

# Developmental abnormalities of NT mouse embryos appear early after implantation

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In mammals, cloning by nuclear transfer (NT) into an enucleated oocyte is a very inefficient process, even if it can generate healthy adults. We show that blastocysts derived from embryonic stem (ES) donor cells develop at a high rate, correctly express the pluripotential marker gene Oct4 in ICM cells and display normal growth in vitro. Moreover, the majority of them implant in the uterus of recipient females. We combine embryological studies, gene expression analysis during gastrulation and generation of chimaeric embryos to identify the developmental origin (stage and tissue affected) of NT embryo mortality. The majority died before mid-gestation from defects arising early, either at peri-implantation stages or during the gastrulation period. The first type of defect is a non-cell autonomous defect of the epiblast cells and is rescued by complementation of NT blastocysts with normal ES or ICM cells. The second type of defect affects growth regulation and the shape of the embryo but does not directly impair the initial establishment of the patterning of the embryo. Only chimaeras formed by the aggregation of NT and tetraploid embryos reveal no growth abnormalities at gastrulation. These studies indicate that the trophoblast cell lineage is the primary source of these defects. These embryological studies provide a solid basis for understanding reprogramming errors in NT embryos. In addition, they unveil new aspects of growth regulation while increasing our knowledge on the role of crosstalk between the extra-embryonic and the embryonic regions of the conceptus in the control of growth and morphogenesis.

**KEY WORDS:** Nuclear transfer, Growth, Gastrulation, Mouse, Trophoblast, Chimaera

## INTRODUCTION

In mammals, the transfer of a cell nucleus into an enucleated oocyte can result in an apparently healthy adult clone. However, the rate of success is low, regardless of the species of origin or the type of donor cells. This is thought to result from incomplete or aberrant reprogramming of donor nuclei (Jouneau and Renard, 2003; Latham, 2004; Solter, 2000).

Whatever the type of the donor cell used, nuclear transfer (NT) embryos can easily be reprogrammed into blastocysts from which pluripotent embryonic stem (ES) cells lines can be derived in vitro (Munsie et al., 2000; Wakayama et al., 2001). More recently, it has been shown that ntES cell lines were very similar to fertilised embryos-derived ES cells in terms of their ability to differentiate in vitro and in vivo, and to colonise the germ-line in chimaera mice (Barberi et al., 2003; Rideout et al., 2002; Wakayama et al., 2005). As NT blastocysts derived from mouse somatic donor cells more often exhibit an abnormal expression pattern of genes compared with NT blastocysts derived from pluripotent ES nuclei (Boiani et al., 2002; Bortvin et al., 2003), these results indicate that in vitro culture is able to either complete the reprogramming process in ICM cells from which ES cells are derived or select the few pluripotent ones.

By contrast, only a few percent of those blastocysts give rise to live pups, despite the fact that most of them can implant after transfer into recipient females (Wakayama et al., 1998; Wakayama et al.,

1999; Wakayama and Yanagimachi, 2001; Zhou et al., 2001). This is observed whatever the type of donor cell used, although the post-implantation developmental arrest of ES-derived NT embryos is lower than that of somatic cell-derived NT embryos (Eggan et al., 2001; Wakayama et al., 1999). In all cases, the period of highest lethality starts after the blastocyst stage. Several histological and molecular placental abnormalities considered to be the major causes of foetal death have been identified after nuclear transfer in different mammalian species (Heyman et al., 2002; Hill et al., 2000; Lee et al., 2004; Wilmut et al., 2002), including the mouse (Suemizu et al., 2003; Tanaka et al., 2001). In the latter species, widespread differences in the expression profile of genes in the liver of control pups, and premature death and obesity of surviving mice have also been reported (Humpherys et al., 2002; Ogonuki et al., 2002; Tamashiro et al., 2002).

Taken together, these data indicate that incomplete reprogramming after nuclear transfer affects more severely the extra-embryonic tissues, which directly participate in the post implantation development, than the embryonic tissues, which remain able to provide at least in vitro pluripotent ES cells. The fact that a high rate of derivation of ntES cell lines is not predictive of a high rate of successful development (Boiani et al., 2005) further supports this view. Although this has practical important consequences for the use of pluripotent ntES cells for therapeutic applications (Jaenisch, 2004), it leaves unresolved the issue of how reprogramming can occur differently in the two compartments of the blastocyst and how this, in turn, will affect the interaction between the different emerging tissues of the conceptus. The concept of epiblast pluripotency encompasses the ability of these embryonic tissues not only to differentiate into different cell lineages but also to establish the embryonic axes and the body plan (Tam et al., 2001). In the mouse, the development of the embryo after the blastocyst stage relies on close relationships between the conceptus and the maternal environment, and within the conceptus, between the

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embryo proper and its surrounding extra-embryonic tissues (Camus et al., 2004). Thus, the fate of the epiblast of NT embryos has to be characterised carefully *in vivo* to ascertain the impact of the cloning procedure on the developmental competence of reconstructed embryos. Despite its importance to development and the need of a basic understanding of reprogramming processes, the early post-implantation period of NT embryos has remained poorly characterised up until now. What is more, no attempt to track down the origin of the defects observed at later stages in the placenta has yet been reported. In the mouse, most of the cells that compose the placenta come from the trophoblast and, more precisely, from the extra-embryonic ectoderm that develops in close contact with the epiblast at early post-implantation stages (Cross, 2005; Rossant and Cross, 2001).

Therefore, we have produced NT embryos and focused our analysis on the development beyond the blastocysts stage. We devised previously specific conditions allowing the production of a high rate of morphologically normal blastocysts using ES cells as donors (Zhou et al., 2001). It allowed us to create an experimental situation where the blastocysts formed a homogenous and apparently normal population, at least for several genes like *Oct4* essential for the immediate peri-implantation survival (Bortvin et al., 2003; Nichols et al., 1998). In this context, we could address clearly the question of the potency of the NT epiblast *in vivo*. The aim of the present study was to pinpoint the embryological origin of the developmental defects encountered during the post-implantation period of NT embryos in terms of stage and tissue affected.

Our results show that growth alteration at early post-implantation stages is the first manifestation of reprogramming defects. It first affects the embryonic tissue and then the trophoblast, but it does not affect the differentiation potential of epiblast cells.

## MATERIALS AND METHODS

### Animals and cells

Oocytes were derived from superovulated B6CB F1 mice. The R1 ES cell line was a generous gift from Andras Nagy (Nagy et al., 1993). The R26.6 ES cell line (129 background), derived from ROSA26 mice, was kindly provided by Philip Soriano (Friedrich and Soriano, 1993). GFP-expressing mice were kindly obtained from Masaru Okabe. They are transgenic mice (B6 background) ubiquitously expressing GFP under a  $\beta$ -actin promoter and cytomegalovirus enhancer (Okabe et al., 1997).

