached the blastocyst stage. From the cell cycle perspective, fertilization or activation induces the exit from meiosis, decondensation of oocyte chromosomes, and the entry into mitosis. Decondensation of oocyte chromatin with subsequent formation of nuclei can be, however, induced at any postgerminal vesicle breakdown meiotic maturation stage. In this article, we discuss the possibility of cleavage of transformed maturing oocytes and whether they can reach the blastocyst stage, from which pluripotent stem cell lines could be derived.

## Introduction

FINAL STAGES OF OOCYTE MATURATION cover the period when germinal vesicle breakdown (GVBD) occurs and chromosomes condense with subsequent formation of the first metaphase spindle. Then a short anaphase-to-telophase transition can be detected, and chromosomes are arranged in metaphase II plate with the first polar body extruded. Here the oocyte awaits the fertilizing sperm. Once the sperm penetrates the oocyte, it initiates the anaphase-to-telophase II transition with accompanying extrusion of the second polar body (Fulka Jr. et al., 1998). This is then followed by extensive oocyte and sperm head chromatin decondensation and formation of a male (paternal) and female (maternal) pronucleus.

Eventually, the metaphase II oocyte can be activated parthenogenetically, but here only a maternal pronucleus (i) is formed in the cytoplasm. DNA replication in pronuclei begins approximately 6–8 h postfertilization, and, once completed, the chromatin condenses and the first mitotic chromosome group is formed. In normally fertilized oocytes, this mitotic group is diploid (McLay and Clarke, 2003). If the extrusion of the second polar body is prevented in parthenogenetically activated oocytes by incubating them in cytochalasin B (or D), two pronuclei are usually formed in the oocyte cytoplasm and the first mitotic group is also diploid. In parthenogenetically activated oocytes with the second polar body extruded, the mitotic group is haploid.

The difference between the first and a second polar body is interesting. The first polar body disappears soon after its extrusion, but the second polar body persists for several embryonic cleavages. The second polar body contains a

pronucleus-like structure, and DNA replication also occurs in it. The length of oocyte maturation is species specific. For example, in the mouse it lasts approximately 10–12 h, bovine oocytes need approximately 20 h, pig oocytes require 40 h, and human oocytes require more than 30 h. The process of oocyte maturation proceeds in ovarian follicles after a luteinizing hormone (LH) surge. If oocytes are, however, isolated from large follicles and cultured under appropriate conditions, they begin to mature and reach metaphase II stage *in vitro* as well. In general, their quality is typically lower when compared to *in vivo*-matured oocytes.

## Oocyte Maturation: The Chromosome (Nuclear) Perspective

Immature oocytes contain a prominent nucleus, the germinal vesicle (GV), in which a specific organelle, the nucleolus, is visible in some species (rodents, pig, humans). This nucleolus is morphologically different from nucleoli that can be found in differentiated cells. In somatic cells, nucleoli contain three compartments—dense fibrillar centers and fibrillar and granular components. These differentiated nucleoli are engaged in many cellular processes, *i.e.*, cell cycle control, transcription, differentiation, etc. On the other hand, nucleoli in fully grown oocytes are spherical and contain only a dense fibrillar material. For this reason, they are called as the “nucleolus precursor bodies” (NPBs). In most developmentally competent oocytes, NPBs are enclosed [surrounded nucleoli (SN) oocytes] with a ring of chromatin (heterochromatin). The developmentally incompetent, fully grown oocytes, where the nucleolus is not surrounded with

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chromatin [nonsurrounded nucleoli (NSN)], are able to mature, but after fertilization they typically cleave only to the two-cell stage (Bonnet-Garnier et al., 2013). As mentioned above, following a gonadotropin surge or after liberation of oocytes from follicles, the process of maturation begins; the nucleus (GV) slowly disappears (in mouse at 1.5 h, in pig at 18 h) and the chromosomes condense. During the first meiotic division, the homologous chromosomes are segregated while the sister chromatids remain still attached to each other (until metaphase II). The sister chromatids are separated during the second meiotic division, which occurs after the oocyte becomes fertilized or activated parthenogenetically (Li and Albertini, 2013).

### Oocyte Maturation: The Cell Cycle Control Perspective

The onset of oocyte maturation is under the control of so called maturation-promoting factor (MPF), which is a heterodimer composed of a catalytic (CDK1) and a regulatory (cyclin B) subunit. The onset of MPF activity is regulated by Wee 1/Myt 1 kinases that phosphorylate CDK1, thus keeping MPF inactive. On the other hand, CDC25 phosphatases dephosphorylate CDK1 and MPF becomes active. The activity of MPF becomes high shortly before the onset of GVBD. High activity persists until the metaphase I stage, with its drop during the anaphase to telophase I transition. Following this transition, MPF activity rises again and declines after fertilization or parthenogenetic activation (Jones, 2004).

### Induced Chromosome Decondensation in Maturing Oocytes

In this article, the term “maturing oocytes” refers to those oocytes in which GVBD has already occurred but have not yet reached the metaphase II stage. As far as we are aware, the first papers dealing with induced chromosome decondensation were published by Clarke and Masui (1983, 1985). These authors treated mouse oocytes at different stages of maturation with the protein synthesis inhibitor puromycin. When puromycin was added to oocytes shortly after GVBD, it prevented the progression of oocyte maturation, and the chromosomes formed a cluster. Nuclei (decondensed chromosomes) in these oocytes are formed only exceptionally. Quite a different situation was, however, observed in oocytes that were more advanced in maturation, *i.e.*, in those oocytes that already reached the metaphase I stage with a well-formed spindle. When these oocytes were treated with puromycin, the first polar body was rapidly expelled and chromosomes that remained in the oocyte cytoplasm decondensed and formed a nucleus with a very visible nucleolus. This nucleus was typically detectable after 6 h. The minimum puromycin treatment time that is necessary for the formation of a stable nucleus was approximately 8–9 h. If shorter, the nucleus broke down and chromosomes recondensed. When metaphase I oocytes treated by puromycin for 9 h were transferred into a puromycin-free medium, their nuclei persisted, but DNA replication occurred only exceptionally in them.

The same results were published also by Hashimoto and Kishimoto (1988). These experiments were later expanded by Clarke et al. (1988). Mouse metaphase I oocytes were treated with puromycin for 9 h and then transferred into an inhibitor-free medium. Nuclei in these oocytes remained very visible

for 8–10 h, but after this interval chromosome condensation occurred. If, however, the oocytes with nuclei were transferred into a medium supplemented with dibutyryl cyclic adenosine monophosphate (dbcAMP), nuclei did not break down and began to replicate DNA. After transfer into oviducts of host mice, they even reached the blastocyst stage. No attempts to establish embryonic stem cell (ESC) lines were performed. Karyotyping demonstrated that these blastocysts were mostly diploid.

Another group of drugs that were used for induced chromosome decondensation were inhibitors of phosphorylation (kinases inhibitors). In general, 6-dimethylaminopurine (6-DMAP) inhibits the maturation progression when applied at all stages post-GVBD. If treatment is sufficiently long, chromosomes (chromatin) decondense and a nucleus is formed. These nuclei were characterized only by electron microscopy (EM), which showed well-developed membrane and the presence of NPBs that were morphologically similar to NPBs found in GVs. DNA replication was not studied (Szollosi et al., 1991).

The effect of another kinase (CDK1) inhibitor butyrolactone 1 (BL1) on metaphase I mouse oocytes has been studied by Fulka Jr. et al. (1999). Under our conditions, GVBD occurred approximately within 60 min after the beginning of oocyte culture (time T1). Well-formed metaphase I spindles were detected after 7–8 h (T7–8) and anaphase-to-telophase I transition was detectable after about 9 h (T9). The oocytes reached the MII stage at 11–12 h after the start of their culture (T11–12). For the BL1 treatment, the oocytes were first cultured with cumulus cells, which were removed by pipetting at T1.5. Those oocytes with GVs were discarded, and the remaining oocytes were cultured further. At T7.5, these oocytes were transferred into BL1-supplemented medium (75–100  $\mu$ M). The oocytes exited from MI normally and the polar body was extruded, but MII spindle was not formed in them. Instead, chromosomes decondensed and formed a very visible nucleus with a prominent nucleolus. These nuclei (lamin A/C-positive) are typically detectable in the cytoplasm approximately after 6 h of incubation in BL1-supplemented medium. We observed an unexpected behavior of oocytes when they were removed from the BL1 medium (approximately after 12 h) and transferred into a normal medium. In this medium, the BL1-treated oocytes cleaved directly into a two-cell embryo-like stage.

This unexpected behavior prompted us to study the effect of BL1 in more detail. The cleavage into a two-cell embryo-like stage suggested that DNA replication occurred in BL1-treated oocytes. This was confirmed when BL1-treated oocytes were incubated with bromodeoxyuridine (BrdU). Interestingly, polar bodies were also positively labeled, and this indicated that they rather represent second polar body-like structures. Moreover, these polar bodies did not disintegrate rapidly and persisted for several further cleavages. Also chromosomes of first mitotic cleavage plates were morphologically similar to chromosomes that can be found in normally fertilized embryos. These results clearly indicated that BL1 treatment of metaphase I oocytes directly converts a meiotic division into a mitotic one. A similar effect was observed when oocytes were incubated in roscovitine, but their response to this drug was very inconsistent.

The detailed examination of chromosomes in BL1-treated oocytes supported the view that BL1 induces a metaphase

II-like chromosome morphology. This was especially striking when we analyzed the presence of REC8, a meiosis-specific cohesin. Similar to normal metaphase II chromosomes, the BL1-treated oocytes showed the loss of REC8 along the chromosome arms and retained only the centromeric REC8. Even more interesting was the behavior of BL1 oocyte-treated chromatin with respect to replication licensing. Although the BL1 oocytes never really reach the metaphase II stage, their chromatin is replication competent. Under normal circumstances, the key licensing factor CDC6 is not present in oocytes and associated with chromosomes until the metaphase II stage. However, in the BL1-treated oocytes, CDC6 can be found on chromosomes as early as 1.5h after the beginning of treatment. This is followed by loading of different minichromosome maintenance (MCM) proteins onto the oocyte chromosomes, which leads to the replication licensing of BL1-treated maturing oocytes.

#### *Development of BL1-treated oocytes*

The first article in which we described the effect of BL1 on metaphase I mouse oocytes was not focused on the development of these cells. We began to study this much later. First, we wanted to know whether these converted oocytes could reach the blastocyst stage. When the experimental conditions were perfected, almost all metaphase I oocytes responded to BL1 treatment, *i.e.*, they formed a nucleus in the cytoplasm and gave off the polar body (80–90%). From these oocytes, approximately 80% cleaved and about 30% reached the blastocyst stage. It must be noted here that this percentage was even higher (more than 50%) in some experiments. The BL1 blastocysts were indistinguishable from blastocysts originating from normally fertilized oocytes. They exhibited the presence of inner cell masses positive for Oct 4 and Nanog, whereas their trophoctoderms were Cdx2 positive. In all cases, these blastocysts were diploid.

Next we tested if we could establish ESCs from them. Interestingly, we found that BL1 blastocysts showed a higher rate of expanded inner cell mass formations when compared with the parthenogenetic blastocysts. This phenomenon cannot be simply explained by the absence of the paternal genome in BL1 blastocysts because it is also absent in the parthenogenetic embryos. Even more interesting is the fact that when the ESC lines were analyzed by low-density arrays, BL1 ESC lines were more similar to lines obtained from fertilized oocytes when compared to parthenogenetic ESC lines. This indicates that some changes must occur during the final phases of oocyte maturation that might lead to the establishment of an oocyte-specific developmental program.

#### *Differentiation of BL1 ESC lines*

*In vitro*-induced BL1 ESC differentiation resulted in the formation of embryoid bodies containing a wide range of cells of ectodermal, mesodermal, and even endodermal origin. When BL1 ES cells were injected into severe combined immunodeficiency (SCID) mice, they formed teratomas. Histological evaluation showed that these teratomas contained a wide range of somatic tissues, again originating from all three embryonic layers. BL1 ESCs were also injected into ICR blastocysts that were transferred to recipient females, and we obtained several chimeric offspring. No germ-line transmission was, however, observed. We cannot

exclude the possibility that the production of more mice and their extensive breeding would result in the birth of such offspring. These findings are interesting with respect to the fact that parthenogenetic ESC lines were originally described to be excluded from tissues originating from mesoderm and endoderm and thus were considered to be inferior to ESCs obtained from fertilized oocytes (Nagy et al., 1987; Paldi et al., 1989). However, more recent reports have shown that even parthenogenetic ESCs can exhibit full developmental and differentiation competence and even contribute to the germ line in chimeric assays (Chen et al., 2009). These findings are indeed very exciting (Fulka et al., 2011).

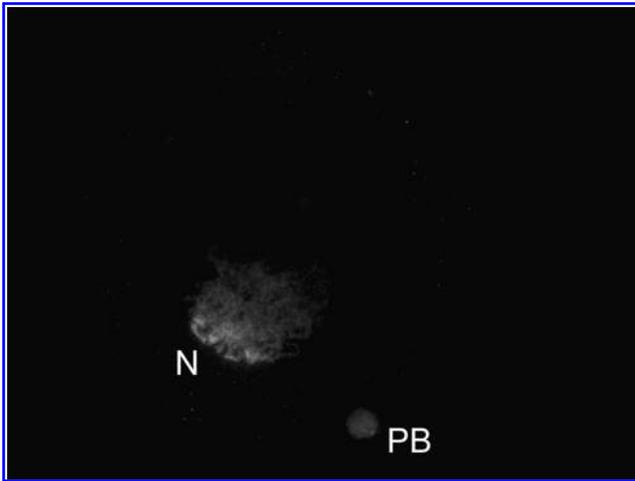
#### *The effect of BL1 on oocytes of other species*

We have also tested the effect BL1 on oocytes of other species. We used bovine oocytes. Compared to the mouse, the main disadvantage here is the lack of transparency of these oocytes. We have transferred metaphase I oocytes into BL1-supplemented medium, and their response to this drug was similar to mouse oocytes. The polar body was extruded and the cytoplasm contained a nucleus that replicated DNA. Further culture of BL1-treated oocytes in synthetic oviductal fluid (SOF) medium resulted in a production of blastocysts with an excellent morphology. Unfortunately, no similar markers that are commonly used in the mouse can be used for labeling of bovine embryos because OCT4 and CDX2 are found in both the inner cell mass and trophoctoderm. Also, there is no method for derivation of ESC lines in bovine.

Logically, the most attractive model for our approach would be human. First, in most *in vitro* fertilization (IVF) clinics, the oocytes that are not metaphase II staged when collected from stimulated ovaries are discarded. Thus, a potentially very valuable material is lost. Second, because offspring would never be born from these oocytes (if they respond to BL1), the possibility of deriving ESC lines from them would represent an ethically acceptable approach. The main disadvantage is that we do not know the exact stage of maturation at which these oocytes are collected (these can be shortly after GVBD, early metaphase I, or late metaphase I).



**FIG. 1.** The nucleus (N) with several nucleoli in a human oocyte that was incubated from metaphase I stage in BL1-supplemented medium. PB, Polar body. Magnification, 600 $\times$ .



**FIG. 2.** DNA replication in nuclei (N) formed in maturing oocytes after BL1 treatment. Note that DNA replication is also detectable in a polar body (PB). Magnification, 600 $\times$ .

The other disadvantage is the low number of oocytes available, and this does not allow us to test, for example, different concentrations of BL1. So, in humans we have applied the scheme that was used in the mouse. Nevertheless, even without testing, human oocytes also responded to BL1—*i.e.*, the polar body was extruded and a nucleus was formed in the cytoplasm. In the mouse, a single nucleolus is typically visible in the nucleus, whereas in humans nuclei contained several nucleoli (Fig. 1). These nuclei also replicated DNA (Fig. 2). Further development of BL1 oocytes was, however, compromised, and only few of them cleaved and exceptionally reached the eight-cell stage (Langerova, unpublished results).

The explanation for this poor development is rather difficult. First, it must be noted that we have used oocytes that were collected along with the population of already matured oocytes (metaphase II), and this may indicate that their quality was somewhat compromised. Second, the oocytes were collected from patients treated for different forms of infertility. Third, it is well known that the quality of human oocytes when compared to oocytes of other species is much lower. For example, the frequency of aneuploidies in human oocytes is very high—up to 60%—compared with 5% in mouse and 10% in cattle and pig (Nagaoka et al., 2012). Also the evaluation of epigenetic changes accompanying the process of maturation and related to the process of chromosomes segregation (acetylation of chromatin) clearly showed, that when compared to other species (mouse), the pattern of labeling is very inconsistent (Langerova, 2013; van den Berg et al., 2011) and this may indicate that the oocytes used might have been developmentally handicapped. Logically, the best solution would be to use high-quality oocytes from paid donors. We believe that further experiments will lead to the production of viable blastocysts from which ESC lines will be established sooner or later.

## Conclusions

The oocyte is an exceptional cell, and the results obtained so far clearly show that even the oocyte (meiotic cell) that did

not reach yet the metaphase II stage can be converted into a mitotic cell without a typical activation stimulus. Moreover, in the mouse, where we can select the best cells, ESC lines can be obtained. Thus, even with the advent of induced pluripotent stem cell (iPSC) technologies, the oocyte remains an attractive model for different studies (Morris and Daley, 2013; Yamanaka, 2012).

## Acknowledgments

This article is dedicated to our friend Keith Campbell, who visited Prague several times and always had a glass of beer with us here. These studies were sponsored by grants JFJR GACR 13-03269S and HF GACR P302/11/P069.

## Author Disclosure Statement

No competing financial interests exist.

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# The ups and downs of somatic cell nucleus transfer (SCNT) in humans

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Received: 2 April 2013 / Accepted: 8 July 2013 / Published online: 24 July 2013  
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**Abstract** Achieving successful somatic cell nuclear transfer (SCNT) in the human and subhuman primate relative to other mammals has been questioned for a variety of technical and logistical issues. Here we summarize the gradual evolution of SCNT technology from the perspective of oocyte quality and cell cycle status that has recently led to the demonstration of feasibility in the human for deriving chromosomally normal stem cells lines. With these advances in hand, prospects for therapeutic cloning must be entertained in a conscientious, rigorous, and timely fashion before broad spectrum clinical applications are undertaken.

