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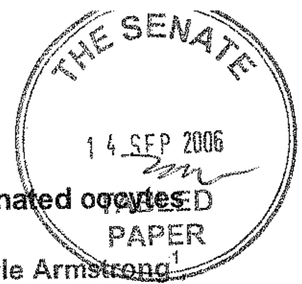

Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes

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Abstract

This paper describes the derivation of a blastocyst following heterologous nuclear transfer (NT) into a human oocyte. It also demonstrates that a major obstacle to continuing research in human NT is the availability of suitable human oocytes. In this study, 36 oocytes were donated by 11 women undergoing four different treatments and their developmental potential was evaluated after NT. The time from oocyte collection to NT seems to be crucial, and only oocytes that were enucleated within 1 h proved successful. After enucleation of oocytes, fusion with undifferentiated human embryonic stem cells and in-vitro culture, early cleavage and blastocyst development of fused complexes was observed. The DNA fingerprinting comparison of the donor cells and derived blastocyst revealed successful heterologous NT, since both oocytes and donor cells were recovered from different patients. It has therefore been demonstrated that NT can be achieved in humans, using heterologous donor nuclei and surplus and donated oocytes. However, if the promise of this new science is to achieve its potential in the foreseeable future, it will be necessary to identify new sources of oocytes that can be used immediately after retrieval.

Keywords: *egg donation, embryo, nuclear transfer, oocyte, stem cells*

Introduction

Application of the technique of somatic cell nuclear transfer to human oocytes and the derivation of stem cells offers the potential of new therapeutic interventions in many diseases (Hochedlinger and Jaenisch, 2003; Hwang *et al.*, 2004, 2005; Stojkovic *et al.*, 2004a). It would enable the production of patient-specific nuclear transfer stem cell (NTSC) lines that could provide additional approaches for studying pathogenesis, new tools for drug screening and the possibility of autologous cell transplantation therapy. However, there are several obstacles to the successful derivation of nuclear transfer (NT) embryos that need to be overcome if the technique is to have a significant medical impact. The major difficulty is the availability of human oocytes (Birmingham, 2003; Stojkovic *et al.*, 2004a). Two remarkable studies have described successful human NT, both published by Hwang *et al.* (2004, 2005). Hwang *et al.* (2004) demonstrated successful derivation of NT embryos and derivation of NTSC line using oocytes recovered from volunteers undergoing ovulation induction. This procedure describes recovery of cumulus–oocyte complexes (COC) from healthy donors undergoing ovulation induction and an *autologous* NT procedure, i.e. the person donating the oocyte that was enucleated was also the source of the donor cell. Meanwhile, the same authors demonstrated that successful and very efficient derivation of human NT embryos and patient-specific NTSC lines could be achieved using a *heterologous* NT strategy (Hwang *et al.*, 2005). However, in both studies, the authors stated that only fresh oocytes recovered from artificially stimulated and fertile volunteers could be used to derive human NT embryos (Hwang *et al.*, 2004, 2005).

The aim of this study was to identify the source of human oocytes with the best potential for the development to blastocysts after enucleation and fusion with the nucleus of a heterologous donor cell. Four sources for human oocytes were used: (i) oocytes that failed IVD (IVF), (ii) oocytes retrieved during the follicle reduction procedure after ovulation induction, (iii) oocytes recovered

during an IVF treatment but not inseminated due to unexpected azoospermia in the partner, and (iv) oocytes recovered after routine ovarian cystectomy procedures. Undifferentiated human embryonic stem cells (hESC) were used as donor cells. Undifferentiated hESC were used as donor cells, since these cells have higher reprogramming ability than adult somatic cells (Wakayama *et al.*, 1999; Humpherys *et al.*, 2002). This strategy allowed us to identify the developmental potential of surplus oocytes after enucleation and NT procedure.

This study reports the outcome from each of these sources and identifies the source that will give the greatest opportunity to achieve success with human NT.

Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

Sources of oocytes

All donated oocytes were obtained from the local IVF clinic (Newcastle Fertility Centre at Life; NFCL) or the Gynaecology Department at the Royal Victoria Infirmary in Newcastle. Appropriate approval was given by the Newcastle and North Tyneside Local Research Ethics Committee and The Human Fertilization and Embryology Authority (HFEA).

