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Short communication

ULTRASTRUCTURAL ANALYSIS OF BOVINE SOMATIC CELL NUCLEAR TRANSFER (SCNT) EMBRYOS DURING THE FIRST CELL CYCLE

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ABSTRACT

At somatic cell nuclear transfer (SCNT), the differentiated somatic cell genome is deprogrammed into a totipotent state of expression by unknown factors in the oocyte. The immediate events of this deprogramming are poorly understood and this study was designed in order to evaluate the global transcription and morphology of this event. Bovine SCNT embryos were produced from starved bovine fibroblasts and fixed at 0.5, 1, 2, 3, 4, 8, 12 and 16 h post-activation (hpa). The SCNT embryos were processed for autoradiography following ^{3}H–uridine incubation and transmission electron microscopy. Likewise, starved and non-starved fibroblasts were processed for autoradiography and TEM. Fibroblasts displayed strong transcriptional activity and active fibrillo-granular nucleoli. None of the reconstructed embryos, however, displayed transcriptional activity. SCNT embryos fixed at 0.5 hpa displayed condensed chromatin invested only in a partial nuclear envelope. Abundant population of somatic cell mitochondria was concentrated around the nuclei at 0.5 to 4 hpa. At 1-3 hpa, a chromatin decondensation and nuclear envelope formation were observed. In all SCNT embryos at 4, 8, 12, and 16 hpa, a complete nuclear envelope surrounding large pronucleus-like nucleus with abundant euchromatic and sparse heterochromatic areas was observed. The first nucleolus-related structures in SCNT embryos were observed at 1.5 hpa and only in the nuclei with a complete nuclear envelope. At 1.5-4 hpa, the nucleolus-related structures appeared either as bodies including large fibrillar centres and a granular component, but no a dense fibrillar component, or as electron-dense spheres, i.e. nucleolus precursor bodies (NPBs). Since 4 hpa, the somatic cell nucleus gained a PN-like appearance and displayed NPBs suggesting an ooplasmic control of development.

Keywords: SCNT; bovine embryo; first cell cycle; nucleus, nucleolus; ultrastructure

INTRODUCTION

There are two basic strategies for the cloning of mammals by SCNT that are able to produce embryos capable of development to term. Both these methods require the removal of the nuclear material from the oocyte and differ only in the way in which the nuclear material of the donor cell is introduced and the subsequent activation of the reconstructed embryos (Armstrong, 2006).

Application of MII-arrested non-activated oocytes is a far more effective for supporting development of the embryos reconstructed with differentiated nuclei than are activated oocytes. Therefore, a success in chromatin remodelling, from differentiated nuclei to the totipotent ones depends on the cell cycle stage of the recipient's cytoplasm. The ability to allow remodelling of chromatin apparently exists in MII-arrested non-activated oocytes and disappears after activation (Kim et al., 2002).

Of prime importance in these events is a cytoplazmatic activity termed maturation /meiosis/ mitosis-promoting factor (MPF) (Campbell et al., 1996). All nuclei that are transferred into the cytoplast with a high

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MPF activity undergo structural and functional remodelling and thus adopt the of behavioural pattern characteristic for the native nucleus. Structural reorganization of transferred nuclei (chromatin condensation/decondesation, nuclear envelope breakdown NEBD/ reformation, reorganization of nucleoli, nuclear swelling) seems to be a prerequisite of undertaking new functions in the heterologous cytoplasm (DNA synthesis, RNA synthesis) (Szollosi et al., 1988; Campbell et al., 1996).

In animal cloning, the highly differentiated donor nucleus must be properly dedifferentiated, cease its own program of gene expression, and express genes required for early embryo development. (Chen et al., 2006). Reprogramming involves a series of molecular events controlling gene expression, and has a profound effect on the nuclear architecture. Evidently the gene expression is silenced during the initial phases of reprogramming, which can be monitored by disassembly of the nucleolus (Misteli et al., 2003). Reprogramming of the donor nucleus is, among other features, indicated by a synthesis of ribosomal RNA (rRNA). The initiation of rRNA synthesis is simultaneously reflected in the nuclear morphology as a transformation of the nucleolus precursor body into a functional rRNA synthesizing nucleolus with a characteristic ultrastructure: fibrillar centres house the enzymatic apparatus for the transcriptional process, the dense fibrillar component carries the primary unprocessed transcript, and the granular component represent processed transcripts associated with proteins in the form of preribosomal particles (Wachtler and Stahl, 1993). This would suggest that the nucleolar morphology can be used as a marker for RNA synthesis and reprogramming of the donor nucleus in the nuclear transfer-derived embryos (Kanka et al., 1999).