**Keywords** Nucleus · Oocyte · Nucleus transfer

Simply stated, SCNT in mammals should in practice be a very straightforward technique. First, cytoplasts are prepared by enucleating the metaphase II stage oocytes, i.e. metaphase II chromosomes are removed from the oocyte. Second, the selected nucleus is introduced into the cytoplast either by direct injection or by induced cell fusion. The reconstructed SCNT products are then activated in ways mimicking fertilization and if everything goes well cloned embryos develop [6].

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*Capsule* This paper is dedicated to the memory of Keith Campbell (1954–2012), a pioneer in the field of somatic cell nuclear transfer.

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Logically, since following the birth of Dolly [7], and the production of additional clones in other mammalian species, there is ample confirmation that Dolly was not an exceptional case, prompting widespread discussion regarding the pros and cons of human SCNT. Given this prospect, it has been commonly accepted and broadly emphasized that the production of cloned human individuals must be banned (reproductive cloning). On the other hand, the production of embryos from which patient compatible embryonic stem cells could be obtained seemed to be acceptable, at least in some countries (therapeutic cloning). However, once the discovery of induced pluripotent stem cell production (iPSC) was realized by the work of Takahashi and Yamanaka [see 8 for review], illustrating that differentiated or even terminally differentiated cells can be converted (dedifferentiated) into a pluripotent state by induced overexpression of four factors (Oct 4, Sox 2, Klf 4, c-Myc; OSKM), greater resistance against the use of SCNT in humans has emerged, including the prospect of therapeutic cloning.

Although many papers on modifications of the original iPSC technology seeking improved efficiency and safety have been published, it should be emphasized that our understanding of the mechanisms by which iPSC elicits dedifferentiation remain woefully incomplete. Instead, it is commonly accepted that the oocyte cytoplasmic milieu is the most direct means to obtaining reprogramming of a differentiated somatic cell nucleus [9, 10]. This fact—the quality of the oocyte cytoplasmic milieu—may well underscore the reason that while research on human SCNT continues, reports are rare in this species relative to the many papers published in other mammals.

## Birth of Dolly

The work that led to the birth of Dolly was antedated by many years of experiments using laboratory and domestic animals to test many methodological combinations and schemes. Initial

experiments used almost exclusively differentiating nuclei of early developing embryos and cytoplasts that were produced by enucleating pronuclear stage embryos. The use of one-cell (pronucleus) stage embryo cytoplasts resulted in completely negative and discouraging results prompting Nobel laureate John Gurdon [11] to state that: “For reasons I have never fully understood, the next major attempts to transplant nuclei in mammals used fertilized eggs as recipients, rather than unfertilized eggs as had proved successful in frogs.”

The first scientist who successfully used cytoplasts produced by enucleation of metaphase II oocytes was Steen Willadsen [12]. In his experiments, blastomeres isolated from 8-cell stage embryos were fused to mature oocyte cytoplasts and several lambs were born. This fundamental scheme, or modifications of it, has been adopted in all subsequent experiments. The trend from this point on was to combine mature oocyte cytoplasts with more and more developmentally advanced cells as donor karyoplasts. In tandem, many technical improvements were realized, such as the piezo injector for nuclear transfer, which collectively accelerated progress in this field [6]. But even after birth of several offspring from cultured inner cell mass cells (ICM) or embryonic fibroblasts, used as a source of somatic cell nuclei for SCNT [13, 14], it was widely assumed that the production of offspring from nuclei of already newborn or adult individuals would be impossible in the near future or not possible at all. This fatalistic opinion amongst the scientific community was not altered even after the birth of Dolly with many prominent scientists within and without the field claiming that Dolly was a rare and unrepeatable case. This attitude was soon to change upon the birth of clones from SCNT in a number of different mammalian species. Concomitantly, and quite logically, discussion and discourse entered the arena of SCNT technology with respect to the prospect of human cloning. For better or worse, the stage was set for the potential of SCNT to become blurred with the manipulation of human reproduction.

### Big problems in humans

As mentioned above, the birth of Dolly was becoming perceived as hardly a chance event having been preceded by many years of empirical testing of different experimental schemes and combinations. The nearly unlimited supply of oocytes and somatic cells enabled scientists to study animal SCNT in more detail, always with the aim in mind to improve its efficiency. In sharp contradistinction, it was recognized and appreciated to be a nearly impossible task to achieve SCNT in humans given the formidable obstacle of supply—where would a sufficient number of oocytes be obtained for cytoplast production?

In laboratory and domestic animals these oocytes can be relatively easily obtained either after induced superovulation

(rodents) or after aspiration of immature oocytes with their subsequent *in vitro* culture (ungulates). Patients in human assisted reproduction clinics are similarly hormonally stimulated to yield many oocytes upon egg retrieval but the mature oocytes are primarily and almost exclusively destined for embryo production and infertility treatment. Moreover, it has become clear that, generally speaking, the quality of human oocytes is lower when compared to oocytes of the other mammalian species studied so far. One of several manifestations for this is the frequency of aneuploidies causing embryonic failure which tends to be very high in human oocytes and rather low in other mammalian species [15]. The reasons for this are not yet fully explained. It is, however evident that the quality of oocytes decreases with the maternal age, and this is for example manifested by the loss of cohesins. Environmental factors (endocrine disruptors, bisphenol A—BPA) also play an important role. It has been also shown that the mitochondrial DNA (mtDNA) content in oocytes is highly variable and ranges from 11.000 to 903.000 mtDNA molecules. Mitochondria are the powerhouse in the cell (production of ATP) but they are also engaged in many other cellular processes [16]. Moreover, analyses of epigenetic characteristics of chromatin (histone methylation and acetylation) clearly show that the population of human oocytes retrieved after controlled ovarian hyperstimulation (COH) is rather inconsistent and variable even between oocytes from the same patients [17] and certainly some other oocyte characteristics will influence the success of SCNT in humans [18]. It should be emphasized, however, that studies of this kind involve predominantly oocytes deemed unsuitable for IVF or ICSI, so it remains unclear whether these oocytes are abnormal *per se*. Human oocytes seem to be also very sensitive to external changes—for example the fluctuation of temperature. Taken together, these confounding factors have limited progress in the human SCNT field and have most recently prompted redirecting the sourcing of human oocytes to those donated by reimbursed and generally younger donors.

### Inconsistent results

In spite of the obstacles mentioned above, several papers reporting SCNT in humans have been published. Not all will be mentioned here, for example the papers by Hwang et al. [19, 20] because these results have been questioned in the literature (for example, see commentaries by David Cyranoski in *Nature*). It is difficult to draw firm conclusions from these papers. In general, embryos produced by NT underwent few cleavage divisions and only exceptionally reached the blastocyst stage [21–24]. It must be noted, however, that cytoplasts were sourced from oocytes that were either immature at the time of egg retrieval and were subsequently *in vitro* matured to metaphase II stage in culture, or

from oocytes deemed unsuitable for IVF. Eventually, enucleated pronuclear stage embryos were used. The first paper with promising results was published by French et al. [25]. These studies reported production of several SCNT blastocysts (23 %) when male fibroblast cell nuclei were introduced into 29 enucleated oocytes obtained from three young donors. When compared to previous reports, a likely reason for success in this instance can be traced to the source of oocytes used for cytoplasm preparation. Oocyte donors were young and demonstrated to have been successful in previous COH stimulation cycles. In light of this, the results published later by Noggle et al. [3] were rather surprising. These authors studied several aspects of human SCNT and concluded that the ordinary NT scheme cannot lead to the production of blastocysts because the reconstructed embryos ceased their development after several cleavages. The reason for arrested development was suggested to be aberrant activation of embryonic genome in reconstructed embryos. Interestingly, when the oocyte genetic material (chromosomes, oocytes were not enucleated) was not removed and nuclei were introduced into intact oocytes, the embryos developed quite well and several embryonic stem cell lines, albeit triploid, were produced. Somewhat surprisingly then, another paper published soon after this report [4] reported successful production of human SCNT blastocysts using the conventional SCNT scheme. No ESC lines were derived from these blastocysts. One telling difference in these latter experiments was the use of in vitro matured oocytes for cytoplasm production. When these three papers are compared, it is very difficult to find a satisfactory explanation for these inconsistent results. Moreover, we can no longer presume that humans are so exceptional or unique such that SCNT would be impossible to achieve in our species.

Or are human oocytes so different when compared to oocytes of other species? Some results indicate that they are, since the frequency of aneuploidies is much higher than in oocytes of other species (mouse 5 %, humans about 50 %) [15]. Again, it must be noted here that these results reflect studies based on analyses of patients treated for different forms of infertility. So, the conclusion is again very difficult. On the other hand, the production of parthenogenetic human ESC lines is a routine procedure and here the oocytes used are not that much different from those that are used for nuclear transfer. The supporting role of the oocyte nuclear material is also not surprising. Essentially, in early days of nuclear transfer experiments the reconstructed embryos only developed when nuclei were injected into non-enucleated zygotes [26, 27]. Thus, one may conclude that more experiments may shed some light on human NT and explain the inconsistent results.

The very recent paper published by Mitalipov's groups confirmed that this is true [5]. In these experiments the oocytes from paid donors were used and some minor, but

probably very essential modifications, were introduced into their NT scheme. First, the oocytes were enucleated in the presence of caffeine and second, after NT the reconstructed embryos were cultured in a medium containing TSA (trichostatin A). In fact, both these treatments were already used in animal NT experiments and improved their efficiency [9]. In general, the NT scheme has been perfected in previous experiments performed by this group and some other technical details can be found in a given paper [5]. Taken together, the efficiency of a given NT scheme was quite high and this was confirmed by a very good embryonic development and high efficiency when ESC lines were established from them. Interestingly, the NT blastocysts exhibited poorly developed trophectoderm but this does not prevent the efficient establishment of ESC lines. In our opinion, there are some additional interesting points mentioned in this paper that may have the implication in a conventional human assisted reproduction. The authors conclude that only those oocytes having a premium quality have a chance to develop well after NT. Second, the best oocytes were obtained from donors from which 10 or less oocytes were collected. Does the same apply to conventional IVF (ICSI)?

## Summary

It has not been our intention to review the scant literature on SCNT in humans with respect to detailing experimental procedures so as to provide a modicum of speculation that might help to explain a dichotomy— that is why there are some studies reporting production of blastocysts whilst others suggested that SCNT is impossible. For non-human primates, failure to produce cloned offspring by SCNT has been reported. However, blastocysts produced by SCNT have been used to derive embryonic stem cell lines [28]. This now applies for humans too. It is also clear, that the human and nonhuman primates present subtle but important distinctions in the physiology of the cell cycle and egg activation but this would not come as such a surprise. It is, however, our opinion that the production of blastocysts in humans by SCNT is and will be very difficult. A key factor seems to be the insufficient number of oocytes available for cytoplasm production, even with the use of donor eggs. Moreover, as noted above, the incidence of genetic and epigenetic instability in human oocytes is disconcerting, further contributing to the overall problem of poor oocyte quality especially as a function of advancing age or environmental factors [15]. Nevertheless, we also do believe that experiments aiming at producing blastocysts by SCNT in humans and primates [28] will certainly accelerate the introduction of some technical improvements, that might be very useful in solving certain problems of assisted human

reproduction, into clinical practice. These approaches are now almost exclusively oriented on the elimination of mutated mitochondrial DNA (mtDNA), that can cause some devastating diseases [29]. It has been demonstrated quite recently in humans that the transfer of pronuclei or spindles, isolated from the cytoplasm containing mutated mtDNA, into healthy cytoplasts seems to be very promising and healthy looking blastocysts were obtained [30–33].

**Acknowledgments** HF is supported from GACR P302/11/P069. JFJR is supported from GACR 13-03269S. DFA is supported by a grant from the ESHE Fund.

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# Chromatin acetylation in human oocytes

## Acetylacja chromatyny w ludzkich oocytach

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### Abstract

**Objectives:** The frequency of aneuploidies in human oocytes is extremely high. It is hypothesized that the cause may be due to abnormal chromatin (histone) acetylation/deacetylation. The aim of our study was to analyze the acetylation/deacetylation pattern in spare human oocytes.

**Materials and Methods:** Human spare oocytes (311), in other words oocytes that were not mature when collected from follicles or control oocytes (bovine, mouse), were fixed with paraformaldehyde and then labeled with antibodies against acetylated histones.

**Results:** Labeling against AcH4/K12 or hyperacetylated H4 showed high intensity of the fluorescence signal in all immature (germinal vesicle staged) and approximately 50% of the maturing (mature) oocytes.

**Conclusion:** In conclusion, the labeling of human oocytes (chromosomes) showed very inconsistent patterns of acetylation/deacetylation, what may suggest they did not reach the metaphase II stage at the time of follicle aspiration, and were epigenetically abnormal. It may also explain the high frequency of chromosomal abnormalities in human oocytes.

Key words: **oocytes / maturation / chromosomes / acetylation / aneuploidies /**

### Streszczenie

**Cel:** Częstość aneuploidii w ludzkich oocytach jest niezmiernie wysoka. Istnieje hipoteza, że jest to związane z nieprawidłową acetylacją/deacetylacją chromatyny (histonów). Celem badania była ocena wzoru acetylacji/deacetylacji w niewykorzystanych ludzkich oocytach.

**Materiał i metoda:** Ludzkie niewykorzystane oocyty (311), innymi słowy oocyty niedojrzałe w momencie pobrania z pęcherzyków lub oocyty kontrolne (krowie, mysie), zatopiono w paraformaldehydzie i oznakowano przeciwciałami przeciwko acetylowanym histonom.

**Wyniki:** Znakowanie przeciwko AcH4/K12 lub hiperacetylacja H4 wykazały wysoką częstość sygnału fluorescencyjnego we wszystkich niedojrzałych (w stadium pęcherzyka germinalnego) i w około 50% dojrzałych oocytach.

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Otrzymano: 12.10.2012  
Zaakceptowano do druku: 15.03.2013

Alena Langerova. *Chromatin acetylation in human oocytes.*

**Wnioski:** Znakowanie ludzkich oocytów (chromosomów) wykazuje wysoce niejednoznaczny model acetylacji/de-acetylacji, co może sugerować, że nie osiągają one metafazy II podziału w momencie aspiracji pęcherzyka i są epigenetycznie nieprawidłowe. Ten fakt może tłumaczyć wysoką częstość nieprawidłowości chromosomalnych w ludzkich oocytach.

Słowa kluczowe: **oocyt /dojrzewanie /chromosom /acetylacja /aneuploidia /**

## Introduction

The frequency of aneuploidies in human oocytes is very high and up to 60% of them have been reported to be chromosomally abnormal [1]. The incidence in humans seems to be much higher than in other mammals: mouse – approximately 5%, cattle – 10%, pig – 10% [2]. It must be, however, emphasized that these estimations often differ, depending on the technique used. The reason why human oocytes are so often chromosomally abnormal remains undiscovered. In fact, the basis for aneuploidies can be established long before they can be detected in fully grown oocytes [3]. Hunt and Hassold [3] defined three developmental phases of oogenesis when the quality of oocytes can be influenced – i/ oogenesis, ii/ follicle formation and iii/ oocyte growth and maturation. The research in this field in humans is mostly oriented towards the final phase of the oocyte development: its maturation covering the period when the oocyte undergoes germinal vesicle breakdown and subsequently reaches the metaphase II stage [4]. Highly dynamic morphological changes and specific histone modifications were reported to occur in mammalian oocytes within that time period. An immature oocyte contains a prominent nucleus (germinal vesicle – GV), in which a well-visible nucleolus can be seen in some species (rodents, humans, pig). A germinal vesicle breakdown (GVBD), in other words the dissolution of GV membrane, disappearance of nucleoli and gradual condensation of chromatin, occurs following a gonadotropin surge or in in vitro cultures. The condensed chromosomes are then arranged in the metaphase I stage (MI) with a well-formed spindle. This stage is followed by a short anaphase to telophase I (A-TI) transition, during which a half of the oocyte chromosome complement is extruded from the oocyte in the form of the first polar body (PBI). The chromosomes are again arranged on a metaphase plate (metaphase II, MII).

In most mammals, the oocyte is released from the follicle at that stage and then fertilized by the sperm. The histone modifications studies are mostly focused on acetylation and methylation, while the most exhaustive experiments were performed in mice. Methylation of chromatin has been reported to be rather constant and positive labeling was detected not only on condensed chromosomes (MI, A-TI, MII), but also in the nuclei [5, 6]. Quite different situation was, however, observed when antibodies against acetylated histones were used. In general, germinal vesicles were consistently positively labeled but as soon as chromosomes condensed (MI, MII), no labeling was detected. The only exception is the anaphase to telophase I (A-TI) transition with a weak signal on chromosomes [7, 8, 9, 10]. It must be noted, however, that a different pattern of labeling was detected in pigs, where chromatin remained acetylated [11].

Interestingly, the oocyte chromosome acetylation has been shown to increase with age of the female and also some IVF (in

vitro fertilization) techniques may be the influencing factor [12, 13, 14]. A clear association between the aberrant acetylation pattern and the unequal segregation of chromosomes has been demonstrated in mice [15, 16]. Whilst in oocytes isolated from young females no positive labeling was detected on condensed chromosomes, in oocytes from old females about half of the metaphases showed a positive signal. The increased acetylation of chromosomes was also detected in oocytes aged in culture (MII). Thus, it is logical to assume that similar situation may exist in humans, too. As mentioned above, human oocytes are often chromosomally abnormal and thus one can expect that the analysis of chromosome acetylation pattern will correlate with aneuploidies. To the best of our knowledge, only two papers on the acetylation in human oocytes were published after the completion of our study [17, 18]. We analyzed the pattern of acetylation in spare human oocytes, i.e. those that did not reach the metaphase II stage at the time of their aspiration from the follicles or eventually remained unfertilized after IVF.

