Differential reprogramming of somatic cell nuclei after transfer into mouse cleavage stage blastomeres

Sigrid Eckardt, N Adrian Leu, Satoshi Kurosaka and K John McLaughlin

Center for Animal Transgenesis and Germ Cell Research, New Bolton Center, University of Pennsylvania, Kennett Square, Pennsylvania 19348, USA

Correspondence should be addressed to K J McLaughlin; Email: kjmclaugh@vet.upenn.edu

Abstract

Mammalian somatic cell cloning requires factors specific to the oocyte for reprogramming to succeed. This does not exclude that reprogramming continues during the zygote and cleavage stages. The capacity or role of zygotic and cleavage stages to reprogram somatic cell nuclei is difficult to assess due to the limited development of somatic cell nuclei transplanted into cytoplasts of these stages. Alternatively, tetraploid embryos have been used to study reprogramming and can be assessed for their contribution to extra-embryonic lineages. When mouse cumulus cell nuclei transgenic for Oct4-green fluorescent protein (GFP) were injected into intact two- and four-cell stage blastomeres, manipulated embryos developed into blastocysts with expression of Oct4-GFP as observed in embryos produced by nuclear transfer into metaphase II oocytes. However, only the latter contributed to extra-embryonic tissues in day 10.5 conceptuses, with the exclusion of the somatic genome in cells originating from transfer into blastomeres already at 5.5 days post conception. Somatic nuclei transferred into cleavage stage blastomeres reinitiated expression of an embryonic-specific transgene, but lacked the extent of reprogramming required for contribution to post-implantation development, even when complemented by an embryonic genome.

Reproduction (2005) **129** 547–556

Introduction

Transfer of a mammalian somatic cell nucleus into the enucleated cytoplasm of a metaphase II (MII) stage oocyte has resulted in development to term and adulthood. The underlying mechanisms that mediate the transition of nuclear function from that of a somatic to a totipotent cell have been summarized as reprogramming. Successful cloning of mammals from somatic cell nuclei apparently depends on factors present in the mature mammalian oocyte. Clones produced with later stage recipient cytoplasm, such as the zygotic cytoplasm, do not develop (McGrath & Solter 1984, Robl *et al.* 1987, Wakayama *et al.* 2000), suggesting that reprogramming of somatic nuclei is either limited to or must be initiated in the ooplasm. This is consistent with events that occur in normal development, where decondensation and reorganization of both male and female gametic chromatin critically depend on maternal factors stored in the oocyte (Burns *et al.* 2003, Wu *et al.* 2003). It is therefore probable that ooplasm-specific factors induce epigenetic changes not only onto gametic chromatin but also onto that of an introduced somatic nucleus.

Although during normal development remodeling of the parental genomes is initiated in the oocyte, it

continues during preimplantation development, including processes such as differential demethylation of the two parental genomes (Monk *et al.* 1987, Rougier *et al.* 1998, Mayer *et al.* 2000, Oswald *et al.* 2000). As late as implantation, major genome-wide epigenetic alterations are still ongoing, as the largely demethylated parental genomes are *de novo* methylated (Dean *et al.* 2001, 2003). In somatic cell clones, it remains unclear whether reprogramming is an all or none process or whether it is a dynamic process initiated by oocyte factors but continuing during development (Jouneau & Renard 2003). Continuation of reprogramming during cleavage stages may either be mediated by maternal factors remaining from the ooplasm or via novel gene expression from a partially reprogrammed somatic nucleus. Observations such as mosaic gene expression in murine and porcine blastocyst stage clones could indicate either differential reprogramming between blastomeres or a differential failure of gene expression (Boiani *et al.* 2002, Park *et al.* 2002). Differential reprogramming between blastomeres would require that modifications occur subsequent to the first cleavage. Reprogramming can be interpreted by several criteria including epigenetic changes, gene expression and development. Assessment of the reprogramming capacity of cleavage stage blastomeres could

in theory be tested by transplantation of a somatic cell nucleus into an enucleated cytoplasm. This approach, however, results in little or no cleavage subsequent to nuclear transplantation, including using nuclei from pre-implantation stages (Tsunoda *et al.* 1987, Solter 2000, Wakayama *et al.* 2000). Alternatively, the ability of cytoplasm other than the oocyte to reprogram somatic cell nuclei has been extensively tested by fusing somatic cell nuclei with intact recipient cells (Tada *et al.* 1997, 2001, Takagi 1997). To determine whether reprogramming of a somatic genome can occur at stages subsequent to the MII oocyte, we transferred mouse somatic nuclei into blastomeres of murine cleavage stage embryos and observed expression of an Oct4-green fluorescent protein (GFP) transgene from the somatic genome during preimplantation development. The ability of the somatic genome in somatic-embryonic tetraploid cells to contribute to post-implantation development was determined in chimeras with diploid embryonic cells.

Materials and Methods

Embryo culture

Unless stated otherwise, micromanipulated and control embryos were cultured in groups of 30 in 20 µl drops of α -minimum essential medium (α -MEM) medium (Sigma, St Louis, MO, USA) placed in 35 mm dishes (Corning, NY, USA). α -MEM was supplemented with bovine serum albumin (BSA; 0.4% (w/v); ICN, Aurora, OH, USA) and filtered on an acetate membrane (0.22 µm; Fisher, Pittsburgh, PA, USA; prewashed with medium) prior to use. Culture drops were overlaid with silicon oil (5 centistokes; Sigma) and incubated in an atmosphere of 5% CO₂ in air at 37°C. An additional 10 µl α -MEM was added to the original culture drop 24 h after micromanipulation. Embryos were assessed at 96 h for blastocyst formation and expression of GFP.

