Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei

Megan J. Munsie*, Anna E. Michalska*, Carmel M. O'Brien*, Alan O. Trounson*, Martin F. Pera* and Peter S. Mountford*†

Pluripotent human stem cells isolated from early embryos represent a potentially unlimited source of many different cell types for cell-based gene and tissue therapies [1–3]. Nevertheless, if the full potential of cell lines derived from donor embryos is to be realised, the problem of donor–recipient tissue matching needs to be overcome. One approach, which avoids the problem of transplant rejection, would be to establish stem cell lines from the patient's own cells through therapeutic cloning [3,4]. Recent studies have shown that it is possible to transfer the nucleus from an adult somatic cell to an unfertilised oocyte that is devoid of maternal chromosomes, and achieve embryonic development under the control of the transferred nucleus [5–7]. Stem cells isolated from such a cloned embryo would be genetically identical to the patient and pose no risk of immune rejection. Here, we report the isolation of pluripotent murine stem cells from reprogrammed adult somatic cell nuclei. Embryos were generated by direct injection of mechanically isolated cumulus cell nuclei into mature oocytes. Embryonic stem (ES) cells isolated from cumulus-cell-derived blastocysts displayed the characteristic morphology and marker expression of conventional ES cells and underwent extensive differentiation into all three embryonic germ layers (endoderm, mesoderm and ectoderm) in tumours and in chimaeric foetuses and pups. The ES cells were also shown to differentiate readily into neurons and muscle in culture. This study shows that pluripotent stem cells can be derived from nuclei of terminally differentiated adult somatic cells and offers a model system for the development of therapies that rely on autologous, human pluripotent stem cells.

Addresses: *Monash Institute of Reproduction and Development, Monash University, 27–31 Wright Street, Clayton, Victoria 3168, Australia. †Stem Cell Sciences Pty Ltd, Level 1, 28 Riddell Pde, Elsternwick, Victoria 3185, Australia.

Correspondence: Peter S. Mountford
E-mail: peter.mountford@stemcellsciences.com.au