Oocytes that failed IVF

The routine clinical IVF protocol involved downregulation with Synarel (Pharmacia) nasal spray 800 μg daily and the addition of Menopur (Ferring, M and S, Langley, Berks, UK) 225 IU daily for a period of about 12 days to induce ovulation. Human chorionic gonadotrophin (NCG) 5000 or 10,000 IU (Serono, Aubonne, Switzerland) was given and oocytes were recovered by transvaginal aspiration 38 h later. Following insemination, oocytes were cultured in G-sperm or G-fert (Vitrolife GIII Media System; Research Instruments, Penryn, Cornwall, UK). Failure of fertilization was identified by the absence of 2 pronuclei on day 1 and confirmed by the absence of cleavage on day 2. Before enucleation, these oocytes were stained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) to confirm that the oocytes were not sperm penetrated. If the woman had given prior written consent, these oocytes, which would otherwise be discarded, were used for research. This was about 48 h after oocyte collection.

In one case, the partner unexpectedly produced an azoospermic semen sample after oocyte retrieval (oocytes failed to inseminate). A second sample 4 h later was also azoospermic. The woman had previously given written consent for the oocytes to be used for research if they failed to fertilize. After further discussion, she donated her oocytes to research for immediate use (7 h after collection).

Oocytes retrieved during the follicle-reduction procedure after ovulation induction

Routine ovulation induction treatment at NFCL involves Nafarelin nasal spray 800 μg daily and Menopur at doses ranging from 75 to 225 IU daily, depending on the response as assessed by estimation of serum oestradiol concentrations and ultrasound imaging of the ovaries. This sometimes results in the growth of three or more follicles. Insemination is then contraindicated because of the risk of multiple pregnancy. If this occurs, it is recommended that follicle reduction is performed. HCG 5000 IU is given and, 38 h later, transvaginal aspiration removes all oocytes in excess of two, which are left in their follicles. Insemination is then carried out. If the woman had previously given written consent, these aspirated oocytes were used for immediate study.

Oocytes obtained from these three groups of patients undergoing ovulation induction had been

matured under in-vivo conditions. They were used for research at time intervals of about 46, 7 and 1 h after oocyte collection respectively.

Oocytes recovered after routine ovarian cystectomy procedures

A further two patients were undergoing routine gynaecological procedures at which there was access to the ovaries at laparotomy. Both patients agreed to donate oocytes aspirated from small ovarian follicles under direct vision. No ovarian stimulation was given preoperatively. The recovered COC were matured *in vitro* in Tissue Culture Medium (TCM) 199 supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.075 IU/l rFSH, 0.5 IU/l rLH, 0.29 mmol/l pyruvate.

Enucleation of oocytes

Cumulus cells of COC were removed by repeated pipetting in 0.1% (v/v) hyaluronidase in TCM-199 medium. Oocytes were enucleated in manipulation medium supplemented with 10% (v/v) FCS and 5 (μg/ml cytochalasin B). Each oocyte was held with a holding micropipette and the zona pellucida (ZP) was partially dissected with a sharp glass capillary to create a slit. The first polar body (PB) and adjacent cytoplasm containing the metaphase II (MII) chromosomes were extruded by squeezing with the needle (Hwang *et al.*, 2004). The removal of DNA was confirmed by DNA staining and visualization under UV light. DNA staining was performed prior to enucleation, so that the nuclear material was visible and therefore simple to remove. The enucleated oocytes were placed in TCM-199 medium supplemented with 10% (v/v) FCS and used for NT.

Source of karyoplast

The donor karyoplast used was from undifferentiated cells from existing human embryonic stem cell line, hES-NCL1. hES-NCL1 cells were cultured under feeder-free conditions (Stojkovic *et al.*, 2005) to avoid the possibility of contamination with mouse feeder cells.