Recipient embryo and donor nucleus collection

Eight- to ten-week old C57Bl/6J $\times$ C3H/HeN female mice (Taconic, Germantown, NY, USA; referred to as B6C3F1) were superovulated with 7.5 U pregnant mare serum gonadotropin (Calbiochem, San Diego, CA, USA) followed 48 h later by 7.5 U human chorionic gonadotropin (hCG; Sioux, Sioux Center, IA, USA). Females were mated to ICR males. Cumulus–oocyte complexes were collected 15 h post-hCG and cumulus cells removed by hyaluronidase (ICN; activity > 5000 IU/mg) at 50 U/ml in HEPES-buffered CZB medium (Chatot *et al.* 1989) at 27°C. The HEPES-buffered CZB medium was modified as follows – 5.56 mM glucose, 5 mM HEPES, 20 mM sodium bicarbonate, 0.1% (w/v) polyvinylpyrrolidone (PVP; 40 kDa; Calbiochem), no albumin – in the presence of protease inhibitors (Sigma; 0.2% (v/v)). After 15–20 min, the cumulus-free oocytes were washed three times in HEPES-buffered CZB medium and then placed in α -MEM culture

medium (see section on Embryo culture). Two-cell stage embryos were collected from the oviducts at 40 h post-hCG. For manipulation at the four-cell stage, embryos were collected 48 h post-hCG from the oviduct and cultured *in vitro* until 90% of the embryos completed the second cleavage. Cumulus cell donor nuclei were isolated from the cumulus–oocyte complexes of superovulated female mice transgenic for Oct4-GFP (transgenic strain referred to as OG2; Szabo *et al.* 2002) or transgenic for ubiquitous GFP (Okabe *et al.* 1997) to visualize gene expression from the somatic nucleus after nuclear transfer. For analysis of contribution to post-implantation development, donor cumulus cells were also derived from C57Bl/6J $\times$ DBA2 F1 mice (referred to as B6D2F1). Cumulus cells were stored at 4°C after adding an equal volume of α -MEM to the hyaluronidase drop.

Animals were maintained and used for experimentation according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Micromanipulation

Enucleation of cleavage stage blastomeres was performed in HEPES-buffered CZB medium (PVP, 40 kDa, 0.1% (w/v); albumin free) in the presence of cytochalasin B (5 µg/ml from ICN) and nocodazole (0.3 µg/ml from Sigma). Blastomere nuclei were removed by using a piezo-driven (PMM 150 FU; PrimeTech, Tsuchiura, Japan) borosilicate enucleation needle (~15 µm), and embryos were left to recover in α -MEM medium for 1–2 h at 37°C with several passages through fresh drops of medium. Somatic cell nuclear transplantation was done in hypertonic (110%) HEPES-buffered CZB medium (supplemented as above with PVP 1% (w/v)). Cumulus cell nuclei were transferred using piezo-driven borosilicate needles as described above. Embryos were processed in batches of ten within a 10-min time window, and left to recover for 1 h at 27°C in a 1:1 part mixture of hypertonic (110%) HEPES-buffered CZB medium (PVP 1% (w/v)) and α -MEM medium prior to embryo culture. In experiments where embryonic karyoplasts were fused to blastomeres, or blastomeres fused together, embryos were exposed to a 1.1 kV/cm DC pulse of 40 µs duration in HEPES-buffered CZB medium (PVP, 40 kDa, 0.1% (w/v); albumin free).

Chimeric diploid-tetraploid embryos resulting from somatic cell nuclear transfer into MII oocytes were produced as follows. Cumulus cell nuclei were transferred into MII oocytes using piezo-driven injection needles (~7 µm) in hypertonic (110%) HEPES-buffered CZB medium (PVP 1% (w/v)). Injected oocytes were left to recover in a 1:1 part mixture of hypertonic (110%) HEPES-buffered CZB medium (PVP 1% (w/v)) and α -MEM medium for 1 h at 27°C prior to incubation in α -MEM medium at 37°C. One to two hours later, they were activated for 6 h in modified (Ca free, 10 mM SrCl₂) M16 medium, supplemented with BSA (0.4% (w/v); ICN), vitamins (1 $\times$; Sigma) in the presence of cytochalasin B (5 µg/ml from

ICN; prepared in dimethyl sulfoxide as a 200× stock solution). The presence of cytochalasin B was required to prevent extrusion of a pseudo polar body. The resulting tetraploid embryos were cultured to the late two-cell stage, and one tetraploid karyoplast was fused to a developmentally synchronous, enucleated blastomere of a B6C3F1 × ICR two-cell stage embryo.

Hoechst staining of embryos

Embryos were stained with Hoechst 3342 (or 33258) (Sigma; 10 µg/ml in α-MEM) for 5 min, then washed in HEPES-buffered CZB medium and mounted in microdrops for photography.

Detection of DNA Synthesis

Subsequent to somatic cell nuclear transfer into one blastomere (at 42 h post-hCG), two-cell stage embryos were incubated with 5-bromo-2'-deoxyuridine (BrdU; BrdU Cell Proliferation Assay Kit; Calbiochem; 1:1000 dilution of supplied stock in α-MEM) for 6 h, washed briefly with α-MEM and treated with acidic Tyrode's solution to remove the zona pellucida. Zona-free embryos were placed on glass coverslips, air dried, fixed in 100% methanol for 20 min at −20°C and post-fixed/denatured with fixative/denaturing solution (BrdU Cell Proliferation Assay Kit) for 15 min at room temperature. Incubation with anti-BrdU antibody (1:100 dilution in antibody diluent; BrdU Cell Proliferation Kit) was performed overnight at 4°C. Coverslips were washed once with phosphate-buffered serum (PBS), incubated with anti-mouse IgG FITC conjugate (F-8771; Sigma; diluted at 1:50 in PBS) for 4–6 h at 4°C and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Metaphase spreads of blastocysts

Chromosome preparations of morulae and blastocyst stage embryos were performed by the air-drying method as described (Tarkowski 1966), with minor modifications. Blastocyst stage embryos were processed without addition of inhibitors to culture media, and slides were stained in 3% Gurr's Giemsa (improved Gurr's Giemsa R66; BDH, Carle Place, NY, USA).