### Production of NT embryos from ES nuclei

Nuclear transfer of ES nuclei into enucleated oocytes was performed as previously described (Zhou et al., 2001). Briefly, mitotic cells were collected from R1 ES cells treated with demecolcin. NT embryos were reconstituted by injection of metaphase nuclei into enucleated oocytes from superovulated B6CB F1 females and then activated in strontium containing CZB medium.

Embryos were either transferred at two-cell stage into pseudo pregnant B6CB F1 females or cultured in M16 medium (Sigma) up to the blastocyst stage. Recipients were sacrificed at different stages and the embryos dissected into PB1 medium containing 10% foetal calf serum. The recovered embryos or foetuses were fixed with 4% paraformaldehyde. They were then dehydrated through graded ethanol series and stored at 20°C. Controls consisted of *in vivo* or *in vitro* fertilised embryos (B6CB F2) cultured for at least 1 day before transfer into recipients.

### Immunofluorescence studies on preimplantation embryos

Embryos were rinsed in PBS and fixed in 4% paraformaldehyde at room temperature for 20 minutes. They were then permeabilised in PBS containing 10% FCS and 0.4% Triton X100 for 20 min at room temperature. Incubation was carried out for 1 hour at room temperature with anti-*Oct4* monoclonal antibody (Becton-Dickinson) diluted 1/100 in

the same solution. After rinsing, the embryos were incubated under the same conditions with anti-mouse IgG coupled to FITC (Jackson). Nuclei were counterstained with propidium iodide (Sigma) before the slides were mounted. Observations were made with an LSM 310 confocal microscope (Carl Zeiss, Germany).

### Blastocyst outgrowths

NT or control embryos were cultured in M16 medium for 4 days. The zona pellucida of the blastocysts was removed in acid tyrode and the embryos were cultured in DMEM with 20% serum on gelatinised four-well plates. Outgrowth formation was followed daily using an inverted microscope and photographed. The areas occupied by the ICM outgrowths were measured on the digitalised images.

### RT-PCR analysis of gene expression on single embryos

Poly A<sup>+</sup> RNA from each single NT embryo was isolated using a Dynabeads mRNA DIRECT kit (DYNAL). The Oligo-(dT)<sub>25</sub> covalently bound to the biomagnetic dynabeads was used to capture mRNA and subsequently as a primer for the reverse transcriptase (Superscript II, GIBCO) to synthesise the first strand of cDNA at 42°C for 1 hour. PCR products were separated on 1.5% agarose gel and visualised with ethidium bromide fluorescence. Our RT-PCR assays were not quantitative; a positive signal shows that the transcript of the gene is detected.

### Volume and mitotic index analyses

Embryos from pre-streak to late streak stages were fixed with 4% paraformaldehyde, then permeabilised with 0.4% Triton X100 before being treated by RNase A (20  $\mu$ g/ml) for 20 minutes at 37°C. The nuclei were then stained with propidium iodide at 10  $\mu$ g/ml in PBS before being mounted on slide with Vectashield mounting medium (Vector). Optical sectioning was carried out using a confocal microscope (LSM 310, Zeiss). From each series of slices, we determined the volume of the epiblast and the extra-embryonic ectoderm using Image Tool software. The number of mitotic figures was also counted on each section. The initial determination of the correlation between the number of cells and the volume was made on a subset of control and cloned embryos using a method adapted from Power and Tam (Power and Tam, 1993). For these experiments, several small areas of the epiblast and the extra-embryonic ectoderm were selected from the optical sections (4  $\mu$ m) of each embryo. The number of cells contained in these areas and the number of cells per unit volume were both determined. The total cell number in each epiblast and extra-embryonic ectoderm was obtained by dividing the volume of each region by the number of cells per unit volume. The equations of the resulting linear curves were then used to calculate the number of cells from each volume subsequently determined.

### Whole-mount *in situ* hybridisation

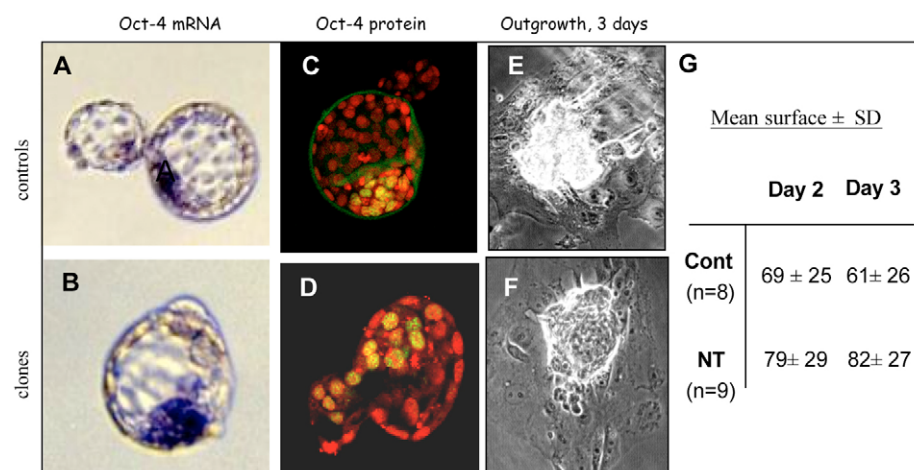
The probe-containing plasmids were transcribed *in vitro* using the Dig-RNA labelling kit (Roche). Whole-mount *in situ* hybridisation was performed essentially as previously described (Wilkinson et al., 1990). Proteinase K and RNase treatments were omitted for embryos up to MS stage. Alkaline phosphatase activity was detected by using BM purple AP substrate (Roche). Embryos were observed and photographed through a SZX binocular (Olympus) coupled to a camera.

Probes for the following genes were obtained as follows: *Oct4*, from A. Smith; *Bmp4*, from B. Hogan (Winnier et al., 1995); *brachyury*, from B. Herrmann (Herrmann et al., 1990); *Eomes* and *Cdx2*, from J. Rossant (Beck et al., 1995; Ciruna and Rossant, 1999), and *Nodal* from E. Robertson (Varlet et al., 1997).

### Generation and analysis of chimaeras

Blastocysts developed *in vitro* from NT embryos were injected with 8-12 R26.6 ES cells and then transferred into pseudo-pregnant females. Conceptuses were recovered at E7 or E19 then fixed and processed for X-gal staining as described by Hogan et al. (Hogan et al., 1994).