Activation and culture of NT embryos

A single donor cell was deposited into the perivitelline space of an enucleated oocyte, the couplets were subsequently placed in a fusion medium comprising 0.26 mol/l mannitol, 0.1 mmol/l $MgSO_4$, 0.5 mmol/l HEPES, and 0.05% (w/v) BSA, and transferred into a cell fusion chamber (0.5 mm gap) after equilibration for 3 min. Fusion was induced using an electro-cell manipulator (Multiporator, Eppendorf, Germany) as previously described (Hwang *et al.*, 2004). Only fused embryos were selected and after reprogramming time (for more details, see Hwang *et al.*, 2004), chemical activation was induced by incubating fusion complexes in G1 medium containing 10 μmol/l calcium ionophore A23187 (CI) for 5 min at 37°C. Reconstructed embryos were then washed thoroughly in G1 medium and further incubated for 4 h in G1 medium supplemented with 2.0 mmol/l of 6-dimethylaminopurine (6-DMAP). At the end of reconstruction, embryos were cultured in G1 medium under mineral oil at 37°C in a humidified atmosphere of 5% CO_2. On day 3, embryos were removed into fresh droplets of culture G2.3 medium and cultured for another 2–3 days at same culture conditions (Stojkovic *et al.*, 2004b).

On days 3 and day 5, early cleavage and blastocyst rates were evaluated. A recovered blastocyst was cultured on a γ-irradiated mouse embryonic feeder (MEF) monolayer (75,000 cell/cm^2) in DMEM supplemented with 10% (v/v) Hyclone defined FCS (Hyclone, Logan, UT, USA) as previously described (Stojkovic *et al.*, 2004b). Unfortunately, after 2 days the blastocyst did not attach to MEF and degenerated. However, one part of blastocyst was used for extraction of single stranded human genomic DNA and amplification of 10 independent DNA markers and the sex chromosomes (Amelogenin) using the Amp*f*/STR kit (Applera, UK). Genescan analysis on the amplified fragments was performed from both samples (part of degenerated NT embryo and donor cells) using the following microsatellite markers: D3S1358, vWA, D16S539, D2S1338,

Amelogenin, D8S1179, D21S11, D18S51, D19S433, TH01, and FGA-then analysed on an ABI 377 sequence detector using Genescan version 3.1.2 and Genotyper version 2.5.2 software (Applied Biosystems, Foster City, CA, USA).

Results

Between September 2004 and February 2005, 309 women who started IVF treatment were asked if they would donate unfertilized oocytes to the NT research project. Of these, 252 patients (82%) agreed. All those women having follicle reduction (n = 4) or gynaecological procedure (n = 2) who were asked to participate agreed to donate oocytes. For this part of the NT project, 11 patients donated 36 spare oocytes.

The presence of the first polar body (PB) was noted in oocytes in all groups demonstrating that they had reached MII stage (**Table 1**).

Table 1. Effects of different sources of human oocytes on cleavage and blastocyst rates after nuclear transfer with undifferentiated human embryonic stem cells. PB = polar body.

Sources of oocytes	*No. of patients*	*No. of oocytes*	*PB (%)*	*Cleaved (%) Day 3*	*Blastocyst (%) Day 5*
Failed fertilization	4	10	7 (70.0)	0	0
Follicle reduction	4	10	8 (80.0)	3 (30.0)	1 (10.0)
Failed to inseminate	1	12	10 (83.3)	0	0
Cysterectomy	2	4	2 (50.0)	0	0
Total	11	36	27 (75.0)	3 (8.3)	1 (2.8)

Oocytes recovered after follicle reduction and failed to inseminate oocytes showed a very higher percentage of PB (**Table 1**, **Figures 1b, c**) and neighbouring MII spindle complex after staining with DAPI (**Figure 1c**). After enucleation and fusion with undifferentiated hESC, only oocytes that were derived after follicle reduction were able to cleave after chemical activation (**Table 1**). On day 3, three embryos with five or more cells were recorded (**Figure 1d**) and one embryo cleaved to the blastocyst stage on day 5 (**Figure 1e**). The blastocyst possessed very well formed inner cell mass (**Figures 1e, f**) and was plated on MEF; however, after 48 h no signs of attachment or proliferation were noted.

