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Early alteration of the self-renewal/differentiation threshold in trophoblast stem cells derived from mouse embryos after nuclear transfer

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Development after nuclear transfer (NT) is subjected to defects originating from both the epiblast and the trophoblast parts of the conceptus and is always accompanied by placentomegaly at term. Here we have investigated the origin of the reprogramming errors affecting the trophoblast lineage in mouse NT embryos. We show that trophoblast stem (TS) cells can be derived from NT embryos (ntTS cells) and used as an experimental *in vitro* model of trophoblast proliferation and differentiation. Strikingly, TS derivation is more efficient from NT embryos than from controls and ntTS cells exhibit a growth advantage over control TS cells under self-renewal conditions. While epiblast-produced growth factors Fgf4 and Activin exert a fine-tuned control on the balance between self-renewal and differentiation of control TS cells, ntTS cells exhibit a reduced dependency upon their micro-environment. Since the supply of growth factors is known to decrease at the onset of placental formation *in vivo* we propose that TS cells in NT embryos continue to self-renew during a longer period of time than in fertilized embryo. The resulting increased pool of progenitors could contribute to the enlarged extra-embryonic region observed in the early trophoblast of *in vivo* grown mouse NT blastocysts that results in placentomegaly.

Introduction

Reprogramming through nuclear transfer (NT) leads to viable adults although with a low efficiency (Campbell et al., 2007). Both the embryonic and extra-embryonic compartments are probably affected by reprogramming errors (Jouneau et al., 2006; Yang et al., 2007; Miki et al., 2009). The blastocyst is composed of the pluripotent inner cell mass (ICM) surrounded by the trophoblast, which is the first differentiated lineage of the embryo (Yamanaka et al., 2006; Arnold and Robertson, 2009). The trophoblast is characterized molecularly by the expression of the transcription factor Cdx2, whereas the ICM express the POU family factor Oct4. The part of the trophoblast not in contact with the ICM (mural trophoblast) differentiates into giant cells that are involved in implantation whereas the polar trophoblast in contact with the ICM remains proliferative (Sutherland, 2003; Rossant, 2004). The trophoblast seems correctly specified and initially functional in NT blastocysts, as most of them express Cdx2 correctly and can implant (Wakayama et al., 1998; Jouneau et al., 2006; Kishigami et al., 2006). However, the quality of these blastocysts is dependent on the origin of the donor cells. In ESNT reprogramming of

pluripotent markers such as Oct4, Sox2, Nodal and Nanog is probably less difficult as they are already expressed in the donor cells. Indeed, ICM from ESNT blastocysts are more alike fertilized ones whereas ICM from SCNT are more abnormal, only a few of them expressing correctly Oct4 (Boiani et al., 2002; Bortvin et al., 2003). Cell number and cell allocation between ICM and trophoblast are also correct in ESNT blastocysts (Zhou et al., 2001; Jouneau et al., 2006). After implantation and during gastrulation (E5–E7) the growth and patterning of the embryo require a tightly controlled cross-talk between trophoblast and epiblast (Ang and Constam, 2004). In our previous study we showed that despite being less abnormal at the beginning, ESNT embryos rapidly display defects affecting first the epiblast and then the trophoblast, leading to early post-implantation lethality (Jouneau et al., 2006). We showed that the specific morphological defects evidenced at E7 in NT embryos, could be rescued by incorporating trophoblast cells from a tetraploid fertilized embryo. Among the described defects, one was characterized by an enlarged extra-embryonic region as the expense of the embryonic one. During fetal stages, as early as E13, the NT placentas become hypertrophic and structural abnormalities of term placentas such as enlarged and abnormally shaped spongiotrophoblast and increase in glycogen cell population have been reported in NT conceptuses derived from both somatic and ES donor cells (Tanaka et al., 2001; Ono and Kono, 2006; Wakisaka et al., 2008). Placentomegaly is a

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feature of NT conceptuses shared by many species (Yang et al., 2007). In ruminants, where placentas are composed of multiple units called placentomes, these units are less numerous but hypertrophic after NT. Large scale gene expression analysis have been performed on placental tissues from both cattle and mouse clones and revealed a large number of deregulated genes, with a higher incidence than reported for the liver of cloned mice (Humpherys et al., 2002; Suemizu et al., 2003; Everts et al., 2008). Despite these molecular description of placentas, no attempts have been made to determine the developmental origin of the trophoblast defects. The aim of the present study was, therefore, to investigate the reprogramming errors affecting trophoblast lineage in mouse NT embryos. For that we decided to take advantage of the possibility to derive trophoblast stem cells (TS) from early trophoblast tissue. TS cells are isolated in vitro from the trophoblast of blastocysts (at E3.5) or implanted embryos (from E6.5 to E8.5). They are derived from the proliferating polar trophoblast and from the extra-embryonic ectoderm (ExE), tissues localized at the direct contact of the epiblast and supposed to maintain a pool of proliferating cells before their differentiation within the forming placenta at E9.5 (Tanaka et al., 1998; Uy et al., 2002; Kunath et al., 2004; Rielland et al., 2008). The Exe will give rise to the chorion and then to the placental labyrinth, whereas progenitors of differentiated derivatives already present in vivo in the ectoplacental cone lying above the ExE will give rise to the spongiotrophoblast of the placenta (Cross, 2005). Since the initial study of Tanaka et al. (1998), TS cells have been largely used as an in vitro model to study the function of genes involved in trophoblast development and function (Rielland et al., 2008). They express a set of markers known to be involved in vivo in the maintenance of the trophoblast, such as *Cdx2*, *Eomes*, *Esrrb*, *Fgfr2* and *Sox2* (Beck et al., 1995; Arman et al., 1998; Tanaka et al., 1998; Tremblay et al., 2001; Avilion et al., 2003; Strumpf et al., 2005). TS cells self-renew under the control of two main signaling pathways, FGF4/Erk1/2 and TGF β /Smad2/3 (Corson et al., 2003; Saba-El-Leil et al., 2003; Erlebacher et al., 2004; Yang et al., 2006). In their absence, TS cells readily differentiate in vitro into different trophoblast derivatives, such as giant cells expressing *Prld1*, spongiotrophoblast expressing *Ascl2* and glycogen cells expressing *Tpbpa* (Hemberger et al., 2004; Hughes et al., 2004; Simmons and Cross, 2005). In vivo both Fgf4 and Nodal, a member of the TGF β family, are secreted by the epiblast (Niswander and Martin, 1992; Conlon et al., 1994) which thus participates in the maintenance of a trophoblast stem cell micro-environment (Goldin and Papaioannou, 2003; Guzman-Ayala et al., 2004).

We first established the in vivo pattern of expression of several genes involved in the maintenance of a trophoblast stem cell micro-environment in NT embryos. Then we showed that TS cells, when derived from NT embryos, exhibited a growth advantage over their fertilized counterparts. Finally we provide evidence that this advantage is still exhibited when the supply of growth factors is reduced, suggesting a functional disconnection of NT trophoblast cells with their micro-environment in vitro. Taken together, our data provide an experimental support for the in vitro analysis of the mechanisms leading to the abnormalities observed during the early post-implantation period of NT trophoblast development in vivo.

