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Unexpected nuclear localization of Cdc25C in bovine oocytes, early embryos, and nuclear-transferred embryos

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Abstract

It is clear from a wide range of studies that the nuclear/cytoplasmic distribution of Cdc25C has important functional consequences for cell cycle control. It is now admitted that in somatic cells, the localization of Cdc25C in the cytoplasm is required to maintain the cell in an interphasic state and that Cdc25C has to translocate to the nucleus just before M-phase to induce mitotic events. We characterized the expression and localization of Cdc25C during oocyte maturation, the first embryo mitosis, and the first steps of somatic cell nuclear transfer (SCNT) in cattle. We demonstrated that Cdc25C was expressed throughout the maturation process and the early development. We clearly showed that Cdc25C was localized in the nucleus at the germinal vesicle stage and during the early development until the blastocyst stage. However, the signal change in blastocyst and Cdc25C became cytoplasmic as is the case in somatic cells. Thus, oocytes and early embryonic cells presented a specific nuclear Cdc25C localization different from the one observed in somatic cells, suggesting that Cdc25C could have a particular localization/regulation in undifferentiated cells. Following SCNT, Cdc25C became nuclear as soon as the nucleus swelled, and this localization persisted until the blastocyst stage, as is the case in *in vitro* fertilized embryos. The Cdc25C nuclear localization appeared to constitute a major change, which could be associated with the reorganization of the somatic nucleus upon nuclear transfer.

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Introduction

Progression through the cell cycle is mediated by cyclin-dependent kinases (Cdks) in complex with cyclins. The activity of cyclin/Cdk complexes is regulated at several levels: expression and proteolysis, binding of inhibitors, phosphorylation/dephosphorylation, and also subcellular localization (Nurse 1990). One of the most important steps at G2-M transition is the activation of Cdc2/cyclinB (M-phase promoting factor) by the dephosphorylation of inhibitory Thr 14 and Tyr 15 residues catalyzed by cell division cycle 25C (Cdc25C), which thus plays a key role in cell cycle progression (Dunphy & Kumagai 1991, Gautier *et al.* 1991, Izumi *et al.* 1992). Cdc25C activity, as for Cdc2/cyclinB, appears to be regulated spatially and the controlled compartmentalization of this factor is crucial to regulate the cell cycle progression. Despite the importance of Cdc25C phosphatase for the cell cycle regulation, the controlled changes of its location are not yet fully understood. Most evidence now shows that in somatic cells, during interphase, Cdc25C has to be localized in the cytoplasm distant from its target Cdc2/cyclinB (Heald *et al.* 1993, Kumagai & Dunphy 1999, Graves *et al.* 2000). The binding of Cdc25C with 14-3-3 protein is responsible for

retaining Cdc25C in the cytoplasm, thereby contributing to the prevention of premature mitotic events (Heald *et al.* 1993, Dalal *et al.* 1999, Kumagai & Dunphy 1999, Lopez-Girona *et al.* 1999). Just before prophase, the 14-3-3/Cdc25C complex dissociates, allowing the translocation of Cdc25C to the nucleus and thus the activation of Cdc2/cyclinB and the entry in M-phase (Dalal *et al.* 1999, Takizawa & Morgan 2000). These findings establish that the translocation of Cdc25C into the nucleus plays an important role in governing the onset of nuclear mitotic events. This relocalization to the nucleus has been likewise described to occur just before G2–M transition at the meiosis resumption in *Xenopus* and starfish oocytes, (Izumi *et al.* 1992, Heald *et al.* 1993, Dalal *et al.* 1999, Kishimoto 1999, Kumagai & Dunphy 1999, Yang *et al.* 1999). Surprisingly, in porcine (Dai *et al.* 2000) and caprine oocytes (Gall *et al.* 2002), a reversed situation has been observed: Cdc25C is localized in the germinal vesicle and transported to the cytoplasm at the resumption of meiosis. The biological significance and regulation of these different localizations remain unclear.

Data on Cdc25C in mammalian oocytes are available only for pig (Dai *et al.* 2000) and goat (Gall *et al.* 2002) and no data exist about Cdc25C expression and

localization in early mammalian embryos. Bovine oocytes and embryos, which have served as an important model for many studies concerning the first steps of development and describing the nuclear remodeling that occurs after somatic cell nuclear transfer (SCNT), provide a relevant opportunity to investigate the mechanisms of specific cell cycle regulations, especially for Cdc25C. The aim of this study was to investigate Cdc25C expression and localization in bovine oocytes, early embryos, and during the SCNT procedure.

