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—Original Article—

Dissecting the role of the germinal vesicle nuclear envelope and soluble content in the process of somatic cell remodelling and reprogramming

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Abstract. Differentiated nuclei can be reprogrammed/remodelled to totipotency after their transfer to enucleated metaphase II (MII) oocytes. The process of reprogramming/remodelling is, however, only partially characterized. It has been shown that the oocyte nucleus (germinal vesicle – GV) components are essential for a successful remodelling of the transferred nucleus by providing the materials for pseudo-nucleus formation. However, the nucleus is a complex structure and exactly what nuclear components are required for a successful nucleus remodelling and reprogramming is unknown. Till date, the only nuclear sub-structure experimentally demonstrated to be essential is the oocyte nucleolus (nucleolus-like body, NLB). In this study, we investigated what other GV components might be necessary for the formation of normal-sized pseudo-pronuclei (PNs). Our results showed that the removal of the GV nuclear envelope with attached chromatin and chromatin-bound factors does not substantially influence the size of the remodelled nuclei in reconstructed cells and that their nuclear envelopes seem to have normal parameters. Rather than the insoluble nuclear lamina, the GV content, which is dissolved in the cytoplasm with the onset of oocyte maturation, influences the characteristics and size of transferred nuclei.

Key words: Nucleus transfer, Oocyte, Selective enucleation

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The most efficient approach to reprogram differentiated cell nuclei to totipotency is nuclear transfer (NT), where nuclei are introduced into enucleated metaphase II (MII) oocytes (cytoplasts), which are subsequently parthenogenetically activated. Overall, the reprogramming is still rather inefficient and even with various modifications, typically, less than 10% of reconstructed mouse embryos reach the term [1–3]. The process of reprogramming and remodelling of the transferred nuclei still remains poorly characterized [4, 5]. In agreement, it is as yet unclear why transferred nuclei can be fully reprogrammed only in MII and not in other types of oocyte cytoplasts, i.e. in germinal vesicles (GV) or metaphase I (MI).

To date, the vast majority of published studies have focused on epigenetic factors. It has been clearly demonstrated that when a somatic cell nucleus is introduced into the MII cytoplast, partial genome-wide demethylation of DNA can be detected and somatic cell histones, such as linker histone H1 or core histones H3.1 and H3.2, are replaced with the oocyte-derived histone variants such as H1oo or H3.3 [6–9]. The exact mechanism of how these processes aid the reprogramming is unknown. The only exception seems to be the level of H3K9me3, as it is commonly accepted that this is the main barrier to successful reprogramming [10]. Nevertheless, the role of other oocyte reprogramming factors is still rather elusive [11].

Besides the epigenetic reprogramming, it has been shown that the transferred nucleus must be efficiently remodelled. This step likely depends on the oocyte nuclear factors and organelles that must be incorporated into the newly formed pseudo-pronuclei (pseudo-PNs). In general, intra-nuclear organelles as well as other nuclear factors are dissolved in the oocyte cytoplasm concomitantly with the germinal vesicle breakdown (GVBD) and it may be thus assumed that they are re-incorporated into newly formed pseudo-PNs. However, what oocyte nuclear components are required for a successful nuclear remodelling and development remains poorly characterized. The only exception are oocyte nucleoli (generally known as nucleolus-like bodies, NLBs) that have been shown to be absolutely essential for a successful chromatin remodelling during normal development as well as after somatic cell nuclear transfer (SCNT) [12–14].

The correct intranuclear organization is critical for nuclear function. The distribution of the nuclear content is neither chaotic nor random. Chromosomes occupying individual territories and specific regions must be properly attached to the nuclear lamina as well as to some nuclear organelles. In embryos, the centric chromatin collapses, fails to be reprogrammed, and embryos cease to develop when NLBs are missing [12, 13]. At the same time, artificially tethering pericentric repeats to the nuclear envelope also result in a developmental arrest [15]. In the context of NT, it has been demonstrated that proper pseudo-PN morphology reflects the developmental potential of

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reconstructed embryos [16, 17].

With the exception of NLBs, the role of individual nuclear subcomponents, including the nuclear envelope, has not been investigated so far. The nuclear envelope has a vital role in nuclear organization as well as in proper nuclear function; not only does it shape the distribution of specific chromosomal regions, but also forms a selective barrier between the nucleus and the cytoplasm [18–20]. This potentially allows the selective import of putative reprogramming factors into the newly formed pseudo-PNs. In the present study, we questioned the extent to which the oocyte nuclear envelope and/or the nuclear soluble content participate in the processes of reprogramming and formation of pseudo-PNs in NT embryos. We showed that these are specifically the soluble GV components that play a major role in the pseudo-PN remodelling and expansion. Moreover, they also induce noticeable reprogramming in the somatic nucleus.

Materials and Methods

Source of oocytes

Immature oocytes were isolated from large antral follicles from PMSG stimulated (5 IU, Intervet, Boxmeer, NL) adult BDF1 mice. The oocytes were released into Human Tubal Fluid (HTF) - Hepes medium (Zenith Biotech, Guilford, CT, USA) supplemented with bovine serum albumin (BSA, 4 mg/ml), and only those oocytes enclosed with compact cumulus were further used. These oocytes were briefly cultured (5% CO_2/95% air, 37°C) in Minimal Essential Medium (MEM) supplemented with BSA (4 mg/ml), gentamicin (50 µg/ml), Na-pyruvate (0.22 mM), and dibutyryl cyclic-AMP (dbcAMP; 150 µg/ml). Then, their cumuli were almost completely removed by pipetting. Some cells were, however, left on the surface of zonae pellucidae and used later for nuclear transfer.