Materials and methods

Production of nuclear transfer mouse embryos

Nuclear transfer was performed as described previously (Zhou et al., 2001; Maalouf et al., 2009). Briefly, NT embryos were reconstructed by injection of metaphase plates of R1 ES cell into enucleated oocytes from superovulated B6CBF1 females. The reconstructed embryos were activated with strontium in CZB medium. A pseudo polar body was expelled so that the reconstructed embryo remained diploid. Embryos were then cultured in M16 medium

(Sigma) at 37 °C and 5% CO₂ up to the blastocyst stage (4 days) or transferred at one or two-cell stage into pseudo pregnant B6CBF1 females before dissection at E3.5, E6.6 or E7.5. All experiments involving animals were carried out with the obligation to observe European regulations on animal welfare.

Isolation and culture of TS cells

TS cells were isolated from blastocysts or extra-embryonic ectoderm (ExE) of both fertilized 129/SVJ embryos and NT embryos reconstructed with ES donor nuclei (R1). NT blastocysts were either developed in vivo and flushed from females 3 days after transfer or in vitro after 4 days of culture in M16 medium (Sigma). 129/SVJ fertilized blastocysts were directly flushed from the uterus at E3.5. Blastocysts were then seeded in 4-well plates on a layer of Mitomycin-inactivated fetal fibroblasts (feeders) in TS medium containing FGF4 (37 ng/ml, Sigma) and heparin (1 μ g/ml, Sigma) at 37 °C, 5% CO₂ as described (Tanaka et al., 1998). ExE from E6.5/7 embryos were isolated from the ectoplacental cone and the epiblast and then separated from the visceral endoderm by enzymatic treatment (trypsin 0.5%, Sigma and pancreatin 2.5%, Sigma) in saline tyrode 10 min at 4 °C (Hogan et al., 1994). Cells from the ExE were then dissociated 10 min at 37 °C in 0.25% trypsin diluted in saline tyrode before culture. TS colonies were kept four passages on feeders with FGF4 at high concentration (37 ng/ml). After that, feeders were replaced by 70% feeder-conditioned medium in a medium referred as TS + EFCM + FGF4, in which FGF4 concentration was lowered to 25 ng/ml (as described in Tanaka et al. (1998)). After adaptation to this new culture system, cell lines were considered as established. For some experiments, conditioned medium was replaced by Activin A (10 ng/ml, R&D System). Differentiation medium is composed by TS medium and the Activin receptor specific inhibitor SB431542 (Sigma) at 10 μ M (Inman et al., 2002).

In situ hybridization on cultured cells and embryos

Cells grown on coverslips were fixed in paraformaldehyde 4% for 1 h at RT. The probe-containing plasmids were transcribed in vitro using the Dig-RNA labeling kit (Roche). Whole-mount in situ hybridization on cells and embryos was performed essentially as previously described (Wilkinson et al., 1990). Permeabilization of cells was done using Triton 1%. Proteinase K and RNase treatments were omitted for embryos up to midstreak stage. Alkaline phosphatase activity was detected by using BM purple AP substrate (Roche). All probes were kindly provided by the lab of Janet Rossant. Cells were counter-stained with Nuclear Fast Red, dehydrated and coverslips were mounted on glass slide using Eukitt mounting medium (EMS).

Immunostaining on cells and embryos

TS cells grown on coverslips were fixed with 4% paraformaldehyde in PBS at room temperature (RT) for 30 min. They were then permeabilized 20 min in PBS containing 5% of fetal calf serum (Gibco) and 0.1% Triton X100 (Sigma) (= PSST). Incubation was carried out overnight at 4 °C with primary antibodies (Cdx2 1:200, Biogenex; P Erk1/2, 1:350, Cell Signaling; Sox2, 1:500, Millipore; β -Catenin, 1:100, Transduction; Fgfr2, 1:200,) diluted in PSST. After rinsing, cells or embryos were incubated 1 h at RT with secondary antibodies as follows: for Cdx2 and β -Catenin: FITC conjugated goat anti-mouse antibody (Jackson, 1:400), for Sox2: TRITC conjugated goat anti-rabbit (Jackson 1:400); for P Erk1/2 and Fgfr2: Biotin-conjugated Donkey anti-rabbit (Upstate, 1:350) followed by Streptavidin-conjugated Texas Red, (Zymed, 1:300). Nuclei were counter-stained with Hoechst (Sigma, 1 μ g/ml). Coverslips were mounted with Citifluor (Biovalley) on a glass slide.

Whole-mount immunostaining of P-Erk1/2 in E6-E7 embryos was performed as described in Corson et al. (2003).

Assessment of proliferation by BrdU incorporation

TS cells grown on coverslips incubated with 100 μ M BrdU during 10 min at 37 °C in TS medium before being fixed in 4% paraformaldehyde in PBS. BrdU incorporation was revealed by immunostaining with an anti-BrdU monoclonal antibody (Roche) at 1/50 one hour at 37 °C followed by a goat anti-mouse antibody coupled to Fluorescein (Jackson) at 1/400 one hour at RT. Coverslips were mounted on slides using Cytifluor (Biovalley).

Observation and imaging of samples

Cells were photographed using a Leitz DMRB microscope (Leica Microsystems, Wetzlar, Germany) with a 40 \times objective equipped with an Olympus DP50 digital camera (Tokyo, Japan). Embryos after whole-mount in situ hybridization were observed and photographed under a Zeiss SZX binocular coupled to a digital camera. Embryos after whole-mount immunostaining were observed under a Zeiss Confocal microscope (LSM 310) and pictures were taken on middle optical sections.

Q-RTPCR

Total RNA were extracted by RNAeasy kit (Qiagen) then treated by DNase I (Roche) at 1 U/ μ g of total RNA and reverse transcribed with hexanucleotides at 50 μ g/ μ g of total RNA (Promega) and Superscript II (Invitrogen). Real time PCR was performed using an ABI Prism 7000 Sequence Detector. All reaction were run in triplicates using cDNA template synthesized from 1 μ g RNA. The thermal cycler program consisted of 45 cycles with SYBRG (Applied Biosystem) detection. Standard curves were established for each gene using serial dilutions of a control TS cDNA and run on the same plate as samples. Ct values were averaged and copy abundance of each gene in each sample were determined from their respective standard curves then normalized to the number of β -Actin copies. Statistical significance was determined by Student test: in control or in NT lines each gene has been compared between control medium and differentiation medium.