Embryos expressing GFP were produced after mating GFP-expressing homozygous B6 males with B6CB F1 females. ICM were isolated by immunosurgery from GFP-expressing blastocysts, as described by Solter and Knowles (Solter and Knowles, 1975). They were briefly incubated with



**Fig. 1. Expression of Oct4 and outgrowth of NT blastocysts.** (A,C,E) In vivo fertilised control embryos. (B,D,F) ES-cell NT blastocysts. (A,B) Whole-mount in situ hybridisation with *Oct4* mRNA probe. (C,D) Immunofluorescent staining using an Oct4 antibody. Overlapping of chromatin staining (red) and Oct4 expression (green) is revealed by a yellow stain. Both Oct4 mRNA and proteins are correctly expressed in ICM of NT embryos. (E,F) Examples of 3-day cultures of control and NT blastocysts. (G) Mean surface occupied by the ICM-derived clusters of cells (arbitrary units). No statistical difference could be detected between outgrowths.

0.5% Pronase and injected into NT or control blastocysts using an injection pipette with an internal diameter of 20 µm. Injected blastocysts were then transferred into pseudo-pregnant recipients.

To produce tetraploid embryos, the blastomeres from fertilised embryos collected at the two-cell stage were electrically fused. After 1 day of culture, they were aggregated with NT or control 4- or 8-cell stage embryos after removal of the zona pellucida by a brief treatment with tyrode acid. The aggregates were then cultured for 24 hours. Chimaeras expressing GFP (in the tetraploid cells) at the morula/blastocyst stage were selected under an inverted fluorescent microscope before transfer into recipient females.

After dissection at E7, chimaeras expressing GFP were fixed with 4% paraformaldehyde, permeabilised and subjected to RNaseA treatment before nuclear staining with propidium iodide. They were then observed under the confocal microscope.

**RESULTS**

**Preimplantation development of ES-cell derived NT embryos**

We have previously shown that ES-cell derived NT blastocysts display normal morphology in terms of cell allocation between the ICM and the trophectoderm (Zhou et al., 2001). In the present study, we examined whether the pluripotential gene marker *Oct4* was correctly expressed in a subset of these blastocysts. Analysis of the expression of Oct4 in ES-cell NT blastocysts both at the RNA (n=14) and protein (n=9) levels showed a normal pattern of expression restricted to ICM (Fig. 1A-D).

Although blastocysts with ‘excellent morphology’ may display abnormal gene expression levels (Mann et al., 2003), we concluded that most of the ES-cell derived embryos could consistently develop to the blastocyst stage. To achieve a more functional view of the developmental potential of these blastocysts, we assessed their ability to grow in culture as an in vitro assay of the peri-implantation period. We found that the ICM cells from almost all NT blastocysts (18 out of 20) grew and formed a typical mass of cells (Fig. 1E,F), the areas of which were not significantly different from those of controls after 2 and 3 days of culture (Fig. 1G).

Finally, the ability of the NT blastocysts to implant and form deciduas was tested after transfer into pseudo-pregnant mice. Most of them (12 out of 14) were found to be pregnant at E7, with two-thirds of the transferred blastocysts implanted (67±19%, n=112). In vitro fertilised embryos used as controls have an implantation rate of 80% (n=40). Under our conditions, we thus conclude that most of the ES-cell derived blastocysts were indistinguishable from normal blastocysts in terms of cell allocation, ICM cell growth ability in vitro and high rate of implantation.

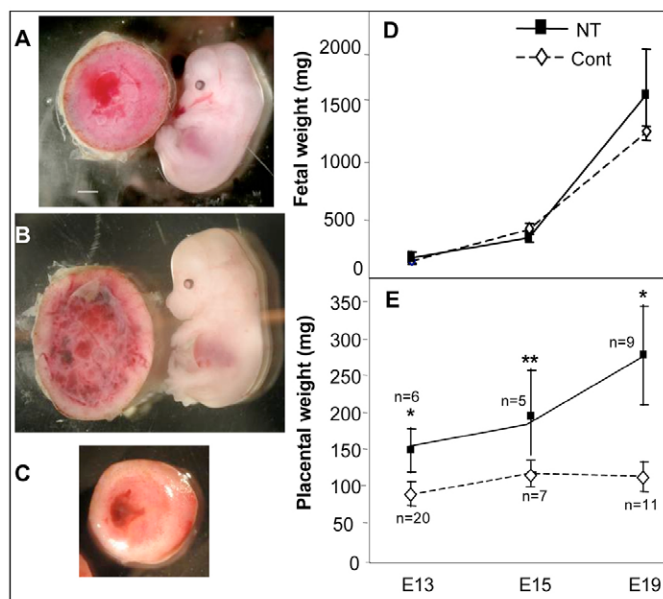
**Main features of the post-implantation development of NT embryos**

Because placental defects are considered to be the main cause of death of clones, we first assessed the post-implantation survival of NT embryos after E10 to cover the placental period of development (Iguchi et al., 1993; Rossant, 1995). NT embryos were transferred at the two-cell stage into pseudo-pregnant females that were dissected at E10, E13, E15 and E19 (Table 1).

At mid-gestation (E10), one third of the implantation sites still contained a conceptus, the remaining ones being composed of only decidual tissue. Half of the foetuses recovered were still alive (beating heart; 16% of the implanted embryos). More death had occurred by E13 and continued to E15. Only 6% of the implanted embryos were still alive at E19 compared with 65% when in vitro fertilised (IVF) embryos were transferred. Abnormally large placentas were always found to be associated with living or dead foetuses at all stages (Fig. 2B). Surprisingly, we found that NT placentas continued to grow after E15, in contrast to what is observed during normal development. At birth, these NT placentas became twice as heavy as controls (Fig. 2E). In addition, placentas totally devoid of foetal membranes and foetuses (Fig. 2C) made up half of the conceptuses recovered between E13 and E19. The ongoing histology of these placentas indicates that they can be considered true placentas as they contain all the tissue subtypes found

**Table 1. Post-implantation development of ES-NT embryos**

		Number of transferred two-cell embryos	Number of implantation sites (% transferred)	Number of conceptus recovered (% of implanted)			
				Total	Living foetuses	Resorbing foetuses	Placentas only
NT	E10	245	106 (43)	33 (31)	17 (16)	16 (15)	0
	E13	80	34 (42)	9 (26)	4 (10)	1 (3)	4 (12)
	E15	97	42 (44)	11 (26)	4 (10)	2 (5)	5 (12)
	E19	463	178 (38)	36 (21)	10 (6)	2 (5)	17 (10)
Cont IVF	E19	30	20 (67)	13 (65)	13 (65)	0	0



**Fig. 2. Growth of the conceptus during foetal stages.** (A) Control conceptus at E13. NT conceptus with a living foetus (B), or consisting of only a placenta with no foetus or membrane present (C). Scale bar: 1 mm. Weight curves of the foetus (D) and the placenta (E) for control and NT conceptus from E13 to term (E19). \* $P < 0.01$ ; \*\* $P < 0.05$ .

in a fully developed placenta (Simmons and Cross, 2005) (A.J., L.M., H. Jammes, V.B., Q.Z. and J.-P.R., unpublished). When present, the foetuses were, however, very similar to controls, although slightly heavier (Fig. 2D).