## Material and methods

Human oocytes, after standard stimulation, were collected by aspiration from follicles and immediately morphologically evaluated. Written consent was obtained from all couples and the study was approved by the Ethical Board of GENNET. In total, we analyzed 311 oocytes obtained from 152 patients, aged from 26 to 38 years, treated at GENNET for a wide range of infertility problems (endometriosis, tubal factors, PCOS - polycystic ovarian syndrome, anovulation, genetic factors, male factors, etc.). The mature oocytes, i.e. the metaphase II staged, were used either for ICSI or were mixed with spermatozoa. The remaining oocytes were briefly cultured (2-3h) in Quinn's Advantage Protein Plus Cleavage Medium (SAGE IVF, Trumbull, CT, USA) at 37°C and 5% CO<sub>2</sub>, during that period some oocytes expelled first polar bodies. Thereafter, zona pellucidae were dissolved by pronase (0.5%) in PBS (phosphate buffered saline) and then the oocytes were fixed with 4% paraformaldehyde (PFA) in PBS for 30min. The oocytes were then kept in PBS at 4°C until immunolabeling. Before labeling the oocytes were permeabilized by Triton X-100 (0.2% in PBS) for 10min and then blocked overnight in PBS supplemented with 1% BSA (bovine serum albumin) and 0.1% TritonX-100 at 4°C. The oocytes were then incubated overnight with one of the following antibodies: anti-acetylated H4/K12 (1:800, Upstate) and anti-hyperacetylated H4 (1:1000, Upstate). These antibodies were extensively characterized in our laboratory [5] or were most widely used in studies analyzing the acetylation/deacetylation processes during oocyte maturation in different mammals (anti-acetylated H4/K12). Following the incubation with one of the above mentioned antibodies, the oocytes were washed several times in PBS/BSA and then incubated for 2h

with the secondary antibody (1:400, donkey-anti rabbit, Jackson Immunoresearch). The oocytes were then washed several times in PBS/BSA, mounted on slides in Vectashield mounting medium with DAPI (Vector Laboratories) and examined under Olympus IX 71 fluorescence microscope. Each labeling was performed several times. Unless otherwise stated, all chemicals were purchased from Sigma.

### Control animal oocytes

In order to exclude some technical problems in labeling, we used bovine and mouse oocytes at different stages of maturation as controls. Bovine oocytes were aspirated from large antral follicles and either fixed immediately (GV stage) or cultured in M199 supplemented with FCS (fetal calf serum, 10%), gentamicin (50 µg/ml), Na-pyruvate (0.2 mM) and Pergonal (0.1 IU/ml, Serono, UK) for 18-20h at 38.5°C/5% CO<sub>2</sub> in the air (maturing – MI, mature – MII) and then fixed. Mouse oocytes were isolated from ovaries of PMSG (pregnant mare serum gonadotropin, 5 I.U.) stimulated mice (BDF1) and either fixed immediately (GV) or matured in MEM supplemented with BSA (4mg/ml), gentamicin (50 µg/ml) and Na-pyruvate (0.2 mM) at 37°C/5% CO<sub>2</sub> in the air for up to 12h and then fixed. The oocytes of both species were treated and labeled similarly as described above for the human oocytes. As the animal oocytes can be easily distinguished from the human oocytes, they were often incubated in the same well.

### Results

The results are summarized in Table I. It must be noted that we analyzed only the oocytes with no signs of degeneration, i.e. round-shaped with homogeneous cytoplasm.

First, we tested the antibody that was specific for AcH4/K12. Controls, i.e. bovine (25) and mouse (42) GV staged oocytes were always positively labeled, whereas in human oocytes 90% (95/105) of the immature oocytes showed positive labeling (Fig 1 a,b). No labeling at all was detected in the remaining ones. 47% of maturing MI stage oocytes were positively labeled (27/58), all telophase I (TI) were positive (Figure 2 a,b), and 49% (22/45) of the metaphase II oocytes showed a positive signal. Bovine and mouse maturing and mature oocytes showed mostly no labeling (90%).

Anti-hyperacetylated H4 was used as the second antibody. In that case, almost all human immature GV staged oocytes were positively labeled (95%; 35/37). About half of the metaphase I (48%; 40/83) and the metaphase II (50%; 9/18) was positive when this antibody was used, whilst the other half was without labeling. All telophase I stage oocytes were positively labeled.

The expected pattern of labeling was detected in controls, i.e. in bovine and murine oocytes. Here, all GV-staged oocytes were positively labeled (bovine – 15, murine – 25), in maturing oocytes no labeling was detected in almost all oocytes (45 – bovine, 56 – murine). Surprisingly, we found evident differences in labeling when comparing the pattern of labeling (AcH4/K12, anti-hyperacetylated H4) between oocytes obtained from the younger and the older patients, (under and over the age of 30, respectively). Similarly, we were unable to find any relationship between the pattern of labeling and a given fertility problem.

In conclusion, when compared to other species tested so far, our results demonstrate that the analysis of acetylation/deacetylation of chromatin in human oocytes gave us rather inconsistent results. At present, it would be very difficult to use this labeling to evaluate the quality of oocytes and to explain the high frequency of aneuploidies in them.

### Discussion

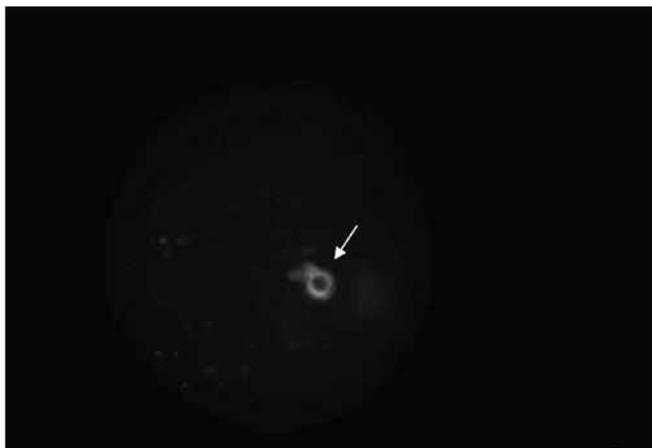
The ovary contains basically three categories of oocytes: growing, intermediate and full-grown. Only full-grown oocytes can undergo germinal vesicle breakdown and reach the metaphase II stage [4]. This, however, does not mean that they are chromosomally normal and developmentally fully competent. For example, it has been convincingly demonstrated that those oocytes where the nucleolus is surrounded with a ring of chromatin are the best ones.

In general, GV (immature) oocytes of all mammalian species labeled so far with antibodies against acetylated histones were positive [19, 20, 21], but the use of these antibodies (anti-AcH4/K12, anti-hyperacetylated H4) also indicates that the extrapolation of the results obtained in laboratory and domestic animals to humans, in order to explain the frequency of aneuploidies, may be risky. Essentially, our results are in agreement with the observations of van der Berg et al. [18], who found that all immature oocytes (GV) labeled with anti-AcH4/K12 antibody and approximately 50% of MI and MII oocytes were positive. Moreover, detailed analyses demonstrate a positive correlation between positive labeling and abnormal spindle morphology. This may consequently lead to unequal distribution of the chromosomal material, either during the exit from the metaphase I or the metaphase II. Their results, however, also show that it will be difficult to find any relationship between the acetylation pattern and aberrant chromosome segregation. AcH4K12 seems to be the most convenient of all antibodies they tested (AcH4K5, AcH4K8, AcH4K12, AcH4K16). These authors also found certain differences in labeling (AcH4/K12) between the oocytes

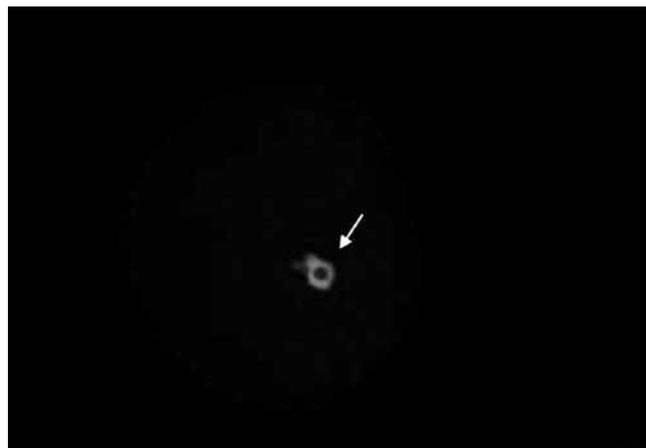
**Table I.** Chromosome Acetylation in Human Oocytes.

Antibody	Oocyte maturation stage – No of oocytes (±; positive/negative labeling) [%]			
	GV	MI	A-TI	MI
AcH4K12	105 (95/10) [95/10]	58 (27/31) [47/53]	2 (2/0) [100/0]	45 (22/23) [49/51]
HyperAcH4	37 (35/2) [95/5]	83 (40/43) [48/52]	3 (3/0) [100/0]	18 (9/9) [50/50]

Abbreviations: GV – germinal vesicle, MI(II) - metaphase I(II), A/TI – anaphase/telophase I, GV category involves both SN (surrounded nucleolus, approximately 60%) and NSN (non-surrounded nucleolus) oocytes. As no differences between these two groups were detected, the data were pooled.



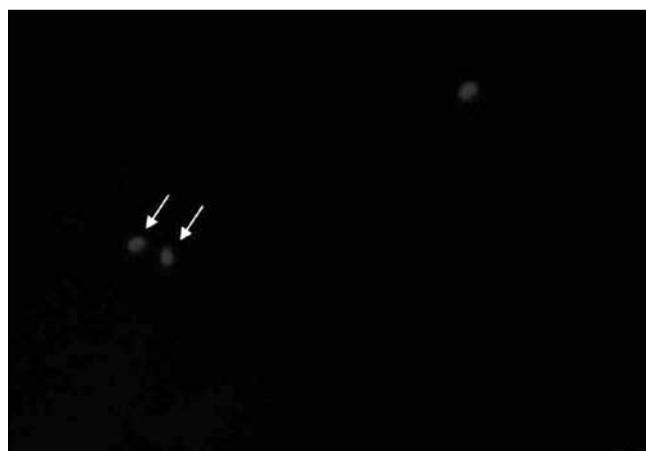
**Figure 1A.** Immature germinal vesicle stage human oocyte with the nucleolus surrounded by chromatin (arrow) labeled with anti-AcH4/K12 antibody. All oocytes showed the positive chromatin labeling. The same situation was observed after labeling with anti-hyperacetylated H4 antibody. x 400.



**Figure 1B.** Parallel DAPI staining.



**Figure 2A.** Telophase I stage human oocyte with a weak chromosome groups (arrows) labeling (AcH4/K12). x 400.



**Figure 2B.** Parallel DAPI staining.

from the young vs. the older patients. The first group contained fewer oocytes that were positively labeled with that antibody. We did not find these differences in our study. That, however, may be explained by different media that had been used for oocyte culture before they were fixed [22]. Slightly different results were reported by Huang et al. [17], who noted positive labeling for all GV-staged oocytes (AcH4K12, AcH3K9).

For AcH3K9 the percentage of positively labeled maturing and mature oocytes was below 50% in in vitro matured oocytes and almost all in vivo matured ones were without labeling. Essentially, the same situation was observed when AcH4K12 antibody was used. The explanation for these differences is very difficult and one cannot exclude that certain technical aspects, i.e. the oocyte culture, handling and labeling, might have influenced the overall pattern of labeling.

In general, the interpretation of our results poses considerable challenges. First, it must be noted that we used oocytes from patients with different fertility problems. Second, we analyzed only those oocytes that did not reach the MII stage at the time of their collection from follicles. The question remains whether these oocytes are somewhat abnormal when compared to more

rapidly maturing oocytes. It has been clearly demonstrated that oocytes maturing faster are, after fertilization, developmentally more competent than those oocytes in which maturation lasts longer [23]. It has been also shown that certain manipulations and culture conditions influence the level of methylation (global DNA methylation and/or histone acetylation) in embryos [20, 24, 14]. Essentially, the labeling intensity in manipulated embryos was significantly higher than in controls. Manosalva and Gonzales [25] demonstrated in mice that the consistency of labeling decreases in GV stage oocytes with an increasing female age.

We hypothesize that there may be two possible explanations: a) human oocytes are distinct when compared to other mammalian species tested so far, b) the prevailing population of human oocytes is abnormal.

It is fairly difficult to determine these issues as human oocytes are primarily used for test tube babies production and spare, high quality oocytes, are available only in exceptional situations.

Clearly, more detailed analysis in this field is necessary to account for the high frequency of aneuploidies in human oocytes and embryos, as well as to develop some ways of their prevention [26, 27].

## Acknowledgements

This work was supported from MZE0002701404.

The author declares no conflict of interest.

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2 września 2013 (pon)  
Łódź

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Prof. H.P. Dietz (Australia)  
Dr med. J. Kociszewski (Niemcy)

Tematyka:

- Wszystko o co chciałeś zapytać, możesz teraz zobaczyć w usg uroginekologicznym np.:
  - optymalizacja doboru operacji,
  - diagnostyka po nieudanych zabiegach.
- Pęcherz nadreaktywny: jak skutecznie leczyć?
- Praktyczne aspekty pessaroterapii w zapobieganiu porodu przedwczesnemu oraz w uroginekologii.
- Wpływ porodu na choroby uroginekologiczne.
- Profilaktyka i diagnostyka poporodowych uszkodzeń dna miednicy.
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—Technology Report—

## Production of Giant Mouse Oocyte Nucleoli and Assessment of Their Protein Content

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**Abstract.** Compared with advanced developmental stage embryos and somatic cells, fully grown mammalian oocytes contain specific nucleolus-like structures (NPB – nucleolus precursor bodies). It is commonly accepted that they serve as a store of material(s) from which typical nucleoli are gradually formed. Whilst nucleoli from somatic cells can be collected relatively easily for further biochemical analyses, a sufficient number of oocyte nucleoli is very difficult to obtain. We have found that isolated oocytes nucleoli fuse very efficiently when contact is established between them. Thus, well visible giant nucleoli can be obtained, relatively easily handled and then used for further biochemical analyses. With the use of colloidal gold staining, we estimated that a single fully grown mouse oocyte nucleolus contains approximately 1.6 ng of protein. We do believe that this approach will accelerate further research aiming at analyzing the composition of oocyte nucleoli in more detail.

**Key words:** Fusion, Nucleolus, Oocyte, Protein content

(J. Reprod. Dev. 58: 371–376, 2012)

The nucleolus is the most prominent structure in a cell nucleus [1]. In developmentally advanced embryos (i.e., blastocysts) and in somatic cells, it contains three basic components: fibrillar centers (FC), a dense fibrillar component (DFC) and a granular component (GC) [2]. Originally, it has been accepted that the nucleolus is exclusively the site of ribosomal RNAs (rRNAs) synthesis, processing and their assembly with ribosomal proteins, but now it is clear that nucleoli play an essential role(s) in many cellular processes [3]. Within the last few years, the composition of somatic cell nucleoli has been characterized, and it is now evident that somatic cell nucleoli contain thousands of proteins [4].

Contrary to differentiated cells, fully grown mammalian oocytes and very early developing embryos prior to major embryonic genome activation do not contain typical tripartite nucleoli. Instead, so-called “nucleolus precursor bodies” (NPBs) are visible in germinal vesicles (GVs) and in pronuclei (PNs) of some mammalian species, i.e., rodents (mouse, rat), humans and pigs [5]. NPBs contain only a dense fibrillar mass. Contrary to somatic cell nucleoli, very little is known about the composition of NPBs. The only protein convincingly demonstrated to be present in them is nucleoplasmin 2 [6]. The function of NPBs in oocytes and embryos has been characterized, especially after the invention of so-called “enucleolation” [7]. This method represents the microsurgical removal of nucleoli from fully grown and growing oocytes as well as from one-cell stage embryos

[8–10]. Thus, it has been convincingly demonstrated that NPBs are dispensable for maturation of fully grown oocytes. The enucleolation method also showed that nucleoli (NPBs) in zygotes are formed from the original oocyte nucleolar material [11]. Further detailed analysis demonstrated that the presence of nucleoli in pronuclei is absolutely essential for normal embryonic development [12–15]. As mentioned above, contrary to somatic cells, the composition of NPBs is almost unknown. The reason for this is the lack of a sufficient amount of material (number of NPBs) that could be then used for further biochemical analyses. Even with the invention of enucleolation, a limited number of NPBs can be collected, they are difficult to handle and they can be very easily lost.

When we have manipulated NPBs, we have found that isolated nucleoli rapidly fused when contact was established between them. This prompted us to exploit this phenomenon for the production of giant NPBs and to assess their protein content. The information on NPB protein content might be useful for further estimation of the number of NPBs needed for different biochemical analyses (e.g., when comparing the level of different nucleolar proteins between somatic cell nucleoli and NPBs).