PCR amplifications were performed with the following primers: *mEomes* (Tm: 60 °C) ccttcaccttctcagagacagatt, tcgatcttagctgggtgat/atcc (Erlebacher et al., 2002), *Ascl2* (Tm: 60 °C) catcccaccctcctaag/ct aggcattagggcaggtttctt (Erlebacher et al., 2002), *Pr13d1* (Tm: 60 °C) gctgacattaaggcagaacc, gaccaagcagggtagtcataatt (Erlebacher et al., 2002), and β -Actin (Tm: 55 °C) gctctggctctcagccat, gccaccgatccacccgct.

Growth curves

ntTS or TS cells (passages 10–15) were seeded in duplicates at a density of 40,000 or 20,000 cells per 4-well plates in 400 μ l of TS +

Table 1
Characteristics of TS derivation from blastocysts (A) and extra-embryonic ectoderm (ExE) (B).

A				
	No of ExE	No of ExE giving TS colonies (%)	No of lines established (% of ExE)	
Cont	9	8 (89) ^e	0 (0)	
NT	26	13(50) ^f	5 (19)	
B				
	No of blastocysts	No of outgrowths (%)	No of outgrowths giving TS colonies (% of blastocysts)	No of derived TS cell lines (% of outgrowth giving TS colonies)
Cont	34	30 (88) ^a	29 (85) ^b	9 (31) ^c
NT	45	40 (89) ^a	33 (73) ^b	19 (58) ^d

Numbers with different superscript letters within a column are significantly different ($P < 0.05$).

Table 2

Number of blastocysts giving rise to TS colonies (appeared/total, %) when cultured in the presence of decreased concentrations (in ng/ml) of FGF4 or Activin A.

	37	37	37	12	0
FGF4	37	37	37	12	0
Activin A	10	5	0	10	10
Cont	17 / 22 (77%) ^a	12 / 22 (54.5%)	8 / 23 (35%) ^b	0 / 20 (0%)	0 / 19 (0%)
NT	18 / 24 (75%)	14 / 20 (70%)	17 / 23 (74%) ^a	10 / 12 (83%)	0 / 15 (0%)

a and b are significantly different ($P < 0.01$).

EFCM + FGF4 medium. Every 2 days, cells were trypsinized, resuspended in 1 ml of medium and manually counted. Experiment was repeated twice.

Cloning assay

ntTS or TS cells (passages 10–20) were seeded in duplicates at a density of 2000 or 1000 cells per 60 mm plate in 2 ml of TS + EFCM + FGF4 and cultured 10 to 15 days. Cells were fixed 30 min in 3.7% formaldehyde in PBS, then washed twice in PBS and stained with crystal violet. Only colonies composed of cells with the characteristic TS morphology were counted. Experiment was repeated three times.

Apo-one Homogeneous Caspase 3/7 Assay

Caspase-3/7 activity assays were performed with the Promega Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Charbonnières, France) according to the manufacturer's protocol. TS or ntTS cells (passages 18–19) were grown in 60 mm diameter plates in TS + EFCM + FGF4 medium. After four days of culture, adherent and floating cells were harvested, centrifuged and resuspended in TS medium. Cells were counted and 100 μ l of cell suspensions or control TS medium was mixed with the same volume of the Apo-ONE Homogeneous Caspase-3/7 reagent. Fluorescent product formation was measured at an excitation/emission wavelength of 499/521 nm, every 6 min over a 120 min period using a Tristar LB 941 plate reader (Berthold, Thoiry, France). Caspase-3/7 activity was expressed as the ratio of the caspase activity (slope of the kinetic in arbitrary units) to the number of cells. Each cell line was tested in duplicate.

Statistical analyses

Student test and Mann–Whitney U test were used for all analyses except for Tables 1 and 2, for which χ^2 test was used.

Results

Trophoblast markers are correctly expressed and maintained in NT embryos

Progenitors of the differentiated cells of the placenta originate from a pool of trophoblast stem cells present in the extra-embryonic ectoderm and ectoplacental cone at E6–7. We investigated whether the trophoblast in NT embryos correctly expressed at this early stage the different genes known to be essential for its maintenance. In our previous study we had shown that some morphological abnormalities were apparent in NT embryos at E6–7 such as enlarged ExE, reduced epiblast and abnormal shape (Jouneau et al., 2006). Such defects may impact the ability of the trophoblast to maintain a favorable trophoblast stem cell (TS) micro-environment (Guzman-Ayala et al., 2004). Transcription factors such as *Cdx2*, *Eomes* and *Essrb* and signaling molecules such as *Nodal* and *Erk1/2* (downstream effector of FGF signaling pathway) need to be expressed correctly (for review, see Rielland et al., 2008; Jouneau et al., 2006). ESNT embryos were dissected at E6 or E7 and subjected to whole-mount immunostaining or in situ hybridization (Fig. 1). More than ten embryos were used for each gene tested, some of them exhibiting abnormal morphologies:

embryos shown in B, H, L and P exhibited an enlarged ExE and/or a reduced epiblast and the NT embryo in E, a vesicular shape morphology (small, rounded, with no visible frontier between epiblast and Exe) (Jouneau et al., 2006). In all cases, *Cdx2* and *Esrrb* were found

to be correctly expressed in the ExE abutting the epiblast and in the chorion (Figs. 1A–F), as shown previously for *Eomes* (Jouneau et al., 2006). The Exe contains the proliferating stem cells of the trophoblast, whereas the ectoplacental cone contains already committed proge-