From these first series of in vivo observations, we concluded that most NT embryos died before the completion of a functional placenta and that abnormal growth of the extra-embryonic tissues was a consistent feature of the development of clones. To better characterise the origin of this earlier death of clones and that of the growth deregulation, we next focused our analysis on the early post-implantation period.

### Most NT embryos initiate gastrulation

Upon implantation, the rapid growth of the epiblast, the extra-embryonic ectoderm (derived from the trophoblast) and the overlying visceral endoderm (derived from the primitive endoderm) leads to the formation and elongation of an egg-cylinder-shaped embryo (Camus et al., 2004; Downs and Davies, 1993; Lewis and Rossant, 1982). The visceral endoderm and extra-embryonic ectoderm do not contribute directly to the embryonic tissues but have an important role in growth and patterning of the epiblast (Ang and Constam, 2004; Beck et al., 2002; Perea-Gomez et al., 2004). In the epiblast, proliferation and morphogenetic movements cooperate to generate the primitive streak and the three primitive germ layers (Lawson et al., 1991; Tam and Behringer, 1997). To characterise the gastrulation pattern in NT embryos, we dissected recipient females at E7 to E8.5 and recorded the length of the primitive streak and the presence of either an anterior neural plate or a headfold, according to Downs and Davis (Downs and Davis, 1993). The presence of somites signified the end of gastrulation. We found that NT embryos were developmentally delayed compared with controls: at E7.5 only a few had reached the neural plate stage, whereas more than half of the controls had (see Table 2). One day later (at E8.5), fewer NT embryos had reached the somite stage compared with controls (Table 2).

**Table 2. Development of ES-cell derived NT embryos at E7.5 and E8.5**

		Controls*	NT
Two-cell transferred		106	94
Implantation sites (%)		86 (85)	442 (47)
Conceptus recovered (% implanted)		65 (79)	177 (40)
		% Embryos	% Embryos
E7.5	Pre-streak	6	24
	Primitive streak	37	62
	NP/HF <sup>†</sup>	57	14
E8.5	Pre-streak	0	27
	Primitive streak	0	14
	NP/HF <sup>†</sup>	8	45
	Somites	92	14

\*Embryos fertilised in vivo and cultured 1 day before transfer.

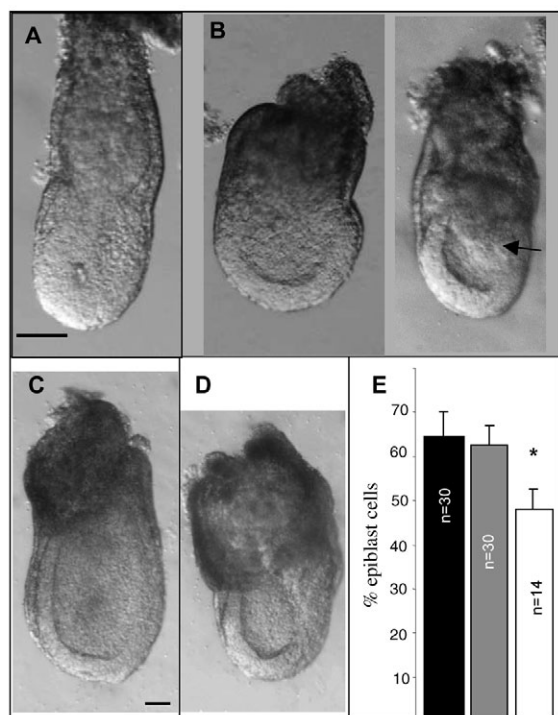
<sup>†</sup>Embryos at neural plate or headfold stages [from Downs and Davies (Downs and Davies, 1993)].

Careful examination of the embryos however revealed that a low proportion (13%, 7/53), recorded as pre-streak stage, had no apparent frontier between the extra-embryonic ectoderm and the epiblast and displayed a vesicular rather than an elongated form with no sign of differentiation of the visceral endoderm layer in the embryonic region (data not shown). This observation was indicative of development which had already been compromised. We further clarified this point by checking for the expression of pluripotency and early differentiation markers using an RT-PCR approach on single embryos. We found that both *Oct4* and *Nodal* (Nichols et al., 1998; Varlet et al., 1997) were absent (data not shown). By contrast, three markers of the differentiation of the visceral endoderm, the early marker *Hesx1* and two late markers *Ttr* and *Afp* (Barbacci et al., 1999; Coffinier et al., 1999; Thomas et al., 1998), were expressed, suggesting that this tissue was differentiating at least to some extent.

The sizes of the other NT embryos were within the normal range at E7 and showed the presence of a clear limit between the extra-embryonic and embryonic regions. A closer morphological examination, however, revealed two abnormal phenotypes: one with an abnormal rounded rather than elongated shape, as illustrated in Fig. 3B. These embryos were, however, capable of initiating gastrulation, as half of them had a visible primitive streak (Fig. 3B, arrow on right). The second one, illustrated in Fig. 3D, was called the 'large Exe' phenotype, and was found at all gastrulation stages in NT embryos. It was characterised by an abnormal ratio between the extra-embryonic and the embryonic regions. These two abnormal morphological phenotypes were the only ones observed at these stages (Table 3). These defects also occurred independently, as some NT embryos displayed both defects.

### Growth defects appear early during gastrulation

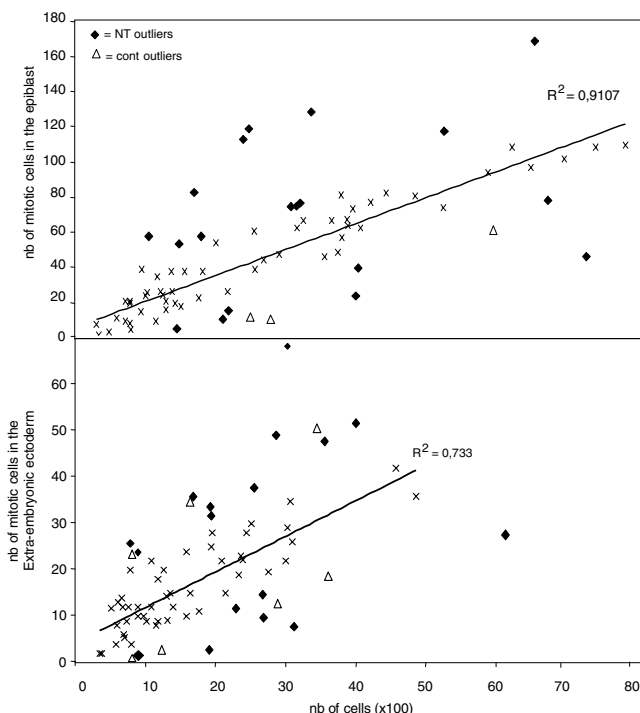
The abnormal shape observed in NT embryos may result from an abnormal elongation of the epiblast, owing to a defect in either cell proliferation or the morphogenesis of the primitive streak. The 'large Exe' embryos may reveal deregulation of the relative growth of the two regions of the embryo. To document this point, the cell number in the epiblast and the extra-embryonic ectoderm of NT embryos was determined. To achieve this, we first measured the volume of the tissues and then estimated the correlation between the volume and the cell number (see the Materials and methods). Surprisingly, the profiles of the regression curves were different for controls and NT embryos (data not shown). In other words, for a given volume, the