Nucleoli were isolated from fully grown oocytes (enucleolation) that were collected from large antral follicles of PMSG-stimulated BDF1 mice (Fig. 1). After isolation, the oocytes were carefully examined, and only those enclosed with several layers of cumulus cells were used for enucleolation. These oocytes were first cultured in MEM with dbcAMP for 30 min, and then their cumulus cells were removed by vigorous pipetting. The oocytes were then cultured at least for 3–4 hours before enucleolation. In total, we used 610 fully grown oocytes with a single nucleolus in the germinal vesicle (GV; Fig. 2A). Those oocytes having more than

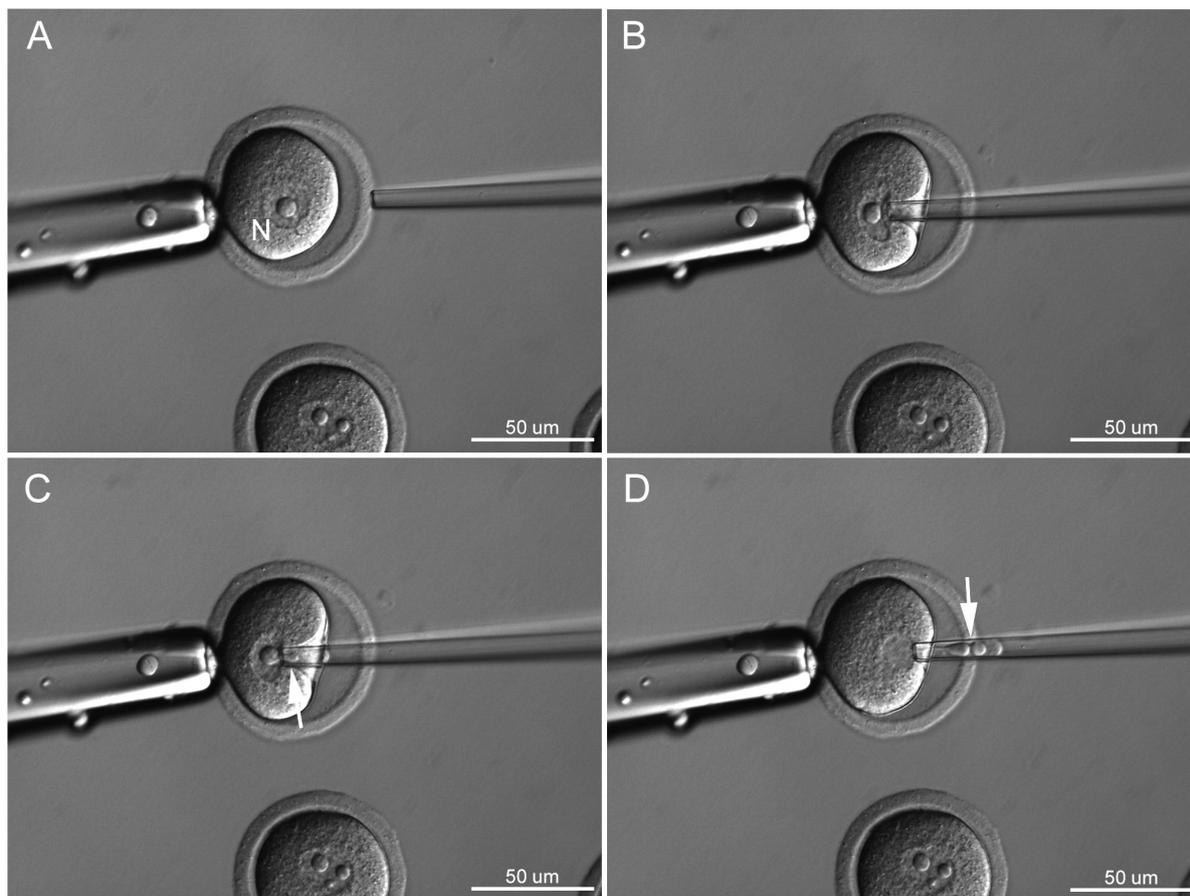
Received: October 7, 2011

Accepted: December 22, 2011

Published online in J-STAGE: February 1, 2012

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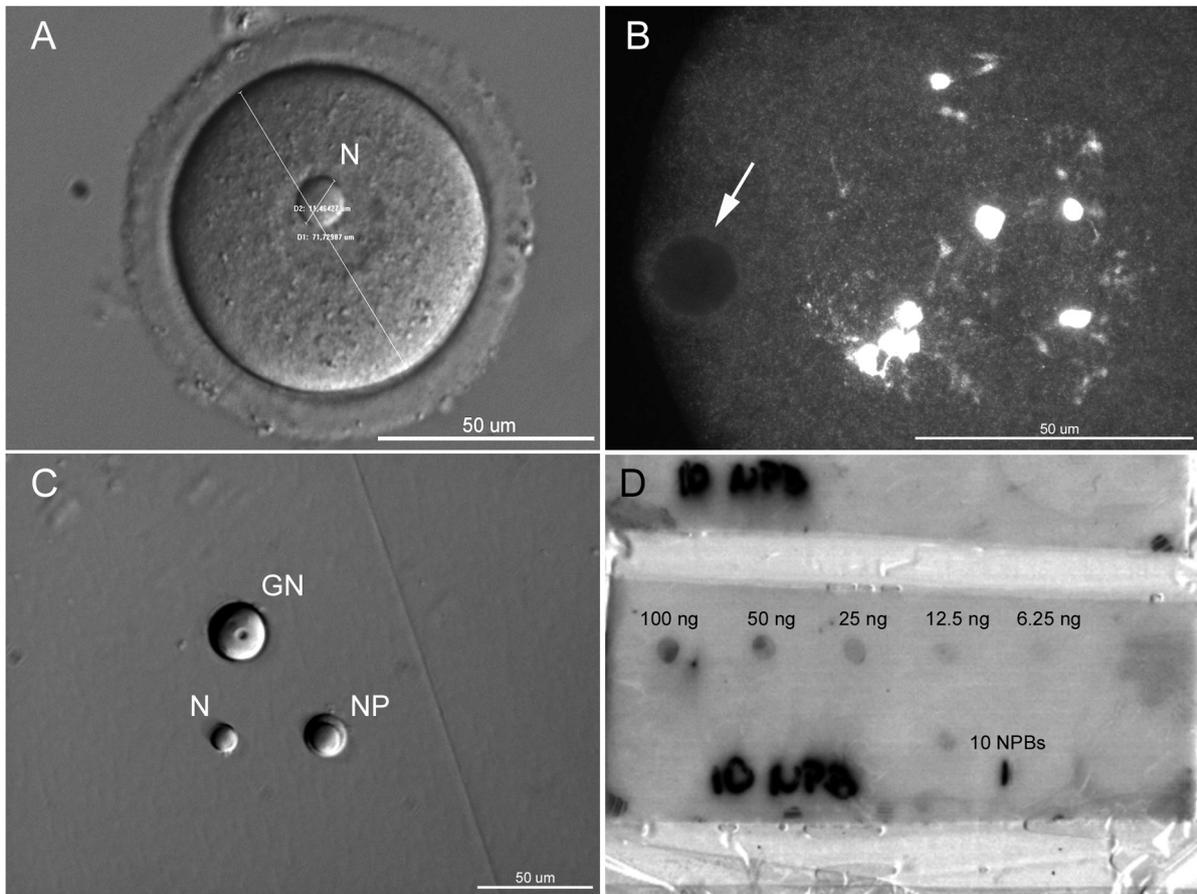


**Fig. 1.** The enucleation procedure. Only GV-stage oocytes containing a single nucleolus precursor body, NPB (N), can be used for enucleation (A). The enucleation pipette is positioned as close as possible to the NPB (B). A mild suction force is applied—the germinal vesicle including the NPB (arrow) is slowly aspirated into the enucleation pipette (C). Finally, the NPB (arrow) penetrates the germinal vesicle membrane (D).

one nucleolus were discarded. We successfully enucleated 505 (83%) fully grown oocytes, and the remaining oocytes were either damaged or their nucleoli could not be completely removed. The nucleoli were isolated in the form of so-called nucleoloplasts, i.e., they were enclosed with a minimal volume of the oocyte cytoplasm surrounded by the oocyte plasma membrane. Some nucleoloplasts were damaged when released from the enucleation pipette (75). The remaining nucleoloplasts (430) were used for the production of giant nucleoli or labeling. As the oocytes used for enucleation contain nucleoli that are enclosed with a ring of chromatin (SN – surrounded nucleoli), we first verified the absence of chromatin contamination of isolated nucleoli. To do that, the isolated nucleoloplasts were kept under zonae pellucidae of the oocytes from which they were isolated. The nucleoloplasts were stuck to enucleated oocytes by a short incubation in phytohemagglutinin. Zonae pellucidae were then dissolved, and the oocyte-nucleoloplast aggregates were fixed and labeled with anti-trimethyl H4/K20 antibody. In the mouse, this antibody detects predominantly the pericentric heterochromatin—a chromatin domain that is specifically associated with NPBs. We evaluated 45 oocyte-nucleoloplast aggregates. In 42 of them, positive labeling was detected only

inside GVs, whilst in nucleoloplasts, there was no labeling (Fig. 2B). In three cases, the labeling was also visible in nucleoloplasts. Additionally, the staining of live oocyte-nucleolus aggregates (57) by Hoechst 33342 gave essentially the same results. In 54 cases, no blue fluorescence was visible in nucleoloplasts. Thus, in almost all cases, the isolated nucleoli do not contain residual chromatin. Finally, it must be noted that ultimate proof that the enucleation does not cause DNA damage was reported by Ogushi *et al.* [11] – when NPBs are returned to previously enucleated oocytes, live mice can be obtained after fertilizing the oocytes.

As mentioned, in some cases, during the enucleation procedure, the nucleolus was split into two parts, which rapidly fused when the nucleoloplast was released from the pipette into the medium. This prompted us to test if nucleoli isolated from several oocytes can also fuse. First, we isolated nucleoli from nucleoloplasts by breaking their membranes with several piezo pulses. These nucleoli, however, rapidly decreased in volume and disappeared in the medium. Thus, we kept them in the form of nucleoloplasts from which they were released immediately before the induction of fusion. So, before the induction of fusion, the nucleoloplast was aspirated into an enucleation pipette and then several piezo pulses were ap-



**Fig. 2.** A mouse oocyte containing a prominent nuclear structure – the nucleolus precursor body, NPB (N). The NPB is approximately 10  $\mu\text{m}$  in diameter; the whole oocyte has a diameter of approximately 70–80  $\mu\text{m}$  (A). From these, NPBs can be microsurgically isolated (enucleolation), and the isolated nucleoloplasts (arrow) are devoid of traces of chromatin, as shown by the lack of anti-trimethyl H4/K20 labeling (B). Isolated nucleoloplasts (NP) can be further manipulated (C)–nucleoli (NPBs) stripped of the oocyte cytoplasmic material can be prepared (N), and fusion can be induced to produce a giant nucleolus (GN). Finally, these giant nucleoli can be analyzed for the protein content. PVDF membranes containing standards (top row) and a sample comprised of 10 NPBs (bottom row) were stained by colloidal gold. This picture also shows that the 3,125 ng of BSA standard cannot be reliably detected (D).

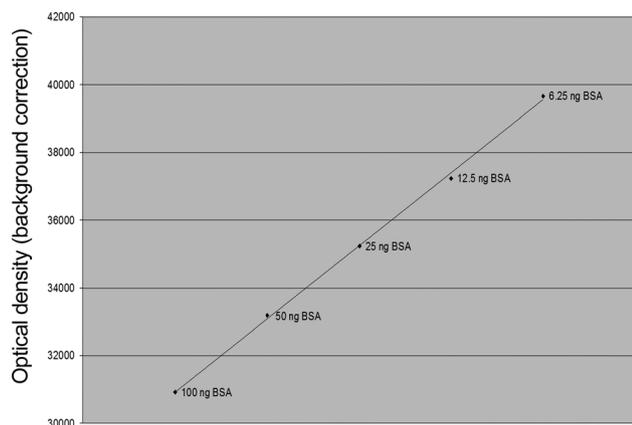
plied. This breaks the nucleoloplast membrane and removes some cytoplasmic debris from the nucleolus. The isolated nucleolus was then released into a manipulation medium. After the next nucleolus was isolated in the same way, it was released into the medium and immediately pushed against the first isolated nucleolus, with which it fused when contact was achieved. Thereafter, additional nucleoli could be added (fused) to the previously obtained giant nucleoli produced by fusion (Fig. 2C). The success of fusion is absolute. In contrast to a single isolated nucleolus, the giant nucleoli are well visible and can thus be relatively easily handled.

The giant nucleoli can be removed from the manipulation medium and used for further analysis.

During previous experiments, we noticed that oocyte nucleoli are sensitive to pepsin digestion. This indicates that NPBs might be composed primarily from different proteins. However, the amount of protein in NPBs is unknown, and common methods used for protein quantification (Lowry, BCA assay, etc.) are not sensitive enough to

reliably detect these small amounts of protein. However, silver or colloidal gold staining is known to detect nanogram quantities of protein. Therefore, we reasoned that it might be feasible to use one of these methods to quantify the protein content of giant nucleoli.

Because the manipulation medium contains bovine serum albumin (BSA), which would ultimately interfere with quantification, we first needed to select a suitable wash solution. After testing several different solutions, we selected phosphate buffered saline (PBS) supplemented with polyvinyl alcohol (PVA), as it does not react with colloidal gold and proteins are not washed through the carrier membrane. Thus, the giant nucleoli (composed of 10 fused NPBs) were quickly washed in PBS/PVA, and stored in 1  $\mu\text{l}$  of this solution in Protein LoBind Tubes and then transferred to a PVDF membrane. At the same time, a protein standard, composed of different solutions with known BSA concentrations, was also spotted onto the membrane. After transfer, the whole PVDF membrane was stained by colloidal gold solution (Fig. 2D). The next day,

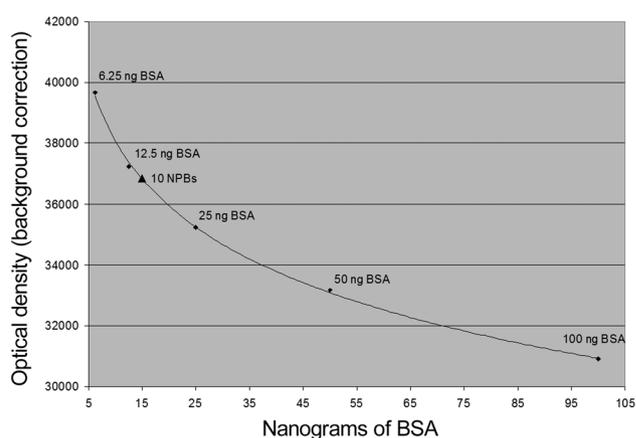


**Fig. 3.** Standard curve construction and standard quality verification. This plot shows an example of the standard curve construction and standard quality assessment (representative PVDF membrane - no. 7). Integrated optical density (IOD) was measured for each standard of known BSA concentration, background correction was applied and IODs were plotted. The standards show a good distribution along the line, which indicates the reliability of the standards within the selected range.

membranes were removed from the staining solution, washed and scanned, and the integrated optical density (IOD) was measured for each standard as well as the sample (Fig. 3). The optical density of the sample was plotted against the standards, and the approximate amount of protein per one oocyte nucleolus was established (Fig. 4). Our analysis indicated that one oocyte nucleolus contains roughly  $1.6 \pm 0.3$  ng of protein. However, it must be noted that the protein content is likely to be higher, as part of the proteins is probably lost during the wash steps. Unfortunately, this cannot be avoided. In our experiment, we labeled both freshly prepared NPB samples as well as NPB samples that were stored at  $-20$  C for at least one month. No differences in sample quality were observed. Thus, the main concern of this procedure is the contamination of NPB samples by BSA originating from the manipulation media.

Our approach describes a simple way to collect a sufficient amount of nucleolar materials from fully grown mammalian oocytes that can then be used for further biochemical analyses. The methods for isolation of a sufficient number of somatic cell nucleoli have already been published, and the isolated nucleoli were used for proteomic analysis. As far as we are aware, no similar approach has been reported for the isolation of mammalian oocyte nucleoli.

The method of oocyte enucleation was invented by Fulka, Jr. *et al.* [7], who demonstrated that nucleoli from fully grown porcine oocytes can be microsurgically removed. Subsequently, essentially the same approach was used for enucleation of mouse oocytes [8, 11, 13]. Oocyte enucleation thus enabled us to clarify the role of nucleoli during the process of oocyte maturation and in early embryonic development. Logically, the next step is a detailed biochemical characterization of NPBs. When compared with somatic cell nucleoli, mammalian oocyte nucleoli are relatively difficult to isolate in sufficient numbers. Their visibility is also limited. Moreover, when collected from the manipulation medium, they can be easily lost, for example, by sticking to a pipette wall. The



**Fig. 4.** The NPB sample protein content estimation. The plot shows the logarithmic dependence of integrated optical density (IOD) on the protein concentration (PVDF membrane no. 7). In this case, the nucleolus sample (obtained by aggregating 10 NPBs) was plotted together with the standard. The NPB sample protein content was estimated to be about 15 ng. Thus, the protein quantity was roughly 1.5 ng of protein per one NPB.

production of giant nucleoli eliminates these problems, as giant nucleoli are well visible and can be easily transferred into a test tube and frozen. However, attention must be paid to some issues. First, the enucleation method requires good equipment and certain skill. Second, the volume of the oocyte cytoplasm enclosing the nucleolus must be kept to a minimum as the nucleoli reinjected into the oocyte cytoplasm disappear (are dissolved) readily. Therefore, we must assume that the same will happen in the nucleoloplast cytoplasm if the volume of cytoplasm is too large. Third, the work required for fusing the isolated nucleoli must be performed very quickly. The isolated nucleoli dissolve in the manipulation medium, so we must keep in mind that some nucleolar material will also be degraded when fusion of nucleoli is performed. Logically, it is thus better to produce relatively smaller giant nucleoli and to freeze them quickly than to produce supergiants from hundreds of nucleoli but lose some nucleolar material. Nevertheless, we do believe that our approach will accelerate the work aiming at characterizing the composition of fully grown mammalian oocyte nucleoli.

## Methods

### Preparation of oocytes

BDF1 (B6D2F1/Crl) females were stimulated with 7.5 IU of PMSG (pregnant mare serum gonadotropin, Calbiochem, EMD Chemicals USA, Gibbstown, NJ, USA). After about 44-46 h, their ovaries were isolated, and oocytes were released from large antral follicles into HTF (Human Tubal Fluid, Zenith Biotech, Guilford, CT, USA) supplemented with 4 mg/ml of BSA (bovine serum albumin). Thereafter, only those oocytes surrounded by several layers of cumulus cells were selected, transferred into MEM (minimal essential medium) supplemented with gentamicin (50  $\mu$ g/ml), Na-pyruvate (0.2 mM), BSA (4 mg/ml) and dbcAMP (150  $\mu$ g/ml; dibutyryl cyclic AMP) and cultured in an incubator at 37 C/5%

CO<sub>2</sub> for 30 min. The cumulus cells were then removed by vigorous pipetting, and only healthy looking oocytes with intact germinal vesicles were further cultured for another 3–4 h. The remaining oocytes were discarded.

