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Sheep cloned by nuclear transfer from a cultured cell line

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NUCLEAR transfer has been used in mammals as both a valuable tool in embryological studies[1] and as a method for the multiplication of 'elite' embryos[2–4]. Offspring have only been reported when early embryos, or embryo-derived cells during primary culture, were used as nuclear donors[5,6]. Here we provide the first report, to our knowledge, of live mammalian offspring following nuclear transfer from an established cell line. Lambs were born after cells derived from sheep embryos, which had been cultured for 6 to 13 passages, were induced to quiesce by serum starvation before transfer of their nuclei into enucleated oocytes. Induction of quiescence in the donor cells may modify the donor chromatin structure to help nuclear reprogramming and allow development. This approach will provide the same powerful opportunities for analysis and modification of gene function in livestock species that are available in the mouse through the use of embryonic stem cells[7].

The cells used in these experiments were isolated by microdissection and explantation of the embryonic disc (ED) of day 9 *in vivo* produced 'Welsh mountain' sheep embryos. The line was established from early passage colonies with a morphology like that of embryonic stem (ES) cells. By the second and third passages, the cells had assumed a more epithelial, flattened morphology (Fig. 1*a*) which was maintained on further culture (to at least passage 25). At passage 6, unlike murine ES cells they expressed cytokeratin, and nuclear lamin A/C which are markers associated with differentiation[8]. This embryo-derived epithelial cell line has been designated TNT4 (for totipotent for nuclear transfer).

The development of embryos reconstructed by nuclear transfer is dependent upon interactions between the donor nucleus and the recipient cytoplasm. We have previously reported the effects of the cytoplasmic kinase activity, maturation/mitosis/meiosis promoting factor (MPF), on the incidence of chromosomal damage and aneuploidy in reconstructed embryos and established two means of preventing such damage[9]. First, the effects of the donor cell-cycle stage can be overcome by transferring nuclei after the disappearance of MPF activity by prior activation of the recipient enucleated MII oocyte[9,10]. Using this approach we obtained the birth of lambs by nuclear transfer during establishment of the cell line (up to and including passage 3). On subsequent culture (passages 6 and 11) no development to term was obtained (see Table 1). From these numbers we cannot conclude that development to term will not be obtained using this method. The lack of development of some control embryos is thought to relate to an infection in the oviduct of the temporary recipient ewe from which 6 were recovered.

An alternative means of avoiding damage due to the activity of MPF is to transfer diploid nuclei into metaphase II oocytes that have a high level of MPF activity[9]. The availability of TNT4 cells allows this approach to be used. In this study a synchronous population of diploid donor nuclei was produced by inducing the cells to exit the growth cycle and arrest in G0 in a state of quiescence. In the presence of a high level of MPF activity the transferred nucleus undergoes nuclear membrane breakdown and chromosome condensation. It has been argued[11] that the developmental potential of reconstructed embryos depends upon the "reprogramming of gene expression" by the action of cytoplasmic factors and that this might be enhanced by the prolongation of this period of exposure. To assess these effects donor cells were fused to oocytes either (1) 4–8 h before activation 'post-activated' or (2) at the time of activation 'fusion and activation' or (3) to pre-activated oocytes 'preactivated'.

During these studies *in vivo* ovulated metaphase arrested (MII) oocytes were flushed from the oviduct of 'Scottish blackface' ewes. The methodology used was as previously described[10] with the following exceptions; oocytes were recovered 28–33 h after injection of gonadotropin-releasing hormone (GnRH), calcium/magnesium-free PBS containing 1.0% FCS was used for all flushing, and recovered oocytes were transferred to calcium-free M2 medium[12] containing 10% FCS and were maintained at 37 °C in 5% CO_2 in air until use. As soon as possible after recovery oocytes were enucleated and embryos reconstructed. At 50–54 h

TABLE 1 Development using unsynchronized TNT4 cells

Donor cell type	Number of morula and blastocysts/total embryos (%)	Number of lambs/embryos transferred
	October 1993–February 1994	
16 cell	6/11 (27.3)	2/6
ED cell	1/15 (6.7)	0/1
ED P1	4/19 (21.0)	1/4
ED P2	1/11 (9.1)	1/1
ED P3	2/36 (5.5)	2/2
	October–December 1994	
16 cell	14/28 (50.0)	0/14
TNT4 P6	9/98 (9.2)	0*/9
TNT4 P11	10/92 (10.9)	0/10