**Fig. 3. Two morphological abnormalities observed in E7 NT embryos.** (A,C) Control embryos at early-streak (A) and mid-streak (C) stages. (B) Two examples of NT embryos with abnormal rounded shape at pre-streak (left) and early-streak stage (right). (D) The normal proportion of the embryonic and extra-embryonic regions is perturbed in some NT conceptuses (Exe phenotype). (E) Mean ratios of the epiblast (=number of cells in epiblast/total cell number, in %) in controls (black), in NT displaying the Exe phenotype (white), and in other NT embryos (grey). Scale bars: 100  $\mu$ m. \* $P < 0.01$ .

number of cells in NT embryos is slightly lower than for controls. Measurements of nuclear diameters revealed that, in fact, NT embryos had larger nuclei and thus, probably, larger cells that could explain this reduction in cell density (data not shown).

The statistically significant ( $P < 0.01$ ) coefficients of determination found for all four linear curves made it possible for us to use the equations to compute the number of cells corresponding to a given measured volume. The mean ratio between the number of epiblast cells and the total number of cells was then calculated for controls and NT embryos. Those previously designated as the ‘large Exe phenotype’ displayed a mean ratio of 48%, instead of 62% and 64% as for other NT and control embryos, respectively (Fig. 3E). The comparison of ‘large exe’ embryos at pre-streak stage with control embryos at the same stage confirmed that the extra-embryonic region of the NT embryos was larger at the expense of the embryonic region (data not shown). For gastrulating stages, this point could not be asserted indubitably owing to the normal variation in the size of



**Fig. 4. Linear regression ( $P < 0.01$ ) of the number of mitotic cells and the total cell number in the epiblast of both NT and control embryos.** Forty-one percent (19/47) of the NT embryos are outliers (black diamonds), with either too high or too low a number of mitotic cells. By contrast, only 10% of the controls (3/29) are outliers (open triangles). The coefficients of determination  $R^2$  were statistically significant ( $P < 0.01$ ).

the embryos during this period of development (Downs and Davies, 1993). However, in any case, the normal balance of the cell number in one compartment in relation to the other is disrupted. Cell number determination for embryos with the abnormal shape revealed that they were within the normal range according to their stage of development (data not shown), suggesting that their poorly elongated shape is not due to a specific proliferation defect but rather to some perturbation of the control of the directed growth of the conceptus along its proximodistal axis.

Cell proliferation was then estimated using the mitotic indexes. The number of mitoses of more than 50 NT embryos and nearly 30 controls were plotted against the number of cells in the epiblast and extra-embryonic ectoderm. In most clones and controls, there was a positive linear correlation between cell numbers and mitoses, with much more significant correlations for the epiblasts (Fig. 4). Interestingly, 41% of the epiblasts of NT embryos were pinpointed as outliers, either displaying too high or too low mitotic indexes (Table

**Table 3. Abnormal phenotypes of NT recovered at E7**

Total	Morphological criteria (n=53 embryos examined)			Abnormal MI in the epiblast (n=47 embryos examined)	
	Vesicular form, retarded	Abnormal shape	‘Exe’ phenotype	Too high MI	Too low MI
43%*	13%	13%	17%	25%	15%

\*The total is not the sum of all columns, as some phenotypes overlap.

3). By contrast, only 10% of the controls were outliers with too low mitotic indexes. No evident correlation was found between the mitotic indexes and the phenotypes of the NT embryos. In the extra-embryonic ectoderms, the mitotic indexes were generally lower, only about half the level of the epiblasts. In addition, the correlation was less significant. Thirty-eight percent of the NT embryos were found to be outliers along with 26% of the control embryos.

In conclusion, this embryological analysis revealed that: (1) developmental defects in NT embryos arose early after implantation and affected some of the embryos recovered at E7 (Table 3); and (2) the types of defects observed were related to growth deregulation in one or both compartments of the conceptus.

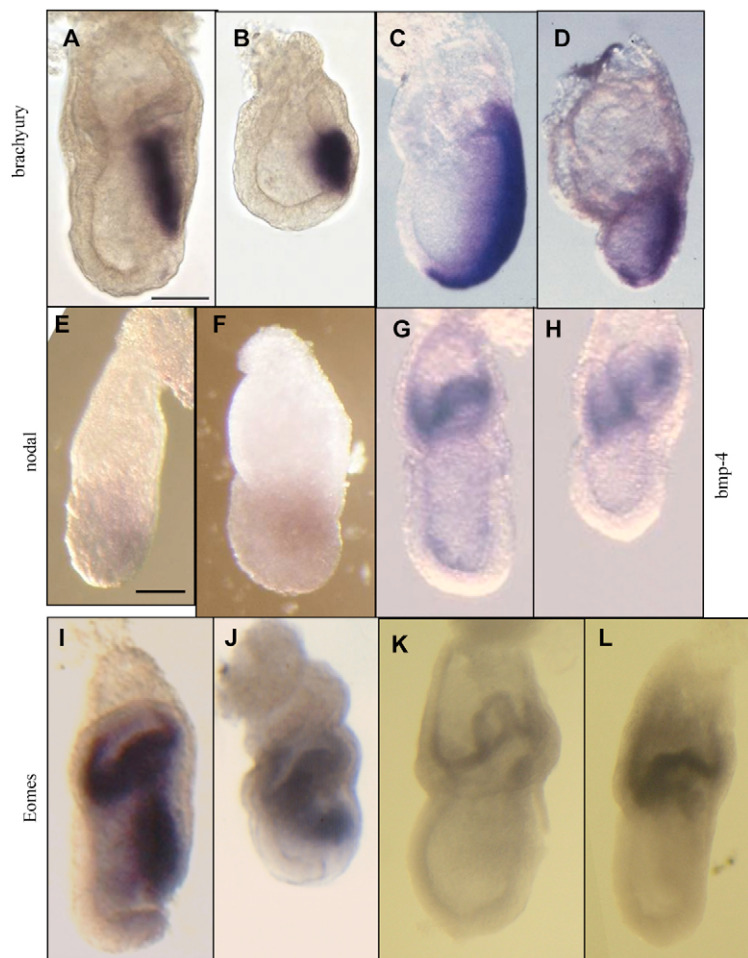
### Molecular pattern of gastrulation in NT embryos