SCNT, selective enucleation (SE), and complete enucleation (CE)

The oocytes were incubated in HTF-Hepes supplemented with dbcAMP and cytochalasin D (7.5 µg/ml) for 15 min, following which, cumulus cell nuclei from their surface were injected into the cytoplasm, as described by Bui *et al.* [21]. The manipulations were performed under Olympus IX 71 inverted microscope with Narishige micromanipulators and PMM piezo microinjector (Prime Tech Ltd., Ibaraki-ken, Japan).

The injected oocytes were washed several times in MEM supplemented with dbcAMP and cultured in this media for another 2–3 h. Next, they were selectively enucleated (SE) as described by Modlinski [22] and Greda *et al*. [23]. Briefly, the GV nuclear envelope along with the associated chromatin was removed from the oocyte but the GV soluble content, including NLBs, was expelled into the oocyte cytoplasm. In some cases, the SE step was omitted and reconstructed oocytes containing both the GV and the somatic cell nucleus were generated (referred to as "SCNT-only"). Alternatively, the whole GV was removed and the somatic cell nucleus was injected into the cytoplast (complete enucleation-SCNT, "CE-SCNT"). The reconstructed oocytes were then cultured in dbcAMP/MEM for 18–20 h and further evaluated.

In some experiments, the reconstructed oocytes were incubated for 18–20 h with cycloheximide (CHXM; 25 µg/ml).

5-Ethynyl uridine (EU) incorporation

To assess the transcriptional activity, SCNT-SE, SCNT-only, and CE-SCNT reconstructed oocytes were generated as described above. After 18–20 h, the samples were incubated with 1mM 5-EU (ThermoFisher Scientific, Prague, CZ) in MEM media supplemented with dbcAMP for an additional time of 2 h (5% CO_2/95% air, 37°C). The EU incorporation was detected by the Click-iT™ RNA Alexa Fluor™ 488 Imaging Kit as recommended (ThermoFisher Scientific).

Immunofluorescence staining of remodelled nuclei

In order to evaluate the nuclear envelope in the remodelled nuclei, the reconstructed oocytes were fixed and labelled with polyclonal antibodies against the lamins A/C and B (both Santa Cruz, Heidelberg, Germany; 1: 200). Briefly, 20 h post manipulation, the manipulated oocytes were fixed in ice-cold methanol for 10 min and then washed several times in phosphate buffered saline (PBS). Next, the samples were permeabilized in Triton X-100 (TX-100, 0.2% in PBS) for 1 h at 22ºC and blocked in PBS/BSA solution (2% BSA/PBS). The samples were then incubated with the appropriate primary antibody diluted in PBS with BSA (1%) and TX-100 (0.1%) overnight at 4ºC, and then washed several times in PBS/BSA followed by incubation for 2 h with the appropriate secondary antibody (Jackson Immunoresearch, Cambridge, UK; 1:600) at room temperature. To better preserve the nuclear morphology, we also used well-characterised monoclonal antibodies that are compatible with paraformaldehyde (PFA) fixation: anti-lamin A/C (Cell Signaling Technology, BioTech, Prague, CZ; 1:200) and anti-lamin B (Abcam, Cambridge, UK; undiluted supernatant). The detailed procedure for immunostaining is provided below. The same results were obtained irrespective of the antibody and/or fixation used.

The remaining antibodies used were as follows: anti-nuclear pore complex (NPC; Covance; BioLegend, Prague, CZ; 1:200), anti-histones H3.3 and H3.1/H3.2 (CosmoBio, Baria, Prague, CZ; 1:1000). Briefly, approximately 20 h post SE, the manipulated oocytes were fixed in 4% PFA/0.2% TX-100 in PBS for 30 min at 4ºC and then washed several times in PBS containing BSA (1%). Following 2 h of incubation in blocking solution (2% BSA/PBS), the samples were incubated with the appropriate primary antibody diluted in PBS with BSA (1%) and TX-100 (0.1%) overnight at 4ºC, and then washed several times in PBS/BSA and incubated for 2 h with the appropriate secondary antibody (Jackson Immunoresearch, 1:600) at room temperature. After extensive washing, the oocytes were mounted in ProLong Gold Antifade Mountant (ThermoFisher Scientific) and examined under the fluorescence microscope Olympus BX61.

mRNA synthesis

H2b-mCherry and *Npm2-Egfp* mRNA were prepared from H2b-mCherry pBlueScript II and Npm2-Egfp pcDNA3.1 (a kind gift from Dr Kazuo Yamagata), respectively, using the mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, ThermoFisher Scientific) according to the manufacturer's recommendation, and injected (200 ng/µl) into the cytoplasm of GV oocytes using the FemtoJet (Eppendorf, Ricany, CZ). The injection was performed in HTF-Hepes media supplemented with dbcAMP, as above. Injected oocytes were further cultured in the presence or absence of CHXM as described. The samples were fixed in 4% PFA, mounted in SlowFade

Diamond mounting media (ThermoFisher Scientific) and examined under the Olympus BX61 fluorescence microscope. Alternatively, GV oocytes were injected with *Npm2-Egfp* mRNA as described above and allowed to synthesize the protein for 4 h to mark the position of NLBs. Next, a cumulus cell nucleus was injected. From this point, CHXM was added to all media (25 µg/ml). Finally, SE was performed and the SCNT-SE reconstructed oocytes were further cultured in media containing CHXM for 18–20 h. The samples were then fixed in 4% PFA and evaluated as above.

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich, Prague, Czech Republic. Each experiment has been repeated at least three times.