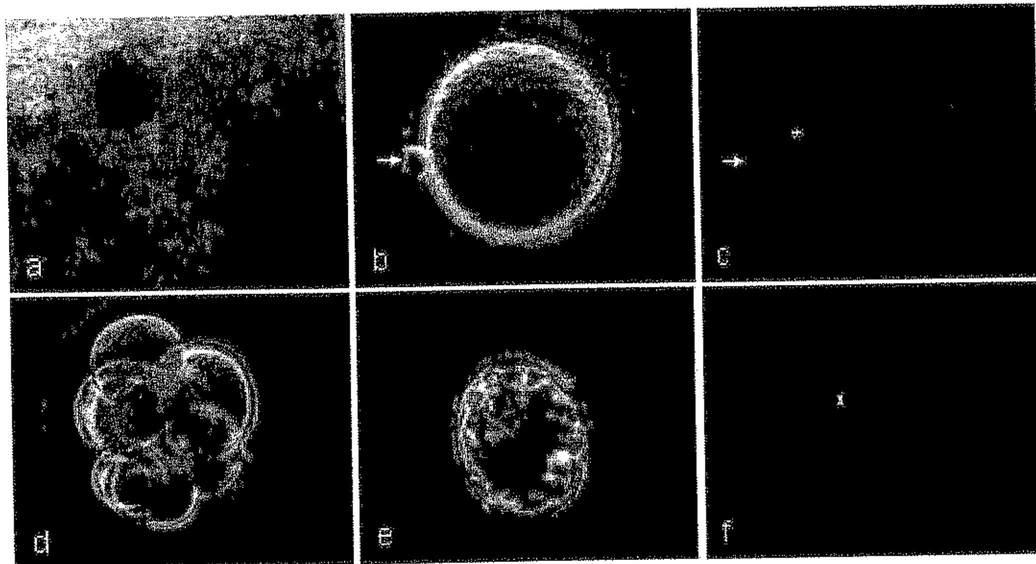

Figure 1. Derivation of human nuclear transfer embryos. Cumulus–oocyte complex (**a**) recovered after follicle reduction. After treatment with hyaluronidase, oocytes were denuded (**b**) and stained with 4,6-diamidino-2-phenylindole (DAPI) (**c**). Note the presence of polar body (**b**, **c**; arrow) and neighbouring spindle complex (**c**; asterisk). After nuclear transfer (NT), early cleavage stage (**d**) and blastocyst (**e**) were observed on day 3 and day 5 (fusio*n* = day 0) respectively. NT blastocyst stained with DAPI (**f**). Note the presence of very well formed inner cell mass (**e**, **f**; cross). The image **c** was merged (UV and bright light). Magnification: ×50 (**a**), ×200 (**e**, **f**), ×400 (**b**–**d**).

The blastocyst then degenerated and disaggregated. One part of the degenerated blastocyst (**Figure 2a**) and the undifferentiated hES-NCL1 (**Figure 2b**) were used for DNA fingerprinting to compare microsatellite markers of both samples. This analysis revealed that both donor cell and NT blastocyst had the same genetic origin (**Figure 2**).