However, no detailed investigations have been carried out on the ultrastructure and transcriptional activity during early genomic deprogramming i.e. first cell cycle in bovine embryos reconstructed by nuclear transfer from bovine fibroblasts to non-activated cytoplast. The objective of this study was to investigate morphological changes and RNA synthesis in the nuclei from cultured starved bovine fibroblast, when introduced into the enucleated metaphase II oocytes.

MATERIAL AND METHODS

Oocyte recovery and *in vitro* maturation

In vitro production of bovine embryos was performed using established protocols (Wrenzycki et al., 2002).

Somatic cell nuclear transfer (SCNT)

Adult female fibroblasts used for the nuclear transfer procedure were established from ear skin samples collected from an abattoir. The tissue was cut and dispersed in 0.1% trypsin solution. The cell suspension was maintained in DMEM-F12. At 90% confluence the cell monolayer was trypsinized and harvested cells were adjusted to the concentration of a 1 million cells/ml and then either frozen in 10% DMSO or returned to the culture. The fibroblasts were induced to enter a period of quiescence (presumptive G_0) by a serum starvation for 3 days (0.5% FCS). Immediately before transfer into an enucleated oocyte, a suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted, resuspended and maintained in TCM-air medium.

For the production of SCNT embryos, metaphase II oocytes were placed into a TCM-air medium containing 5 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B for 8 min. Oocytes were enucleated by aspirating the first polar body and the metaphase II plate. Single fibroblast was transferred into the perivitelline space of the recipient oocyte using a 30μm pipette. Oocyte-fibroblast cell couplets were electrically fused by 1-2 DC pulses of 0.7 kV for 30 μsec in a 0.285 M mannitol based medium containing 0.1mM $MgSO_4$ and 0.05% BSA with an Eppendorf Multiporator machine (Hamburg, Germany). Fused cell hybrids were chemically activated by 5 μM ionomycin (Sigma) for 5 min followed by a 3-4 h incubation in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma). After activation, the embryos were washed and cultured in 30 μl droplets of SOFaa medium supplemented with 0.4% BSA at 39°C in 5% O_2, 5% CO_2 and 90% N_2 in Modular incubator chambers (ICN Biomedicals, Inc., Aurora, No. 615300, Ohio, USA). The embryos were collected at 0.5 hours post activation (hpa); 1 hpa; 1.5 hpa; 2 hpa; 3 hpa; 4 hpa; 8 hpa; 12 hpa and 16 hpa.

^{3}H-Uridine Incubation for Autoradiography

Embryos harvested at the above defined time points were labeled by ^{3}H-uridine (sp. act. 962 GBq/mmol; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) at a final concentration of 4 MBq/mmol (Laurincik et al., 2000) for 20 min in a gas-equilibrated culture medium. After incubation with the radioactive precursor, the specimens were repeatedly washed in ^{3}H-uridine-free culture medium and fixed as described below.

Processing for Light Microscopic Autoradiography and Transmission Electron Microscopy

After labelling with the radioactive precursor, the embryos were fixed in 3% glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.2). Subsequently, the specimens were washed in buffer, post-fixed in 1% OsO_4 in 0.1 M Na-phosphate buffer, embedded in Epon, and serially sectioned into semi-thin sections (2 μm). Every

second section was stained with basic toluidine blue and evaluated by bright field light microscopy. Selected semi-thin sections were re-embedded according to Hyttel and Madsen (1987) and processed for ultra-thin sectioning (70 nm). The ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined on a Philips CM100 transmission electron microscope. Selected unstained semi-thin sections were processed for an autoradiography to detect a total RNA synthesis and nucleolus-associated RNA synthesis (Laurincik et al., 2000).

RESULTS AND DISCUSSION

The processes taking place in the reconstructed embryos immediately after the fusion of the recipient ooplast (cytoplast) and the donor nucleus (karyoplast) are still poorly understood. Information on the mechanism accompanying chromatin remodelling could help to increase the success of the production of cloned animals. A nature of the oocyte-derived factors responsible for reprogramming is largely unknown, although it is clear from activation studies that their existence is transitory. From the point of view of the normally fertilized oocyte, their limited persistence is undoubtedly sufficient for the task of rapidly demethylating the incoming paternal DNA, but the highly differentiated state of transplanted somatic donor karyoplast may be more problematic (Armstrong, 2006).