Most of the defects described above did not prevent the initiation of gastrulation. The formation of the primitive streak and the patterning of the embryo require finely regulated crosstalk between the extra-embryonic ectoderm and adjacent epiblast (Ang and Constam, 2004; Brennan et al., 2001). *Nodal* in the epiblast, and *Cdx2*, *Eomes* and *Bmp4* in the extra-embryonic ectoderm are essential players in this process (Fujiwara et al., 2002; Russ et al., 2000; Strumpf et al., 2005; Vincent et al., 2003). *Brachyury*, a T-box transcription factor, is first expressed in the extra-embryonic ectoderm abutting the epiblast, then in the posterior epiblast where the primitive streak forms and finally in the primitive streak cells (Perea-Gomez et al., 2004; Thomas et al., 1998). The expression of this marker at the onset of gastrulation is indicative of the correct establishment of the

anteroposterior polarity. As an abnormal shape of the egg cylinder or a disproportionate extra-embryonic ectoderm may perturb this crosstalk and the establishment of morphogenetic gradients, we analysed the expression of these different genes by whole-mount in situ hybridisation in NT embryos displaying these different phenotypes (Fig. 5).

Embryos with abnormal shapes had normal spatial patterns of expression of *brachyury* (Fig. 5B) and *Eomes* (Fig. 5J). Embryos with the 'Exe' phenotype expressed *Nodal* (Fig. 5F) and *Bmp4* (Fig. 5H), as in controls. In the NT embryo expressing *Bmp4*, histological examination confirmed that the size of the posterior amniotic fold in the extra-embryonic region was indicative of a late-streak stage, whereas the elongation in the size of the streak was that of a mid-streak stage (data not shown). This discrepancy correlates the respective size of the extra-embryonic and embryonic regions of the conceptus in such NT embryos with the 'large Exe' phenotype. At the advanced stage, this developmental gap was even larger, as illustrated by the embryo in Fig. 5D. *Brachyury* was still expressed in the primitive streak of the reduced embryonic region. The presence of a fully expanded amniotic fold and an allantoic bud were features of a neural plate embryo, whereas the epiblast was still at late-streak stage (Downs and Davies, 1993). Localisation of *Cdx2* in the extra-embryonic ectoderm was correct in the different NT embryos analysed (Fig. 5L).

Thus, despite defects in growth and morphology, NT embryos displayed a pattern of gene expression that was correct in both the epiblast and extra-embryonic ectoderm. In the 'large Exe'



**Fig. 5. Molecular characterisation of NT embryos with abnormal morphologies.** Whole-mount in situ hybridisation showing the expression of *brachyury* (A-D), *Nodal* (E,F), *Bmp4* (G,H), *Eomes* (I,J) and *Cdx2* (K,L) in primitive-streak stage (A,B,E-L) and neural-plate stage (C,D) embryos. (A,C,E,G,I,K) Controls; (B,F,J) NT embryos with abnormal shape; (D,H) NT embryos displaying the 'large Exe' phenotype. Scale bars: in A, 100  $\mu$ m for A,B, 50  $\mu$ m for C,D; in E, 100  $\mu$ m for E-L.

**Table 4. Development of chimaeras generated by injection of ES cells into cloned blastocysts**

	Number transferred	Number of implantation sites	Number of embryos (% implanted)	Chimaeras	
				Total	Abnormal morphology (%)
Cont+ES E7	30	20	18 (72)	15 (83)	0
NT +ES E7-8	80	53	37 (70)	22 (59)	36

**Table 5. Mid-gestation and term development of ES chimaeras**

		Number of embryos transferred	Number of conceptus	Chimaeras	
				Alive (% chimaeras)	Resorbing (% chimaeras)
Cont+ES	E10	2	1	8	0
	E19	101	3	18	NA
NT+ES	E10	10	2	7	9
	E19	57	5	1 (50%)	1 (50%)

NA, not applicable. Natural delivery occurs for fosters transferred with control chimaeras.

phenotype, there was a discrepancy in the advancement of gastrulation between the epiblast and the extra-embryonic ectoderm, with the latter being later than the former.

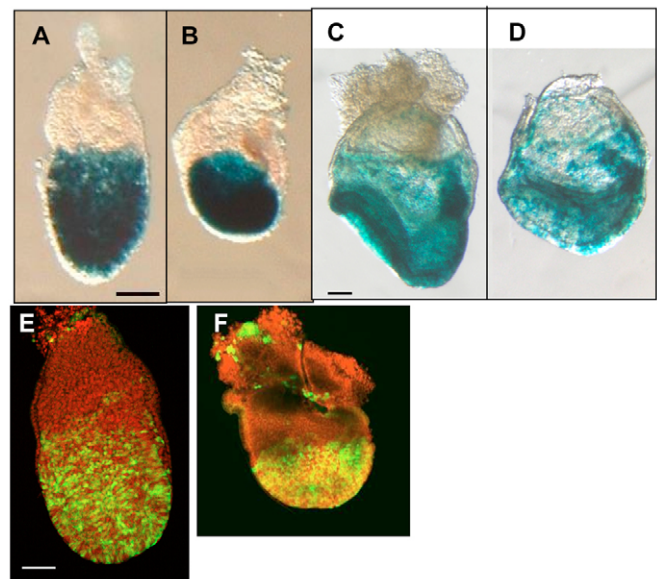
### Normal ES or ICM cells do not rescue the developmental defects of NT embryos

The abnormal phenotypes that we characterised during the gastrulation of NT embryos perhaps resulted from defective functioning of the extra-embryonic or embryonic lineages (or both). Such defects, when occurring in the epiblast, can be remedied by injecting normal ES cells into NT blastocysts. In the resulting chimaeras, ES cells incorporate in the ICM and contribute mainly to epiblast derivatives (Beddington and Robertson, 1989). To confirm the contribution of ES cells to the embryo, we used R26.6 ES cells that constitutively expressed the reporter *lacZ* gene (Friedrich and Soriano, 1993). Injected blastocysts were transferred into recipients and conceptuses recovered at different stages (Tables 4, 5; Fig. 6A–D).

At E7–8, the rate of embryos recovered from the implantation sites (70%) was significantly higher (*t*-test,  $P < 0.025$ ) than with non-injected NT blastocysts (40%, see Table 2). Peri-implantation lethality was thus rescued, indicative of a non-cell autonomous defect in the epiblast of the NT embryos that died before E7. At all developmental stages examined *lacZ*-expressing cells were found in all epiblast-derived tissues intermingled with clone-derived unstained cells. However, chimaeras displaying abnormal morphology were found at a similar rate as in non-injected NT embryos (see Tables 3 and 4). The same types of abnormalities were observed between chimaeric and non-chimaeric conceptuses, as shown in Fig. 6B (abnormal shape) and Fig. 6D ('large Exe' phenotype). Observation of pups at mid-gestation (E10) and term (E19) further confirmed the absence of rescue by the injected ES cells: the rate of live foetuses was similar to that of implanted non-injected NT blastocysts (Table 5, compare with Table 1). These experiments revealed that the rescue was only partial, suggesting that the extra-embryonic tissues were deficient in the NT conceptuses.