We demonstrated that Cdc25C was expressed throughout maturation and early embryonic development. We observed that Cdc25C was exclusively nuclear in oocytes at the germinal vesicle stage. Unexpectedly, Cdc25C is persistently nuclear during the early development until the blastocyst stage. We observed that during the first steps of SCNT, Cdc25C became nuclear as soon as the nucleus swelled and remained nuclear during the early development.

Results

Expression of Cdc25C and 14-3-3 protein in bovine oocytes and during early embryonic development

At different times during *in vitro* maturation and after *in vitro* fertilization, oocytes and embryos were lysed, and Cdc25C and 14-3-3 proteins were analyzed through immunoblotting using anti-Cdc25C antibody and anti-14-3-3 antibody.

In bovine oocytes at the GV stage, Cdc25C was detected at 55 kDa as a doublet that corresponds to the inactive nonphosphorylated forms of Cdc25C. After 7 h of culture, germinal vesicle breakdown (GVB) had occurred and the majority of the 55 kDa form was converted to a 70 kDa form, corresponding to the hyperphosphorylated active form of Cdc25C. At the metaphase II stage, Cdc25C was observed at 70 kDa. The amount of Cdc25C decreased between GVB and metaphase II stages (Fig. 1A). After fertilization, when oocytes were at the pronucleus stage, Cdc25C shifted down from the 70 kDa active form to the 55 kDa inactive form (Fig. 1B). During the early development, Cdc25C was detected mostly in a nonactive 55 kDa form (Fig. 1B). An equivalent number of oocytes and embryos were used per lane. We observed that the amount of Cdc25C increased from the pronucleus stage to the blastocyst stage. This was probably a consequence of the increase in the number of cells. With a specific anti-active-Cdc25C that is described to recognize essentially the hyperphosphorylated active form of Cdc25C, we detected Cdc25C at 70 kDa at the metaphase II stage as expected and as described above but also a weak labeling at 70 kDa during development, indicating an activation of Cdc25C by hyperphosphorylation during early development (Fig. 1C).