#### *Oocyte enucleation*

The nucleoli from oocytes were isolated exactly as described in our previous papers [7, 11]. Briefly, the oocytes were transferred into 10 µl droplets of KSOM/HEPES (Zenith Biotech, Guilford, CT, USA) supplemented with cytochalasin D (7.5 µg/ml), BSA (4 mg/ml) and dbcAMP (150 µg/ml). The droplets were covered with paraffin oil. The basic micromanipulation procedure was essentially the same as that described by Kishigami *et al.* [16]. The manipulation was performed on an Olympus IX-71 stage with the help of Narishige micromanipulators. The oocytes were stabilized with a holding pipette. The injection (enucleation) pipette fixed to piezo unit (Prime Tech, Tsuchiura, Ibaraki, Japan) first penetrated through the zona pellucida and then was slowly pushed against the GV membrane. As soon as the tip was in close vicinity to the nucleolus, a very mild suction was applied. The nucleolus typically penetrates through GV membrane and is slowly translocated into the oocyte cytoplasm. A nucleolus enclosed by a minimum volume of the cytoplasm and surrounded by an oocyte plasma membrane (nucleoloplast) was obtained by withdrawing the enucleation pipette from the oocyte. The nucleoloplasts are then either expelled from the pipette into the medium or left under zonae pellucidae (Hoechst staining, immunolabeling). As soon as a chosen number of nucleoloplasts was prepared, nucleoli were isolated from them. A single nucleoloplast was slowly aspirated into the enucleation pipette, and then several high frequency pulses were applied. This breaks the membrane and concomitantly removes some cytoplasmic debris from the nucleolus. The nucleolus was then released from the pipette. Next, another nucleolus that was prepared in the same way was pushed against the first nucleolus. Typically, as soon as minimal contact was established between these nucleoli, fusion occurred very rapidly. The procedure was repeated until a giant nucleolus with the desired amount of nucleolar material was collected.

#### *Verifying the absence of chromatin incorporation around isolated nucleoli*

As mentioned above, nucleoli in the best oocytes are surrounded closely by the chromatin (SN – surrounded nucleolus). In order to verify whether this chromatin is or is not attached to isolated nucleoli in nucleoloplasts, two approaches were used. First, oocytes with nucleoloplasts under zonae pellucidae were stained with Hoechst 33342 (20 µg/ml) for 20 min at 37 C. Then they were washed several times in HTF/BSA and examined under a fluorescence microscope.

The second approach used labeling with anti-trimethyl H4/K20 antibody. Briefly, the enucleolated oocytes with nucleoloplasts under zonae pellucidae were incubated in PBS supplemented with phytohemagglutinin (200 µg/ml). This sticks the nucleoloplast to the oocyte. Zonae pellucidae were then dissolved in acid Tyrode's solution, and then oocytes with nucleoloplasts were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed specimens were then stored in PBS (4 C). Before labeling,

the oocyte-nucleoloplasts aggregates were permeabilized with Triton-TX100 (0.2%) in PBS for 30 min and then blocked 1 h (4 C) in 1% BSA/PBS. Samples were then incubated with anti-trimethyl H4/K20 antibody (Abcam, Cambridge, UK) overnight at 4 C, washed several times in PBS/BSA and incubated with Cy2 conjugated secondary antibody (Jackson ImmunoResearch, Newmarket, UK) for 2 h (RT). After this incubation and several washes in PBS/BSA, samples were mounted on slides in VECTASHIELD (Vector Laboratories, Peterborough, UK) and evaluated under a fluorescence microscope.

#### *Assessing the protein content in nucleoli*

Oocyte nucleoli (NPBs) were isolated and fused as described. Samples of 10 NPBs were taken for analysis. A giant nucleolus was removed from the manipulation medium containing BSA and quickly washed in phosphate buffered saline (PBS) supplemented with 0.1% polyvinyl alcohol (PVA). The washed giant nucleolus was transferred into an Eppendorf Protein LoBind tube (Eppendorf, Prague, Czech Republic) under a stereomicroscope, snap-frozen in liquid nitrogen and analyzed or stored at –20 C until analysis. Standards were prepared from a BSA solution containing 2 mg/ml protein (Pierce, Rockford, IL, USA) by serial dilution at the following concentrations: 100, 50, 25, 12.5, 6.25 and 3.125 ng/µl. A PVDF membrane (Bio-Rad, Prague, Czech Republic) was activated by a brief incubation in methanol. The membrane was washed in distilled water and then in Towbin Transfer Buffer containing 20% methanol. Excess buffer was removed by vacuum. One microliter of each standard was spotted onto the membrane; finally, the sample was transferred onto the membrane, and the membrane was stained by colloidal gold solution as described by the manufacturer (Bio-Rad, Prague, Czech Republic). The next day, the membrane was washed in distilled water and scanned. Signal quantification was performed using the Image-Pro software (Media Cybernetics, Bethesda, MD, USA). Parameters collected were the area and integrated optical density (IOD), and samples of the background were also analyzed. Background intensity was subtracted, and standard concentrations of proteins were plotted against the signal. In total, we evaluated 9 PVDF membranes, each containing a single sample equivalent to 10 NPBs (this number of NPBs was estimated to be the lowest amount of material sufficient for reliable analysis by the colloidal gold staining method). From this, the unknown protein concentration of the sample was calculated. Results are given as the average ± standard deviation.

Unless stated otherwise, all chemicals were purchased from Sigma.

### Acknowledgments

This study was supported by GACR P302/11/P069. HK was supported by a Research Fellowship for Young Scientists.

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# Transplantation of nucleoli into human zygotes: not as simple as expected?

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Received: 1 February 2011 / Accepted: 29 March 2011 / Published online: 8 April 2011  
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## Introduction

Mammalian oocytes and one-cell stage embryos contain prominent nuclear organelles, the nucleolus precursor bodies (nucleoli), that are structurally and functionally different when compared to nucleoli in more developmentally advanced embryos and somatic cells. The function of these organelles, called the nucleolus precursor body—NPB(s), is not known and it is commonly accepted that they might serve as the storage site of material from which fully functional nucleoli are gradually formed. When evaluated ultrastructurally, NPBs are exclusively composed from dense fibrillar material. On the other hand, fully differentiated nucleoli contain dense fibrillar, fibrillar and granular components and are involved in many cellular processes, i.e. transcription of ribosomal genes, ribosome biogenesis, cell cycle progression and proliferation, gene silencing and so on. The differentiated nucleolus contains

about 300 proteins [1]. The composition of NPBs is unknown—the only protein that has been demonstrated to localize to NPBs without any doubts is nucleoplasmin 2 [2].

Nucleoli (NPBs) are well visible in growing and fully grown oocytes of some mammals, i.e. mouse, rat, pig human, etc. On the other hand, they cannot be observed for example in native bovine, sheep and rabbit oocytes. In most developmentally advanced fully grown oocytes, that are competent to undergo germinal vesicle breakdown and reach metaphase II stage, nucleoli are surrounded with a ring of chromatin. In less developed oocytes, the chromatin is dispersed in the nucleoplasm [3]. Similar chromatin attachment can be seen also in pronuclei. It is not fully and completely understood why contact of NPB/chromatin is so important from the developmental point of view.

From a human assisted reproduction point of view an interesting observation has been made by Tesarik and co-workers [4]. These authors found that the pattern of distribution of nucleoli as well as their number in pronuclei of one-cell stage human embryos reflect developmental potential of these embryos and can be used as a non-invasive tool for selecting the best embryos. These observations were confirmed by others. But again, why zygotes with various patterns differ developmentally and why some of them have a certain pattern and in others the pattern is different remains to be explained. Evidently, these results however indicate the importance of nucleoli even in one cell stage embryo. This has been confirmed by Burns et al. [5] who produced knock-out NPM2 (nucleoplasmin 2) mice. Nucleoplasmin 2 is important in *Xenopus* as it helps to decondense sperm DNA. In contrast, it does not play the same role in the mouse. In *NPM 2<sup>-/-</sup>* mice the sperm head decondenses in the oocyte cytoplasm but in pronuclei no nucleoli can be detected. Development of these embryos is compromised and is typically arrested at the two-cell stage.

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*Capsule* Development of one-cell stage human embryos with low number of nucleoli and their unequal distribution in pronuclei is compromised. Thus, it has been suggested that the transplantation of extra-nucleolar material may possibly rescue these embryos. Recently published results, however, show that this might be more complicated than expected.

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Nevertheless, some knock-out female mice gave birth to offspring. Interestingly, the typical nucleoli cannot be seen in immature *NPM 2<sup>-/-</sup>* oocytes, but surprisingly, their maturation was apparently normal and they reached metaphase II stage.

### Manipulating the mammalian oocyte nucleoli

In 2003 Fulka, Jr. et al. reported that the nucleolus can be removed from fully grown porcine oocytes by micromanipulation—the enucleolation [6]. The same approach has been shown (enucleolation) to work well in the mouse too and probably in some others mammals where oocyte nucleoli are clearly visible [7, 8]. Nucleoli can also be removed from pronuclei of one-cell stage embryos (Figs. 1a–d). Here, however, the method is more complicated and the complete enucleolation is only successful when each pronucleus contains just a single nucleolus. The enucleolation thus gave us an unrivalled opportunity to study the function of NPBs in more detail. Thus, it has been showed that oocyte nucleoli are not essential for the initiation and completion of oocyte maturation. The manipulated and control oocytes reached metaphase II at the same frequency. The enucleolated oocytes can be parthenogenetically activated or fertilized but their pronuclei do not contain the typical nucleoli and the resulting embryos usually do not cleave beyond the two-cell stage. As expected, the detailed analysis of these embryos indicated defects in transcription. The embryo nucleolus is thus of maternal (oocyte) origin [9, 10].

An interesting technical observation has been made by Fulka and Fulka, Jr. who found that if the nucleolus (NPB) is transferred into the oocytes (zygote) cytoplasm it is rapidly translocated into the nucleus—germinal vesicle (GV)—or into pronuclei (PNs). Thus, it is not necessary to inject the nucleoli directly into nuclei and this greatly facilitates the micromanipulation procedure and largely reduces the oocyte or zygote damage. Therefore, the authors suggested that it might be possible, by simple injection, to rescue human zygotes having an abnormal pattern or number of nucleoli in PNs [11]. The recently published papers however showed that this will be more complicated than expected.

### Nucleoli in oocytes and one—cell stage embryos

As pointed out earlier, the NPBs play an important structural role both in oocytes and in zygotes by helping to organize the chromatin in these cells.

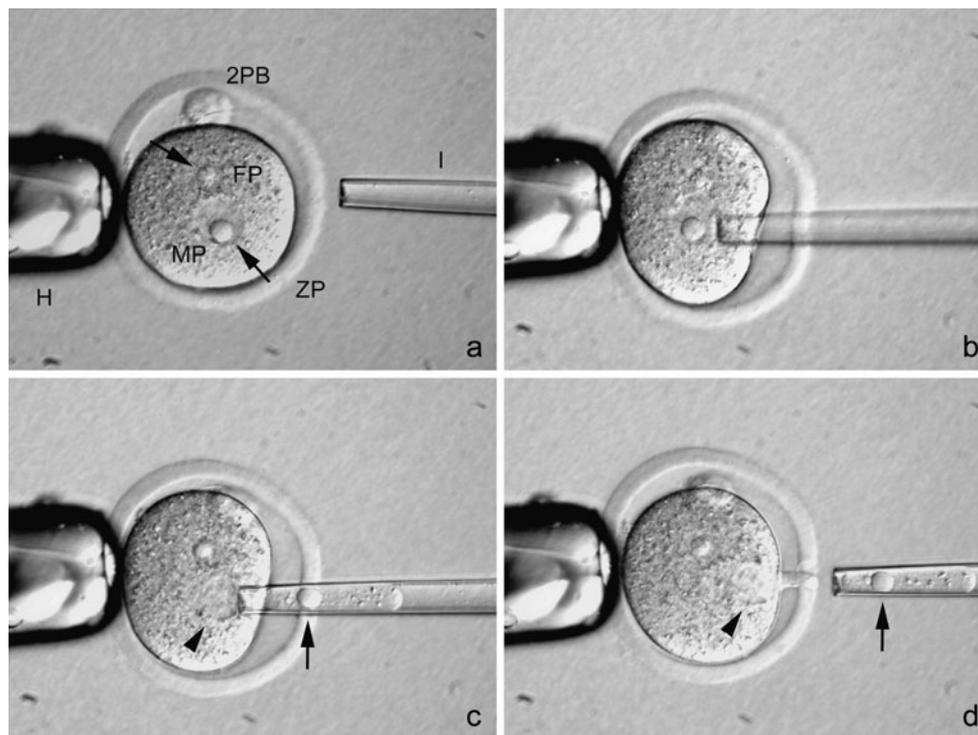
Recently, several experiments demonstrated that in zygotes, especially the pericentric heterochromatin is

associated with the NPBs. This association is typical for transcriptionally inactive embryonic stages. However, upon the activation of the embryonic genome the centromeres are re-localised from NPBs to chromocenters that are typical for somatic cells. This process is probably important for the functional maturation of centromeres and pericentric heterochromatin. This, in turn, is necessary for example for the correct segregation of chromosomes during subsequent cell divisions and in consequence, for embryonic development [12, 13].

It has been documented that after fertilization, the sperm chromatin, that is originally organized mostly by protamines, undergoes decondensation and protamines are gradually replaced by histones originating from the oocyte cytoplasm. As a result, the epigenetic information carried by covalent histone modifications must be newly set. In contrast, the oocyte does not exhibit extensive replacement of histones (except for the linker histone, for review see [14]). Therefore we might expect the parental genomes to be epigenetically unequal shortly after fertilization. Indeed this had been extensively documented in the past years both on the whole genome scale and also specifically for centric and pericentric chromatin. Probst and colleagues [15] showed that while the female pronucleus exhibits the presence of histone modifications typical for centric and pericentric heterochromatin (H3K9me3, H3K27me3 and H4K20me3), these modifications are absent from the male pronucleus. Even at the two-cell embryonic stage, the paternal pericentric chromatin is devoid of H3K9me3 [16].

Interestingly, irrespectively of this epigenetic difference between the male and female pronucleus, in both pronuclei the pericentric heterochromatin has been shown to gradually associate with the NPB as it forms [15, 16]. However, at the two-cell stage, this distribution is lost and the centromeres do no longer associate with the NPBs, instead they become gradually organized to chromocentres—an organization typical for somatic cells [16]. Probst and colleagues [17] has shown that this re-localization into chromocentres is dependent upon transcription of the major satellite repeats. These authors show that in the mouse embryos the transcription of these repeats starts at late zygotic stage and that these repeats are mainly derived from the paternal genome. This is followed by a burst of transcription of these sequences at the time of major genome activation (in the mouse at the two-cell stage) with its subsequent down-regulation. Interestingly, further analysis showed that this process is parent-specific with the forward major satellite transcript mainly originating from the paternal genome. Finally, these authors show that interference with this process leads to developmental arrest.

This observation was confirmed by two more recent articles. In the first one, Santenard and colleagues [12] show that histone H3.3 plays a major role in pericentric



**Fig. 1** a–d The enucleation of one-cell stage mouse embryo. **a** One-cell pronuclear stage embryo where each pronucleus contains just a single nucleolus is stabilized with a holding pipette (H). The enucleation (I— injection pipette is on the right. ZP—zona pellucida, 2 PB—second polar body, FP—female pronucleus (maternal), MP—male pronucleus (paternal), arrows—nucleoli. **b** The injection pipette first penetrates through zona pellucida and its tip is pushed into a close vicinity of the pronucleus from which the nucleolus has to be removed. Thereafter a very gentle suction is applied. **c** This mild suction preferentially aspirates the nucleolus that penetrates the pronuclear membrane and moves into the embryo cytoplasm (arrow). In the pronucleus no nucleolus can be seen (arrowhead). Note, that the

heterochromatin formation. When a mutation is introduced at the position 27 (Lysine) of histone H3.3, embryos show aberrant transcription of the pericentric sequences leading to errors in chromosome segregation and finally to developmental arrest. Interestingly, a similar observation was made by Baumann and colleagues who showed that maternal loss of alpha thalassemia/mental retardation X-linked syndrome (ATR-X) protein leads to abnormal chromosome morphology and severe reduction of fertility. It was shown that ATR-X, that belongs to the SNF2-family remodeling complexes, together with Fas death domain-associated protein (DAXX) localize to the pericentric chromatin domains during oocyte prophase I arrest. Furthermore, the authors show that maternal loss of ATR-X abrogates DAXX localization and leads to centromeric and chromosomal instability. What might be the link between these observations?

ATR-X and DAXX have previously been shown to localize to telomeres in embryonic stem cells. Moreover, newer results show that ATR-X-DAXX complex is required for the deposition of H3.3 at telomeres [18]. Therefore, one

injection pipette actually did not penetrate directly into the embryo cytoplasm. **d** By increasing the distance between both pipettes, the nucleolus enclosed with an embryo membrane and a minimum volume cytoplasm—arrow (nucleoloplast) can be completely removed from the embryo. Arrowhead—the enucleolated paternal pronucleus. Those embryos from which nucleoli were removed from both pronuclei do not develop beyond the 2–4 cell stage. The enucleation of either maternal or paternal pronucleus resulted in compromised embryonic development with a minimum of abortive blastocysts produced. The nucleolus can be removed similarly from immature (GV-stage) oocytes

might imagine that if ATR-X and DAXX localize to pericentric chromatin in prophase I arrested oocytes they might carry out similar process—that is the deposition of H3.3 histone. H3.3 (and specifically H3.3 K27) in turn seems to be important for the transcriptional regulation of these repetitive sequences [13]. Finally, interfering with this process will lead to non-functional centromeres and pericentric heterochromatin and thus to abnormal and unstable chromosomes and developmental arrest.