In the present study, a nuclear transfer (SCNT) was followed by a period, when chromosomes were densely packed and thus transcriptionally inactive (Fig.1, B). From 1-2 hr post activation (hpa), a gradual

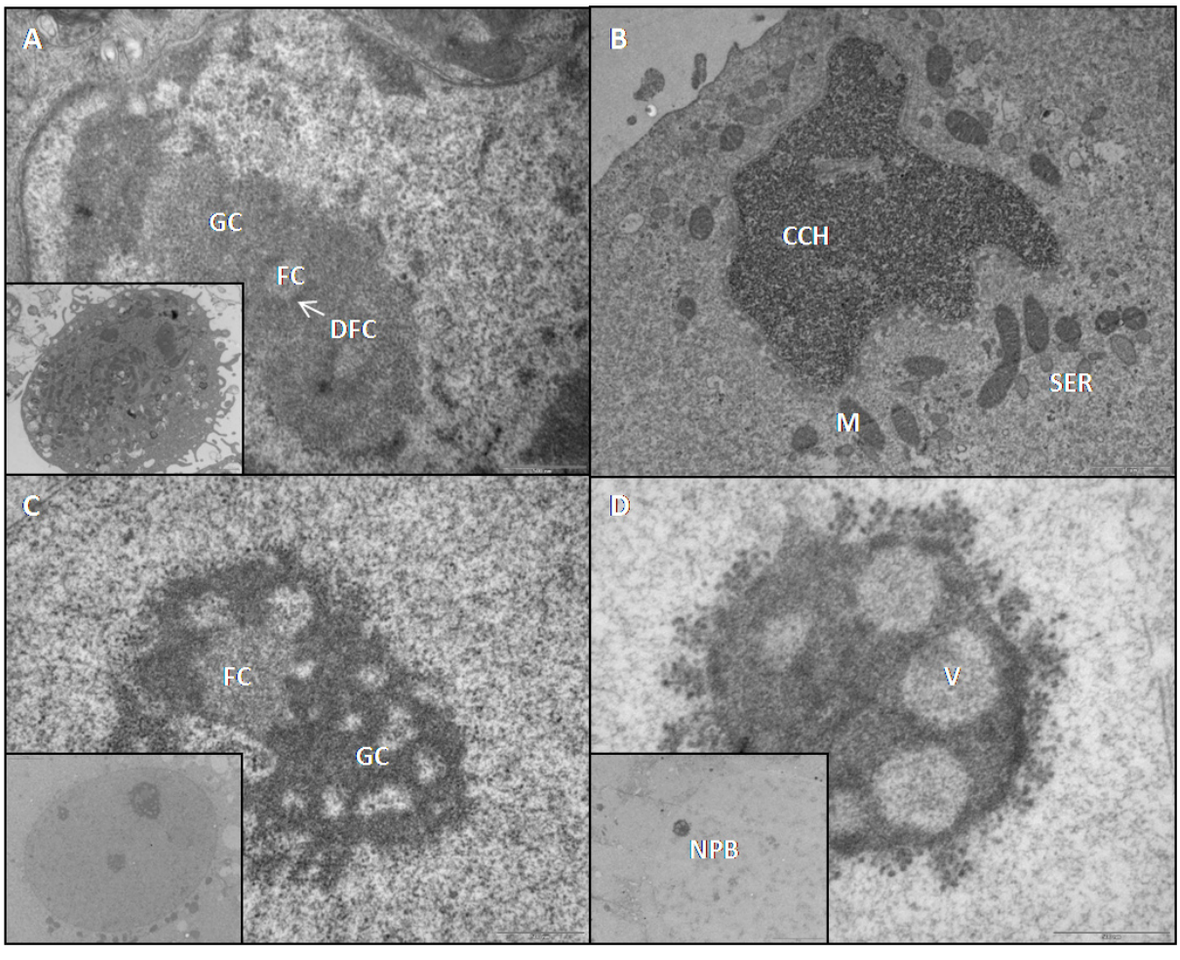

Fig. 1: Transmission electron micrographs of fibroblast (A) and somatic cell nuclear transfer (SCNT) bovine embryos at 0.5 hpa (B), 4 hpa (C), 12 hpa (D)