Embryonic outgrowths

Blastocyst stage embryos were placed on a confluent layer of mitomycin C-inactivated feeder fibroblasts (neomycin resistant, leukemia inhibitory factor expressing mouse fibroblasts) in 48-well plates (Falcon Becton Dickinson, Franklin Lakes, NJ, USA). Feeder cells and embryos (outgrowths) were cultured in Dulbecco's minimum Eagles' medium (Speciality Media SLM-220B; 4.5 g/l glucose supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.5 mM β-mercaptoethanol, 14% fetal bovine serum (HyClone, Logan, UH, USA) and 50 U/ml penicillin–streptomycin). Outgrowth formation was

defined by the spreading of trophoblast cells from the attached blastocyst.

In vivo development of embryos

Four-cell stage embryos were transferred into the oviducts of ICR females (Taconic) that were pseudopregnant as determined by the presence of a copulatory plug subsequent to mating with vasectomized ICR males, on the day of plug detection (0.5 days post coitum (d.p.c.)). At 10.5 d.p.c., females with a weight increase consistent with a pregnancy were killed. Fetuses were recovered, and tissue samples from fetus, placenta and yolk sac removed for glucose phosphate isomerase (GPI-1) isozyme gel electrophoresis. Fertilized embryos were homozygous for the allele encoding the B electrophoretic form of GPI-1, whereas somatic nuclei were either heterozygous for B and C alleles or for A and B alleles. This allowed assessment of the level of chimerism by GPI-1 isozyme electrophoresis (Nagy & Rossant 1993, Hogan 1994).

Results

Experimental design: somatic cell nuclear transfer into cleavage stage blastomeres

To assess whether preimplantation stage-specific gene expression occurs from somatic nuclei when transplanted into cleavage stage blastomeres, we transferred mouse cumulus cell nuclei into the cytoplasm of murine cleavage stage blastomeres and monitored expression of the preimplantation stage-specific Oct4-GFP transgene from the somatic genome. Oct4-GFP transgene expression parallels that of the germline-specific transcription factor Oct4 (Yoshimizu *et al.* 1999). Oct4 is silent in somatic cells, but expressed during preimplantation development and in the germ lineage (Pesce *et al.* 1998). In embryos developing from oocytes fertilized with Oct4-GFP transgenic sperm, Oct4-GFP expression is typically observed subsequent to the four-cell stage (weak expression at the eight-cell stage), is upregulated upon compaction, ubiquitous in the morula, becomes inner cell mass (ICM) restricted in the late blastocyst, and has been used as a marker of reprogramming in mouse somatic cell cloning (Boiani *et al.* 2002, 2003).

Oct4-GFP expression from somatic nuclei after transfer into intact blastomeres

A single cumulus cell nucleus was transplanted into one blastomere of a late two-cell (Fig. 1a), four-cell (Fig. 1b) and eight-cell stage mouse embryo (Fig. 1c). As a control for Oct4-GFP expression, a nucleus from an Oct4-GFP transgenic two-cell embryo was transferred into one blastomere of a developmentally synchronous non-transgenic two-cell stage embryo (Fig. 1, control). To compare with gene re-expression in MII oocyte recipients, individual cumulus cells were also transferred into intact MII oocytes

Embryo		Cultured n	M/B n (%)	Oct4-GFP positive n (%)
ctrl		51	43 (84)	43 (100)
1a		328	310 (95)	227 (73)
1b		206	190 (92)	129 (68)
1c		46	46 (100)	21 (46)
1d		40	29 (79)	29 (100)
1e		75	66 (88)	52 (79)

Figure 1 Preimplantation development and Oct4-GFP expression in embryos subsequent to somatic cell nuclear transfer into cleavage blastomeres or MII oocytes. M/B = morula/blastocyst stage; ctrl = control, transfer of nucleus from two-cell stage Oct4-GFP transgenic embryo into blastomere of synchronous non-transgenic embryo. (a–c) Transfer of Oct4-GFP transgenic cumulus cell nucleus into two-, four- and eight-cell blastomeres of non-transgenic embryos. #Data for four-cell embryos include three different manipulation techniques that had similar outcomes and are summarized: transfer of one cumulus cell nucleus into one of four blastomeres (depicted: $n = 13$, 13/13 M/B (100%), seven GFP-positive (54%)), transfer of one cumulus cell each into two of four blastomeres ($n = 84$, 80/84 M/B (95%), 57 GFP-positive (71%)) and fusion of two of four blastomeres, removal of one of two embryonic nuclei from fused blastomeres and transfer of one cumulus cell nucleus ($n = 66$, 59/66 M/B (89%), 39 GFP-positive (66%)). (d) Transfer of Oct4-GFP transgenic cumulus cell nucleus into MII oocyte; (e) same as (d), when developed to the two-cell stage, transfer of nucleus into enucleated blastomere of synchronous non-transgenic embryo.