Received: **11 May 2000**
Revised: **23 June 2000**
Accepted: **23 June 2000**

Published: **11 August 2000**

Current Biology 2000, 10:989–992

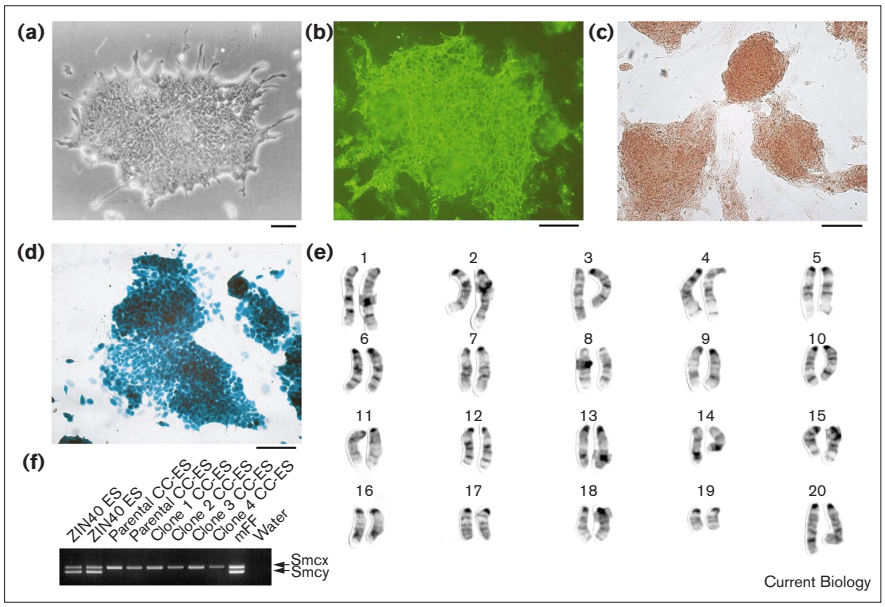
0960-9822/00/$ – see front matter

Results and discussion

Blastocysts were generated by substituting cumulus cell nuclei for the genetic material of mature oocytes using a modification of the method described previously [6]. The cumulus cells were collected from transgenic mice (ZIN40) that ubiquitously express a nuclear-localised *lacZ* reporter gene and demonstrate distinctive blue staining when treated with X-gal substrate [8]. As recipient oocytes were collected from a non-transgenic strain (B6D2F1), the presence of the *lacZ* transgenic marker was used to demonstrate nuclear contribution of the cumulus cell nuclei following transfer [9]. In 21 replicate experiments, 39% (362/926) of manipulated oocytes survived injection and enucleation. Following strontium activation, 75% (270/362) of the reconstructed oocytes cleaved to the two-cell stage after 24 hours in culture. After 96 hours in culture, 10 (3%) morphologically normal cumulus cell (CC)-derived blastocysts formed and were transferred into media supplemented with leukaemia inhibitory factor (LIF) for ES cell isolation. Eight blastocysts hatched and formed obvious inner cell mass outgrowths, with three displaying putative ES cell morphology in initial passages. One cell line, which we call the parental CC-derived ES (CC-ES) cell line, was established and continued to display characteristic mouse ES cell morphology when grown in the presence or absence of a fibroblast feeder layer in media supplemented with LIF (Figure 1a). Four sub-lines were established from 48 individual, mechanically isolated cells from the CC-ES cell line.

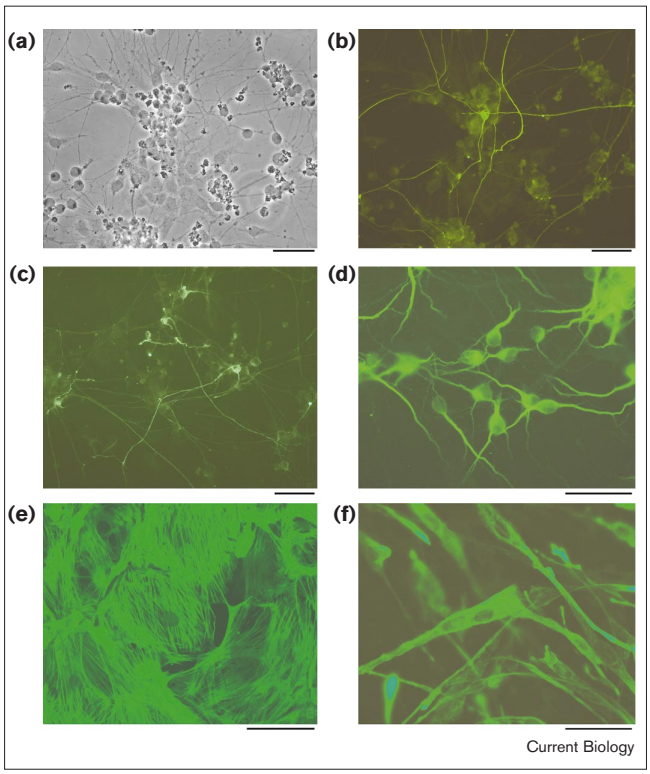
All CC-ES cell lines examined showed typical ES cell morphology (Figure 1a) and expressed characteristic mouse ES cell markers, including stage-specific embryonic antigen-1 (SSEA-1; Figure 1b) and alkaline phosphatase activity (Figure 1c). Oct4 mRNA was highly expressed in undifferentiated ES cells and downregulated in differentiated ES cell cultures (data not shown). All CC-ES cell lines were demonstrated to have been derived from a reprogrammed somatic nucleus by distinctive blue X-gal-stained nuclei (Figure 1d) and a normal female (40,XX) karyotype (Figure 1e). In addition to the cytological examination, the parental CC-ES cells and clonally derived sub-lines were shown to lack a Y-chromosome-specific PCR product (Figure 1f). No other female ES cells have been grown in our laboratory. The rate of ES cell derivation from CC-derived blastocysts was not statistically different ($p = 0.77$) from routine ES cell isolation with respect to the sample size analysed.

Figure 1



Characterisation and confirmation of CC-ES cells. Parental CC-ES cells displayed **(a)** morphology that is characteristic of mouse ES cell colonies, **(b)** SSEA-1 expression and **(c)** alkaline phosphatase activity. **(d–f)** Nuclear contribution was confirmed by (d) distinctive X-gal-stained nuclei, (e) female (40,XX) karyotype, and (f) the absence of Y-chromosome-specific PCR product (lower band) in parental CC-ES cells and four clonally derived CC-ES cell sub-lines. Male ZIN40 ES cells, male foetal fibroblast (mFF) cells and no DNA (water) controls are also shown. The PCR primers detected the mouse *Smcy* gene and its X-chromosome homologue *Smcx*. The scale bars represent 100 μm.