Results

First, we wanted to better understand the nature of the material that is removed during the SE procedure. Thus, we prepared and stained intact control and selectively enucleated oocytes for the nuclear envelope components. As shown in Fig. 1, the nuclear envelope as well as the DNA are removed during SE (Fig. 1a, top row). Concomitantly, the soluble nuclear content including NLBs is expelled from the GV into the cytoplasm as visualized by NPM2-EGFP fusion protein (Fig. 1a, bottom row). Next, we wanted to know the remodelling capacity of these cytoplasts when a somatic nucleus is transferred into them. To do this, we adopted an experimental scheme where somatic cell nuclei are first introduced into a GV oocyte by injection (SCNT) followed by SE to remove the insoluble i.e. chromatin and nuclear envelope-bound GV components. We reasoned that performing the SCNT first would allow an immediate incorporation of the released nuclear components eliminating a possible partial or full degradation of GV components in the cytoplasm.

In our initial experiments, from 923 oocytes injected with a single cumulus cell nucleus, 762 survived (83%). Next, the reconstructed oocytes were subjected to SE. From the total of 762 selectively enucleated oocytes, 103 underwent lysis during this procedure (103/762; 14%). After 20 h of culture, 566 of the manipulated cells contained a clearly visible large nucleus with prominent NLBs (566/659; 86%; Fig. 1b). The remaining SCNT-SE oocytes contained condensed chromosomes or small nuclei (93/659; 14%). As evident from Fig. 1b, the gross morphology and size of the newly formed nuclei did not substantially differ when compared to GVs (Fig. 1b, bottom row).

Because the GV nuclear envelope is removed during SE, we further analysed the composition of the nuclear envelope of the remodelled somatic nuclei. Twenty hours post SCNT-SE, we labelled the enlarged nuclei with specific antibodies against the nuclear lamina as well as the components of the nuclear pore complex (NPC). In total, we used 157 reconstructed oocytes for immunofluorescent labelling. In all the cases, the nuclear envelope of the enlarged nuclei exhibited characteristics that are typical of normal cells as well as of GV oocytes. The remodelled envelopes were positive for lamin A/C (25 methanol and 32 PFA fixation; 57/57; 100%; Fig. 2), lamin B (34 methanol and 28 PFA fixation; 62/62; 100%), and the nuclear pore complex (38/38; 100%) (Fig. 2a and 2b).

This indicates that apart from the components that are stably incorporated in the GV nuclear envelope, oocytes contain a substantial soluble fraction of at least some nuclear envelope proteins.

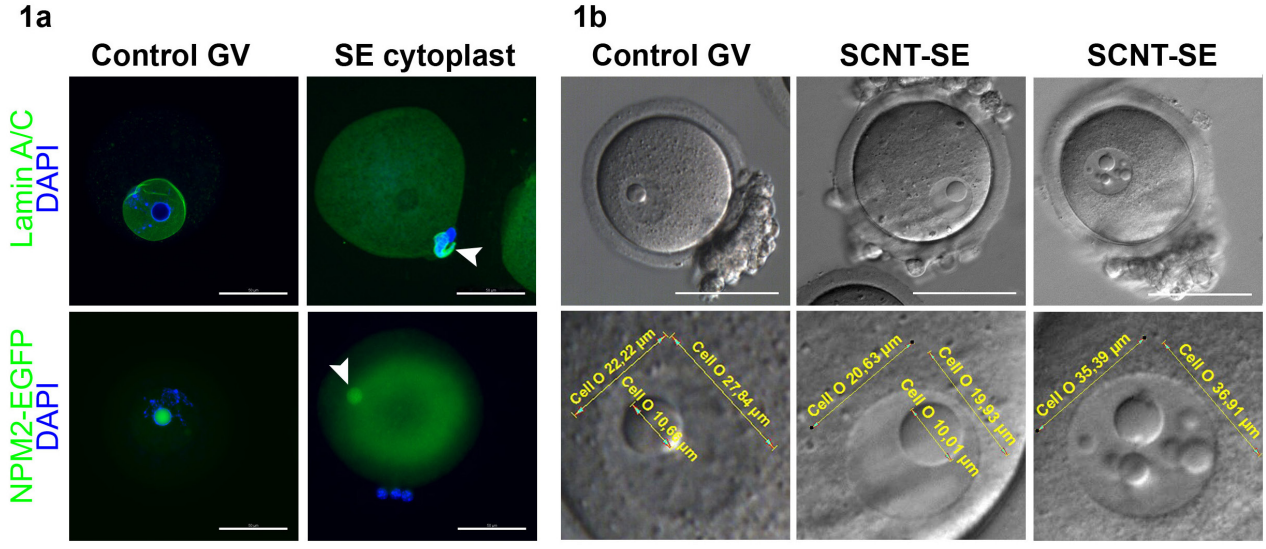

Fig. 1. Selective enucleation procedure and the effect of a selective enucleation (SE) cytoplast on the somatic nucleus remodelling. 1a, top row: During SE, the nuclear envelope, together with the germinal vesicle (GV) DNA is removed (visualized by anti-Lamin A/C antibody – green; DNA - blue). Here, the removed DNA and nuclear envelope were left under the zona pellucida to facilitate their labelling (SE cytoplast; arrowhead). 1a, bottom row. During the removal of the GV nuclear envelope, the soluble GV content together with nucleolus-like bodies (NLBs) is released into the cytoplasm. Here, the GV oocytes were allowed to express NPM2-EGFP (green) prior to SE. NPM2-EGFP localizes to NLBs in control oocytes. After SE, the NLB mass can be detected in the cytoplast where it partially disperses. 1b, top row: The top row shows the gross morphology of the somatic nuclei after 20 h of remodelling in the SE cytoplast. Note the resemblance of the remodelled nuclei to GVs. 1b, bottom row: The diameter of the remodelled somatic nucleus, as well as of NLBs, does not grossly differ when compared to GVs. Scale bar: 50 µm.