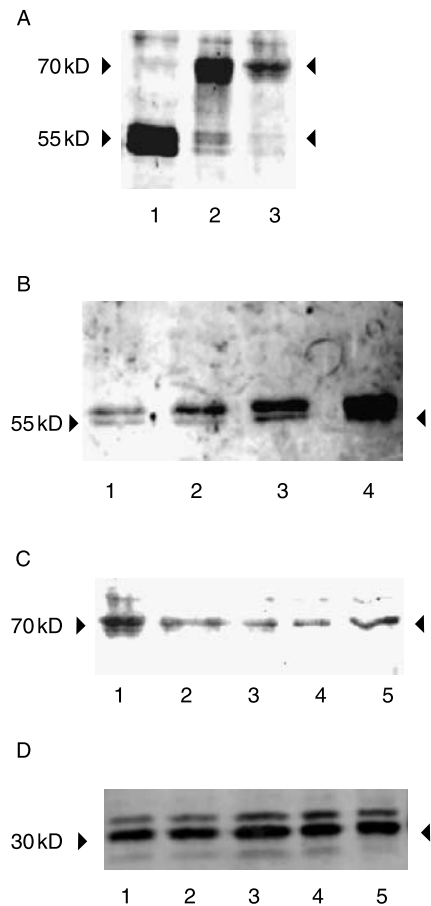


Figure 1 Immunodetection of Cdc25C and 14-3-3 protein. Each lane corresponds to 30 oocytes or embryos (A) Cdc25C during meiotic maturation. Lane 1, GV-stage oocytes; lane 2, GVB-stage oocytes; and lane 3, MII-stage oocytes. At the GV stage, Cdc25C was detected as a doublet at 55 kDa. When GVB occurred, Cdc25C shifted from 55 to 70 kDa. At the MII-stage, only the 70 kDa form was detected. (B) Cdc25C during early development after *in vitro* fertilization. Lane 1, pronucleus stage; lane 2, 4-cell stage; lane 3, 8/16-cell stage; and lane 4, blastocyst stage. After fertilization, when oocytes were at the pronucleus stage, the 70 kDa protein returned to its 55 kDa form. During the early development, Cdc25C was detected at 55 kDa. (C) Active Cdc25C during the early development after *in vitro* fertilization. Lane 1, MII-stage oocytes; lane 2, 4-cell stage; lane 3, 8/16-cell stage; lane 4, morula stage; and lane 5, blastocyst stage. During the early development, phosphorylated Cdc25C at 70 kDa was detected at a weak level. (D) 14-3-3 protein during the early development after *in vitro* fertilization. Lane 1, GV-stage oocytes; lane 2, MII-stage oocytes; lane 3, pronucleus stage; lane 4, 8/16-cell stage; and lane 5, blastocyst stage. During early development, 14-3-3 protein was detected at 30 kDa.

The 14-3-3 protein expression was examined in oocytes and during development and was specifically detected at 30 kDa as expected from the GV stage to the blastocyst stage (Fig. 1D). We did not detect any modification of its expression or electrophoretic mobility during the time course of meiotic maturation and development.

Cdc25C localization during bovine oocyte maturation and early development

The intracellular distribution of Cdc25C was examined by indirect immunofluorescence using the polyclonal antibody. At the GV stage, Cdc25C was essentially localized in the germinal vesicle, and no cytoplasmic staining was observed (Fig. 2A). Nuclear staining persisted until GVB occurred. As the nuclear envelope completely dissolved, Cdc25C staining spread throughout the cytoplasm and remained distributed in the whole cell until the metaphase II stage (not shown). After *in vitro* fertilization, at the pronucleus stage, Cdc25C was localized in the two pronuclei (Fig. 2B). From the two-cell stage to the morula stage, Cdc25C was systematically localized in the nucleus (Fig. 2C–E). When mitosis was observed in a blastomere, no specific staining around the spindle was detected. At the compact morula stage (day 6), Cdc25C is ever more nuclear (Fig. 3A and B). At day 7 of *in vitro* development, at the blastocyst stage, Cdc25C was no longer localized in the nucleus but was detected in the cytoplasm (Figs 2E and 3C and D). At the blastocyst stage, between 6 and 7 days, a modification in the Cdc25C localization took place. We did not observe a difference between the cells of the inner cell mass and the trophoblastic cells concerning the localization of Cdc25C.

Cdc25C localization during early development after somatic nuclear transfer

Cdc25C intracellular distribution was examined by indirect immunofluorescence, both in the fibroblasts used for nuclear transfer and during early development after nuclear transfer (Fig. 4).

In fibroblasts, Cdc25C was essentially detected throughout the cytoplasm and was particularly abundant around the nucleus (Fig. 4A).

In our SCNT procedure, 20% of the donor nuclei underwent premature chromatin condensation (PCC) and 80% remained intact within 2 h after the electric pulse. Before 7 h, regardless of the behavior of the foreign nucleus (PCC or not), Cdc25C was not evidenced in or around the nucleus transferred (Fig. 4B).

Seven hours after fusion, the embryos showed an enlarged nucleus and Cdc25C was essentially localized inside the nucleus (Fig. 4C). Twenty hours after fusion, the transferred nucleus appeared to be similar to a pronucleus and Cdc25C was nuclear (Fig. 4D). During the 2-cell, 4-cell, and 16/32-cell stages, labeling was essentially nuclear (Fig. 4E–G). At the blastocyst stage, Cdc25C was mainly localized in the cytoplasm as in *in vitro*-fertilized embryos (Fig. 4H)

Discussion

Using Western blotting and immunofluorescence analyses, we investigated the expression, phosphorylation, and localization of Cdc25C in bovine oocytes and early embryos. We showed that Cdc25C was expressed throughout the maturation process as well as during the early development. Cdc25C was detected at 55 and 70 kDa, which correspond to the nonphosphorylated inactive form and the hyperphosphorylated active form respectively, as previously described in different cell types (Millar *et al.* 1991, Jones & First 1995, Dalal *et al.* 1999, Gall *et al.* 2002). At the GV stage, Cdc25C was detected at 55 kDa; during G2–M transition, at meiosis resumption, Cdc25C became hyperphosphorylated as evidenced by a clear shift from 55 to 70 kDa. The shift from 70 to 55 kDa was associated with a return to the interphase state after fertilization. These results are in accordance with what had previously been reported in goat and *Xenopus* oocytes and in somatic cells