(Fig. 1d and e), and the resulting embryos either left intact as tetraploid embryo (Fig. 1d) or, at the two-cell stage, one nucleus was transferred to an enucleated blastomere of a synchronous embryo to produce a chimeric embryo comparable with group 1a. The majority of two-cell stage embryos receiving a cumulus cell nucleus expressed Oct4-GFP at the blastocyst stage (Fig. 1a, 73% and Fig. 2a). Similarly, 68% of embryos receiving cumulus cell nucleus transplants at the four-cell stage were positive for Oct4-GFP expressed from the somatic genome (Fig. 1b and Fig. 2b). The frequency of Oct4-GFP expression in blastocysts resulting from cleavage stage nuclear transfer was similar to that of chimeric blastocysts produced by cumulus cell nuclear transfer into MII oocytes (Fig. 1e and Fig. 2e). All control blastocysts (Fig. 1, control and Fig. 2f), and those resulting from MII recipients (Fig. 1d and Fig. 2d) expressed the Oct4 transgene. When somatic cell nuclei were introduced into eight-cell stage blastomeres, expression of Oct4-GFP at the blastocyst stage was observed in a substantial proportion

of embryos although less frequently than at the two- and four-cell stages (Fig. 1c, 46% and Fig. 2c). Lysis of blastomeres occurred very frequently after somatic cell nuclear transfer into eight-cell stage blastomeres and possibly contributed to the lower proportion of GFP-positive blastocysts, even though embryos with any indication of lysis were carefully excluded. In a parallel series of experiments, mouse cumulus cell nuclei from Oct4-GFP transgenic mice were transferred into enucleated blastomeres of non-transgenic (B6C3F1 $\times$ ICR) embryos at the late two-cell and at the four-cell stage. Transfer into late two-cell cytoplasm was performed on one of two blastomeres (Fig. 3a). To facilitate manipulation of embryos at the four-cell stage, two four-cell stage blastomeres were fused and subsequently enucleated (Fig. 3b). The resulting embryos (Fig. 3a and b) were chimeric, with one blastomere containing only the injected somatic nucleus. As a control for effects of manipulation, a karyoplast containing an embryonic two-cell stage nucleus from an Oct4-GFP transgenic embryo

Figure 2 Blastocyst stage chimeric embryos resulting from somatic cell nuclear transfer into intact cleavage stage blastomeres (a–c), from somatic cell nuclear transfer into MII oocytes (d and e) and from exchange of embryonic nuclei (f). In (a–e), GFP fluorescence indicates Oct4-GFP expression from the transplanted somatic genome. (a) Blastocysts resulting from transfer of somatic nucleus into two-cell stage blastomeres (Fig. 1a). (b) Blastocysts resulting from transfer of somatic nucleus into four-cell stage blastomeres (Fig. 1b). (c) Blastocysts resulting from transfer of somatic nuclei into eight-cell stage blastomeres (Fig. 1c). (d) Blastocysts resulting from somatic cell nuclear transfer into MII oocytes (Fig. 1d). (e) Same as (d), when developed to the two-cell stage, transfer of nucleus into one enucleated blastomere of synchronous non-transgenic embryo (Fig. 1e). (f) Control, transfer of Oct4-GFP transgenic two-cell stage nucleus into blastomere of synchronous non-transgenic embryo.

was transplanted into one enucleated blastomere of a developmentally synchronous two-cell stage embryo (Fig. 3, control). All blastocysts developing from control chimeras produced by the exchange of embryonic two-cell stage nuclei expressed Oct4-GFP in a mosaic pattern, consistent with a contribution from both two-cell stage blastomeres in subsequent preimplantation development (Fig. 3, control). The blastomere constructs of cumulus cell nuclei in enucleated late two-cell stage cytoplasm (Fig. 3a) or fused four-cell cytoplasm (Fig. 3b) invariably

failed to cleave after transplantation of a cumulus cell nucleus, as observed by the presence of a large, non-cleaving blastomere in subsequent stages that was often still visible at the blastocyst stage, peripherally attached to the embryo. The electrofusion treatment of blastomeres did not contribute to developmental arrest since the rate

Embryo		Cultured n	M/B n (%)	Oct4-GFP positive n (%)
ctrl		23	21 (91)	21 (100)
3a		72	61 (85)	1*
3b		40	35 (88)	1*

● somatic cell nucleus, Oct4-GFP transgenic ○ blastomere nucleus
 ● blastomere nucleus, Oct4-GFP transgenic

Figure 3 Development and Oct4-GFP expression in embryos subsequent to somatic cell nuclear transfer into enucleated cleavage stage blastomeres. M/B = morula/blastocyst stage; ctrl = control, transfer of Oct4-GFP transgenic two-cell stage nucleus into enucleated blastomere of synchronous non-transgenic embryo. (a and b) Transfer of Oct4-GFP transgenic cumulus cell nucleus into enucleated two- and four-cell blastomeres of non-transgenic embryos. The asterisks indicate two embryos in which Oct 4 gene expression from the somatic nucleus was observed in the uncleaved, manipulated blastomere when the embryos were at the blastocyst stage.

Figure 4 Oct4-GFP expression in embryos subsequent to somatic cell nuclear transfer into enucleated cleavage stage blastomeres and onset of Oct4-GFP in embryos after somatic nuclear transfer into intact two-cell stage blastomeres. (a and b) Expression of Oct4-GFP at the blastocyst stage in arrested blastomere after transfer of Oct4-GFP transgenic cumulus cell nucleus into enucleated two- (a) and four-cell (b) blastomeres of non-transgenic embryos. (c–e) Oct4-GFP expression in somatic-embryonic hybrid cells of eight-cell stage blastomeres, after somatic nuclear transfer into intact two-cell stage blastomeres.

of blastocyst formation of control embryos in which two two-cell stage blastomeres were fused and one nucleus removed subsequently did not differ from unmanipulated embryos (data not shown). In two manipulated embryos, Oct4 gene expression from the somatic nucleus commenced at the same time as in control transgenic embryos (asterisks in Fig. 3a and b and Fig. 4a and b), suggesting that cytoplasmic factors can initiate embryonic gene expression from the somatic cell nucleus.