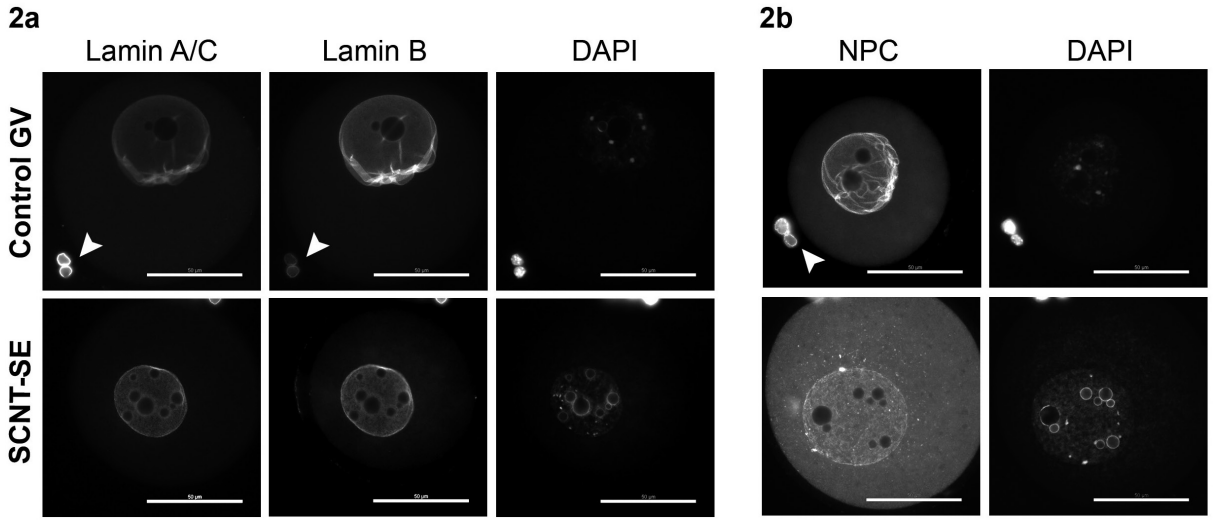

Fig. 2. Cumulus cell nuclei are efficiently remodelled in selective enucleation (SE) cytoplasts. 2a, top row: Intact non-manipulated germinal vesicle (GV) oocyte typically shows the presence of both types of lamins, i.e. lamin AC and B, in their nuclear envelopes. Cumulus cells used as donors are also positive for the above nuclear envelope components (arrowhead). 2a, bottom row: After somatic cell nuclear transfer (SCNT)-SE, the remodelled somatic nuclei are markedly enlarged and exhibit the presence of the nuclear envelope markers. 2b, top row: As expected, control GV oocytes as well as somatic cumulus cells (arrowhead) exhibit the presence of nuclear pore complexes (NPC) in their nuclei. 2b, bottom row: NPCs can also be detected in the SCNT-SE remodelled nuclei. Scale bar: 50 μm.

Alternatively, *de novo* synthesis of nuclear components might be necessary to allow the expansion of the transferred somatic nuclei. To discriminate between these possibilities and to investigate which of the analysed nuclear components are available to the somatic nucleus in the form of a protein, we used CHXM to block proteosynthesis in the reconstructed oocytes. To verify that the CHXM concentration used indeed efficiently inhibits proteosynthesis, control GV oocytes were injected with histone *H2b-mCherry* mRNA and cultured for 20 h in the presence or absence of this drug. As expected, control oocytes (71/75; 95%) readily synthesized the H2B-mCHERRY and this was incorporated into their chromatin. By contrast, the CHXM-treated oocytes (69/77; 90%) showed no fluorescence confirming the absence of proteosynthesis (Fig. 3a, top and middle rows). Next, we incorporated the CHXM treatment into the SCNT-SE manipulation scheme, starting from the time of somatic cell injection. Even in the absence of proteosynthesis, the somatic nuclei were remodelled and were able to uptake the GV components that were released into the cytoplasm upon SE, as indicated by the nuclear import of EGFP-tagged NLBs (16/16; 100%; Fig. 3a, bottom row). As evident from Fig. 3a, bottom row and Fig. 3b, the gross morphology of the remodelled nuclei was not affected by the CHXM treatment. When the presence of nuclear envelope components was analysed, the CHXM-treated SCNT-SE oocytes were positive for lamin B and the intensity did not differ dramatically between the treated and non-treated groups (52 treated *vs*. 34 non-treated oocytes; Fig. 3b, middle row). Likewise, the same result was obtained when the presence of NPCs was analysed (45 treated *vs*. 38 non-treated oocytes; Fig. 3b, bottom row). However, when the same experiment was performed for lamin A/C, an evident drop in the labelling intensity was observed in the CHXM-treated group (69 treated *vs*. 25 non-treated oocytes; Fig. 3b, top row). This indicates that while oocytes contain relatively large soluble lamin B and NPC pools together with the insoluble nuclear envelope-bound fraction, the vast majority of lamin A/C is incorporated in the GV nuclear lamina and is newly synthesized during the nuclear remodelling. At the same time, this also demonstrates that new components are introduced into the somatic nuclear envelope indicating active remodelling.

The import and incorporation of the NLB material into the transferred somatic nuclei even in the presence of CHXM shows that the nuclear import in the remodelled nuclei is functional (Fig. 3a, bottom row). Moreover, the above results also indicate that the soluble GV content, along with NLBs, is critical for the somatic nucleus expansion and remodelling.

To test this more directly, we injected somatic cell nuclei into GV oocytes, but these oocytes were not subsequently selectively enucleated (SCNT-only), or into cytoplasts from which the whole GV was removed prior to NT (complete enucleation; CE-SCNT). These reconstructed oocytes were further cultured for approximately 20 h in the presence of dbcAMP. In the SCNT-only group, the reconstructed oocytes contained two nuclei: the GV and the injected somatic cell nucleus. In these reconstructed oocytes, GVs remained unchanged and the somatic nuclei underwent only a moderate enlargement (Fig. 4a and b, top row). No structures equivalent to NLBs were detectable in the somatic nuclei. Essentially, the same results were obtained when cumulus cell nuclei were injected into oocytes from which the whole GVs were removed (Fig. 4a and b, bottom row). These findings are in agreement with previous studies [24, 25]. However, this is the first direct demonstration that the soluble GV content critically influences the expansion of the transferred nucleus.