Extra-embryonic tissues are composed of the trophoblast and the primitive endoderm. Alteration in the latter could have negative consequences on the growth of the embryo, because at early stages it provides the embryo with nutrients (Bielinska et al., 1999). As ICM cell derivatives are found in both the epiblast and the primitive endoderm, we conducted chimaera experiments by

injecting GFP-expressing ICM cells from fertilised embryos into control or NT blastocysts (Table 6). The epiblast, its derivatives and the visceral endoderm were found to be chimaeric (Fig. 6E). As for injected ES cells, a partial rescue was observed, with a high rate of embryos found in the deciduas at E7. The same morphological abnormalities were, however, observed at similar rates (Table 6; Fig. 6F). Altogether, these results refute a primary source of abnormalities in the epiblast or the visceral endoderm, and point to the extra-embryonic ectoderm lineage as the defective tissue.



**Fig. 6. Developmental defects at E7 are not rescued by incorporation of normal cells into the epiblast and the visceral endoderm.** (A,D) Chimaeras made by injection of normal *lacZ*-expressing ES cells into blastocysts. Normal NT chimaeras at early-streak (A) and headfold (C) stages. (B) NT chimaera with an abnormal shape; (D) NT chimaera with a 'large Exe' phenotype. (E,F) Early-streak stage chimaeras made by injection of normal GFP-expressing ICM cells into NT blastocysts. The chimaera in F displays a 'large Exe' phenotype (as well as an abnormal shape), in comparison with the one in E. The nuclei are stained in red by propidium iodide. Cytoplasmic GFP appears green. Scale bars: 100  $\mu$ m.

**Table 6. Development to E7 of chimaeras generated by injection of ICM cells into blastocysts**

	Number transferred	Number of implantation sites	Number of embryos (% implanted)	Number of chimaeras (%)	Number of abnormal non chimaeras (%)	Number of abnormal chimaeras (%)
Controls	46	42	31 (74)	18 (58)	1 (8)	2 (11)
NT	75	43	31 (72)	13 (42)	10 (56)	6 (46)

**Table 7. Development to E7 of aggregation chimaeras (2n embryo + 4n GFP-expressing embryo)**

	Number of implantation sites (%)	Number of embryos recovered			Number of normal embryos with epiblast from 2n embryo only (%)		
		Total (% implanted)	GFP 4n embryo	Non-GFP 2n embryo	Chimaeras	With GFP cells in Exe	±GFP cells in VE
Controls	36 (46)	24 (67)	1	11	12	4 (100)	11 (92)
NT	82 (42)	42 (51)	13	8	21	10 (91)	7 (64)

Exe, extra-embryonic ectoderm.  
VE, visceral endoderm.

### Tetraploid cells in the extra-embryonic ectoderm can rescue the development of NT embryos at E7

We investigated the development of NT-derived epiblast cells when associated with a normal trophoblast in the same embryo. To do so, eight-cell stage NT or control embryos were aggregated with four-cell stage tetraploid, GFP-expressing embryos. We used this approach because diploid cells have a strong tendency to colonise the epiblast and its derivatives preferentially, and, by contrast, tetraploid cells are restricted to the extra-embryonic tissues (for a review, see Eakin and Behringer, 2003). We checked for the presence of both types of cell (GFP positive and negative) in the chimaeric embryos at morula stage before transferring them into fosters. Upon recovery at E7, we found half of the implanted embryos of each group (control and NT) to be chimaeric (Table 7). Only a subset of these chimaeras, however, had the expected contribution of GFP-expressing, tetraploid cells in the extra-embryonic ectoderm. Most of them had normal morphology,

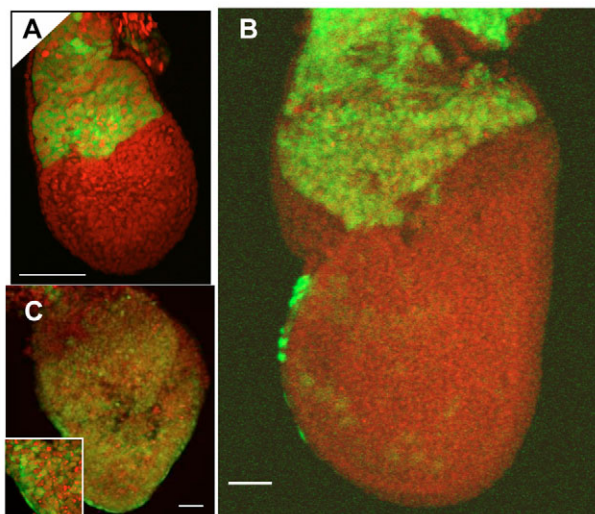
suggesting that the tetraploid extra-embryonic cells remedied the defects (Fig. 7A,B; Table 7). In some of the embryos, the presence of non GFP-expressing, extra-embryonic ectoderm cells indicated that NT derived cells had not been overcome by the tetraploid cells.

We also noticed that tetraploid embryos were capable of developing without the diploid partner, although all of them were abnormal (Fig. 7C). Such embryos were found more numerous in the NT group (62%, 13/21) than in the control one (8%). Altogether, these results indicate that the tetraploid extra-embryonic ectoderm cells have compensated for the defects arising at gastrulation, but not for the earlier ICM defects.

### DISCUSSION

In the present study, we investigate the developmental fate of reprogrammed nuclei following their transfer into recipient enucleated oocytes (i.e. cloning). It is usually admitted that failures in the development or function of the placenta are the main causes of foetal and peri-natal death of NT embryos (Wilmut et al., 2002). However, few if any results have been provided about the first stages and tissues which are affected during development of NT embryos. Here, we provide the first detailed analysis of the post-implantation development of NT embryos.