Temporal regulation of Oct4-GFP expression in nuclear transfer embryos

In chimeric embryos receiving Oct4-GFP transgenic cumulus cell nuclei at the two-cell stage, temporal regulation of Oct4-GFP expression was similar to control manipulated embryos (Fig. 1a and control and Fig. 3, control): Weak Oct4-GFP signal was observed in blastomeres at the eight-cell stage (Fig. 4c–e), and strong signal in cells of morula and blastocyst stage embryos. GFP signal in nuclear transfer embryos was weaker than in control embryos, and fewer cells expressed the transgene (in control chimeric embryos (Fig. 1, control and e), as expected, approximately 50% of cells expressed the transgene). In embryos receiving a nuclear transplant at the eight-cell stage, at which Oct4 is already being expressed, we did not observe immediate Oct4-GFP expression from the somatic genome. Weak Oct4-GFP was first visible at the late morula/early blastocyst stage, and a strong signal apparent at the mid-blastocyst stage, suggesting that the somatic genome Oct4 promoter is not immediately activated.

Presence of the somatic genome in blastocysts not expressing Oct4-GFP

More than 70% of blastocysts formed from those embryos receiving a somatic cell nucleus into an intact blastomere at the two- or four-cell stage expressed Oct4-GFP. To ascertain whether blastocysts not expressing Oct4-GFP contained hybrid cells with an embryonic and a somatic genome, or whether they had expelled the transplanted somatic genome, we assessed the presence of tetraploid cells in blastocysts using metaphase spreads (Tarkowski 1966). In GFP-expressing and non-expressing hybrid embryos produced by cleavage stage nuclear transfer, both diploid and tetraploid nuclei were detected at a similar frequency (Table 1). In non-manipulated control

embryos, tetraploid cells were never detected, suggesting that spontaneous formation of tetraploid cells during pre-implantation development is rare and that the additional genome in the nuclear transfer embryos originated from the introduced somatic cell nucleus. The presence of tetraploid cells in GFP-negative embryos indicated that, despite the persistence of the somatic genome at the blastocyst stage, Oct4-GFP expression was not initiated in somatic-embryonic hybrid cells.

Since somatic cell nuclear transfer into two-cell stage blastomeres involved transfer of a nucleus at the G0 stage of the cell cycle into a blastomere in G2 phase, we ascertained whether the somatic genome replicated prior to cleavage to the four-cell stage by measuring BrdU incorporation. Since the embryonic genome has already replicated at the time of nuclear transfer, any BrdU incorporation must be due to replication of the somatic genome. Embryos receiving somatic cell nuclear transplants at the two-cell stage were always negative for BrdU staining, suggesting that the somatic genome did not replicate but formed a hybrid nucleus with one of the newly formed four-cell nuclei. Consistent with this hypothesis, in embryos receiving a somatic cell nucleus transplant at the two-cell stage, Oct4-GFP expression was usually visible in two of eight blastomeres at the eight-cell stage (Fig. 4c–e).

Developmental potential of somatic-embryonic tetraploid cells

Blastocysts resulting from transfer of somatic cell nuclei into cleavage stage blastomeres were chimeras, composed of normal embryonic diploid and somatic-embryonic hybrid tetraploid cells arising from the injected blastomere. When combined with normal embryos or embryonic stem cells to form chimeras, tetraploid cells exhibit limited ability to contribute to embryonic development but can form extra-embryonic tissues (Kaufman & Webb 1990, Nagy *et al.* 1990, James *et al.* 1995). We assessed if somatic-embryonic tetraploid cells, produced by somatic cell nuclear transfer into cleavage stage blastomeres, could contribute to development in chimeras with normal embryos. Chimeric embryos resulting from transfer of cumulus cell nuclei into two- and four-cell stage blastomeres (Fig. 5a and b) were transferred into recipient female mice, and fetuses recovered at 10.5 d.p.c. As a control, we transferred chimeras of embryonic-diploid and

Table 1 Ploidy of metaphase spreads from manipulated and control blastocyst stage embryos.

Embryo type	No. of metaphases counted (n)	Diploid n (%)	Tetraploid n (%)	Aneuploid* n (%)
<i>In vivo</i> fertilized	9	8 (89%)	0	1 (11%)
NT into 2-cell blastomere				
GFP positive	28	12 (43%)	8 (29%)	8 (29%)
GFP negative	10	5 (50%)	3 (30%)	2 (20%)
NT into 4-cell blastomere				
GFP positive	5	2 (40%)	2 (40%)	1 (20%)
GFP negative	7	6 (86%)	1 (14%)	0

* Aneuploid: 32; 36; 39; 42; 47 and > 80 chromosomes counted.

Embryo		Fetuses n/transf.	Somatic genome contribution to fetus	yolk sac	placenta	No exp.
ctrl		10/25	0	4	6	1
5a		27/74	0	0	0	3
5b		12/36	0	0	0	1
5c		10/24	0	2	4	2

● somatic cell nucleus, Oct4-GFP transgenic ○ blastomere nucleus ⊗ blastomere nucleus, Oct4-GFP transgenic

Figure 5 Contribution of somatic-embryonic hybrids to fetuses at 10.5 d.p.c. ctrl = control, transfer of Oct4-GFP transgenic two-cell stage nucleus into blastomere of synchronous non-transgenic embryo; No exp. = number of experiments. (a and b) Somatic cell nuclear transfer into intact two- (Fig. 4a) and four- (Fig. 4b) cell stage blastomeres. (c) Somatic cell nuclear transfer into MII oocyte, transfer of tetraploid nucleus at two-cell stage into an enucleated blastomere of synchronous two-cell stage embryo.

embryonic-tetraploid cells that were produced by transfer of a two-cell nucleus into one blastomere of a developmentally synchronous two-cell stage embryo (Fig. 5, control). We also assessed the contribution of hybrid cells constructed with somatic nuclei and MII oocytes to post-implantation development (Fig. 5c). Although previous studies have produced tetraploid clones by transfer of somatic cell nuclei into intact MII oocytes, the post-implantation developmental potential of these embryos has not been reported. Cumulus cell nuclei were transferred into non-enucleated MII oocytes and these subsequently activated. At the two-cell stage, the resulting tetraploid nucleus was removed from one blastomere and transferred to one enucleated blastomere of a synchronous, late two-cell stage normal embryo (Fig. 5c), creating a chimeric embryo with diploid embryonic and tetraploid cells.