Given the enlargement of the SCNT-SE nuclei and their overall

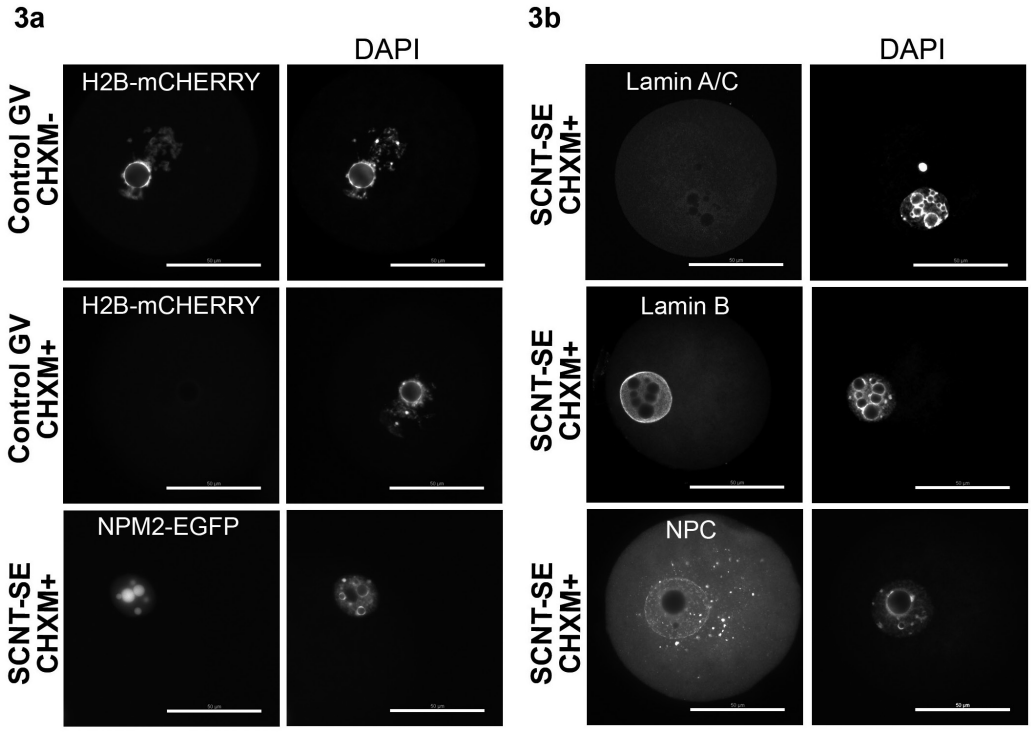

Fig. 3. The effect of proteosynthesis block on the nuclear remodelling. 3a, top row: In the absence of cycloheximide (CHXM), control germinal vesicle (GV) oocytes synthesize H2B-mCHERRY. This is incorporated into their chromatin as expected. 3a, middle row: When CHXM is added, the proteosynthesis is blocked, and no H2B-mCHERRY is produced. 3a, bottom row: Nevertheless, the nuclear import is active even in the presence of CHXM, and somatic nuclei takes up the GV material as shown by the incorporation of nucleolus-like bodies (NLBs) (visualized by NPM2-EGFP). 3b: When cycloheximide is added, the somatic nuclei are remodelled, but the nuclear envelope composition changes. While Lamin A/C becomes virtually undetectable, Lamin B and NPCs can still be found in the nuclear envelopes. This indicates that these components are present in excess in the GV oocytes and in the soluble form, i.e. not bound in the GV nuclear envelope. Scale bar: 50 μm.

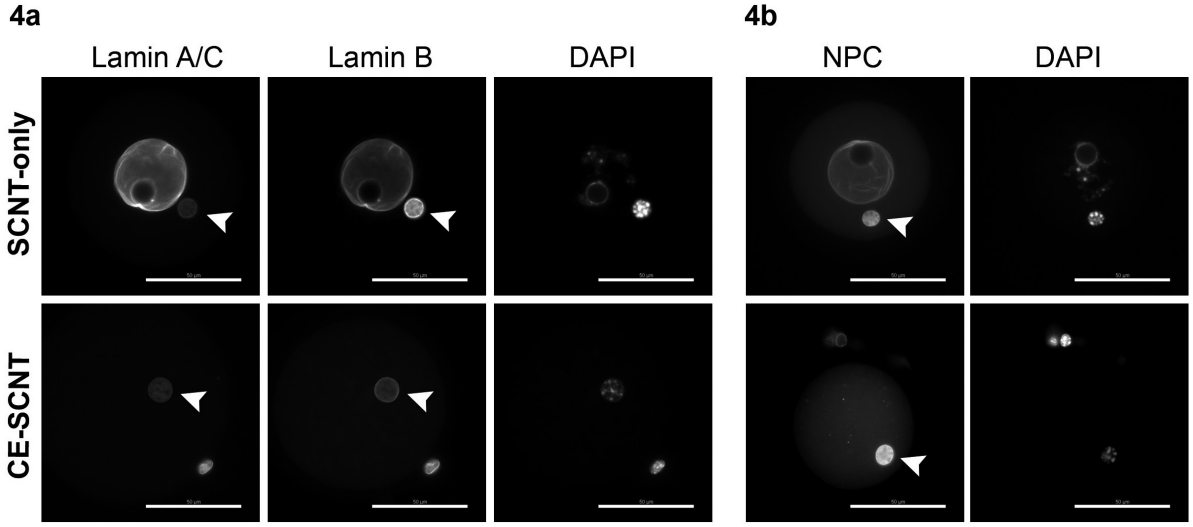

Fig. 4. The effect of different types of cytoplasts on the somatic cell nucleus remodelling. 4a and 4b, top row: When the somatic cell is fused with an intact germinal vesicle (GV) oocyte (somatic cell nuclear transfer (SCNT)-only), marked enlargement is not observed in the somatic nucleus (arrowhead) even after 20 h of culture. As expected, both the nuclei, i.e. the somatic nucleus and GV, show the presence of both lamin types (4a) and NPC can also be detected (4b). 4a, bottom row: Essentially the same result is obtained when the whole GV is removed prior to somatic cell nucleus transfer (CE-SCNT). Again, only a moderate enlargement of the somatic nucleus occurs after 20 h of culture (arrowhead). For reference, a few somatic cells were left on the surface. Scale bar: 50 μm.