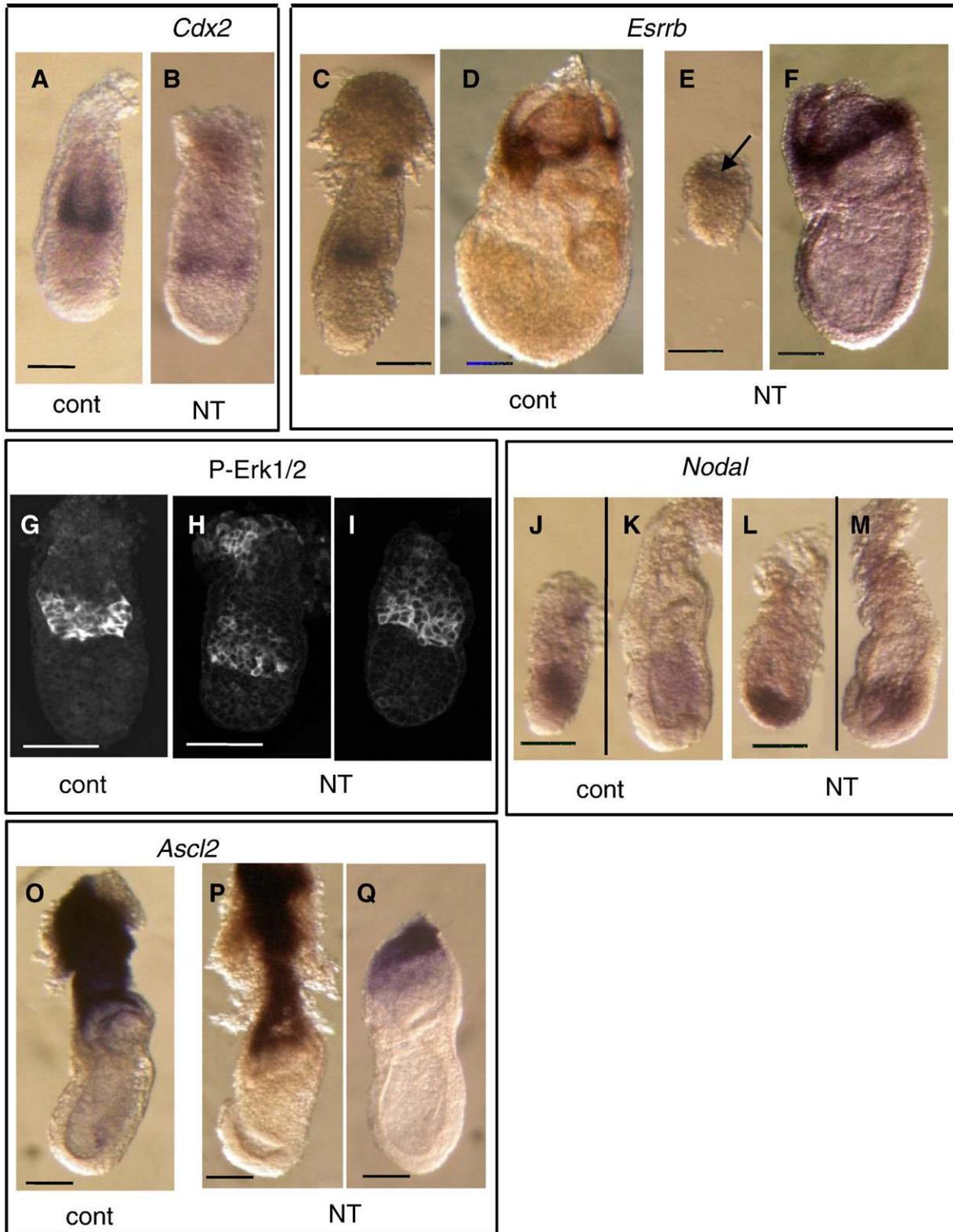


Fig. 1. Correct expression of different markers controlling the extra-embryonic ectoderm fate in NT embryos. Whole-mount in situ hybridization showing expression of *Cdx2* (A, B), *Esrrb* (C–F), *Nodal* (J–M) and *Ascl2* (O–Q). Whole-mount immunostaining showing the presence of phosphorylated-Erk1/2 (G–I). Note the reduced epiblast and/or extra-embryonic region in NT embryos in B, H, L and P and the vesicular shape in E. The arrow in E indicates the weak but positive staining for *Esrrb*. Controls were dissected at E6.5 except for D, which is at E7.5. NT embryos were dissected at E7.5 except for H and I (E6.5). Scale Bars: 100 μm.

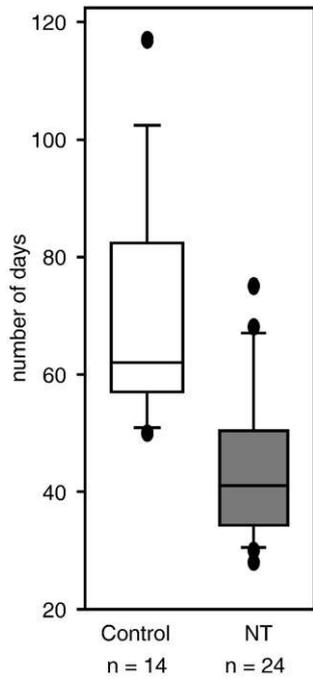


Fig. 2. ntTS cell lines are established more rapidly than control TS lines. Box plots showing the time from P0 to the fourth passage of TS cells. The lines correspond to the median. Cells are considered to be established after passage 4. The mean time for ntTS cells is significantly shorter than for cont TS cells ($P < 0.001$).

nitors that express *Ascl2* instead of *Cdx2*, *Essrb* and *Eomes* (Guillemot et al., 1994). We verified that *Ascl2* domain of expression was also restricted to the ectoplacental cone in NT embryos (Figs. 1K–M). Then

we examined the presence of the phosphorylated activated form of Erk1/2 and the expression of *Nodal*. Immunostaining of P-Erk1/2 revealed its expression in the proximal ExE in NT embryos as expected (Corson et al., 2003) and compared to control, the domain of expression was apparently more extended distally (compare Figs. 1G and I). *Nodal* expression domain corresponded to the epiblast (Figs. 1J–M) and in NT embryos with a reduced epiblast (see the embryo in Fig. 1H) the *Nodal* expression domain was reduced accordingly.

Our data indicate that the early trophoblast patterning is not dramatically affected in NT embryos. We then decided to isolate trophoblast stem cells from NT embryos to examine whether they would exhibit properties different from control ones.

TS cells can be derived from NT embryos at a higher efficiency than in controls

We first collected ExE tissues from ESNT embryos with the different morphologies as described above. The ExE were dissociated and cultured on a feeder layer in the presence of FGF4. We then checked the appearance of colonies of cells with the characteristic TS morphology (epithelial colony with smooth edges and cells with a small nucleus/cytoplasm ratio, see Tanaka et al., 1998). Only 50% of NT ExE gave rise to TS colonies compared to 88% for controls ($P < 0.05$) (Table 1A). However, it was not possible to correlate TS colony formation and the stage or morphology of the initial NT ExE (data not shown). This indicates that despite an apparently correct in vivo micro-environment, some NT embryos are already too compromised to give rise to TS colonies. This fits well with the fact that half of the NT embryos die between E7 and E10 (Jouneau et al., 2006). Nevertheless, five ntTS lines were obtained from twenty-six NT ExE, among them one line from a vesicular shape embryo (Table 1A and data not