The contribution of the different genomes of the diploid and tetraploid components to fetus, yolk sac and placenta was determined by analysis of electrophoretically distinct GPI-1 isoenzymes encoded by the *Gpi-1* alleles of different mouse strains used (Nagy & Rossant 1993). The recipient two- or four-cell stage embryos were of GPI-1 isotype BB (B6C3F1), while the transplanted somatic or embryonic nuclei were either GFP-1 BC (129B6F1) or GPI-1 AB (B6D2F1). Recipient ICR females used were of the BB isotype.

Of 25 control chimeras with embryonic tetraploid cells transferred, ten fetuses were recovered at 10.5 d.p.c. (Fig. 5, control). Contribution from the transplanted embryonic nucleus was detected in the placenta of six fetuses, and

contribution to the yolk sac in four of these. In contrast, in chimeric embryos containing somatic-embryonic tetraploid hybrid cells produced by somatic cell nuclear transfer into cleavage stage blastomeres, contribution of hybrid cells to post-implantation tissues at 10.5 d.p.c. was never observed (Fig. 5a and b). In contrast, somatic-embryonic hybrid cells resulting from transfer of somatic nuclei into MII oocytes did contribute to extra-embryonic tissues: Four of ten fetuses exhibited contribution from the transferred somatic cell genome to yolk sac and/or placenta (Fig. 5c). Therefore, despite expression of Oct4-GFP from the somatic cell nucleus in chimeric blastocysts, the somatic-embryonic tetraploid component created by transfer into cleavage stage blastomeres was not functionally equivalent to an embryonic tetraploid or to somatic-embryonic tetraploid cells resulting from transfer into MII oocytes.

To determine at which stage exclusion of somatic-embryonic tetraploid hybrid cells resulting from blastomere nuclear transfer occurred subsequent to the blastocyst stage, at which significant contribution is observed, we generated chimeras with hybrid cells (Fig. 5a and c) using somatic cell nuclei transgenic for an ubiquitously expressed GFP transgene (Okabe *et al.* 1997). Embryos were transferred to recipients, and GFP-positive blastocysts were also placed on feeder cell layers to produce *in vitro* outgrowths. During *in vitro* culture, the number of GFP-expressing outgrowths from somatic-embryonic hybrid embryos resulting from blastomere nuclear transfer decreased (Fig. 6), and many outgrowths contained only few (less than five) GFP-expressing cells (six out of ten

Figure 6 Contribution of somatic-embryonic hybrids in *in vitro* outgrowths and fetuses at early post-implantation stages. Numbers on bars = total number of embryos.

outgrowths at 53 h). GFP-positive cells were located in both ICM and trophectoderm of outgrowths. In a few outgrowths (four out of ten), a substantial number of GFP-expressing cells were detected after 3 days of culture, suggesting that somatic-embryonic hybrid cells can develop to the *in vitro* equivalent of peri-implantation stages.

Fetuses from *in vitro* transfers were recovered at various stages between 5.5 and 9.5 d.p.c. While chimeric fetuses resulting from somatic cell nuclear transfer into MII oocytes (Fig. 5c) frequently expressed GFP (Fig. 6; four out of seven and five out of five fetuses recovered at 5.5 and 6.5 d.p.c. respectively, from a total of 2×21 (42) morula stage embryos transferred), GFP-expressing cells were only detected in a single 5.5 d.p.c. fetus resulting from somatic nuclear transfer into a two-cell stage blastomere (Fig. 6; 17 fetuses recovered at 5.5 d.p.c., from 50 four-cell stage embryos transferred). In this embryo, a small number (two to four) of GFP-positive cells were located in the visceral endoderm. In later stage fetuses from this experimental group, no GFP-expressing cells were detected (Fig. 6).

Thus, tetraploid cells produced with embryonic nuclei, and those produced by somatic cell nuclear transfer to MII oocytes and serial transfer at the two-cell stage can contribute to extra-embryonic development, whereas tetraploid cells resulting from somatic cell nuclear transfer into two- and four-cell stage blastomeres are excluded from development between the late blastocyst/peri-implantation stage and early post-implantation development.

Discussion

Here we have reported that transplantation of somatic cell nuclei into intact cleavage stage embryonic blastomeres resulted in stage-specific re-activation of the germline and embryonic-specific Oct4-GFP transgene from a somatic

genome. However, despite the contribution of the somatic genome in tetraploid cells throughout preimplantation development, hybrid cells are excluded during early post-implantation development. These results indicated that preimplantation stage contribution and embryonic gene re-expression from a somatic nucleus occurs after somatic cell nuclear transfer into cleavage stage blastomeres, but is exclusive of reprogramming sufficient for contribution to post-implantation development.