Development of ovine embryos reconstructed by nuclear transfer of unsynchronized cells during isolation and after establishment of the TNT4 line to enucleated preactivated ovine oocytes. P, Passage number; ED, embryonic disc. For embryo reconstruction, donor oocytes were placed into calcium-free M2 containing 10% FCS, 7.5 μg ml^{-1} Cytochalasin B (Sigma) and 5.0 μg ml^{-1} Hoechst 33342 (Sigma) at 37 °C for 20 min to aspirate. A small amount of cytoplasm enclosed in plasma membrane was removed from directly beneath the 1st polar body using a glass pipette (~20 μm tip external diameter). Enucleation was confirmed by exposing this karyoplast to ultraviolet light and checking for the presence of a metaphase plate. At 34–36 h after GnRH injection enucleated oocytes were activated. Following further culture for 4–6 h in TC199, 10% FCS a single cell was fused. All activations and fusions were accomplished as previously described[10,17] in 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.0005 mM $CaCl_2$[17]. For activation a single DC pulse of 1.25 kV cm^{-1} for 80 μs and for fusion an AC pulse of 3 V for 5 s followed by 3 d.c. pulses of 1.25 kV cm^{-1} for 80 μs were applied. All oocyte/cell couplets were cultured in TC199, 10% FCS 7.5 μg ml^{-1} Cytochalasin B (SIGMA) for 1 h following application of the fusion pulse and then in the same medium without Cytochalasin until transferred to temporary recipient ewes. Reconstructed embryos were cultured in the ligated oviduct of a recipient 'blackface' ewe until day 7 after reconstruction. All morula and blastocyst stage embryos were transferred to synchronized recipient blackface ewes for development to term.

* A single pregnancy was established but subsequently lost at about 70–80 days.

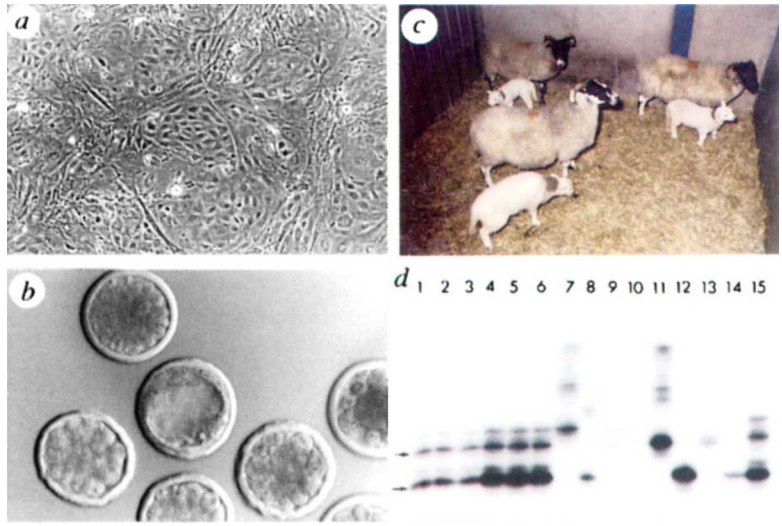

FIG. 1 Production and characterization of the TNT4 cell line and the offspring produced by nuclear transfer from TNT4 cells. *a*, Morphology of the TNT4 cell line at passage 6. *b*, Group of embryos including a single blastocyst on day 7 after reconstruction. *c*, Group of three Welsh mountain lambs produced by nuclear transfer with surrogate Scottish blackface ewes. *d*, Autoradiogram showing the alleles generated following amplification of the microsatellite FCB266 (ref. 18). Lanes 1–6 are from, respectively, TNT4 cells and the five lambs generated by nuclear transfer. Both lambs and cells display an identical pattern, revealing 2 alleles (arrowed) at 114 and 125 bp. Lanes 7–15, nine randomly chosen Welsh mountain sheep, none of whom show an identical pattern to the nuclear transfer group. Lambs and TNT4 cells were also identical at six further microsatellite loci: MAF33, MAF48, MAF65, MAF209, OarFCB11, OarFCB128, OarRCB304 (data not shown). The nine unrelated random control animals showed extensive variation at all of these loci.