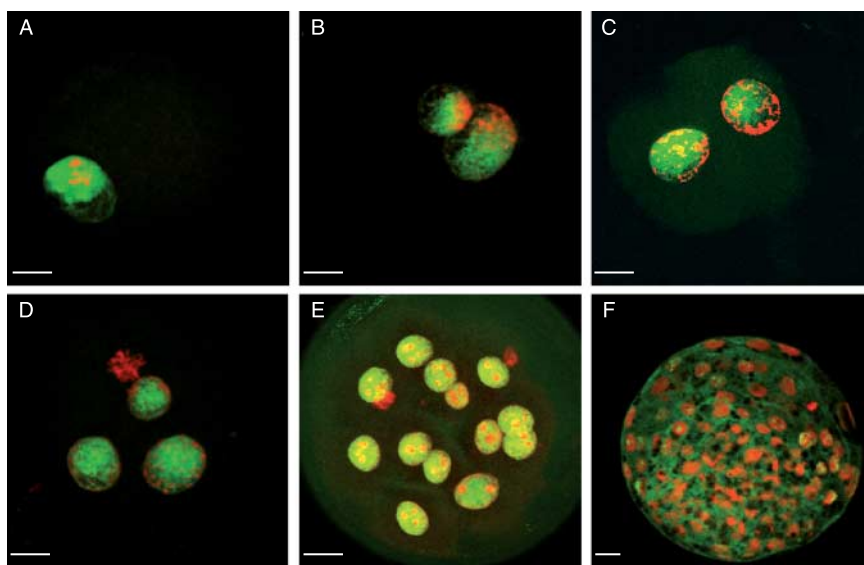


Figure 2 Immunolocalization of Cdc25C in oocytes and embryos. Each oocyte and embryo was double stained for Cdc25C (polyclonal antibody, FITC; green) and for chromatin (propidium iodide, red). (A) GV-stage oocytes; (B) pronucleus stage (18 h), (C) 2-cell stage (30 h), (D) 4-cell stage (40 h), (E) 8/16-cell stage (96 h), (F) blastocyst stage (7 days). At the GV stage, Cdc25C was localized in the germinal vesicle. At the pronuclear stage, Cdc25C was localized in the two pronuclei. From the two-cell stage to the morula stage, Cdc25C was localized in the nucleus. At the blastocyst stage, Cdc25C was detected in the cytoplasm. Bars = 20 μ m.

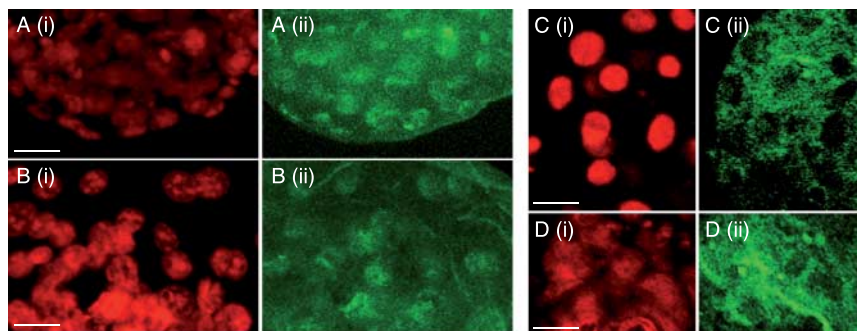


Figure 3 Immunolocalization of Cdc25C in morula and blastocyst stages. Each embryo was double stained for Cdc25C (FITC: A(ii), B(ii), C(ii), D(ii)) and for chromatin (propidium iodide: A(i), B(i), C(i), D(i)). (A, B) Compact morula stage (6 days) and (C, D) blastocyst stage (7 days). At the compact morula stage, Cdc25C is even clearly nuclear. The signal changed at the blastocyst stage and Cdc25C became clearly cytoplasmic. Bars = 20 μ m.