We have observed that embryos receiving a cumulus cell nucleus transplanted into a single blastomere at the two- or four-cell stage develop normally to the blastocyst stage with frequent contribution of somatic-embryonic tetraploid cells. Consistent with previous studies examining nuclear transfer into post-meiotic cytoplasts, the somatic genome cannot support preimplantation development in a cleavage stage cytoplasm in the absence of the blastomere nucleus. After transfer into intact cleavage stage blastomeres, the embryonic and the transplanted cumulus cell nucleus integrated to form one tetraploid nucleus after the first division, as verified by Hoechst staining (data not shown) and analysis of metaphase spreads. Apparently, somatic cell-specific gene expression from the somatic genome is either compatible with, or not detrimental to, preimplantation development. Alternatively, it may be silenced subsequent to somatic cell nuclear transfer at preimplantation stages. Silencing of somatic specific gene expression has been frequently observed in hybrid cell models (Takagi 1997) and also subsequent to somatic cell nuclear transfer (Bortvin *et al.* 2003). The observation that somatic-embryonic hybrid cells express Oct4-GFP stage and cell type specifically from the cumulus cell genome could indicate that reprogramming events are occurring on the somatic genome, resulting in embryonic-specific gene expression. Remarkably, both blastocyst stage embryos with somatic-embryonic hybrid cells produced by transfer at cleavage stages and by somatic cell nuclear

transfer into non-enucleated MII oocytes or enucleated MII oocytes (Boiani *et al.* 2002) expressed Oct4-GFP, while developmental potential and post-implantation contributions were strikingly different. Only hybrid cells produced by transfer to MII oocytes contributed to post-implantation development.

It is possible that, subsequent to nuclear transfer into blastomeres, the somatic genome, as part of a somatic-embryonic hybrid nucleus, undergoes epigenetic modifications during preimplantation development, such as demethylation and changes in heterochromatin distribution. However, even in the event of such changes, it is also possible that the cumulus cell genome does not undergo reprogramming *per se*, but rather that the Oct4 promoter of the somatic genome becomes accessible for transcription when at a developmental stage that expresses high levels of Oct4 from the embryonic allele, such as the morula or blastocyst. We observed that embryos that did not express Oct4-GFP subsequent to receiving somatic cell nuclear transplant contained tetraploid cells at the blastocyst stage, suggesting that Oct4-GFP expression from the somatic genome does not occur by default in somatic-embryonic tetraploid nuclei. This is consistent with the stage-specific re-expression of Oct4-GFP observed from a somatic genome, regardless of whether the nucleus was transferred into a two-, four- or eight-cell stage blastomere, although in eight-cell stage embryos, chromatin configuration changes from a less repressive to a more repressive state (Thompson *et al.* 1995).

Considering the observed incompatibility of somatic-embryonic tetraploid cells resulting from cleavage stage nuclear transfer with any contribution post implantation, it appears that the somatic genome does not undergo reprogramming or enough reprogramming which is required for post-implantation development. If the somatic genome was partially reprogrammed or remained dormant, some contribution of hybrid cells rather than none would have been expected due to the presence of the embryonic component. One explanation for the failure is that the somatic genome continues to express some somatic cell-specific genes as indicated in diploid nuclear transfer embryos at early stages (Gao *et al.* 2003), and that this gene expression may be tolerated during preimplantation but not post-implantation development. Thus, in somatic-embryonic blastomere hybrid cells, either functional reprogramming does not occur, or occurs at a level at which post-implantation contribution of cells with a somatic genome is precluded.

Expression of Oct4 has been used extensively as a marker of reprogramming of somatic cell nuclei in diverse experimental systems. Preimplantation stage mouse somatic cell clones frequently exhibit abnormal Oct4 expression patterns or fail to activate Oct4 and related embryonic genes (Boiani *et al.* 2002, Bortvin *et al.* 2003), suggesting insufficient reprogramming of the somatic donor nucleus. In somatic cell clones, re-expression of Oct4 reflects a gene expression change of the somatic gen-

ome that occurs upon transfer to an enucleated MII oocyte, in the absence of any other genome. In contrast, activation of Oct4 gene expression from a somatic genome occurs much more frequently in other experimental systems that have been used to study the induction of epigenetic changes on somatic nuclei, such as hybrid cells between somatic cells and embryonic germ (EG) or ES cells (Tada *et al.* 1997, 2001). While the thymocyte genomes in hybrid cells between thymocytes and ES cells almost always express Oct4 and exhibit features similar to the epigenotype of an ES cell nucleus, hybrid cells only rarely contribute to fetuses which may either be due to their tetraploid nature or limited developmental potential. Activation of Oct4 gene expression was observed in cells of certain somatic cell lines when placed in physical contact with compacting mouse preimplantation embryos (Burnside & Collas 2002). Oct4 transcription from mammalian somatic cell nuclei was also detected after transfer of nuclei into amphibian oocytes (Byrne *et al.* 2003). The developmental potential of these Oct4-expressing nuclei, however, has not been assessed. Our observation that cleavage stage blastomeres can instate timely expression of Oct4-GFP from a somatic cell genome, but do not acquire developmental competence post implantation suggests that in a hybrid system, particularly those not using an MII ooplasm, gene expression alone is not a valid indicator for reprogramming. Although changes in gene expression or epigenetic modification such as methylation have been used as indicators for reprogramming of somatic nuclei, they should ultimately be interpreted in the context of the 'reprogrammed' cell to contribute to development.

Our observations have shown that, in a cell hybrid system using embryonic blastomeres and somatic cell nuclei, gene expression changes and functional reprogramming are not equivalent. The analysis of epigenetic and gene expression changes in cell hybrid systems thus may have limited applicability as a tool to assess the reversal of differentiation and the acquisition of pluripotency, in contrast to somatic cell clones produced with MII ooplasm where, in some instances, adequate reprogramming has been effected. The study of somatic-embryonic tetraploid cells, however, proved to be extremely useful. Our observation of the post-implantation contribution of somatic cell nuclei-MII oocyte tetraploid cells validates these tetraploid constructs as a model to evaluate reprogramming changes. Compared with clones produced by somatic cell nuclear transfer into MII oocytes, the similarity in Oct4 gene expression but difference in developmental capacity may provide a tool with which to delineate epigenetic changes that determine gene expression versus the reprogramming associated with production of a totipotent state.