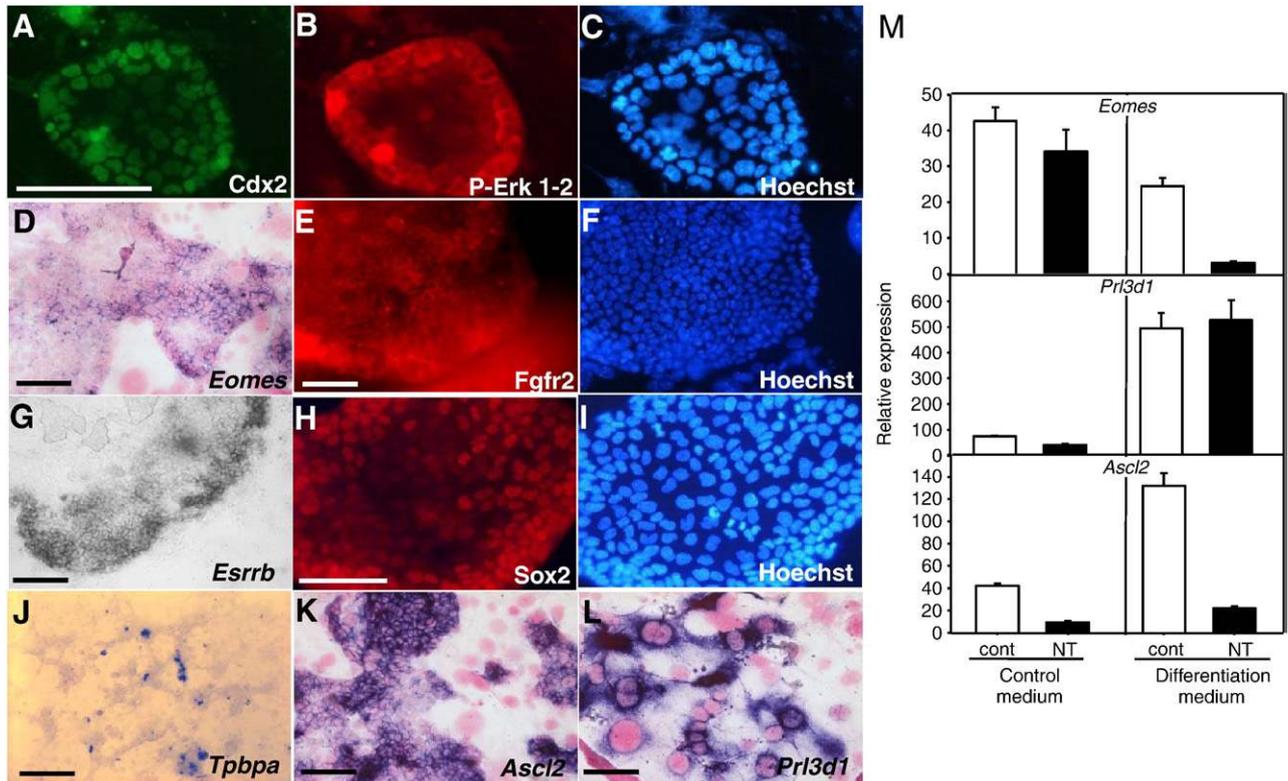


Fig. 3. ntTS cells express the expected markers during self-renewal (A–I and M) and differentiation (J–L and M). Immunostaining for A–C, E, F, H and I. Nuclei were stained in blue. In situ hybridization for D, G, J–L. Scale bar: 100 μ m. (M) Q-PCR analysis of *Eomes*, *Ascl2* or *Prl3d1* expression in cont- and ntTS cells cultured 5 days in TS + EFMC + FGF4 (control medium) or TS + SB431542 10 μ M (differentiation medium). Relative level of expression was expressed as percentage of Actin. The histograms represent the mean value (\pm sd) for two control and two NT cell lines. For each gene within one cell line expression was compared between “control medium” and “differentiation medium” and the differences were all statistically significant ($P < 0.01$).

shown). By contrast, no TS lines were obtained from the 9 control Exe used. It seems, therefore, that the derivation of a TS cell line from the primary colonies is efficient for NT embryos. We next further investigated this phenomenon by deriving TS cells from NT blastocysts.

NT and fertilized blastocysts were explanted on a feeder layer with FGF4. After four days they formed outgrowths which after dissociation were able to give rise to TS cell colonies. Our control blastocysts were directly collected in vivo at E3.5 as the strain of mice we used (129 SV/J) was very refractory to in vitro culture. We, therefore, tested whether the in vitro culture of NT embryos could influence their ability to give rise to TS colonies. For that, in a separate series of experiment, we checked the proportion of blastocysts giving TS colonies and the number of TS colonies for NT embryos transferred into pseudo pregnant females for three days before flushing ($n=30$). No difference was detectable between these embryos and those cultured only in vitro, thereby excluding any bias due to in vitro culture.

We observed a similar proportion of NT and control blastocysts forming an outgrowth and then giving rise to TS colonies (Table 1B). However, in vitro expansion of these colonies into a cell line highlighted a first difference between control and ntTS cells. Only 31% of control outgrowths gave rise to TS lines whereas more than half (58%) of NT outgrowths did ($P<0.03$, Table 1B). Moreover, we estimated the time in days from the passage 0 (dissociation of the outgrowth) to the passage 4 when TS cells were considered to be established (see Materials and methods). We observed a significant decrease of establishment time (mean = 43 days) in NT cultures compared to controls (mean = 70 days) (Fig. 2, $P<0.001$).

In conclusion, TS cells were easily established from both pre- and post-implantation NT embryos and the time required for the derivation of TS cell from NT blastocysts was strikingly shorter than for controls. We, therefore, decided to further investigate the properties of these TS cell lines.

ntTS cells can differentiate properly

We first verified the correct expression of different markers expressed by TS cells during self-renewal such as *Cdx2*, *Eomes*, *Esrrb*, *Sox2*, *Fgf2* and Phospho-Erk1/2. As expected, the established ntTS cell lines expressed all these genes and the Erk1/2 pathway was efficiently activated (Figs. 3A to I). Removal of FGF4 and Activin from the medium should lead to down-regulation of these markers and differentiation into giant cells expressing *Prl3d1*, spongiotrophoblast expressing *Ascl2* and glycogen cells expressing *Tpbpa* (Simmons and Cross, 2005). We demonstrated the correct differentiation ability of ntTS cells by in situ hybridization for *Ascl2* (Fig. 3J), *Tpbpa* (Fig. 3K) and *Prl3d1* (Fig. 3L). Moreover, we correlated by Q-PCR the down-regulation of *Eomes* with the up-regulation of *Ascl2* and *Prl3d1* (Fig. 3M). In conclusion, the established NT lines are true TS cells with the ability to self-renew and differentiate properly.

Proliferative ability differs between ntTS and control TS lines

In order to understand why ntTS cells were established more rapidly than controls, we investigated the proliferation ability of two ntTS cell lines in comparison with two control TS lines, all being derived from blastocysts. We first estimated the increase in cell number during 8 days in culture (Fig. 4A). ntTS cells began to proliferate 24 h after plating while a latency period of 48 h was evident for the control TS cells. In addition to this initial delay in the onset of proliferation, control TS cells displayed a longer doubling time, as evidenced by the separation of the curves on Fig. 4A. After 72 h of culture, the doubling time was 24 h for NT lines and 33 h for control lines. As a result, the increase in cell number is higher in ntTS cultures than controls after 8 days of culture. We concluded that the early ntTS growth advantage exhibited during establishment in culture was maintained along passages.

The percentage of TS cells in S phase was estimated by BrdU incorporation (Fig. 4B). The mean ratio of cells in S phase ranged between 50 and 60% after only 10 min of BrdU uptake, showing that TS cells are highly proliferative cells. In addition, we observed that the border of the colonies always proliferated more actively than the center, whatever the cell origin, control or clones (62% vs 48%, $P<0.005$). The slower proliferation in the center could be due to contact inhibition of proliferation, a property of non-transformed cells (Zhao et al., 2007). We compared separately the proliferation rate in the border and the center between control and ntTS cell lines. No significant differences were detected (Fig. 4B). We conclude that ntTS cell growth advantage cannot be explained just by an increased number of proliferating cells.