We have chosen ES cells as donors for two reasons: first, a high rate of blastocysts with both a normal morphology and allocation of cells to the first two lineages can be obtained in an easy and reproducible manner (Zhou et al., 2001), which is not the case for somatic cells; second, ES-cell derived embryos faithfully recapitulate the expression of Oct4 and related genes (Bortvin et al., 2003). In the present study, we have confirmed that all ICM cells of ES-cell derived blastocysts express the protein Oct4 and we have shown, using an in vitro outgrowth assay, that they can proliferate very similarly to controls. After transfer into foster mice, the rate of implantation of NT embryos is high but half of them resorb completely before E7. This result further confirms a previous study which showed that the pattern of expression of Oct4 is not a reliable indicator of developmental competence in vivo (Boiani et al., 2005). We have shown that this peri-implantation lethality could, however, be rescued partially by the injection of wild-type ES or ICM cells into NT blastocysts, as we found a higher number of deciduas containing embryos at the time of dissection (E7). As trophoblast proliferation is an essential process for the initiation of implantation and is controlled by the ICM (Chai et al., 1998; Copp, 1978), our results are indicative of a lack of production of some trophic factors by the embryonic compartment (the ICM) of



**Fig. 7. Tetraploid cells can rescue abnormal morphologies of NT embryos at E7. (A,B)** Normal chimaeras made by aggregation of a NT embryo and a tetraploid, GFP-expressing, embryo developed from a fertilised zygote. **(C)** Tetraploid embryo developed after disappearance of NT blastomeres. The embryo in A is at early streak stage; the embryo in B is at late-streak stage. (Inset) Higher magnification of C showing that all epiblast cells express GFP. Scale bars: 100  $\mu$ m.



the NT embryos. No such proliferation defect was observed in the *in vitro* outgrowth assays, indicating some compensation by similar growth factors already present in the serum-containing medium. We propose that a non-cell autonomous defect arising in the early epiblast lineage of NT embryos is mainly responsible for the first peak of embryonic resorption occurring around the time of implantation and before E7.

Once implanted, a fraction of the NT embryos exhibit a marked delay in their growth, and their epiblast is severely compromised, as shown by the simultaneous absence of expression of the key markers *Oct4* and *Nodal*. By contrast, their visceral endoderm shows some signs of differentiation. These embryos can be considered as the remnants of those with a proliferation defect in the ICM. The remaining ones gastrulate, and the expression profile of a panel of gastrulation markers indicates no obvious impairment of differentiation in the epiblast-derived tissues. This observation is in agreement with the ability to derive and differentiate ES cells *in vitro* from NT blastocysts (Munsie et al., 2000; Wakayama et al., 2001). Consistently, the NT embryo-derived cells are not excluded from the embryonic lineages after the injection of normal ES or ICM cells into NT blastocysts and formation of the chimaeric epiblast. However, and in spite of an apparently normal initiation of gastrulation, anomalies in the growth of the conceptus rapidly appear. The alteration in the ratio of extra-embryonic/embryonic compartment sizes, which characterises a group of implanted but obviously abnormal NT embryos ('large Exe' phenotype) is not remedied by the inclusion of wild-type ES or ICM cells. By contrast, during normal development, the growth of the conceptus is tightly regulated between the two compartments during the rapid expansion of the egg cylinder (Downs and Davies, 1993; Lawson et al., 1991). As development proceeds, abnormal growth of the trophoblast of NT embryos is consistently observed during the foetal stages, leading to placentomegaly (Fig. 2). In contrast to normal development (Iguchi et al., 1993), NT placentas continue to grow after E15 and some placentas even keep growing after the disappearance of the foetus. We suggest that these conceptuses that contain only trophoblast tissue come from embryos with the 'large Exe' phenotype. Trophoblast stem cells are thought to give rise to all the differentiated trophoblast derivatives of the placenta (Tanaka et al., 1998), and Uy and Gardner (Uy and Gardner, 2002) have shown that only a few stem cells reside in the extra-embryonic ectoderm *in vivo*. An increased proliferating activity of this small compartment of trophoblast stem cells could be sufficient to give the trophoblast a growth advantage in the NT embryos with the 'large Exe' phenotype. The growth characteristics of the trophoblast stem cell of NT embryos are under investigation. Preliminary data indicate that they have the ability to grow faster than controls during the early culture period. Placentomegaly is a consistent feature of the development of NT embryos, and has been observed both in ES and somatic NT embryos (Suemizu et al., 2003; Tanaka et al., 2001). Our ongoing histological analysis of the ES NT-derived placenta shows several similarities with that of somatic cell derived placentas (Tanaka et al., 2001). Moreover, the comparison of the transcriptome of NT placentas revealed that more than 70% of the deregulated genes were common to both ES and somatic cell NT placentas (Humpherys et al., 2002). This suggests that the placental defects observed after both somatic cell- and ES-derived NT have a common origin in the trophoblast stem cell lineage.

The development of diploid/tetraploid aggregates to E7 indicates that the first abnormal morphological phenotypes arising in NT embryos are rescued by the presence of tetraploid extra-embryonic ectoderm cells. The use of tetraploid cells in the trophoblast would

be useful in more deeply evaluating the developmental potential of the epiblast derivatives themselves at later (foetal) stages of the development of NT embryos.

Changes in the shape of the embryo should modify the establishment of morphogenetic gradients and the crosstalk between the epiblast and the extra-embryonic ectoderm. The robustness of developmental patterning (Eldar et al., 2004) helps them to develop further, and we have thus observed that they can initiate gastrulation. However, as only half of the remaining foetuses are still alive at E10, we suggest that they correspond to those with normal phenotypes at E7. The rest are either degenerated (those that do not gastrulate) or dead (those with morphological or growth defects).

Several recent studies have unveiled multiple roles of the crosstalk between the extra-embryonic tissues and the epiblast in regulating their growth and differentiation (Donnison et al., 2005; Guzman-Ayala et al., 2004; Rodriguez et al., 2005; Strumpf et al., 2005). In this regard, our analysis of the development after nuclear transfer reveals a new aspect of this crosstalk: it appears that the extra-embryonic ectoderm somehow controls the harmonious growth and shape of the whole embryo and the relative number of cells in both regions. This finding is in agreement with the analysis of the transcriptome of extra-embryonic tissues from normal embryos, which revealed that a lot of genes expressed specifically at E7.5 are cell cycle regulators and growth factors (Hemberger et al., 2001). We therefore hypothesise that the analysis of the transcriptome of this tissue in NT embryos will help to confirm these new functions for the extra-embryonic ectoderm.

Altogether, our results indicate that the epiblast is either not affected or affected merely in its role of sustaining trophoblast proliferation. By contrast, the trophoblast lineage rapidly becomes abnormal, leading to severe deregulation of the growth of the embryo. As suggested by Boiani and colleagues (Boiani et al., 2005), the reprogramming process may follow different paths in the two lineages. At least one epigenetic process, the remethylation of the genome that occurs soon after the blastocyst stage, is indeed different in the two regions of the conceptus (Monk et al., 1987; Santos et al., 2002). The result is an asymmetric pattern of methylation, with the trophoblast lineage remaining hypomethylated in comparison with the epiblast. Recent results in cattle show that, despite their low developmental potential *in vivo*, NT blastocysts closely resemble *in vivo*, but not *in vitro*, fertilised embryos in term of their global patterns of expressed genes (Smith et al., 2005). This also suggests that reprogramming errors mainly affecting the trophoblast lineage must arise during the epigenetic modifications after the blastocyst stage, and supports the view that reprogramming should be viewed essentially as a multistep process.

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