Acknowledgements

The authors thank Jeffrey R Mann for the Oct4-GFP transgenic mice, Marianne Friez for technical assistance and Patricia Labosky for advice on post-implantation embryos. This

work was supported, in part, by the Marion Dilley and David George Jones Funds and the Commonwealth and General Assembly of Pennsylvania (to K J M, S E and N A L) and NIH funding R01-HD-44066-01A1 (to K J M). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Boiani M, Eckardt S, Scholer HR & McLaughlin KJ 2002 Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes and Development* **16** 1209–1219.
- Boiani M, Eckardt S, Leu NA, Scholer HR & McLaughlin KJ 2003 Pluripotency deficit in clones overcome by clone–clone aggregation: epigenetic complementation? *EMBO Journal* **22** 5304–5312.
- Bortvin A, Eggan K, Skaletsky H, Akutsu H, Berry DL, Yanagimachi R, Page DC & Jaenisch R 2003 Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. *Development* **130** 1673–1680.
- Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ & Matzuk MM 2003 Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science* **300** 633–636.
- Burnside AS & Collas P 2002 Induction of Oct-3/4 expression in somatic cells by gap junction-mediated cAMP signaling from blastomeres. *European Journal of Cell Biology* **81** 585–591.
- Byrne JA, Simonsson S, Western PS & Gurdon JB 2003 Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. *Current Biology* **13** 1206–1213.
- Chatot CL, Ziomek CA, Bavister BD, Lewis JL & Torres I 1989 An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* **86** 679–688.
- Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E & Reik W 2001 Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *PNAS* **98** 13734–13738.
- Dean W, Santos F & Reik W 2003 Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. *Seminars in Cell and Developmental Biology* **14** 93–100.
- Gao S, Chung YG, Williams JW, Riley J, Moley K & Latham KE 2003 Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. *Biology of Reproduction* **69** 48–56.
- Hogan BL, Beddington R, Costantini F & Lacy E 1994 In *Manipulating the Mouse Embryo – A Laboratory Manual*, Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- James RM, Klerkx AH, Keighren M, Flockhart JH & West JD 1995 Restricted distribution of tetraploid cells in mouse tetraploid ↔ diploid chimaeras. *Developmental Biology* **167** 213–226.
- Jouneau A & Renard JP 2003 Reprogramming in nuclear transfer. *Current Opinion in Genetics and Development* **13** 486–491.
- Kaufman MH & Webb S 1990 Postimplantation development of tetraploid mouse embryos produced by electrofusion. *Development* **110** 1121–1132.
- McGrath J & Solter D 1984 Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development *in vitro*. *Science* **226** 1317–1319.
- Mayer W, Niveleau A, Walter J, Fundele R & Haaf T 2000 Demethylation of the zygotic paternal genome. *Nature* **403** 501–502.
- Monk M, Boubelik M & Lehnert S 1987 Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* **99** 371–382.
- Nagy A & Rossant J 1993 Production of completely ES cell derived fetuses. In *Gene Targeting*, vol. 126, pp 147–178. Ed. AL Joyner. Oxford: IRL.
- Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E, Markkula M & Rossant J 1990 Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110** 815–821.
- Okabe M, Ikawa M, Kominami K, Nakanishi T & Nishimune Y 1997 'Green mice' as a source of ubiquitous green cells. *FEBS Letters* **407** 313–319.
- Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W & Walter J 2000 Active demethylation of the paternal genome in the mouse zygote. *Current Biology* **10** 475–478.
- Park KW, Lai L, Cheong HT, Cabot R, Sun QY, Wu G, Rucker EB, Durtschi D, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB & Prather RS 2002 Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. *Biology of Reproduction* **66** 1001–1005.
- Pesce M, Gross MK & Scholer HR 1998 In line with our ancestors: Oct-4 and the mammalian germ. *Bioessays* **20** 722–732.
- Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B & First NL 1987 Nuclear transplantation in bovine embryos. *Journal of Animal Science* **64** 642–647.
- Rougier N, Bourc'his D, Gomes DM, Niveleau A, Plachot M, Paldi A & Viegas-Pequignot E 1998 Chromosome methylation patterns during mammalian preimplantation development. *Genes and Development* **12** 2108–2113.
- Solter D 2000 Mammalian cloning: advances and limitations. *Nature Reviews: Genetics* **1** 199–207.
- Szabo PE, Hubner K, Scholer H & Mann JR 2002 Allele-specific expression of imprinted genes in mouse migratory primordial germ cells. *Mechanisms of Development* **115** 157–160.
- Tada M, Tada T, Lefebvre L, Barton SC & Surani MA 1997 Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO Journal* **16** 6510–6520.
- Tada M, Takahama Y, Abe K, Nakatsuji N & Tada T 2001 Nuclear reprogramming of somatic cells by *in vitro* hybridization with ES cells. *Current Biology* **11** 1553–1558.
- Takagi N 1997 Mouse embryonal carcinoma cell–somatic cell hybrids as experimental tools for the study of cell differentiation and X chromosome activity. *Cancer Genetics and Cytogenetics* **93** 48–55.
- Tarkowski AK 1966 An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* **5** 394–400.
- Thompson EM, Legouy E, Christians E & Renard JP 1995 Progressive maturation of chromatin structure regulates HSP70.1 gene expression in the preimplantation mouse embryo. *Development* **121** 3425–3437.
- Tsunoda Y, Yasui T, Shioda Y, Nakamura K, Uchida T & Sugie T 1987 Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. *Journal of Experimental Zoology* **242** 147–151.
- Wakayama T, Tateno H, Mombaerts P & Yanagimachi R 2000 Nuclear transfer into mouse zygotes. *Nature Genetics* **24** 108–109.
- Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick SL & Matzuk MM 2003 Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nature Genetics* **33** 187–191.
- Yoshimizu T, Sugiyama N, De Felice M, Yeom YI, Ohbo K, Masuko K, Obinata M, Abe K, Scholer HR & Matsui Y 1999 Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. *Development, Growth and Differentiation* **41** 675–684.

Received 30 January 2005

First decision 1 February 2005

Accepted 1 February 2005