Thus to summarize, the above mentioned articles show that the association of centric/pericentric repeats with the NPB might reflect a functional as well as epigenetic “maturation” of these sequences.

#### Transplanting the nucleolus into one cell stage embryos

An interesting observation was made by Ogushi and Saitou in 2010 [19]. These authors have transplanted the NPBs from GV stage oocytes into different oocyte maturation

stages or one-cell stage embryos and examined the development of such manipulated oocytes or embryos. When fertilized, the enucleolated and NPB re-transplanted oocytes at the GV or metaphase II stage had the ability to develop up to the blastocyst stage, on the other hand, when the NPB was re-injected into one-cell stage embryos, originating from previously enucleolated oocytes, the developmental ability of these embryos was severely reduced. These experiments show that the NPB might undergo progressive changes in composition and/or function between the oocyte (GV) and zygote stage. Thus, it is possible that NPBs originating either from oocytes or zygotes are not equivalent albeit they have seemingly identical structure and appearance. The other possibility is that NPBs start to function very soon after fertilization and their transplantation into more advanced zygotes does not allow them to fulfill their typical role(s).

The detailed analysis of higher order chromatin showed that the NPB is the major organizing structure both in oocytes and in early embryos. Thus, the higher order chromatin structure of zygotes originating from enucleolated oocytes showed severe defects in organization (especially with respect of centromeres) although the global level of several histone modifications, that were investigated, was apparently not altered. These findings are not surprising taking into account the recently published articles.

Interestingly, Ogushi and Saitou [19] also show alteration of duration of the first mitotic cell cycle in embryos derived from enucleolated oocytes. Whether this observation reflects defects in centromere and pericentric heterochromatin establishment and function is currently unknown. As pointed out above, two recently published articles highlight the role of epigenetic mechanism operating in establishment of functional centromeres and pericentric heterochromatin in oocytes and early embryos [12, 13]. However, in somatic cells, the nucleolus is also known to play an important role in cell cycle progression by sequestering certain cell cycle involved proteins therefore the explanation for the observed lag in development to the two-cell stage might be the absence of some protein that localizes to the NPB. Unfortunately, as pointed out earlier the protein composition of NPBs is currently unknown and due to technical limitations will be very difficult to establish.

## Summary

To summarize, it is clear that NPBs play an important structural and chromatin organizing role, especially the pericentric heterochromatin and centromeres that associate with this nuclear body. Recent results demonstrate the role

of epigenetic mechanisms in establishment of pericentric heterochromatin and centromere function. However, as the protein composition of this unique structure is unknown it is also not known to what extent NPBs play an active role in this process. Ogushi and Saitou [19] have demonstrated that zygotic and GV-stage oocyte NPBs are not equivalent in composition and/or function. Exchanging zygotic NPBs for oocyte NPBs in mouse one-cell stage embryos lead to a lag in cell cycle progression and severe developmental arrest. These experiments clearly demonstrate that the proposed nucleolus transplantation therapy will not be trivial. In other words, the mentioned experiments indicate that during oocyte growth and early embryonic development the composition and/or function of the NPB changes, even though the NPB appears morphologically equivalent in these cells. These subtle changes might therefore prevent the proposed micromanipulation therapy (exchange of NPBs between oocyte and zygote or transfer of additional NPB material originating from oocytes to zygotes).

**Acknowledgements** JFJr's lab is supported from MZe 0002701401 and GACR 523/09/1878, HF is supported from GACR P302/11/P069. PL research is partially funded by NextGene, VII FP, project n.244356.

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## Brief report

# An observational study of assisted reproductive technology outcomes in new European Union member states: an overview of protocols used for ovarian stimulation

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#### Key words:

Assisted reproductive technology – Audit – Europe – Recombinant human follicle-stimulating hormone

Accepted: 22 December 2009; published online: 3 February 2010  
Citation: Curr Med Res Opin 2010; 26:819–25

## Abstract

#### Background:

The development of new fertility treatment options has facilitated individualized assisted reproductive technology (ART) protocols to improve outcomes. Manufacturing improvements to recombinant human follitropin alfa have allowed precise dosing based on mass (filled-by-mass; FbM) rather than bioactivity (filled-by-bioassay; FbIU). Continued monitoring and reporting of follitropin alfa treatment outcomes in routine clinical practice is essential.

#### Objective:

To provide an overview of the frequency of different controlled ovarian-stimulation protocols used in *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles in new European Union member states, and to provide post-registration efficacy and safety data on follitropin alfa.

#### Research design and methods:

A 2-year, prospective, observational, multicentre, Phase IV study conducted at ART clinics in the Czech Republic, Estonia, Latvia, Lithuania, Poland, Slovakia and Slovenia. Women aged 18–47 years undergoing ovarian stimulation with follitropin alfa for conventional IVF or ICSI were eligible for inclusion. The main treatment outcome was cumulative clinical pregnancy rate. Data were analysed descriptively.

#### Results:

Clinical pregnancy outcomes were available for 4055 of 4085 (99.3%) patients. In total, 1897 (46.8%) patients used follitropin alfa FbIU; 2133 (52.6%) used follitropin alfa FbM. Clinical pregnancy was achieved by 39.5% (1603/4055) of patients. A greater proportion of patients with polycystic ovary syndrome achieved a clinical pregnancy than those with endometriosis (41.8% vs 37.8%, respectively). A higher cumulative pregnancy rate was observed with the use of follitropin alfa FbM than follitropin alfa FbIU (41.3% vs 37.8%, respectively;  $p = 0.02$ ).

#### Conclusions:

This study represents the most comprehensive audit of individualized ART in clinical practice in Central and Eastern Europe. Overall, clinical pregnancy was achieved by 39.5% of patients after stimulation with follitropin alfa. The use of follitropin alfa FbM resulted in a higher cumulative pregnancy rate than did the FbIU formulation. However, limitations of the study include the observational and non-comparative study design, and descriptive nature of statistical analyses; furthermore, the study was not designed to make direct comparisons between the success rates of different ovarian-stimulation protocols.

## Introduction

The development of recombinant gonadotrophins for ovarian stimulation, gonadotrophin-releasing hormone (GnRH) antagonists for pituitary down-regulation and various preparations of progesterone for luteal phase support have expanded the available treatment options for assisted reproductive technology (ART). The availability of a wide range of products facilitates the use of individualized ART treatment protocols according to the patient's clinical characteristics<sup>1</sup>.

Administration of follicle-stimulating hormone (FSH) during ART leads to the development of multiple ovarian follicles<sup>2</sup>. Because of the variability of batch quality, doses of FSH are conventionally based on assessment of bioactivity (measured in international units [IU])<sup>3</sup>. This bioactivity assay has limited precision and involves cumbersome procedures for data generation and interpretation. Improvements to the manufacturing process of recombinant human (r-h) follitropin alfa have facilitated the development of a product with highly consistent activity and excellent batch-to-batch consistency<sup>4</sup>. This allows dosing of follitropin alfa based on the more precise measurement of protein mass in micrograms (filled-by-mass; FbM) rather than the inherently variable biological assay (filled-by-bioassay; FbIU).

The efficacy and safety profile of follitropin alfa in clinical trials is well established<sup>5-7</sup>. Nonetheless, it is important to monitor the efficacy and safety of both follitropin alfa FbIU and FbM in routine clinical practice<sup>8</sup>. Observational studies or post-registration surveillance provide a valuable method of assessing the use of new reproductive therapies in a routine clinical setting<sup>9</sup>. Regular assessment and reporting of ART outcomes also allows comparison and sharing of effective clinical practice methods between different clinics and, indeed, countries.

The prevalence of infertility in Central and Eastern European countries has been highlighted previously<sup>10</sup>. Sanocka and Kurpisz recommended greater international co-operation between specialized centres of excellence<sup>10</sup>. To date, there is very little documentation of outcomes in ART across this region, but the recent admission of several Central and Eastern European countries to the European Union (EU) offers an opportunity to examine and to share the best practice and treatment protocols.

A European consortium (The European IVF-monitoring [EIM] programme) collects *in vitro* fertilization (IVF) data from pre-existing national registers in 22 countries<sup>11</sup>. However, the collection of ART treatment outcomes is hampered by variable definitions, coverage, methods of data collection and validation between different countries<sup>9,11,12</sup>. This is exemplified by the unavailability of national registry data from Bosnia, Croatia, the Czech Republic, Romania and Slovakia for the recent

annual European Society of Human Reproduction and Embryology (ESHRE) ART report<sup>13</sup>.

The aim of this observational study was to give an overview of the frequency of different controlled ovarian-stimulation (COS) protocols used in IVF and intracytoplasmic sperm injection (ICSI) cycles, and to gather efficacy and safety data on a tailored approach to multi-follicular stimulation using follitropin alfa during cycles of ART in routine clinical practice in seven Central and Eastern European countries. Furthermore, we were able to compare descriptively the efficacy of follitropin alfa FbIU and FbM because the FbM formulation was made available for routine clinical practice while this study was in progress.

## Patients and methods

### Study design

ART clinics in the Czech Republic, Estonia, Latvia, Lithuania, Poland, Slovakia and Slovenia participated in this prospective, observational, multicentre, Phase IV study. The study was conducted from December 2003 to March 2006 inclusive. All participating clinics conducted at least 200 ART cycles per year. Clinics were encouraged to recruit a number of consecutive patients, and manage them according to their routine clinical practice.

### Patients

Women aged 18–47 years who were scheduled to undergo multi-follicular ovarian stimulation with follitropin alfa (r-hFSH; GONAL-f, Merck Serono S.A. – Geneva, Switzerland [an affiliate of Merck KGaA, Darmstadt, Germany]) for conventional IVF or ICSI were eligible for inclusion in the study. Each patient was eligible for enrolment during multiple treatment cycles, according to centre-specific criteria. Patients provided written informed consent for ART treatment. Ethical approval or informed consent was not required for analysis of patient data.

### Interventions

COS using follitropin alfa was performed according to the standard practice at each clinic. Additional treatments such as pituitary suppression using GnRH analogues (agonists or antagonists), luteinizing hormone (LH) supplementation, follicular maturation triggered by human chorionic gonadotrophin (hCG) and progesterone for luteal phase support were available according to the usual practice at each individual clinical. Treatment was, thus, individualized for each patient. Patients self-administered medication.

## Reporting

Physicians completed a specified case report form for each treatment cycle, which recorded: baseline data, including age, body weight, height, date of birth, smoking habit, cause of infertility and number of previous stimulation cycles; treatment information, including dose of r-hFSH, use of GnRH agonist or antagonist, duration of pituitary down-regulation, use of FSH, LH, hCG or progesterone drugs; pre-treatment hormone concentrations, including FSH, LH, oestradiol and progesterone; clinical outcomes in terms of numbers of follicles, oocytes retrieved, embryos transferred, embryos cryopreserved, cancelled cycles or biochemical/clinical pregnancy (defined as a positive hCG test/positive foetal heart beat, respectively); and the occurrence of adverse events such as ovarian hyperstimulation syndrome (OHSS), miscarriage, extra-uterine or multiple pregnancy (defined as >2 embryos on week 12 ultrasound scan).

## Data analysis

As this was a non-comparative, observational study, all statistical analyses were descriptive. No confirmatory statistical testing was applied. Summary statistics included mean, standard deviation (SD), median and range values for continuous variables, and frequencies and percentages for categorical variables. The Mann-Whitney U or chi-squared tests were used to compare data from groups of patients. The resulting *p*-values are regarded as descriptive rather than tests of hypotheses and, thus, no pre-specified significance level or alpha correction was applicable.

## Results

### Patient disposition and baseline characteristics

Twenty-five centres participated in the study: 12 in the Czech Republic, five in Poland, three in Slovakia, two in Slovenia and one each in Estonia, Latvia and Lithuania. A total of 4085 patients were included in the study. The achievement of a clinical pregnancy was not clearly defined for 30 (0.7%) patients, of whom 24 received follitropin alfa FbM and six received other formulations. These patients were excluded from statistical analyses.

Baseline demographic and disease characteristics of the 4055 evaluable patients are shown in Table 1. Mean (SD) age of patients was 31.8 (4.4) years; 72.9% (2956/4055) were ≤34 years. Patients in Slovenia and Slovakia had the highest and lowest mean (SD) ages (33.1 [4.3] and 31.0 [3.9] years), respectively. Male (1993/4055, 49.1%) and tubal factors (1328/4055, 32.7%) were the most common causes of infertility. Pregnancy rates decreased as the

**Table 1.** Baseline characteristics overall and by pregnancy outcome at ART clinics in the Czech Republic, Estonia, Latvia, Lithuania, Poland, Slovakia and Slovenia.

	Total number (%) of patients ( <i>n</i> = 4055)	Number (%) of patients clinically pregnant
<b>Age in years</b>		
≤34	2956 (72.9)	1263 (42.7)
35–38	751 (18.5)	266 (35.4)
>38	307 (7.6)	56 (18.2)
No data	41 (1)	18 (43.9)
<b>Infertility</b>		
Primary	2584 (63.7)	1026 (39.7)
Secondary	1395 (34.4)	536 (38.4)
No data	76 (1.9)	41 (53.9)
<b>Cause of infertility*</b>		
Tubal factor	1328 (32.7)	514 (38.7)
Polycystic ovary syndrome	316 (7.8)	132 (41.8)
Ovulatory dysfunction	671 (16.5)	235 (35.0)
Endometriosis	563 (13.9)	213 (37.8)
Male factor	1993 (49.1)	805 (40.4)
Other	624 (15.4)	197 (31.6)

\*More than one cause of infertility could be documented for each patient.

number of cycles/attempts increased and rates varied from 29.0% in Slovenia to 55.4% in Latvia, with a mean pregnancy rate of 39.5%.

### Treatment characteristics

Treatment characteristics and clinical pregnancy rates are shown in Table 2.

#### Stimulation

Overall, 1897 (46.8%) patients used follitropin alfa FbM and 2133 (52.6%) patients used follitropin alfa FbIU; in 25 cases (0.6%) the formulation was not recorded (Table 2). Follitropin alfa was administered using a prefilled pen device in 641 (15.8%) cases a single-dose vial in 520 (12.8%) cases and a multi-dose vial in 530 (13.1%) cases; in 2364 (58.3%) cases the mode of application was unknown. The mean total follitropin alfa administered was 2078.75 FbIU and 1921.74 IU FbM.

LH supplementation was received by 1005/4055 (24.8%) patients, while 1351/4055 (33.3%) of patients did not receive LH (Table 2); for 1699 (41.9%), these data were not recorded. The majority (672/1005, 66.9%) of patients who received LH were treated with r-hLH, but approximately one-third (331) received human menopausal gonadotrophin. The mean (SD) number of administrations of r-hLH was 1.03 (0.27) (75 [20.25] IU) and was given for a mean (SD) duration of 4.74 (2.92) days.

#### GnRH analogue

GnRH agonists were used for pituitary suppression in 49.0% (1987/4055) of patients (Table 2). The antagonist

**Table 2.** Clinical pregnancy rates by ART treatment characteristics in ART clinics in the Czech Republic, Estonia, Latvia, Lithuania, Poland, Slovakia and Slovenia.

	Number (%) of patients (n = 4055)	Number (%) of pregnancies by treatment option	p-value for difference in pregnancy rate
<b>FSH formulation</b>			
Follitropin alfa FbM	1897 (46.8)	784/1897 (41.3)	0.022*
Follitropin alfa FbIU	2133 (52.6)	806/2133 (37.8)	
No data	25 (0.6)	13/25 (52.0)	
<b>Type of GnRH analogue</b>			
Cetrorelix acetate 0.25 mg	1118 (27.6)	445/1118 (39.8)	NS†
Cetrorelix acetate 3 mg	827 (20.4)	260/827 (31.4)	
GnRH agonist	1987 (49.0)	836/1987 (42.1)	
No data	123 (3.0)	62/123 (50.4)	
<b>Use of LH supplementation</b>			
Yes	1005 (24.8)	408/1005 (40.6)	NS‡
No	1351 (33.3)	562/1351 (41.6)	
No data	1699 (41.9)	633/1699 (37.3)	
<b>Ovulation trigger</b>			
r-hCG	1535 (37.9)	575/1535 (37.5)	NS§
u-hCG	1043 (25.7)	420/1043 (40.3)	
None, or no data	1477 (36.4)	608/1477 (41.2)	
<b>Luteal support</b>			
Vaginal progesterone gel 8%	251 (6.2)	121/251 (48.2)	0.061
Intramuscular progesterone	1333 (32.9)	552/1333 (41.4)	
None, or no data	2471 (60.9)	930/2471 (37.6)	

ART, assisted reproductive technology; FbM, filled by mass; FbIU, filled by bioassay; FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; r-hCG, recombinant human chorionic gonadotrophin; u-hCG, urinary hCG. All p-values were calculated using the chi-squared test.

\*For difference between follitropin alfa FbM and FbIU formulations.

†For difference between cetrorelix acetate 0.25 mg and GnRH agonist.

‡For difference between groups with or without LH supplementation.

§For difference between r-hCG and u-hCG.

cetrorelix acetate was used in 48.0% (1945/4055) of patients; 1118/1945 (57.5%) received cetrorelix acetate 0.25 mg and 827/1945 (42.5%) received 3 mg.

### Ovulation triggering

A total of 2578 (2578/4055, 63.6%) patients received treatment to trigger ovulation (Table 2). Overall, 1535/4055 (37.9%) patients received r-hCG (Ovitrelle, Merck Serono S.A. – Geneva), of which the majority (1291/1535) received a 250 mcg dose, whilst others (78/1535) received 6500 IU; 1043/4055 (25.7%) received urinary hCG (u-hCG) preparations; for 1477 (36.4%), no data were available. Body weight was significantly greater in the group who received r-hCG compared with those receiving u-hCG to trigger ovulation (66.5 vs 62.5 kg;  $p < 0.0001$ ).