Figure 2

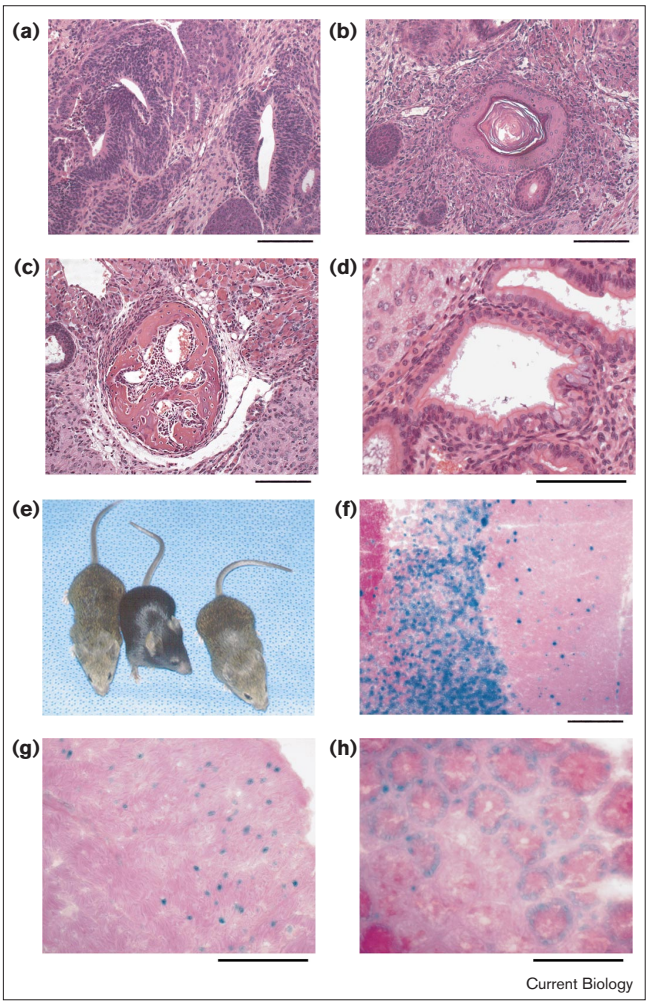


(a–d) Neural and **(e,f)** myogenic cells derived by *in vitro* differentiation of clonally derived CC-ES cells. Morphology was confirmed by indirect immunofluorescence to the neuron-specific proteins (b,c) neurofilament 160 and 68 kDa and (d) MAP-2a,b, and the muscle-specific proteins (e) alpha and gamma actin and (f) desmin. The scale bars represent 50 μm.

Pluripotency of the parental CC-ES cell line and two of the clonally derived sub-lines was investigated *in vitro* and *in vivo*. All CC-ES cell lines were found to differentiate readily into neurons and beating muscle *in vitro* at a similar frequency to control ES cells. Morphological observations were confirmed by indirect immunofluorescence in differentiated CC-ES cell cultures using neuronal- and muscle-specific antibodies (Figure 2). When CC-ES cells were injected into the testis capsule of immunocompromised mice, all injection sites (12/12) yielded *lacZ*-positive teratocarcinomas with extensive differentiation into all three embryonic germ layers (Figure 3a–d), including neural epithelium and stratified squamous epithelium (ectoderm); muscle, cartilage and bone (mesoderm); and ciliated secretory epithelium (endoderm).

Restoration of pluripotency to the somatic nucleus was further demonstrated by somatic cell contribution in chimaeric foetuses and pups. Four out of six midgestation (10 days post coitum; 10 dpc) foetuses contained X-gal-positive cells. One foetus appeared to be composed solely of CC-ES cell derivatives (data not shown). Six out of 14 pups were identified as chimaeric by obvious coat and eye colour (Figure 3e). Multi-lineage CC-ES cell contribution was confirmed by *lacZ* genomic PCR (data not shown) and microscopic analysis of X-gal-stained frozen sections. All organs analysed by X-gal staining, including cerebellum, heart, gut (Figure 3f–h), liver, lung, kidney, skeletal muscle, thymus and spleen, showed extensive contribution of CC-ES-derived cells. Detailed analysis will, however, be required to exclude cell-type-specific restrictions in the developmental potential of CC-ES cells.