(Kumagai & Dunphy 1992, Hoffmann *et al.* 1993, Hoffmann & Karsenti 1994, Galaktionov *et al.* 1995, Izumi & Maller 1995, Karaïskou *et al.* 1998, Pérez-Mongiovi *et al.* 2000, Gall *et al.* 2002), and indicated that, in bovine oocytes the G2–M transition during maturation process is accompanied by the Cdc25C activation due to hyperphosphorylation. During the early development, Cdc25C was mostly expressed in the 55 kDa nonphosphorylated form until the blastocyst stage and only a weak amount of active phosphorylated Cdc25C was detected. Indeed, the Cdc25C activity is undetectable during interphase and become measurable only as cells enter mitosis. During the early development, at any given time, only 10–15% of embryonic blastomeres are in M-phase, thus it is not surprising to detect only very little phosphorylated Cdc25C. Furthermore, the hyperphosphorylation of Cdc25C occurring during early prophase of mitosis is a fast transitory phenomenon not easily evidenced.

It is clear from a wide range of studies that the nuclear/cytoplasmic distribution of Cdc25C has important functional consequences for cell cycle control, but a complete understanding of its localization and spatio-temporal regulation is missing. During the G2-phase, in somatic cells as well as in *Xenopus laevis* and starfish oocytes, Cdc25C is required in the cytoplasm and it translocates to the nucleus shortly prior to M-phase to trigger M-phase events (Izumi *et al.* 1992, Heald *et al.* 1993, Dalal *et al.* 1999, Kishimoto 1999, Kumagai & Dunphy 1999, Lopez-Girona *et al.* 1999, Yang *et al.* 1999). Our results on cultured bovine fibroblasts confirmed this somatic cytoplasmic localization. But in bovine oocytes, Cdc25C was exclusively found in the nucleus at the germinal vesicle stage. This particular localization is similar to what had previously been observed in pig and goat oocytes (Dai *et al.* 2000, Gall *et al.* 2002). Cdc25C thus appears to be differently located in both the germinal and somatic cells in mammals. These results suggest that although the same set of proteins drives mitosis and meiosis, different regulatory strategy could be involved during mitosis and meiosis. Surprisingly,

after *in vitro* fertilization in cattle, Cdc25C was localized in the two pronuclei (male and female) and during the early development, strong nuclear localization of Cdc25C in all blastomeres was detected until blastocyst stage. Preliminary experiments on mouse embryos indicated the same behavior of Cdc25C during the early development (unpublished data). All the results presented above demonstrate that Cdc25C, contrary to what is known in somatic cells, is always located in the nucleus in oocytes and embryonic cells, until the blastocyst stage, even during interphase. We could postulate that these two conditions of Cdc25C, somatic and embryonic, could undergo different regulatory strategies. In somatic cells, it has been described that during interphase, Cdc25C is found predominantly in the cytoplasm associated with 14-3-3 protein. This association is responsible for retaining Cdc25C in the cytoplasm, thereby contributing to the prevention of initiation of mitotic events. At G2-M transition, the Cdc25C/14-3-3 complex dissociates, which may allow Cdc25C to accumulate in the nucleus and to promote mitosis (Nebrada & Ferby 2000, Takizawa & Morgan 2000, Duckworth *et al.* 2002). We have observed that 14-3-3 protein was expressed in bovine oocytes and during the early development, indicating that permanent nuclear Cdc25C localization was not due to an absence of 14-3-3 protein. One possibility is that 14-3-3 protein becomes modified during oogenesis and early development, reducing its binding activity and causing its dissociation from Cdc25C, which results in a permanent nuclear localization. Another suggestion is that Cdc25C-binding sites for 14-3-3 protein, regulated by phosphorylation, may be defective during the oogenesis and early development.

At the blastocyst stage, the localization of Cdc25C changed drastically and cytoplasmic localization became obvious, as was the case for somatic cells. We did not observe any differences for the localization of Cdc25C between inner cell mass and trophoblastic cells. The cytoplasmic localization of Cdc25C appears in the blastomeres at the blastocyst stage,