### Luteal support

Vaginal progesterone 8% (Crinone, Merck Serono S. A. – Geneva) gel was used for luteal support in 251/4055 (6.2%) of patients; 1333/4055 (32.9%) received luteal support from intramuscular progesterone (Table 2). The mean (SD) number of vials used was 29.63 (23.50). Either no luteal support was given, or no data was available, for 2471/4055 (60.9%) patients.

### Fertilization

Fertilization was achieved using conventional IVF for 1704 (42.0%) and ICSI for 2319 (57.2%) patients. The fertilization technique used was unknown for 32 (0.8%) patients.

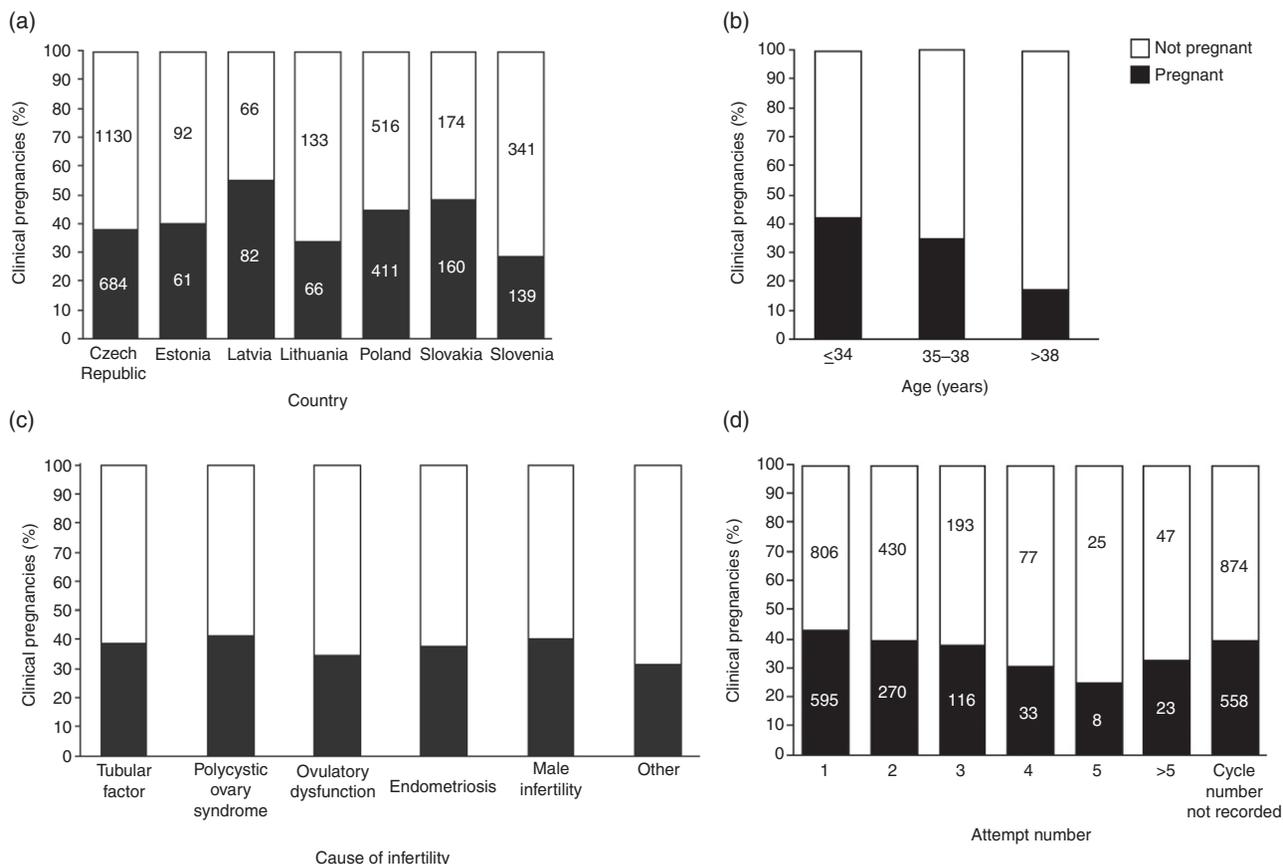
### Treatment outcomes

#### Oocyte retrieval and embryo transfer

The mean (SD) number of oocytes retrieved during cycles of IVF was 10.4 (6.9), and the mean (SD) number of embryos transferred was 1.9 (0.8). The mean (SD) number of oocytes retrieved during cycles of ICSI was 9.6 (6.4), and the mean (SD) number of embryos transferred was 2.0 (0.7).

#### Pregnancy outcomes

The clinical pregnancy outcome was available for 4055 patients. Overall, 39.5% (1603/4055) of patients became pregnant. Patients who became pregnant had a lower baseline weight than those who did not become pregnant (63.9 vs 65.1 kg) and, for those with data available, a slightly lower mean body mass index (22.7 [n = 493] vs 23.1 [n = 750], respectively;  $p = 0.015$ , Mann–Whitney U test).



**Figure 1.** Proportion of clinical pregnancies by (a) country, (b) age, (c) cause of infertility and (d) cycle attempt number in ART clinics in seven new European Union member states.

A higher pregnancy rate was observed with the use of follitropin alfa FbM (784/1897, 41.3%) than follitropin alfa FbIU (806/2133, 37.8%;  $p = 0.022$ ; Table 2). The mean follitropin alfa consumption per cycle was 1987.5 IU for patients undergoing pituitary suppression with a GnRH antagonist, and 2085.0 IU for those receiving GnRH agonist treatment. When the FbM formulation was used, mean FSH consumption was 1921.74 IU per cycle ( $n = 1896$ ; data unavailable for one patient), compared with 2078.75 IU per cycle for FbIU ( $n = 2133$ ).

In total, 40.6% (408/1005) of patients who received LH supplementation became pregnant, compared with 41.6% (562/1351) of patients who did not receive any LH supplementation ( $p = 0.6251$ ).

Pregnancy outcomes were further analysed according to baseline demographic factors: country of origin, age, cause of infertility and treatment cycle number. The proportion of patients achieving a clinical pregnancy ranged from 29.0% (139/480) in Slovenia to 55.4% (82/148) in Latvia (Figure 1a). The proportion of patients aged  $\leq 38$  years who achieved a clinical pregnancy was 41.2% (1529/3707), whereas only 18.2% (56/307) of those aged  $> 38$  years became pregnant (Figure 1b). In total, 41.8% (132/316) of women with infertility caused

by polycystic ovary syndrome and 37.8% (213/563) of those with endometriosis achieved a clinical pregnancy (Figure 1c). Of the pregnancies for which the cumulative treatment cycle number was available (Figure 1d), 93.9% (981/1045) occurred within three treatment cycles.

### Safety

OHSS was experienced by 270/4055 (6.7%) patients in the study. There were 234/4055 cases of grade I–II OHSS (5.8%) and 36/4055 cases of grade III–IV OHSS (0.9%). Of the 270 patients who developed OHSS, 146 (54.1%) achieved a clinical pregnancy in subsequent treatment cycles. Of the 1603 clinical pregnancies, 17 (1.1%) were extra-uterine, 201 (12.5%) resulted in spontaneous miscarriage, 17 (1.1%) were electively aborted and 256 (16.0%) were multiple pregnancies.

### Discussion

This study provides the most comprehensive collection of data on IVF and ICSI cycles in clinical practice in Central and Eastern Europe to date. Indeed, it is estimated that

data in this observational study represent approximately 10% of all IVF and ICSI cycles performed in the seven countries during the study period. As such, the data represent a snapshot of routine, day-to-day, clinical practice at the time of the study. Thus, the ART cycles included in the study are considered representative of those performed nationally. The use of follitropin alfa in this large population led to a mean cumulative clinical pregnancy rate of 39.5% in cycles of IVF and ICSI.

During the study, the use of follitropin alfa FbIU was discontinued when the FbM formulation was launched in these countries. In this study, the pregnancy rate was significantly higher in patients who received follitropin alfa FbM for follicular stimulation than in those who received follitropin alfa FbIU (although this observational study was not specifically designed to address this issue). This confirms the results from previous studies demonstrating that more consistent therapeutic responses are seen with the FbM formulation than with the FbIU formulation<sup>3,5</sup>.

Similar pregnancy rates were achieved with the use of GnRH agonists and the GnRH antagonist cetrorelix acetate at 0.25 mg for pituitary suppression. There was a trend towards use of a lower total dose follitropin alfa per cycle when used in combination with GnRH antagonists than agonists. Similar pregnancy rates were observed in patients who did or did not receive LH supplementation in this study. A good pregnancy rate (37.5%) was observed with the use of r-hCG for triggering of ovulation. However, unlike previous interventional studies<sup>14,15</sup>, the pregnancy rate was slightly lower among patients who received r-hCG than u-hCG preparations. Patients who were treated with r-hCG had a significantly higher mean body weight than those who received other therapies for ovulation triggering, which may have affected the observed pregnancy rate. Interestingly, there was a trend for higher pregnancy rates with the use of vaginal progesterone gel for luteal support compared with other vaginal or intramuscular preparations.

No major safety concerns with the use of follitropin alfa were identified in this observational study. The rate of spontaneous abortion was 12.5%, which is in line with the accepted rate of 18% for spontaneous abortion<sup>16</sup>. The favourable safety profile may reflect the previously proven high purity and consistency of the follitropin alfa preparation obtained from the recombinant production process.

The efficacy and safety of follitropin alfa FbM described here are consistent with the findings of a previous observational study reported by Lass *et al.*<sup>17</sup>. This study of 1427 patients who received follitropin alfa FbM achieved a clinical pregnancy rate of 29.2% per cycle with a low rate of serious adverse events.

Our data demonstrate a large difference in cumulative pregnancy rates between countries. An almost two-fold greater pregnancy rate was observed in Latvia than in

Slovenia. However, comparisons of pregnancy outcomes between different countries are extremely complex; data are often incomplete, generated through different methods of data collection and use partly different definitions. Furthermore, factors such as population characteristics, ethical and reimbursement issues, and national legislation will contribute to the overall pregnancy rate from ART<sup>13</sup>. Population characteristics are a major determinant of pregnancy rates in clinical studies<sup>8,18,19</sup>. The highest live birth rates have been reported in women aged 25–30 years with a sharp decline in older women<sup>18</sup>. Indeed, women who received infertility therapy in Slovenia in this study were older than those from other countries.

The availability of ART services differs widely between countries<sup>8</sup>. There is considerable diversity in regulatory and funding schemes for infertility, which reflects their different historical, cultural, social, political, economic and religious values<sup>8</sup>. Furthermore, national reimbursement policies determine the number of funded cycles that women will receive. As our study data are not expressed per cycle, there could be large differences in the number of cycles each woman received depending on her country of origin. Interestingly, the utilization of fertility services in Slovenia (in terms of the number of cycles per population) is one of the highest on ESHRE patient registries<sup>20</sup>.

National policies regarding the number of embryos that can be transferred during a cycle of ART vary across Europe. Although studies suggest that this is likely to account for only a small part of the difference in treatment outcome, such policies will contribute to the overall pregnancy rates<sup>21,22</sup>.

There is a need for improved data collection, monitoring and reporting of ART services and patient outcomes within the EU, particularly with regard to the new member states. To achieve an objective assessment of outcome, and to optimize treatment protocols, large patient groups are needed. Many countries are now establishing national reporting systems, on which regional and world reporting rely. A trend towards greater efficacy of fertility treatments over time has become clear<sup>11</sup>. Other national and regional audits of fertility outcomes have been conducted or are in progress, such as the EIM programme<sup>20,23</sup>. However, this large audit does not include all of the countries that were described in our study.

We must also acknowledge the limitations of our own study. The observational and non-comparative study design, and the descriptive nature of statistical analyses limits the conclusions that can be drawn, and the study was not designed to make direct comparisons between the success rates of different ovarian-stimulation protocols. A variety of treatment protocols were included in our analysis because of the nature of this non-interventional study. Furthermore, we relied on accurate data from patient charts recorded by clinicians. Such source data is often

limited, and in this study, led to incomplete data for some important variables.

Nonetheless, our study provides a valuable overview of COS protocols used in new EU member states and a useful source of information on clinical pregnancy rates in routine practice in Europe following ovarian stimulation with follitropin alfa. These observational surveillance data confirm the findings of earlier randomized clinical trials<sup>24</sup>.

## Conclusion

This observational study conducted in clinical practice in seven Central and Eastern European countries confirms the known efficacy and safety profile of follitropin alfa. These valuable data will also enable clinics in the new EU member states to optimize their treatment protocols.

## Transparency

### Declaration of funding

Regional Merck Serono offices sponsored this study, and assisted with data analysis and provided feedback on the manuscript. Merck Serono S.A. – Geneva, Switzerland (an affiliate of Merck KGaA, Darmstadt, Germany), funded the editorial support provided to the authors and the publication of this article.

### Declaration of financial/other relationships

All the authors have disclosed that they have no relevant financial relationships.

Peer reviewers may receive honoraria from CMRO for their review work. Peer Reviewer 1 has disclosed he is a former employee and share holder in AstraZeneca Ltd. Peer Reviewer 2 has disclosed that he has no relevant financial relationships.

### Acknowledgements

The authors thank Zbigniew Braszkiewicz PhD, Evaldas Brazaitis MD, Tonko Mardesic MD and Kurt Marhardt MD for their support and coordination of the study. The authors would also like to thank Joanna Brown DPhil of Caudex Medical (financially supported by Merck Serono S.A. – Geneva, Switzerland, an affiliate of Merck KGaA, Darmstadt, Germany) for her assistance with the writing of this manuscript.

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—Review—

## How to Repair the Oocyte and Zygote?

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**Abstract.** The supply of human oocytes is very limited. This restricts not only certain assisted reproduction procedures in IVF clinics where recipients wait for oocytes from donors, but also development of some promising approaches, like therapeutic nuclear transfer with subsequent derivation of patient compatible embryonic stem cells. Moreover, in some patients, collected oocytes exhibit certain specific defects, and logically, we can expect that after fertilization, the embryos arising from these defective oocytes may not develop or that their development might eventually be compromised. For this reason, an increased effort to determine how to repair oocytes is evident in the literature. In general, abnormalities (defects) can be detected in different oocyte components, the zona pellucida, cytoplasm, nucleus (chromosomes) and nucleolus. Whereas defects of a nuclear component are impossible (nuclear DNA) or very hard to repair (nucleolus), zona pellucida abnormalities and cytoplasm defects (for example, if containing mutated mitochondrial DNA, mtDNA) can be repaired in some cases with the help of micromanipulation schemes. In the present article, we will briefly outline the current methodological approaches that can be used to repair the oocyte or one-cell stage embryo.

**Key words:** Cytoplasm, Embryo, Micromanipulations, Nucleolus, Nucleus, Oocyte, Zona pellucida

(J. Reprod. Dev. 55: 583–587, 2009)

### Biological Material—Oocytes and One-Cell Stage Embryos (Zygotes)

Oocytes and one-cell stage embryos are most commonly used in model animal experiments and are also considered to be the most convenient biological material for relevant therapies in humans. The reason for this is their large size (in mammals, typically about 100  $\mu\text{m}$ ) and the relative ease of access to this material. The important aspect is that if the oocyte or one-cell stage embryo is repaired, the resulting cell will be, in theory, normal. This will not be the case, if for example, two-cell stage embryos are manipulated. In this case, both blastomeres must be manipulated to obtain a normal embryo. Logically, this is more complicated from a technical point of view, and thus this approach is not used.

The oocytes can be collected either as immature with a prominent nucleus containing one or more nucleoli (germinal vesicle, GV), maturing (with condensed chromosomes, metaphase I, anaphase I or telophase I) or mature (metaphase II). Oocytes are enclosed with a glycoprotein coat known as the zona pellucida. Here, we speculate that only so-called fully-grown oocytes can be used for manipulations because only these cells are able to undergo germinal vesicle breakdown (GVBD) and reach metaphase II (MII) stage. Less advanced oocytes (growing) do not normally undergo GVBD in culture, and if they do, they only reach the metaphase I (MI) stage [1]. Because, mature (metaphase II) oocytes are almost exclusively collected from ovaries in human assisted reproduction, one may expect that this stage would be most commonly used for manipulations. Moreover, use of these oocytes is also advanta-

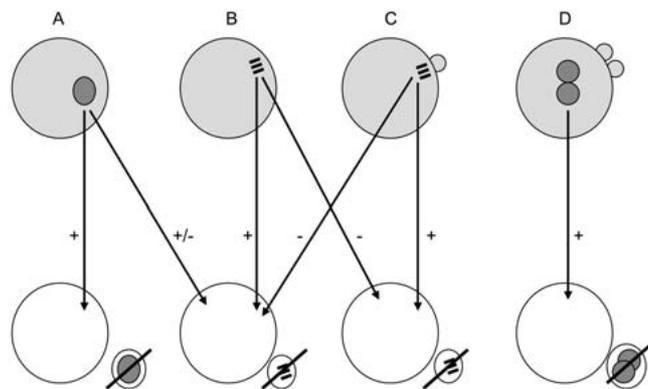
geous because they are fully developmentally competent due to maturation in their natural environment, follicles. It is well documented that developmental competence decreases in oocytes that are collected as immature (GV staged) and thereafter cultured *in vitro* [2]. The crucial period to attain full developmental competence seems to be the period of GVBD. If this process occurs in follicles and oocytes are then aspirated from them and matured *in vitro* up to MII, their developmental potential seems to be equivalent to that of oocytes completely matured *in vivo* [3]. However, the choice of the oocyte maturation stages for manipulation depends on the type of problem that must be solved [4].

Eventually, fertilized oocytes (zygotes) [5] with sufficiently visible pronuclei (PN, male/paternal and female/maternal) can be used for manipulation (Fig. 1).

### Technical Equipment for Oocyte and Zygote Therapy

Compared with somatic cells, oocytes and one-cell stage embryos are relatively large, but they still cannot be simply manipulated by hand because they can be easily destroyed. This is true especially for immature oocytes (GV stage) that are extremely fragile. Thus, the oocytes and zygotes must be treated very cautiously to prevent not only their destruction but also to eliminate some negative effects (temperature fluctuation) that may, for example, increase the frequency of aneuploidies.