METHODS. Groups of 4–6 microdissected embryonic discs were cultured on feeder layers of mitotically inactivated primary murine fibroblasts in Dulbeccos Modified Eagles medium (GIBCO) containing 10% fetal calf serum, 10% newborn serum and supplemented with recombinant human leukaemia inhibition factor (LIF). After 5–7 days of culture, expanding discs were treated with trypsin and passaged onto fresh feeders yielding 4 similar lines. At passage 12 of the 2*n* chromosome complement of 54 was observed in 31 of 50 spreads, the remaining aneuploid spreads are thought to be artefacts of preparation. For microsatellite analysis genomic DNA was extracted from whole blood, tissue culture cells or fetal tissues using a puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, USA). The PCR analysis of microsatellites was carried out using an end-labelled primer ([γ-^{32}P]ATP). All other aspects of labelling and thermal cycling conditions were as described elsewhere[17].

after GnRH injection, reconstructed embryos were embedded in agar and transferred to the ligated oviduct of dioestrus ewes. After 6 days the embryos were retrieved and development assessed microscopically (see Fig. 1*b*).

The development of embryos reconstructed using quiescent TNT4 cells and 3 different cytoplast recipients is summarized in Table 2. No significant difference was observed in the frequency of development with high and low passage number donor cells or with cytoplast recipient type used (results were analysed by the marginal model in ref. 13). All embryos that had developed to the morula/blastocyst stage were transferred as soon as possible to the uterine horn of synchronized final recipient ewes for development to term. Recipient ewes were monitored for pregnancy by ultrasonography. Ewes that were positive at day 35 were classified as pregnant (Table 3). A total of eight fetuses were detected in seven recipient ewes including a single twin pregnancy. A total of five phenotypically female Welsh mountain lambs were born from the Scottish blackface recipient ewes (Fig. 1*c*). Two of these lambs died within minutes of birth and a third at 10 days; the remaining two lambs are apparently normal and healthy (8–9 months old). Of the remaining 3 fetuses, one was lost at about 80 days of gestation, and a second was lost at 144 days of gestation. The third fetus was thought to be a twin pregnancy and was either misdiagnosed or lost at an unknown time. Microsatellite analysis of the cell line, fetuses and lambs showed that all of the female lambs were derived from a single cell population (Fig. 1*d*).

TABLE 2 Development to morula and blastocyst stage of ovine embryos reconstructed using quiescent TNT4 cells and 3 different cytoplast recipients (January–March 1995)

Experiment number	Cytoplast type: TNT passage number	Number of morulae and blastocysts/total number of embryos recovered (%): Post-activated	Activation and fusion	Preactivated
1	6	4/28	6/32*	–
2	7	1/10	1/26*	–
3	13	0/2	–	2/14
4	13	0/14	0/11	–
5	11	1/9	–	0/9
6	11	1/2	9/29***	–
7	12	–		6/45*
8	13	3/13*		–
Total		10/78 (12.8%)	16/98 (16.3%)	8/68 (11.7%)

Development to the morula and blastocyst stage of ovine embryos recovered on day 7 after reconstruction by nuclear transfer of quiescent TNT4 cells at different passages into 3 cytoplast recipients. To induce quiescence, TNT4 cells were plated into feeder layers in 29-cm^2 flasks (GIBCO) and cultured for 2 days, the semiconfluent exponentially growing cultures were then washed three times in medium containing 0.5% FCS and cultured in this low-serum medium for 5 days. Embryos were reconstructed using preactivated cytoplasts as previously described (Table 1) and by two other protocols. (1) post-activation, as soon as possible after enucleation a single cell was fused to the cytoplast in 0.3 M mannitol without calcium and magnesium, to prevent activation. Couplets were washed and cultured in calcium-free M2, 10% FCS at 37 °C, 5% CO_2 for 4–8 h. Thirty minutes before activation the couplets were transferred to M2 medium, 10% FCS containing 5 μM Nocodazole (SIGMA). Following activation the reconstructed zygotes were incubated in medium TC199, 10% FCS, 5.0 μM Nocodazole for a further 3 h. (2) Preactivation, at 34–36 h after GnRH injection a single cell was fused to an enucleated oocyte. The same pulse also induced activation of the recipient cytoplast. All activations and fusions were accomplished as described in Table 1 unless otherwise stated.

* Denotes number of pregnancies following transfer of morula and blastocyst stage embryos to synchronized final recipient ewes.