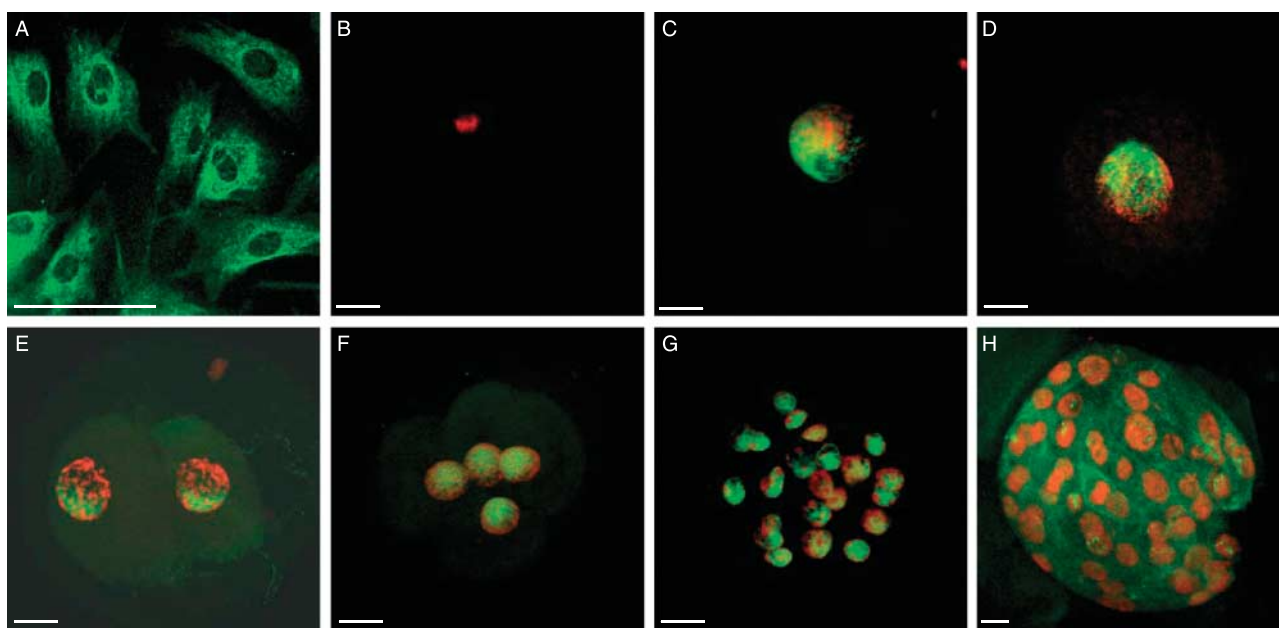


Figure 4 Immunolocalization of Cdc25C after somatic cell nuclear transfer. Each oocyte and embryo was double stained for Cdc25C (FITC, green) and for chromatin (propidium iodide, red). (A) Bovine fibroblasts; (B) 2 h after fusion; (C) 7 h after fusion; (D) 20 h after fusion, pronucleus stage; (E) 2-cell stage (26 h); (F) 4-cell stage (34 h); (G) 16/32-cell stage (100 h); and (H) blastocyst stage (7 days). In fibroblasts, Cdc25C was essentially detected in the cytoplasm. Some patches of Cdc25C were observed in the nucleus. No detectable labeling was observed in the nucleus 2 h after fusion. The nucleus had swelled 5 h post transfer and Cdc25C appeared inside the nucleus. From the one-cell stage to the morula stage, Cdc25C was localized in the nucleus. At the blastocyst stage, Cdc25C was localized essentially in the cytoplasm. Bars=20 μ m.

at the same time as the first differentiations. Altogether, these data suggest that Cdc25C could have a specific localization/regulation in undifferentiated cells when compared with the somatic differentiated ones. It was therefore interesting to observe the behavior of Cdc25C in the SCNT procedure during which a somatic nucleus is set into a germinal cytoplasm. The SCNT is performed using oocytes as the cytoplasm recipient and somatic cells as the nucleus donor. The reconstructed embryo is then artificially activated by different protocols in order to initiate the embryonic development. In successful experiments, the donor nucleus is supposed to be reprogrammed so that it matches embryonic characteristics compatible with a full-term development of the reconstructed embryo (review *Campbell et al. 2005*). The reprogramming of a donor nucleus is a complex process that involves remodeling of nuclear structures, epigenetic modifications of the chromatin, and expression of transcriptional regulators (*Vignon et al. 2002, Alberio et al. 2006*). It is not yet known how the somatic cell cycle regulation is modified to fit with an embryonic regulation. The premise is that if the donor nucleus is successfully reprogrammed, it first must be remodeled to a state similar to that of a pronucleus and the somatic regulation of Cdc25C has to turn to an embryonic regulation. Upon nuclear transfer into an enucleated oocyte, a massive nuclear swelling