resemblance to a normal GV, we wished to further characterize the effect of the soluble GV components on the transferred somatic nuclei. During SE, tightly-bound chromatin factors are likely removed together with the DNA. For this reason, the extent of potential functional somatic nucleus reprogramming is unknown. First, we focused on the transcriptional silencing. In mammals, full-grown GV oocytes are transcriptionally inactive and inefficient transcriptional silencing of the transferred nuclei might represent a barrier to reprogramming [26, 27]. To evaluate the transcriptional activity in the remodelled nuclei, we incubated the samples with 5- EU. While active transcription can be readily detected in the donor cumulus cells (Fig. 5a, top row), the SCNT-SE remodelled nuclei were negative (43/46; 94%) (Fig. 5a, bottom row). This indicates that the soluble GV fraction is able to elicit changes in the transcriptional status of the transferred nuclei. To investigate this further, we used SCNT-only and CE-SCNT reconstructed oocytes as controls (Fig. 5a, top and bottom row, respectively). In both these experimental groups, ongoing transcription was detected in the somatic nuclei even 20 h post transfer. Thus, only the SE cytoplasts were able to efficiently terminate transcription in the transferred somatic nuclei (SCNT-SE: 57/58; 98%, CE-SCNT: 0/37; 0%, SCNT-only: 0/54; 0%). This result, together with the minimal enlargement of the somatic nucleus in the presence of GV, also indicates that the exchange of nuclear components between GVs and somatic nuclei is rather limited.

Next, we studied whether the SE cytoplast can also alter epigenetic characteristics of the transferred somatic nuclei. When MII oocytes are used as a source of cytoplasts, oocyte-specific histone variants are incorporated into the donor cell chromatin. In agreement with the results of Akiyama and colleagues [28], GV oocytes exhibited the presence of the histone variant H3.3 in their chromatin and were negative for H3.1/H3.2 (Fig. 6a, top row). By contrast, cumulus cells showed high levels of histones H3.1/H3.2, but were deprived of the histone variant H3.3 (Fig. 6b, top row). Labelling of the SCNT-SE remodelled nuclei with antibodies specific for H3.3 showed that this histone variant is efficiently imported into the transferred nuclei (41/46 scored positive; 89%; Fig. 6a, bottom row). However, H3.1/H3.2 histones were often not completely removed from the somatic cell chromatin (38/52 scored positive; 73%; Fig. 6b, bottom row). This was especially evident at chromatin blocks that failed to disperse (Fig. 6b, bottom row, arrowheads). Next, we examined whether H3.3 can be detected in SCNT-only or CE-SCNT reconstructed oocytes (Fig. 6c, top and bottom rows, respectively). In total, we scored 39 SCNT-only and 27 CE-SCNT reconstructed oocytes. In both the cases, the level of H3.3 in the transferred somatic nuclei was virtually undetectable even after 20 h of culture. Not surprisingly, high levels of the somatic histones H3.1/H3.2 were present in the somatic nuclei (Fig. 6d, top and bottom, rows, respectively). In summary, these results show that the GV soluble content can induce H3.3 incorporation in the transferred somatic nucleus; the GV DNA and tightly associated factors removed during SE are rather dispensable for this process. However, while there was some level of histone exchange in the remodelled somatic nucleus, the epigenetic remodelling was not complete.

Collectively, these results demonstrate that while the soluble GV fraction is necessary for the structural remodelling, epigenetic reprogramming, and transcriptional silencing of the transferred nuclei, it is likely insufficient for a full reprogramming as the transferred nucleus does not completely recapitulate the GV oocyte chromatin state.