We have then examined whether a difference in apoptotic rate could explain the difference in proliferation between ntTS and control TS cells. The caspase 3/7 activity, one of the mediator of apoptosis, was measured in the cultures under self-renewing conditions. No significant difference between ntTS and control TS lines was detected (Fig. 4C).

In order to compare the self-renewal ability of TS cells, we assessed their clonogenic activity by estimating their plating efficiency (Fig. 5). Only colonies composed of cells with a characteristic TS cell morphology were counted, as the others were obviously composed of giant cells and other differentiated cells. We found that ntTS cell

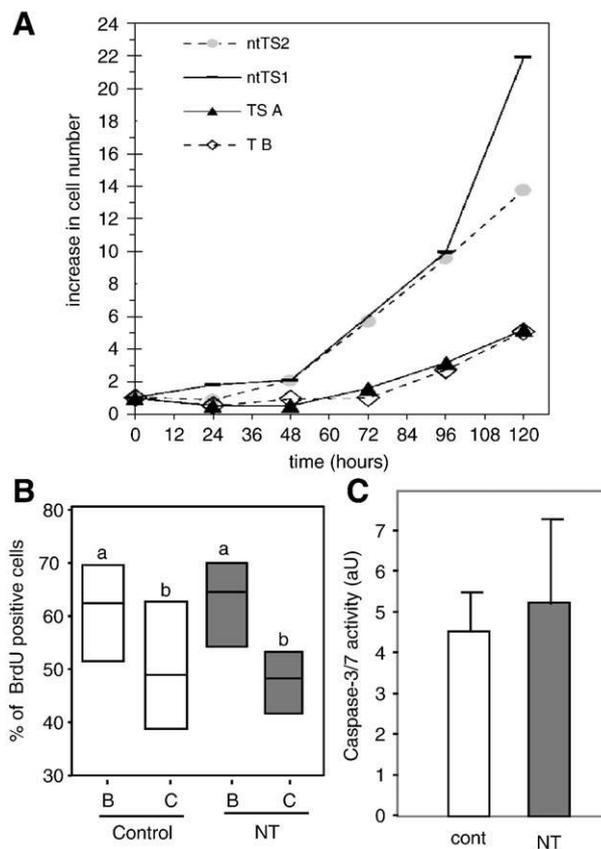


Fig. 4. ntTS cells self-renew more efficiently than controls (A) TS cells were seeded at low density in TS + EFCM + FGF4 medium and counted every 2 days. The increase in cell number was estimated from four replicates. (B) Box plots showing the percentages of TS cells in S phase estimated by the number of BrdU positive cells. At least 300 cells were counted in colonies from six different TS cell lines (6 for controls 6 for NT). Border "B" (the first three layers of cells) and centers "C" of the colonies display significantly different proliferation rate (a vs b, $P<0.01$). By contrast, no difference was found between NT and controls. (C) Caspase-3/7 activity is comparable between TS and ntTS cell lines. TS and ntTS cells were cultured four days and levels of caspase-3/7 activity were determined as described in Materials and methods. Values are the mean of two control and two NT cell lines. They are expressed in arbitrary units (aU) as the ratio of the slope of the caspase activity kinetic to the number of cells.

lines exhibited a higher rate of colony-forming cells (CFC) than controls (15% vs 7%, $P < 0.001$).

To conclude ntTS cell lines have an establishment time shorter than control and this growth advantage seems to be maintained during culture probably due to an increased number of cells with the capacity of self-renewal. We verified the karyotype of these ntTS lines and found that they were euploid, so their peculiar properties cannot be correlated to aneuploidy.

ntTS cells exhibit a decreased dependence upon FGF4 and Activin/Nodal compared to controls

During derivation of TS cells, ntTS cell lines were established more easily from primary colonies than controls (Table 1B). We observed that the loss of control cultures happened mainly at passage 4 when the FGF4 concentration was lowered (from 37 to 25 ng/ml) and the feeder layer replaced by conditioned medium (see Materials and methods) causing cells to differentiate. Interestingly, fewer NT cultures were lost during this change in culture system, suggesting their greater robustness regarding variations of the environment. To investigate whether this could be due to a modified behavior towards growth factors of trophoblast cells from NT embryos, we cultured NT and fertilized blastocysts in the presence of decreasing concentrations of FGF4 or Activin. Twelve days after the outgrowth dissociation, we then counted the number of Cdx2-positive colonies (Table 2 and Fig. 6). In control medium containing 37 ng/ml of FGF4 and 10 ng/ml of Activin A, no significant difference was observed in the number of blastocysts able to give rise to TS colonies between NT and controls and this rate (75–77%) was similar to that obtained with cultures in TS + EFCM + FGF4 (compare Tables 1B and 2). By contrast, a higher mean number of colonies was obtained in NT cultures compared to control cultures (16 and 7, respectively, Fig. 6, $P < 0.05$). A reduced supply of Activin and even its removal did not affect the appearance of TS colonies from NT blastocysts and reduced only marginally the mean number of colonies, from 16 with Activin 10 ng/ml to 9 without Activin ($P = 0.2$). By contrast, the absence of Activin in the cultures of fertilized blastocysts provoked a reduction by half of both the rate of TS colony appearance (77% to 35%, $P < 0.005$) and the mean number of colonies (7 to 3, $P < 0.002$). Moreover, only ntTS colonies were able to

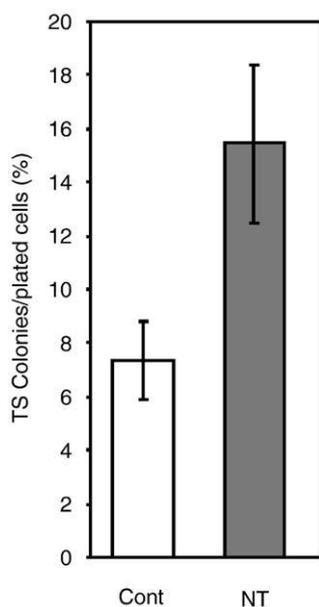


Fig. 5. ntTS cell lines have a higher rate of colony-forming cells (CFC) ($P = 0.001$). Cells were plated at low density and TS colonies derived from single cells were counted. Experiment was repeated three times.