accompanied by chromatin decondensation occurs. This reflects an exchange of proteins, which is an important aspect of remodeling. During our cloning procedure, 80% of transferred nuclei retained their initial state and 20% underwent nuclear envelope breakdown, lamina disassembly, and premature chromatin condensation (PCC). Interestingly, we could generate up to 38% of development to the blastocyst stage with such a protocol (*Vignon et al. 2002; present studies*). This suggests that reprogramming of the somatic nucleus is not directly linked to the occurrence of PCC upon nuclear transfer; this was also demonstrated in ovine species where the frequency of development to the blastocyst stage did not differ whatever be the rate of PCC (*Lee & Cambell 2006*). The somatic nucleus appeared to be deprived of Cdc25C until 7 h after fusion, whatever be the morphological remodeling (PCC or not). From 7 h post fusion, transferred nucleus (PCC or not) underwent nuclear swelling that resembled the conditions occurring at pronucleus formation after fertilization. At this stage, every foreign nucleus presented a dense Cdc25C labeling similar to that observed in embryos after *in vitro* fertilization. These results indicate that, whatever the behavior of the foreign nucleus (PCC or not), Cdc25C is translocated to the nucleus. The translocation to the nucleus of Cdc25C during SCNT is thus a specificity of the oocyte cytoplasm.

We characterized here one of the main modifications that affect a foreign nucleus at its introduction into an oocyte cytoplasm through nuclear transfer. It appears from this study that such a translocation is a robust process participating in nuclear reorganization. Although the biochemical basis for this reorganization is largely unknown, Cdc25C could be one of the oocyte factors associated with the somatic chromatin reorganization.

The nuclear localization of Cdc25C persisted until the blastocyst stage, at which point Cdc25C localization changed and all the blastomeres presented a cytoplasmic Cdc25C localization. The blastomeres of the embryos, resulting from nuclear transfer, presented a nuclear localization of Cdc25C similar to that observed in embryos after *in vitro* fertilization. The present study provides evidence that the embryonic and trophoblastic cells from the cloned embryos are similarly regulated, for Cdc25C, when compared with those from a fertilized embryo. Finally, we show that the developmentally regulated change in Cdc25C localization is not related to the occurrence of PCC in the somatic nucleus after its transfer into an oocyte. This suggests that such remodeling events can take place without the complete loss of the somatic nuclear envelope.

Further studies are under investigation to evaluate more precisely the roles of Cdc25C during the early development, which might explain a particular nuclear localization and to determine whether Cdc25C could be one of the oocyte factors implicated to allow the return of a differentiated nucleus to pluripotency.

Materials and Methods

Oocyte collection and *in vitro* maturation

Cattle ovaries were obtained from a slaughterhouse and transported to the laboratory in sterile PBS at 35 °C within 3 h. The detailed protocols have been described elsewhere (Heyman *et al.* 1994). Briefly, cumulus oocyte complexes (COCs) were aspirated in TCM 199 (Sigma) from 3 to 6 mm diameter follicles. Compact COCs with several dense layers of cumulus cells were selected and washed three times in TCM 199. Some oocytes (at the germinal vesicle stage) were mechanically denuded and used for western blot or immunofluorescence analysis. Maturation was performed in culture dishes (Nunc Delta, Gibco) in TCM 199 supplemented with 10% fetal calf serum (FCS; Life Technologies), 10 µg/ml porcine follicle-stimulating hormone/luteinizing hormone (Stimufol, Merial, Lyon, France), and 1 µg/ml estradiol 17-β, for 22 h at 39 °C in a humidified atmosphere of 5% CO₂ in air. Under our experimental *in vitro* conditions, we obtained maturation rates of 90%. At the end of the maturation period, *in vitro* matured oocytes were used for *in vitro* fertilization (IVF), SCNT, or analyzed using Western blot and immunofluorescence.