A- Starved fibroblast with fibrillo-granular nucleoli with fibrillar centres (FC), dense fibrillar component (DFC) and granular component (GC); B- Condensed chromatin (CCH) of the donor cell at 0.5 hpa closely surrounded by somatic cell cytoplasm, as indicated by somatic mitochondria (M) and smooth endoplasmic reticulum (SER); C- Pronucleus-like nucleus in SCNT embryo at 4 hpa displaying nucleolar structures with fibrillar centres (FC) and granular component (GC); D- SCNT embryo at 12 hpa with nucleolus precursor bodies (NPBs) displaying multiple peripheral vacuoles (V), surrounded by large electron-dense granules associated with condensed chromatin

chromatin decondensation appeared and nuclei displayed large euchromatic and sparse heterochromatic area. In our experiments we did not observe nuclear envelope breakdown (NEBD) and chromatin condensation (CCA) in five embryos from 1.5-3 hpa when nuclei from fibroblasts, presumably arrested in GO/G1, were introduced into MII cytoplasm. These results are similar to those of Dominko et al. (1999) and Ouhibi et al. (1996). Many factors may explain these results. First, all nuclei that are transferred into the cytoplast with a high MPF activity undergo nuclear envelope breakdown and chromosome condensation, however MPF is predominantly associated with the spindle, and the enucleation will decrease its activity. This logically decreases CCA activity too (Fulka et al., 2001). On the other hand, the fact that the somatic cytoplast was introduced together with a somatic nucleus into the non-activated bovine cytoplast, as indicated by increased population of somatic mitochondria around the reforming nuclei during the 0.5-3 hpa (Fig. 1, B), may be taken as an indication that this process of remodelling is a sequential process depending on cytoplasmic factors from somatic cell and on energy supply from the somatic cell. This feature may hamper the import of ooplasmic factors to the somatic cell nucleus during this period which, at least from the partial lack of the nuclear envelope, may represent a particularly permissive period for the deprogramming. However, the presence of a spherical pronucleus-like nucleus and similar NPB-like structures in SCNT embryos from 4 hpa (Fig. 1, C) suggest that nuclear deprogramming occurs within these first hours after activation (Petrovicova et al., 2008).

Our ultrastructural study renews previous electron-microscopy observations of Lavoir et al. (1997), Kanka et al. (1999) and Baran et al. (2002) describing nucleolar changes in embryonic and somatic NT embryos during the first cleavage stages. The first nucleolus-related structures were observed in SCNT embryos at 1.5 hpa and only in the nuclei with a complete nuclear envelope. At 1.5 to 4 hpa, the nucleolus-related structures appeared either as bodies including large fibrillar centres and a granular component, but no dense fibrillar component, or as electron-dense spheres, i.e. nucleolus precursor bodies (NPBs) (Fig. 1, C). The most prominent nuclear entities in the remaining embryos at 4, 8, 12, and 16 hpa were NPBs. Interestingly, at 12 hpa, NPBs displayed multiple peripheral vacuoles (Fig. 1, D) whereas at 16 hpa the NPBs lacked of vacuolization. This sequential remodelling can be interpreted by a presence of the factors from the somatic cytoplast after 4 hpa by oocyte-derived factors (Petrovicova et al., 2008)

As has been demonstrated in previous studies, nucleolar transformation is associated with the first detectable embryonic RNA synthesis as shown by an incorporation of ^{3}H- uridine. Thus, there is a clear correlation between nucleologenesis and nuclear RNA synthesis (Kopecny et al., 1989; Hyttel et al., 1998; Laurincik et al., 2000). Our observations showed that RNA synthesis was readily detected in starved bovine fibroblast before fusion (Fig.1, A). However, in the nuclear transfer reconstructed embryos, whatever the morphological appearance of the nucleoli, as previously described, no RNA was detected. These results clearly show that in reconstructed embryos the structural transformation of the nucleolus after fusion was not accompanied by the incorporation of ^{3}H- uridine, and, therefore, nucleolar function was apparently disturbed.

In conclusion, mechanism, by which foreign nuclei are remodelled in the enucleated oocytes cytoplasm and whether the introduced nucleus is fully reprogrammed, is still not elucidated. However, basing on general appearance and the morphology of the nucleus, as well as the presence of nucleolus precursors bodies, we suppose that the process of remodelling is completed just at 4 hpa. In later time points the overall morphology was similar to the nucleus seen in pronuclei and, therefore, we assume that by this time, the nucleolus finally comes under the control of the factors contained in the ooplasm.

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