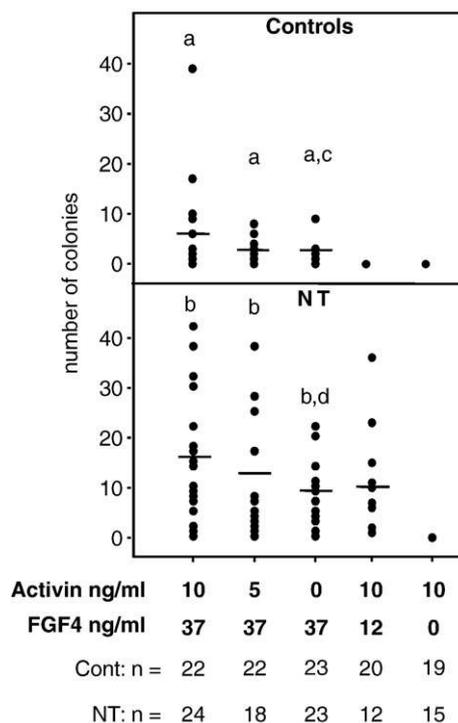


Fig. 6. Vertical dot plots showing the number of Cdx2 positive colonies appearing after dissociation of the blastocyst outgrowths in the presence of various concentrations of FGF4 or Activin A. Each dot represents the number of colonies for one outgrowth. The concentration of both factors in the medium and the corresponding number of control and NT blastocysts used are indicated. Different superscript letters mean significantly different results (*t*-test or Mann–Whitney *U* test): a vs b ($P < 0.05$), c vs d ($P < 0.002$) and a vs c ($P < 0.02$). The lines indicate the mean number of colonies in each condition.

be maintained with a lower concentration of Activin, as 3 ntTS lines out of 10 cultures were established and none from controls in the presence of 5 ng/ml Activin (data not show). A reduction in the supply of FGF4 was sufficient to completely prevent the emergence of Cdx2 positive colonies in control cultures whereas NT cultures were not initially affected. However, no TS lines could be established from these NT cultures. In absence of FGF4 we never observed TS colonies in the two types of culture. The few emerging colonies rapidly differentiated into giant cells.

In conclusion, in control cultures, the formation and number of TS colonies were correlated to the concentration of Activin in the medium and strictly dependent on a high concentration of FGF4. By contrast, in NT cultures, the formation and number of TS colonies were not initially dependent on the presence of Activin and were not affected by a reduction of FGF4 in the medium, even if they were still dependent upon its presence.

Discussion

In this study we have investigated the cellular and developmental origin of the reprogramming errors affecting the NT embryo development.

In our previous study, we showed that morphological defects such as enlarged ExE are caused by some defects in the trophoblast (Jouneau et al., 2006). A common feature of all living NT conceptuses (whatever the donor cell) is placental hypertrophy arising as early as E13. Indeed, a recent study has clearly demonstrated that placentomegaly after nuclear transfer arises when embryonic donor nucleus comes from any embryonic cells older than 8-cell stage (Ono and Kono, 2006).

We derived TS cells from NT embryos, in order to get an in vitro model to study the homeostasis of the trophoblast.

Our results show that the competency of NT embryos to give rise to TS cell colonies and lines is higher than that of control fertilized embryos and that the ntTS cells have a growth advantage over fertilized TS cells as indicated by the following panel of features: (i) a reduced mean time required to establish the ntTS lines (time between colony formation after dissociation of the blastocyst outgrowth and the fourth passage); (ii) a higher rate of cells forming colonies (CFC) in ntTS lines as shown by the higher clonogenic activity; (iii) a reduced latency after passaging before proliferation resumes; (iv) a tendency to resist differentiation and to keep self-renewal when the supply of growth factors is reduced. The growth advantage of ntTS cells *in vitro* correlates well with our previous finding *in vivo*: in chimeras composed of 2n NT cells and 4n fertilized cells, we observed that the resulting Exe at E7 was often chimeric, whereas in control 2n fertilized/4n fertilized cells, the diploid cells were excluded from the Exe. This shows that the growth advantage of trophoblast cells in NT embryos overrides that of the tetraploid cells in the Exe (Eakin and Behringer, 2003; Jouneau et al., 2006). This study of chimeras has recently been prolonged by Miki et al. who observed that placentas derived from 2n NT/4n fertilized cells were indeed mainly composed of cells of NT origin (Miki et al., 2009).

Increased stemness of ntTS cells

During TS cell establishment only a proportion of cells are real stem cells with the ability to expand and self-renew. The other cells are already committed to a differentiated derivative or will divide asymmetrically to give rise to only one daughter stem cell (Clarke and Fuller, 2006). Indeed, at each passage a small proportion of TS cells will differentiate spontaneously into giant cells or spongiotrophoblast (Tanaka et al., 1998 and our results). Such a property makes TS cells quite similar to adult stem cells, such as HSC, which maintain the balance between self-renewal and differentiation so as to keep stable the pool of stem cells (Wilson et al., 2004). The tendency of ntTS outgrowths to give rise to more colonies when Activin A and FGF4 are supplied at the optimal concentration, together with their higher ratio of CFC, may indicate a shift of the balance towards self-renewal rather than differentiation. In hES cell cultures, where the ratio of CFC is in the same range as in TS cells, the CFC assay has been considered to be a good indicator of the undifferentiated status of the culture (O'Connor et al., 2008). The colonies appearing after the dissociation of the outgrowth are probably clonal, as they arise from cells dispersed at a low density in the plate. The fact that the number of colonies is higher for NT cultures reflects either an increased proliferation rate of trophoblast cells during outgrowth phase or a higher number of TS cells in the blastocyst, or both. The initial number of TS cells *in vivo* is not known at the blastocyst stage. Only one detailed embryology study addressed this question in the Exe (Uy et al., 2002). This study has shown that few cells, about 1% of the Exe, have the potential to generate a TS colony. This is in agreement with the view of TS cells being localized in a niche *in vivo* (Jones and Wagers, 2008). At blastocyst stage, it is admitted that the polar trophoblast in contact with the ICM (the source of mitogenic Fgf4) is the reservoir of proliferating cells that will expand to give rise to the ExE after implantation (Kunath et al., 2004). The mural trophoblast that lines the blastocoel will give rise to the trophoblast giant cells, but before implantation they are still diploid and not irreversibly committed as they can resume proliferation if supplied with FGF4 (Gardner et al., 1973; Rassoulzadegan et al., 2000). When the whole blastocyst outgrowth is cultured with FGF4, then it is expected that both polar and mural trophoblast cells can give rise to TS cells. To address this question, it would be necessary to separate the polar from the mural trophoblast and to dissociate the cells before culture and counting of clonal TS colony.

Whereas NT blastocysts can give rise to primary TS colonies as efficiently as fertilized embryos, this capacity is lower after implantation, as less Exe from NT embryos can give rise to TS colonies compared to control Exe. This suggests that subtle defects not detectable by the panel of markers examined may be detrimental for the survival of the embryos and, more specifically, for the derivation of TS cells. Between the late blastocyst and the early implantation stages occurs the remethylation of the genome, ending with the epiblast being hypermethylated compared to the trophoblast (Monk et al., 1987; Santos et al., 2002). An abnormally high methylation in the trophoblast has been shown in bovine NT blastocysts and it is believed to be the same in the mouse, although no experimental evidence has yet been brought up (Dean et al., 2001; Kang et al., 2002; Dean et al., 2003). Such epigenetic defects may be responsible for the lower rate of colony formation from NT Exe compared to controls.