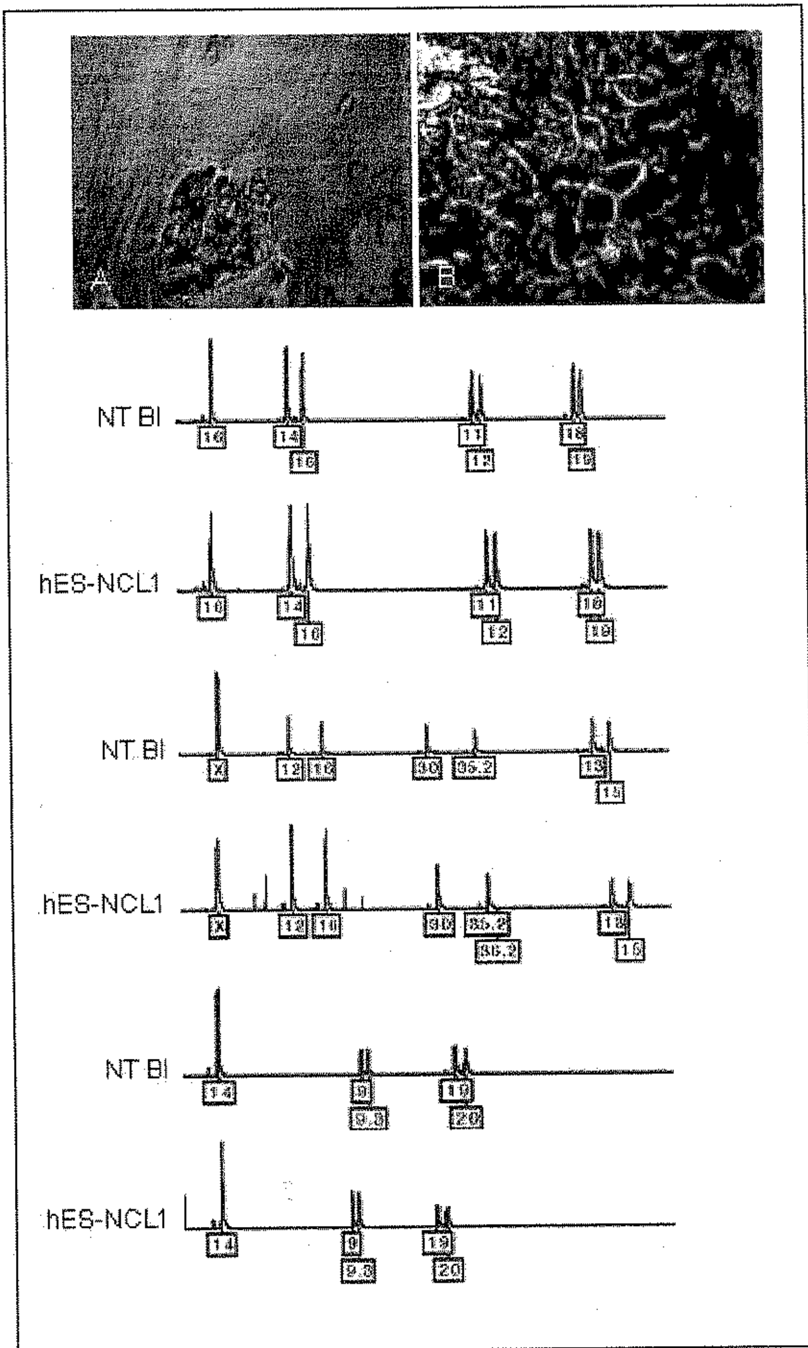

Figure 2. DNA fingerprinting analysis using nuclear transfer blastocyst (NT BL; **a**) and undifferentiated hES-NCL1 cells (**b**). The following microsatellite markers were compared between NT blastocysts and hES-NCL1 (donor cells): D3S1358 (chromosome location 3p21), vWA (chromosome location 12p12-pter), D16S539 (chromosome location 16q22–24), D2S1338 (chromosome location 2q35–37.1), Amelogenin (chromosome location Xp:22.1–22.3 and Y:p11.2), D8S1179 (chromosome location 8q24.1–24.2), D21S11 (chromosome location 21q21.1), D18S51 (chromosome location 18q21.3), D19S433 (chromosome location 19q12–13.1), TH01 (chromosome location 11p15–15.5), and FGA (chromosome location 4q28). Magnification: ×200 (**b**), ×400 (**a**).

Discussion

This study describes the derivation of human NT blastocyst using human oocytes and heterologous donor karyoplasts, since both oocyte and donor cell were recovered from different patients. It was demonstrated that oocytes recovered after follicle reduction possess developmental potential to cleave after the NT procedure which involved enucleation and fusion with undifferentiated hESC. Undifferentiated hESC were used as donor cells, since these cells have higher reprogramming ability than adult somatic cells (Wakayama *et al.*, 1999; Humpherys *et al.*, 2002). Inadequate reprogramming and epigenetic status of the donor nucleus after NT are thought to be the principal reasons for the developmental failure of most NT embryos (Hochedlinger and Jaenisch, 2003; Hwang *et al.*, 2004). Epigenetic modifications that are specific to the differentiated cell, such as DNA methylation and histone modifications, and the chromatin structure, suggest that the state of differentiation of the donor cell directly affects the efficiency of NT (Wakayama *et al.*, 1999; Hochedlinger and Jaenisch, 2003). This might explain why, in contrast to the results of NT involving somatic donor cells, the results of NT involving embryonic stem cells are more efficient (Hochedlinger and Jaenisch, 2003). Meanwhile, Hwang *et al.* (2005) very efficiently derived human NT embryos and patient-specific NTSC lines by heterologous NT. For this study, the authors used donor cells recovered after skin biopsy and fresh oocytes recovered from volunteers after ovarian stimulation. For this study, oocytes that are usually discarded were used.