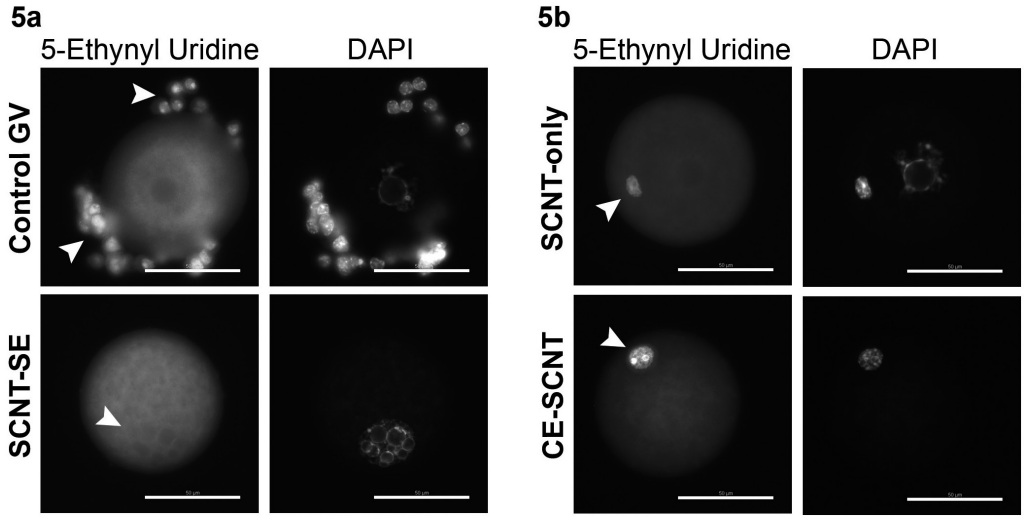

Fig. 5. The transcriptional activity in intact, somatic cell nuclear transfer (SCNT)-selective enucleation (SE), SCNT-only and complete enucleation (CE)-SCNT reconstructed oocytes. 5a, top row: While the full-grown germinal vesicle (GV) oocytes are transcriptionally inactive, the donor cumulus cells (arrowheads) are transcriptionally active as shown by the incorporation of modified uridine (5-Ethynyl Uridine). 5a, bottom row: When SCNT-SE remodelled nuclei are subjected to the incorporation assay, no transcription is detected. This indicates that the released GV components are able to induce transcriptional silencing in the transferred nuclei. 5b: Ongoing transcription in the somatic nuclei can be detected when the soluble GV content is not available in the transferred nuclei. Scale bar: 50 μm.

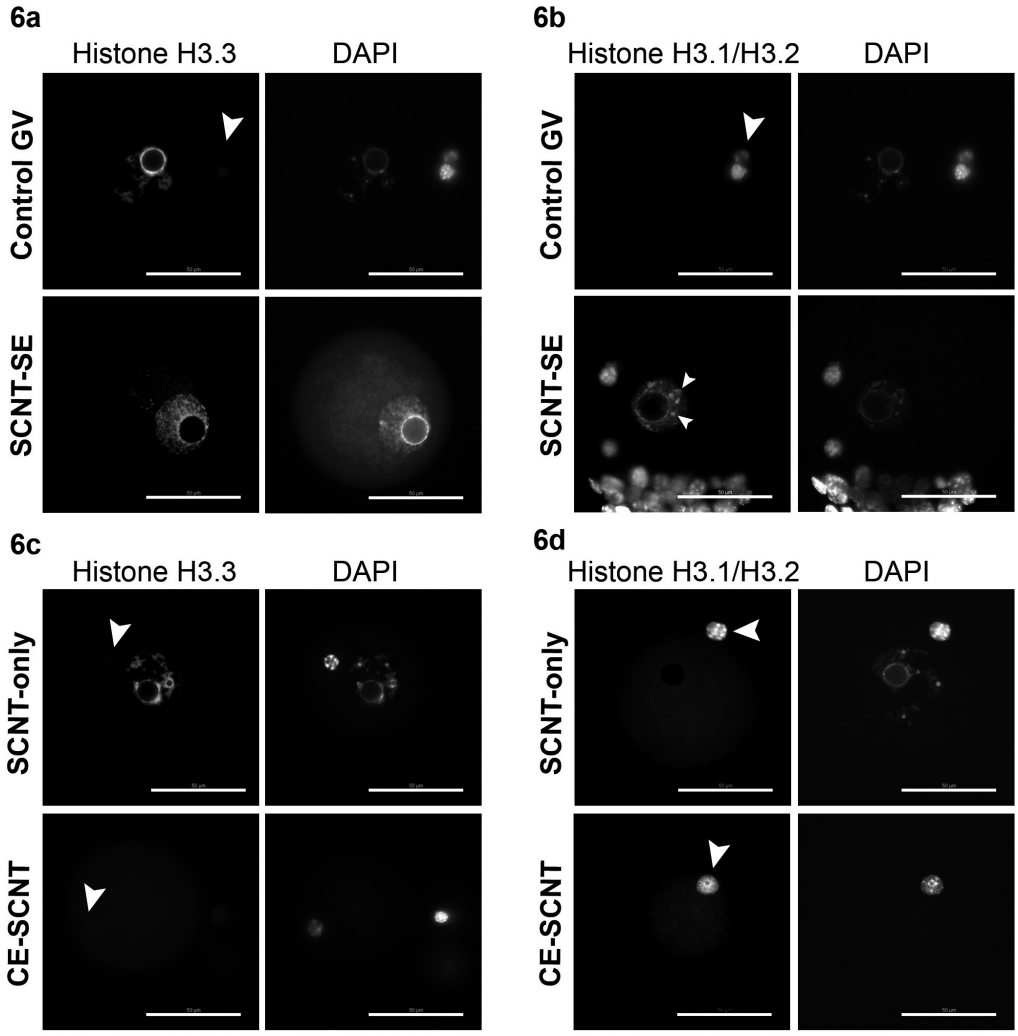

Fig. 6. The soluble nuclear fraction is able to induce favourable reprogramming/remodelling parameters in the transferred nuclei. 6a, top row: The histone variant H3.3 is abundant in oocytes. By contrast, the cumulus cells show highly reduced levels of this variant (arrowhead). 6a, bottom row: When somatic cell nuclear transfer (SCNT)-selective enucleation (SE) reconstructed oocytes are analysed, the histone variant H3.3 can be readily detected in the remodelled nuclei. 6b, top row: In contrast to H3.3, the histones H3.1 and H3.2 are virtually undetectable in germinal vesicles (GVs), but they are abundant in somatic cells (arrowhead). 6b, bottom row: Although the histone variant H3.3 is incorporated into the SCNT-SE nuclei, at the same time, the core histones H3.1 and H3.2 remain associated with the somatic chromatin even after a prolonged incubation. This is especially evident at those chromatin regions that fail to be efficiently reprogrammed (arrowheads). 6c: The histone variant H3.3 is not efficiently incorporated into somatic nuclei (arrowhead) transferred to either intact (SCNT-only) or completely enucleated (complete enucleation (CE)-SCNT) GV oocytes. 6d: As expected, high levels of histones H3.1/H3.2 can be detected in somatic nuclei (arrowheads) after their transfer into intact (SCNT-only) or completely enucleated oocytes (CE-SCNT). Scale bar: 50 μm.