Among the essential equipment is the stereomicroscope with 20 to 60 $\times$  magnification (Olympus, Leica, Nikon) under which oocytes and zygotes can be collected from flushing media and transferred into culture media where they can be cultured or different solutions in which they can be manipulated.



**Fig. 1.** Possible combinations of nuclear material transfer from abnormal to normal cytoplasm in mammalian oocytes and zygotes. Light grey, abnormal cytoplasm (for example, with those containing mutated mtDNA); white, normal cytoplasm. +, very good results. +/-, inconsistent results. -, negative results. A: transfer of GVs (germinal vesicles) from abnormal into normal cytoplasm. B: transfer of MI (metaphase I) groups. C: transfer of MII (metaphase II) groups. D: transfer of pronuclei.

For manipulation, an inverted microscope (Olympus, Nikon, Leica) with differential optics (Nomarski, Hoffman) equipped with micromanipulators and injectors is absolutely essential. The instruments, i.e., the holding and injection pipettes, can be either purchased from different companies or made by the person performing the manipulation. In this case, some additional equipment is required—pipette pullers, a microforge or a grinding wheel (Narishige).

Because of their relatively large size, transfer of nuclear material (GV, PNs) is typically done by electrofusion (Eppendorf, BTX). Thus, this material is not injected directly into the oocyte cytoplasm. On the other hand, the isolated metaphase chromosome groups can be directly injected into the donor oocyte cytoplasm.

The culture (M199, MEM, M16, KSOM, etc.) and manipulation media (M2, HTF, etc.) can be purchased from different companies (Millipore, Sigma, etc.).

It is strongly recommended studying manuals [6, 7] describing manipulation of mammalian eggs and embryos, setting up the essential manipulation equipment and preparation of holding and injection pipettes. Moreover, some training and experience with laboratory (mouse) or domestic animals oocytes (pig, cattle) and embryos is very advantageous.

### Approaches to Repair the Oocytes and Zygotes

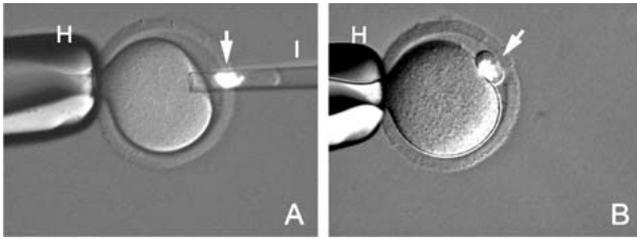
#### *Transfer of germinal vesicles (GV) and storage of GVs*

In human assisted reproduction, mature oocytes, i.e., metaphase II stage oocytes, are almost exclusively collected. However, the possibility of oocytes of other stages of maturation being used for some specific manipulations cannot be excluded. Before the oocyte starts to mature, it contains a prominent nucleus, known as the GV. GVs can be easily detected in rodent or human oocytes without cumulus cells. These cumulus cells are easily removed by pipetting with a glass pipette having an opening slightly smaller or

approximately of the same diameter as an oocyte enclosed in the zona pellucida. Before manipulation, incubation of immature oocytes in culture media (5% CO<sub>2</sub>, 37 C) for about 30 min is recommended. This incubation facilitates removal of cumulus cells and reduces the risk of damage during manipulation. The oocytes are then transferred into manipulation medium droplets under paraffin oil (10–20  $\mu$ l) supplemented with cytochalasin B (D) (5–7.5  $\mu$ g/ml) and incubated for about 10 min before being manipulated. The holding pipette stabilizes the oocyte in a fixed position. The most crucial step is penetration of the zona pellucida. Most typically, an opening can be made either by a laser beam, by applying several piezo pulses through the injection (enucleation-transfer) pipette, by cutting the zona with a sharp glass needle or eventually by penetrating it with a beveled injection pipette tip. The perivitelline space must be sufficiently large because only the zona pellucida must be exposed to a laser beam or piezo pulses. If the oocyte membrane lies very close to the zona pellucida, a laser beam or piezo pulses easily destroy the oocyte.

Once the opening in the zona pellucida is made, the injection pipette is introduced through it into the vicinity of the GV. The pipette opening must be sufficiently large (20–30  $\mu$ m) because a smaller diameter can release nucleoli (or some GV materials) from the GV. Even with an optimal pipette diameter, aspiration must be performed very slowly. Once the GV is inside the injection pipette, it can be easily separated from the oocyte cytoplasm by increasing the distance between both pipettes. Once the GV (or nuclear material in general) is separated from the cytoplasm, it becomes a so-called “karyoplast.” This means that the GV karyoplast also contains a certain volume of the oocyte cytoplasm (including mitochondrial DNA) enclosed with an intact oocyte membrane. The karyoplast is then transferred under the zona pellucida of another oocyte that has been previously enucleated (an enucleated oocyte is called a “cytoplast”) in the same way. The products of the manipulations are then briefly cultured in a convenient maturation medium (30 min), transferred into a fusion chamber with two wire electrodes, oriented in the chamber so that the membranes to be fused are parallel to the electrodes and then exposed to fusion pulses (1.5–2.0 kV per 50–100  $\mu$ sec/1–2 times). The space between the electrodes is usually filled with an isotonic solution (mannitol, glucose). The electropulsed cells are then washed several times in the culture medium; they are subsequently cultured in the same medium. Fusion between the karyoplast and cytoplast can be typically detected after about 30 min, with usually more than 75% of the cells fused. The reconstructed oocytes are then cultured *in vitro*, and they commonly reach the metaphase II stage at the same frequency as non-manipulated cells [8]. This approach works well in the mouse, and it has also been tested with human oocytes, where 73% of the manipulated cells fused and 62% of those cells reached the MII stage. After ICSI (intracytoplasmic sperm injection), about half of sperm-injected oocytes contain both pronuclei, and subsequent embryonic development is also good [9, 10].

It may be possible that, especially in human assisted reproduction, the recipient oocytes (cytoplasts) are not immediately accessible for transfer of GV-karyoplasts isolated from the patient’s oocytes. We have found that these karyoplasts can be efficiently stored by vitrification in liquid nitrogen, but before that



**Fig. 2.** Transfer of a chromosome group. Chromosomes are first removed from the oocyte cytoplasm (2A), the karyoplast is transferred under the zona pellucida of another oocyte that was enucleated previously (2B) and introduction of the chromosomes into the cytoplasm then induced by electrofusion. Eventually, the chromosomes can be directly injected into the cytoplasm. H, holding pipette; I, injection pipette; arrow, chromosome group. Combined light and fluorescence picture.

they must be transferred into evacuated zonae pellucidae so that they can be found after thawing. Compared with freezing of intact oocytes, the GV-karyoplasts have an excellent survival rate and can be fused to freshly prepared cytoplasts [11]. Eventually, the GV-karyoplasts can be cultured *in vitro* because GVBD does not occur in them, probably due to limited volume of cytoplasm they contain and, as a consequence, their inability to produce some crucial cell-cycle factors responsible for GVBD induction.

It may well happen that cytoplasts of more advanced stages are available for GV transfer. We and others have found that reconstructed oocytes can reach the MII stage only when GVs are transferred into cytoplasts obtained from oocytes before they enter the anaphase-telophase I transition. In this regard, it has been reported that reconstructed oocytes can complete maturation (MII) but with widely varying results [4, 12]. Clearly, some additional experiments are necessary [13]. The transfer of GV karyoplasts into more advanced oocyte cytoplasts (MII) does not support oocyte maturation. The GV chromosomes condense but are arrested in a metaphase I-like stage with no transition into metaphase II.

#### *Transfer of chromosomes*

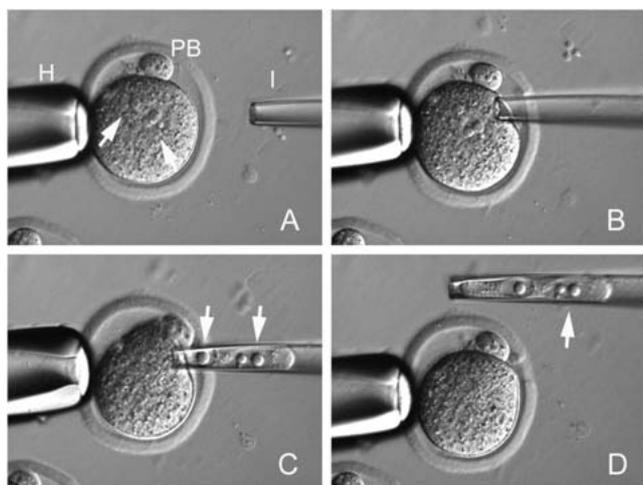
The most typical sign of maturation is germinal vesicle breakdown which is, when the nuclear membrane is dissolved. This is accompanied by chromosome condensation and disassembly of nucleoli. The period of germinal vesicle breakdown differs between species (30–60 min in the mouse, 15–18 h in the human). The chromosomes are then arranged gradually in the metaphase plate at MI, which is followed by the anaphase to telophase I transition. Maturation is then arrested at metaphase II, where the oocytes are subjected to *in vitro* fertilization or ICSI or used for nuclear transfer. The anaphase to telophase I transition is rather short, and oocytes at these stages are not frequently used for manipulations.

The manipulation steps are essentially the same as for immature oocytes (Fig. 2). The advantage here is that the oocytes are more refractory to damage and the injection pipette diameter can be smaller (10–15  $\mu\text{m}$ ). The disadvantage is the very poor visibility of the chromosome groups. Thus, it is necessary to stain the oocytes with some vital DNA stains (Hoechst) and to enucleate them under

short exposure to UV light [8]. It is not yet fully clear whether exposure to UV has any negative effects. It is also possible to use more sophisticated approaches like the PolScope optics and software to image birefringent structures (spindle) [14]. Under the PolScope optics, a spindle with chromosomes can be detected as a bright area in the oocyte cytoplasm. The isolated chromosome groups (karyoplasts) can be electrofused to donor cytoplasts or eventually directly injected into them with a Piezo injector [15]. Because in the latter case there is no nuclear membrane enclosing the chromosome group and the volume of the cytoplasm is thus reduced or the cytoplasm may be almost absent, this approach seems to be almost ideal if we want to reduce the introduction of mutated mitochondrial DNA into the donor cytoplasm. The disadvantage of this approach is that it requires considerable micromanipulation skill and some oocytes are still destroyed. If we are working with true metaphase I stage karyoplasts (the chromosome group is enclosed with the minimum volume of oocyte cytoplasm and membrane), these karyoplasts must be introduced into cytoplasts rather soon because they often undergo cleavage in culture. Concerning the possibility of asynchronous transfer, the results are inconsistent. Thus, MI chromosome groups are recommended to be transferred only into MI oocyte cytoplasts, and MII chromosome groups are recommended to be transferred only into MII oocyte cytoplasts. If MII chromosome groups are used for transfer by fusion, one must be cautious not to activate the cytoplasts parthenogenetically during manipulation. This possibility can be eliminated by manipulating the oocytes in  $\text{Ca}^{2+}$ -free medium. As far as we are aware, metaphase chromosome group (MI, MII) transfer has only been only tested in the mouse, with very promising results and live offspring obtained [16, 17].

#### *Transfer of pronuclei*

The exchange of pronuclei between zygotes was the first approach demonstrating the power and efficiency of manipulation procedures [5]. From the technical point of view, this method does not differ from GV or chromosome group transfer (Fig. 3). As pronuclei are relatively large, the diameter of the enucleation pipette is approximately the same as for GV stage oocytes. This also means that pronuclei cannot be directly injected into donor cytoplasts. Instead, electrofusion or some alternative approaches must be used (Sendai virus, polyethylene glycol induced fusion or mechanically induced fusion) [5, 18]. The important factor here is that zygotes are rather resistant to damage. However, when compared with the previously mentioned approaches, there is a certain ethical aspect that must be taken into consideration. In the case of human zygotes, the pronuclei must be transferred into cytoplasts that have had their pronuclei removed. This actually means destruction of a recipient zygote (embryo, new life), and this will certainly not be acceptable to some ethics or religious groups [19]. To avoid this complication, the recipient oocytes could be parthenogenetically activated, then enucleated and used as recipient cytoplasts. Alternatively, zygotes that have been abnormally fertilized (mono, tripronuclear) could also be used as recipient cytoplasts [20]. The question that remains is whether they will be available when the patient embryo needs to be manipulated.



**Fig. 3.** Microsurgical removal of pronuclei from the zygote. The zygote contains two distinct pronuclei (3A). The injection pipette first penetrates through the zona pellucida, and its tip is pushed close to both pronuclei (3B). Thereafter, both pronuclei are slowly aspirated into the injection pipette (3C). Both pronuclei are completely removed from the cytoplasm by increasing the distance between the pipettes (3D). H, holding pipette; I, injection pipette; arrows, pronuclei; PB, second polar body.

#### Cytoplasmic transfer

Injection of a certain volume of cytoplasm from a donor was originally designed to improve the developmental potential of embryos in women who experienced repetitive embryonic development failure. The volume of injected cytoplasm typically did not exceed 15% of the total oocyte cytoplasm volume. Thus, unlike the above-discussed experimental schemes, this approach cannot be used for elimination of mutated mitochondrial DNA, but it can supply the embryo with some essential organelles (including mitochondria) and molecules that are necessary for driving early embryonic development. As an increased frequency of certain abnormalities has been detected after birth in children, the use of this technique has been questioned, and it is not commonly used at present [20].

#### Destruction of mutated mitochondrial DNA

Because it is commonly accepted that the above-mentioned approaches will be used especially for elimination of mutated mitochondrial DNA, we will discuss here some aspects that may influence the output of a given procedure. First, in all the approaches described above, a certain volume of cytoplasm containing the donor mitochondria will be transferred along with the nuclear material. So far, it is unclear how this mitochondrial population will replicate in the reconstructed cells. The best solution would be to destroy this mitochondrial population and then to transfer the mitochondria-free nuclear material. Some encouraging results from SCNT (somatic cell nuclear transfer) demonstrate the feasibility of this approach. For example, in previous research, the somatic cell mitochondrial population was destroyed by incubating these cells in ethidium bromide. These cells were then fused to enucleated oocytes. The reconstructed embryos developed nor-

mally, and in the case of intergeneric SCNT (sheep cytoplasm/caprine SC), they even reached more advanced embryonic stages [21, 22], compared with controls. The same approach is difficult to use in humans due to time constraints and, in the case of ethidium bromide, safety reasons, but some recent publications indicate other alternative methods. Franciosi *et al.* [23] found that centrifugation of immature oocytes significantly reduces the number of mitochondria enclosing the nucleus (GV).

However, even in this case, it is necessary to know how the introduced mitochondria will be distributed in reconstructed cells [24, 25]. It has been demonstrated that the speed of mitochondria distribution is cell-cycle dependent [14]. Distribution in cells with nuclei is quite rapid, while in cells with condensed chromosomes is rather slow. This must be taken into account because it would not be desirable for the reconstructed cell to cleave before the introduced mitochondria are dispersed and thus produce two daughter cells, where one would be enriched with a population of mitochondria from the karyoplast while the other would contain only a mitochondrial population from the cytoplasm. This might eventually result in blastocysts with an inner cell mass (later embryo, offspring) containing the population of mutated mitochondria and a trophectoderm containing the normal mitochondrial population from the cytoplasm [25]. Moreover, some very recent results clearly show that resolution of the problem of replication of residual mitochondria will not be as simple as expected. Thus, there is a chance that these residual mitochondria (for example, those containing mutated mtDNA) will preferentially replicate and that the replication of normal mitochondrial population will be limited [26].

#### Zona pellucida manipulations

The following is a brief overview of some other approaches that can be used to repair certain defects in oocytes. Sometimes spermatozoa do not adhere to the zona pellucida, so they cannot penetrate it and thus fertilize the oocyte. In these cases, a spermatozoon can be injected directly into the oocyte cytoplasm (ICSI). This approach is very commonly used in human assisted reproduction. In some cases, the zona pellucida is too hard for unknown reasons and cannot be penetrated even with an ICSI pipette. In these cases, an opening can be made in the zona pellucida using a laser beam, and the sperm can then be injected into the oocyte cytoplasm. Logically, opening facilitates the hatching process of the developing embryo (blastocyst) from the zona pellucida [27].

#### Manipulating nucleoli

It has been demonstrated that the number and distribution of nucleoli in pronuclei can serve as an indicator of the developmental competence of human embryos [28]. The feasibility of manipulating mammalian oocyte nucleoli was first demonstrated by Fulka, Jr. *et al.* [29]. Subsequently, this study has been extended by Fulka *et al.* [30] and Ogushi *et al.* [31]. Taken together, mammalian oocyte nucleoli can be efficiently removed from fully grown immature oocytes. The oocyte nucleolar material is, however, absolutely essential for early embryo development. Oocytes with nucleolar deficiency can be rescued if nucleolar material from another oocyte is injected into them. Thus, the developmental potential of embryos with no nucleoli or a very low number of nucleoli would

be improved if some nucleolar material is injected into them.

### Conclusion

In this paper, it was not our intention to describe all aspects that are associated with the potential use of micromanipulation approaches that can be used for elimination of mutated mitochondria in mammalian oocytes and zygotes. These have already been discussed in detail in several articles and books [for example, 32]. From a technical point of view, the approaches outlined above have been almost perfected in animal models (especially in the mouse), but it remains to be determined whether some of the side effects that may be associated with their possible use in humans can be excluded. Several recent experiments clearly document how sensitive the early mammalian embryo is, and it is suspected that the manipulation methods will also influence the expression of some genes and consequently affect the children born via these methods [33]. Clearly, some additional experiments are necessary here. The use of these approaches is, however, very wide and promising. With them, we can, for example, eliminate or almost eliminate mutated mtDNA, prevent oocyte aneuploidies, improve the quality of oocytes with poor morphology or low number of mitochondria [34–36] and eventually solve some problems with causes that are not yet known [37, 38]. Clearly, one must take the risk and compare it with the potential benefits.

### Acknowledgements

JF Jr's lab is supported by GACR 523/09/1878.

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