Oocytes matured *in vivo* (oocytes with failed fertilization or failed insemination) were also able to fuse with donor cells, but not to cleave. The reason for this could be that these oocytes were too old. They were used for the NT procedure about 46 h and 7 h after recovery respectively. Oocytes recovered after cystectomy were matured under in-vitro conditions. None of the fused complexes derived from these three groups developed, which suggests that agedand in-vitro matured oocytes possess low developmental potential after NT. This corroborates results achieved using primate oocytes (Simerly *et al.*, 2004), where the authors demonstrated that oocytes with failed fertilization were not able to cleave after NT. This is probably because enucleation is optimally performed prior to MII arrest (Hwang *et al.*, 2004; Simerly *et al.*, 2004). The application of other enucleation techniques such as manual bisection of similarly obtained oocytes using an ultra-sharp splitting blade (hand-made cloning; Vajta *et al.*, 2005) do not result in reconstructed NT embryos (data not shown). Whether this is the consequence of missing the appropriate time window for enucleation, or the enucleation procedure which causes spindle defects resulting from centrosome and motor deficiencies (Tesarik and Mendoza, 2003; Simerly *et al.*, 2003, 2004), deserves further investigation.

Using the current protocol, only oocytes recovered after follicle reduction showed developmental potential after enucleation and NT. Supernumerary preovulatory follicular reduction is an approach to avoid multiple pregnancies in ovulation induction cycles (Albano *et al.*, 2001). These oocytes were used for enucleation immediately after recovery, and it was noted that the MII spindle complex was located very close to the first PB, which suggested that some of the oocytes might even be at the prometaphase II stage (Hwang *et al.*, 2004). Fresh prometaphase II or MII oocytes have been successfully enucleated using the squeezing method (Hwang *et al.*, 2004, 2005), whereas in the present case, the DNA spindle complex was extruded through a small slit in the ZP. After fusion with undifferentiated hESC, these oocytes cleaved and one developed up to blastocyst. DNA fingerprinting analysis confirmed that the reconstructed NT blastocyst was not of parthenogenetic origin. This is very important, since some studies (Cibelli *et al.*, 2001; Zavos, 2003) claimed successful derivation of human NT embryos but did not provide convincing evidence that these were truly derived from NT and were not simply activated or fragmented oocytes (Surani, 2003).

On the basis of these results, options have been reviewed for obtaining in-vivo matured MII oocytes for immediate donation to research. Follicle reduction is not the desired outcome of ovulation induction treatment, so there is no clinically acceptable option to increase the number of such volunteers. The recruitment of volunteer donors for ovulation induction causes ethical concerns. Although the serious risks associated with ovulation induction are low, severe ovarian hyperstimulation syndrome occurs in 1–2% of all cases (Mathur *et al.*, 2005), and there is a significant risk of life-threatening thrombosis (Stewart *et al.*, 1997). An alternative source is when excess oocytes are retrieved during routine IVF treatment. For instance, if there are >12 oocytes, the pregnancy rate would not be impaired if, say, two oocytes were randomly donated to research. It is already known that couples undergoing fertility treatment are willing to participate in this research (Bjuresten and Hovatta, 2003; Choudhary *et al.*, 2004). Assuming that appropriate informed consent is given, it is believed that such a strategy would be an ethically acceptable way forward to progress this important research.

In summary, it has been demonstrated that heterologous NT can be successfully achieved if carried out in oocytes immediately after in-vivo maturation and recovery. Adult somatic cells will now be used as donor cells, and more will be learned about oocyte physiology, the roles of mitotic spindles, cell cycle, asynchronies, mitochondrial and cytoplasmic incompatibilities, nuclear reprogramming/epigenetic, and gene expression during early human development (Dean *et al.*, 2001; Tesarik *et al.*, 2001; Santos *et al.*, 2003; Simerly *et al.*, 2004; Takeuchi and Palermo, 2004; Tian, 2004). The ultimate goal is to establish robust protocols for deriving NTSC lines. The latter can be subsequently used for 'tailor made' cell-replacement therapy to cure several debilitating human diseases, avoiding the potential immunological rejection of transplanted cells (Hochedlinger and Jaenisch, 2003; Edwards, 2004; Nagy, 2004; Gurdon 2005). They will also be powerful tools for studying pathogenesis of different human diseases.

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Editor's note

In terms of priority, the Editor wishes to stress that the paper from Stojkovic *et al.* was received here on 16 May 2005. As far as we can gather, the work of Hwang *et al.* was received by *Science* on 15 March 2005. This means that the work of Hwang *et al.* should receive priority for originality. We also thank Drs Stojkovic *et al.* for requesting the delay of publication of their Abstract on our web site in order to give Dr Hwang's team full credit for their obviously better results.

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