Discussion

For somatic cell nucleus transfer, the metaphase II cytoplasts are most commonly used. After the injection of somatic nuclei their nuclear envelope is dissolved and chromosomes condense. Following activation, the chromosomes gradually decondense, a new distinct nuclear envelope is formed, and nucleoli (NLBs) become visible in the newly formed nuclei (pseudo-PNs) [29]. It has been previously demonstrated that NLBs originate from the oocyte nucleolar material that is dispersed in the cytoplast with the onset of GVBD, and newly reassembled when pseudo-PNs are formed [12]. The NLBs are absolutely essential for further SCNT embryo development. Without them, the reconstructed embryos cleave just once or twice. What other oocyte (GV) components are used in the process of pseudo-PNs formation and the extent to which the nuclear components are recycled is unknown.

The GV nuclear envelope also disassembles with the onset of GVBD and its components can thus be expected to also be essential for the pseudo-PN formation. When enucleated GV stage cytoplasts are fertilized, the sperm head enlarges but never fully decondenses

to the size that would be comparable to normal pronuclei [30, 31]. Similarly, when enucleated immature oocytes are used as cytoplasts for SCNT, the introduced nuclei get slightly enlarged but do not reach the size that would be comparable with the size of normal SCNT pseudo-PNs. Moreover, these pseudo-PNs do not contain NPBs [24]. In these experiments, the whole GV was removed and thus, the effect of individual nuclear sub-components or sub-structures could not be analysed. Here, we show that somatic nuclei can markedly enlarge and expand in the absence of the original GV nuclear envelope. The critical nuclear sub-fraction is the soluble GV content that is released into the cytoplasm upon SE. Therefore, in contrast to our expectations, our results show that the GV nuclear envelope is rather dispensable for the appropriate pseudo-PN size and formation.

The expansion of the somatic nuclei can theoretically occur in two ways: either the nucleus incorporates the released GV components within the original somatic nucleus envelope or the somatic nuclear envelope is modified by the SE cytoplast. In the first case, it would be expected that there would have been a decrease in the signal intensity of the nuclear envelope components. However, this was not the case. Also, the CHXM experiments do not support this possibility. The difference in incorporation of Lamin A/C when proteosynthesis is inhibited clearly shows that the nuclear envelope becomes altered in the SE cytoplast and that new components are incorporated.

Given the results and the overall resemblance of the somatic nuclei to GVs, we assessed additional functional parameters of the remodelled nuclei. It is known that full-grown GV oocytes are transcriptionally inactive. The transcriptional silencing is achieved by two mechanisms: 1) the release of RNA polymerase II from the GV chromatin and 2) its degradation [25, 26]. The previous experiments showed that the cytoplasm of GV oocytes is not able to efficiently terminate ongoing RNA polymerase II-dependent transcription in the transferred somatic nuclei [25]. However, intact GV oocytes were used as recipients. The reconstructed oocytes thus contained two nuclei: the GV and the somatic nucleus. Our present data indicate that the exchange of factors between the GV and the introduced somatic nucleus is rather limited and we, therefore, wanted to know whether the soluble GV fraction could induce transcriptional silencing in the somatic nucleus. Indeed, transcriptional silencing was only detected in SE cytoplasts. However, the exact factor(s) and the mechanism(s) remains to be identified.

We also examined the ability of the GV soluble content to induce epigenetic reprogramming in the somatic nuclei. It has been convincingly documented, that GVs contain some reprogramming factors and that the somatic nuclei are more or less reprogrammed under their influence [21, 32]. These factors become available to the transferred somatic nuclei upon GVBD. However, whether exactly the same set of reprogramming factors is found in GV oocytes, MII oocytes, and MII cytoplasts is unknown; although it could be assumed that the factors are not bound to MII chromosomes since these are removed during enucleation. These factors still remain to be elucidated and are the subject of intensive research. In any case, it has been demonstrated that specific histone variants might aid or block the reprogramming process. While H3.3 histone variant has been shown to promote the reprogramming process, histones H3.1 and H3.2 block it by "safe-guarding" the cell identity [33–36]. There are additional differences; it is well known that while H3.1/H3.2 incorporates are the major replication-associated histone variants, H3.3 can be incorporated by specialized histone chaperones in a replication-independent manner (for review, see [37, 38]). Here we show that the histone H3.3 is incorporated into the remodelled SCNT-SE nuclei. This observation is not surprising given that a constant turnover of histones in full-grown transcriptionally inactive GV oocytes was described [9]. However, the absence of H3.3 incorporation in SCNT-only and CE-SCNT reconstructed samples is rather puzzling because H3.3 was described to be incorporated in a transcription-dependent manner [39]. However, it is possible that since full-grown GVs are themselves transcriptionally inactive, the appropriate histone chaperone is missing. In any case, the SE cytoplast was the only one to induce efficient H3.3 incorporation into the somatic nucleus. It should be noted that the reconstructed oocytes were extracted during the fixation. Therefore, H3.3 might have been imported into the somatic nuclei in SCNT-only and CE-SCNT reconstructed oocytes. However, in the absence of incorporation into chromatin or formation of a stable complex, the proteins are not efficiently detected. However, the histone exchange in the SCNT-SE nuclei is not complete and the remodelled nuclei do not fully recapitulate the GV chromatin state, as indicated by the persisting histone H3.1/H3.2 in some chromatin regions. Whether the detected H3.3 incorporation is indeed beneficial for the reprogramming remains to be formally tested.

In the context of current knowledge of factors that aid the reprogramming, we showed that the SE cytoplast can induce several potentially favorable changes to the transferred somatic nuclei. However, it remains to be formally tested whether the elicited modifications are indeed beneficial.

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