Conversely, the ability of a NT embryo to give rise to TS colonies *in vitro* is not directly predictive of its survival potential *in vivo*. A clear demonstration of this is the fact that we have been able to derive a TS cell line from a compromised embryo classified as "vesicular shape". We showed previously that such embryos do not express pluripotent epiblast markers, such as Nodal and Oct4 (Jouneau et al., 2006). They probably also do not express Fgf4, as its expression is controlled by Oct4 (Yuan et al., 1995; Ambrosetti et al., 1997). As mentioned above for the mural trophoblast, the supply of both FGF4 and Activin during the *in vitro* culture has allowed the trophoblast of the vesicular embryo to resume proliferation. It suggests that these vesicular embryos still contain living TS cells. It would be interesting to try to derive TS cells from epiblast-deficient embryos, such as Nodal mutants (Guzman-Ayala et al., 2004; Camus et al., 2006; Mesnard et al., 2006). It would tell whether the robust competency of the trophoblast to maintain TS cells in absence of epiblast signaling is a consequence of nuclear transfer or could be also observed in genetically manipulated epiblast-deficient embryos.

Reduced dependency of ntTS cells towards their environment

We then investigated the degree of dependency of ntTS cells upon the two essential growth factors FGF4 and Nodal/Activin. In the fertilized embryo, maintenance of Cdx2 expression requires the presence of both FGF4 and Activin/Nodal (Guzman-Ayala et al., 2004; Georgiades and Rossant, 2006). Our data demonstrate that the balance between self-renewal and differentiation in normal trophoblast cells is controlled by a fine-tuned threshold of the concentration of these two growth factors. Control blastocysts can still give rise to a few Cdx2 positive colonies when Activin is absent, but at a lower rate. It can be explained by the potential source of TGF β in the serum (Erlebacher et al., 2004) and the property of FGF4 alone to prevent ExE to differentiate into EPC cells expressing *Ascl2* (Guzman-Ayala et al., 2004). However, these primary colonies cannot be maintained and established into a TS cell line if Activin is absent or even reduced. The putative source of TGF β in the serum is, therefore, not sufficient to replace the need for Activin in long-term cultures. A dose-dependent effect on pluripotency has been shown previously in human ES cell cultures (Vallier et al., 2005). With either Activin only or FGF2 only, pluripotency of hES cells is initially maintained, but lost progressively with passages. When we reduced the supply of FGF4, no Cdx2-positive TS colonies were formed from control blastocysts. By contrast, NT trophoblast cells are able to partly escape the control exerted by growth factors. First, the appearance and number of TS colonies per NT blastocysts do not depend on Activin concentration and ntTS lines have been established under half the normal supply of Activin. Second, TS colonies can appear at a normal rate when FGF4 supply is reduced, although no lines could be established. Such a

lower dependency is in agreement with the ability to derive TS cells from NT embryos with a reduced epiblast.

Overall our results show that ntTS cell line derivation and functional NT placenta development are possible even though embryonic factors necessary to their development are not in adequate concentrations, because of a modified sensitivity of the NT trophoblast to its environment.

Hypotheses concerning trophoblast development in NT conceptuses

Placental formation starts after E8.5 by the occlusion of the ectoplacental cavity (between chorion and ectoplacental cone) followed by the fusion of the allantois and the chorion (Downs, 2002). This occlusion has been shown to mark the loss of possibility to derive TS cells (Uy et al., 2002). At the same time, *Cdx2* and *Essrb* expression is highly reduced and *Nodal* and *Fgf4* expression is confined to the node and posterior primitive streak, respectively (Conlon et al., 1994; Beck et al., 1995; Bueno et al., 1996; Luo et al., 1997). Attenuation of *Fgf4*-induced Erk signaling releases the repression of the nuclear activity of an Ets-family transcription repressor, Erf, which in turn promotes trophoblast differentiation and probably participates in the occlusion of the ectoplacental cavity (Corson et al., 2003; Papadaki et al., 2007). We hypothesize that in the chorion at E8.5, when both FGF and Nodal signaling are reduced, the ability of ntTS cells to self-renew will resist to this reduction of growth factors. Differentiation would therefore occur later than in control embryos, when embryonic factors would become totally absent. Such a delayed differentiation is in agreement the results from Wakisaka-Saito et al. who have shown that most NT conceptuses at E10.5, when they have some trophoblast tissue, are still at the chorionic stage with no labyrinth and a reduced or absent spongiotrophoblast layer (Wakisaka-Saito et al., 2006). Longer maintenance of TS cells can therefore explain the delayed differentiation and morphogenesis of the placenta, however, it does not completely explain the onset of placentomegaly as early as E13 and the fact that NT placentas continue to grow after E15 whereas controls do not (Jouneau et al., 2006). Placentomegaly could be induced as an adaptive response to placental dysfunction, as it has been suggested for enlarged placentomes developed after NT in bovine (Constant et al., 2006). One hypothesis is that the progenitors generated by these TS cells would also somehow inherit an increased proliferation potential. Alternatively, overgrowth of the placenta may be induced in response to signals emitted by the NT fetus. A recent study by Miki et al. has indeed underlined such role of the embryonic part of the conceptus (Miki et al., 2009). They observed that chimeric embryos composed of 2n fertilized/4n NT embryos, where the trophoblast is of NT origin, exhibited a placenta with normal size. Conversely, the NT embryo part is by itself not able to induce placentomegaly, as shown by the reverse chimera experiment where 2n NT ICM were injected into 4n fertilized blastocysts (Amano et al., 2002). Altogether, these results combined with ours indicate that abnormal interactions between both parts of the NT conceptus lead to placentomegaly. In other terms, an early defective cross-talk between epiblast and trophoblast would be counteracted by a reduced dependency of the latter tissue and later the fetus would send signals to the placenta leading to its hypertrophic development, so as to fulfill the fetal nutrient needs. As all surviving fetuses have a hypertrophic placenta, this feature may be a condition of their survival.

At last, another player contributing to the placentomegaly associated with the development of cloned mouse embryos, namely the uterus, must not be forgotten especially in the light of recent studies in the bovine species. In this species, the uterine endometrium can sense the type of trophoblast it interacts with, fertilized and NT trophoblast eliciting different responses from the uterus (Bauersachs et al., 2009; Mansouri-Attia et al., 2009). Therefore it is conceivable that the growth of the NT placenta could be further enhanced by the

uterus that would overproduce growth-enhancing hormones or mediators.

ES cells have been derived from NT or fertilized embryos and shown to be equivalent functionally and molecularly (Brambrink et al., 2006; Wakayama et al., 2006). By contrast, we have shown in the present study that ntTS cells exhibit some properties of maintenance and proliferation strikingly different from their fertilized counterparts which make them a valuable in vitro tool. Genome-wide molecular comparison of these cell lines is now ongoing and will help to understand the reprogramming errors affecting the extra-embryonic lineage after nuclear transfer.

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