TABLE 3 Induction of pregnancy and further development following transfer of morula and blastocyst stage embryos reconstructed from quiescent TNT4 cells

Cytoplast type	Post-activated	Activation and fusion	Preactivated
Total number of morula and blastocyst stage embryos transferred	10	16	8
Total number of ewes	6	9	4
Number of pregnant ewes (%)	1 (16.7)	5 (55.5)	1 (25.0)
Number of fetuses/total embryos transferred (%)	2/10 (20.0)	5/16 (31.25)	1/8 (12.5)
Number of live births	1	3	1
Passage number of cells resulting in offspring	1 × P11	1 × P6, 2 × P11	1 × P13

Induction of pregnancy following transfer of all morula/blastocyst stage reconstructed embryos to the uterine horn of synchronized final recipient blackface ewes. The table shows the total number of embryos from each group transferred, the frequency of pregnancy in terms of ewes and embryos (in the majority of cases 2 embryos were transferred to each ewe and a single twin pregnancy was established (using the 'post-activated' cytoplast)) and the number of live lambs obtained.

Because of the seasonality of sheep a direct comparison of all of these methods of embryo reconstruction has not yet been made. The success of the later studies may be due to a number of factors. First, quiescent nuclei are diploid and therefore the cell-cycle stages of the karyoplast and cytoplast in both the 'post-activation' and 'fusion and activation' methods of reconstruction are coordinated. The preactivated cytoplast will accept donor nuclei from G0, G1, S and G2 cell-cycle phases. Second, the G0 phase of the cell cycle has been implicated in the differentiation process and the chromatin of quiescent nuclei has been reported to undergo modification[14]. As a result the chromatin of quiescent donor nuclei may be more readily modified by oocyte cytoplasm. The TNT4 cells resemble several cell lines derived previously in sheep[15] and also pigs[16]. It remains to be determined whether comparable development is obtained with other such lines or other cell types. At the present time we are unable to differentiate the mechanisms involved and report that the combination of nuclear transfer and cell type described here support development to term of cloned ovine embryos from cells that had been in culture through up to 13 passages. As cell-cycle duration was about 24 h, this period of culture before nuclear transfer would be sufficient to allow genetic modification and selection if procedures comparable to those used in murine ES cells can be established.

The production of cloned offspring in farm animal species could provide enormous benefits in research, agriculture and biotechnology. The modification by gene targeting and selection of cell populations before embryo reconstruction coupled to the clonal origin of the whole animal provides a method for the dissemination of rapid genetic improvement and/or modification into the population □

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Midbrain development induced by FGF8 in the chick embryo

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VERTEBRATE midbrain development depends on an organizing centre located at the isthmus, a constriction in the embryonic mid/hindbrain region[1–3,28]. Isthmic tissue grafts transform chick caudal forebrain into an ectopic midbrain that is the mirror image of the normal midbrain[4]. Here we report that FGF8 protein has the same midbrain-inducing and polarizing effect as isthmic tissue. Moreover, FGF8 induces ectopic expression in the forebrain of genes normally expressed in the isthmus, suggesting that the ectopic midbrain forms under the influence of signals from a new 'isthmus-like' organizing centre induced in the forebrain. Because *Fgf8* itself is expressed in the isthmus, our results identify FGF8 as an important signalling molecule in normal midbrain development.

Fgf8 is expressed in the isthmus of the developing mouse brain[5–8]. Because FGF8 has inducing activity in another developmental system (the limb[9]), we sought to determine whether FGF8 provides the midbrain-inducing activity of an isthmus graft in the chick. We first confirmed that *Fgf8* is expressed in the chick isthmus (Fig. 1*a*). Next, we determined the effects of implanting a bead soaked in recombinant FGF8 (FGF8-bead) into the caudal diencephalon (prosomere 2, p2, as defined in ref. 10; Fig. 1*b*) of chick embryos at stages 9–12 (ref. 11). An early effect of isthmus grafts is induction in the host neuroepithelium of *Engrailed*-2 (*En2*) expression[1,4], an early marker of mes/rhombencephalic development[12–14]. When an FGF8-bead was implanted, ectopic *En2* RNA was detected caudal to the zona limitans intrathalamica (ZL), a transverse boundary separating dorsal and ventral thalamus anlagen (p2/p3 boundary[10]), in all embryos assayed 22–26 h later ($n = 10$; Fig. 1*c*). Control beads soaked in phosphate-buffered saline (PBS-beads) did not induce *En2* expression ($n = 11$; not shown).

In experimental embryos surviving to E5–E16 (stages 25–42) the diencephalon caudal to the ZL (p1 and p2; ref. 10) was transformed from its normal fate of rostral pretectum and dorsal thalamus to ectopic midbrain ($n = 17/18$; Fig. 2). Control embryos implanted with PBS-beads that survived to E4–E10