IVF and development

Twenty-two hours after the onset of maturation, oocytes were co-incubated with heparin-capacitated frozen-thawed spermatozoa in Tyrode's albumin lactate pyruvate medium for 18 h according to the standard technique routinely used in the laboratory (Revel *et al.* 1995). After IVF, presumptive zygotes were cultured in microdrops of 50 µl B2 medium (CCD, Paris, France) with 2.5% FCS, seeded with Vero cells (Menck *et al.* 1997). The droplets were overlaid with mineral oil (M8410, Sigma) and incubated for 7 days at 39 °C under 5% CO₂. Cleavage was assessed at day 2 after insemination and blastocyst formation was evaluated at day 7. Under our experimental *in vitro* conditions, 87% of zygotes cleaved and 58% reached the blastocyst stage. For immunofluorescence and Western blot analyses, zygotes and embryos were analyzed at 18 h, 30 h, 40 h, 96 h, 6 days, and 7 days, which corresponded to the pronucleus, 2-cell, 4-cell, 8/16-cell, compact morula, and blastocyst stages respectively.

Somatic nuclear transfer

Somatic cloning was performed as described elsewhere (Vignon *et al.* 2000). Donor cells from adult skin biopsies were cultured over 4–10 passages. The fibroblasts were grown in 60 mm Petri dishes in order to obtain a quiescent population of cells on the day of SCNT. The cells were trypsinized, pelleted, and resuspended in fresh TCM 199. After the maturation, oocytes were denuded from cumulus cells and enucleated at 22 h. A single fibroblastic cell was fused to enucleated metaphase II oocytes with a double electric pulse of 2.0 KV/cm for 30 µs. Reconstituted embryos were activated with 10 µg/ml cycloheximide and 5 µg/ml cytochalasin B for 5 h and then cultured in B2 medium as described above. Under these conditions, 96% of the stimulated oocytes formed pronuclei. Cleavage and blastocyst rate were 80 and 45% respectively. For the immunofluorescence analysis, reconstituted embryos were collected at 2 h, 7 h, 20 h, 26 h, 34 h, 100 h, and 7 days, which corresponded to the intact foreign nucleus, swollen foreign nucleus, pronucleus, 2-cell, 4-cell, 16/32-cell, and blastocyst stages respectively.

Western blot analysis

Oocytes and embryos (set of 30) were lysed in SDS-sample buffer and polypeptides were separated using 10% SDS-PAGE, as previously described by Laemmli (1970). Stained proteins of known molecular mass (range: 14–200 kDa, Amersham) were run simultaneously as standards. The electrophoretically separated polypeptides were transferred onto a PVDF membrane (Hybond-P PVDF; Amersham). The membrane was blocked by incubation in PBS 1/1000 Tween-20 (PBS-T; Prolabo, VWR, Fontenais-sous-Bois, France) containing 4% nonfat dry milk and incubated with the first antibodies (rabbit polyclonal anti-Cdc25C, diluted 1/300; Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse monoclonal anti-active-Cdc25C, diluted 1/300, Upstate Biotechnology; and rabbit polyclonal anti-14-3-3, diluted 1/1000, Santa Cruz Biotechnology) diluted in PBS-T 4% nonfat dry milk, for 2 h at room temperature. The blot was then washed three times with PBS-T and incubated in PBS-T 4%

nonfat dry milk, containing the peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1/5000; Biosys Vector Lab. Inc, Burlingame, CA, USA) and washed extensively in PBS-T. Peroxidase activity was revealed using the ECL-Plus Western blotting detection system as per the manufacturer's instructions (Amersham). Immunoreaction signals were analyzed using an image analysis system (Advanced Image Data Analyzer software, LAS 1000 camera; Fujifilm, FSVT, Courbevoie, France). Each experiment was repeated at least five times.

Immunofluorescence

Denuded oocytes, IVF embryos, and reconstituted embryos were fixed in Recta Pur methanol (Prolabo, VWR) at -20°C for 10 min. Fibroblasts were grown on glass coverslips and fixed in cold methanol as above. The samples were washed several times in PBS and then incubated for 1 h in PBS supplemented with 2% BSA, which were then incubated for 2 h with the first antibody (rabbit polyclonal anti-Cdc25C, diluted 1/300; Tebu, Santa Cruz Biotechnology) and rinsed for 1 h by extensive washes in PBS-BSA. The cells, oocytes, and embryos were then incubated for 1 h in fluorescein isothiocyanate-labeled anti-rabbit IgG (Biosys) diluted 1/500 in PBS-BSA. The samples were then exposed for a few minutes to propidium iodide (10 $\mu\text{g}/\text{ml}$; Invitrogen), mounted in a PBS-glycerol mixture (City University, London) and observed under confocal microscopy (Zeiss LSM 310).

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