Figure 3



CC-ES cell differentiation *in vivo*. Histological examination (haematoxylin and eosin staining) of parental CC-ES teratocarcinomas show the presence of ectoderm: **(a)** neural epithelium and **(b)** stratified squamous epithelium; mesoderm: **(c)** muscle and bone; and endoderm: **(d)** ciliated epithelium. Extensive somatic cell contribution of parental CC-ES cells in chimaeric pups (left and right) was indicated by **(e)** coat colour and confirmed by distinctive X-gal-stained nuclei in **(f)** cerebellum, **(g)** heart and **(h)** gut (counterstained with periodic acid schiff or PAS). The scale bars represent 100 μm.

This report describes the first isolation of pluripotent stem cells from adult somatic cell nuclei. Although 'ES-like' cells have been isolated from bovine nuclear transfer embryos derived from foetal fibroblast nuclei [10], the mouse somatic-cell-derived ES cell research described here offers a more accessible and diversified model system for *in vitro* and *in vivo* investigation of nuclear reprogramming and developmental competence. For example, ES cell lines may be established from different donor cell types and evaluated for possible restrictions in pluripotency using established *in vitro* and *in vivo* ES cell differentiation regimes. Such analyses may help explain the limited development of nuclear transfer blastocysts derived from neuronal nuclei compared with cumulus and Sertoli cell nuclei [6,11]. These studies could also be enhanced through the use of established transgenic mouse lines, which harbour developmentally regulated or ubiquitously expressed reporter genes to monitor reprogramming at the molecular level or visualise cellular contribution in chimaeric embryos [9]. Similarly, mutant mouse lines with specific gene deletions or genetic modifications could be used as a nuclear donor source to investigate the role of specific genes in nuclear reprogramming.

Possibly the greatest potential for combining nuclear reprogramming with ES cell derivation lies in the development of autologous human pluripotent stem cells for cell-based gene and tissue therapies. ES cells derived from somatic cell transplantation could restore function to diseased or damaged tissues, or be genetically altered before transplantation to deliver gene therapy. Transplantation studies in the mouse have shown that ES cell-derived cardiomyocytes [12], neural precursors [13], haematopoietic precursors [14] and insulin-secreting cells [15] can survive and function in recipient animals. Nevertheless, at least two independent and significant challenges must be met in order to achieve this goal in human medicine: first, the identification of a suitable donor nuclei source and the enhancement of nuclear reprogramming efficiency for so-called therapeutic cloning; and, second, the establishment of robust pluripotent stem cell culture and *in vitro* differentiation systems. The research described here provides proof-of-principle for human therapeutic cloning in a model system that should play a significant role in achieving at least the first of these important goals.

Supplementary material

Additional methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Andrew French, Jacqui Johnson and Ben Reubinoff for technical assistance; Teija Peura for advice; Jeremy Buzzard, Greg Tesch and Wei Mu for histology; Gary Dawson for karyotype analysis; Rob Daniels for Oct4 RT–PCR; and Austin Smith for comments on the manuscript. Supported by Australian Research Council Post Graduate Award (Industry), National Health and Medical Research Council (Australia) and Stem Cell Sciences Pty Ltd.

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: **Embryonic stem cell lines derived from human blastocysts**. *Science* 1998, **282:**1145-1147.
2. Reubinoff BE, Pera MF, Fong C-Y, Trounson AO, Bongso A: **Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro***. *Nat Biotech* 2000, **18:**399-404.
3. Smith AG: **Cell therapy: in search of pluripotency**. *Curr Biol* 1998, **8:**R802-804.
4. Solter D: **Dolly is a clone — and no longer alone**. *Nature* 1998, **394:**315-316.
5. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH: **Viable offspring derived from fetal and adult mammalian cells**. *Nature* 1997, **385:**810-813.
6. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R: **Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei**. *Nature* 1998, **394:**369-374.

7. Wakayama T, Yanagimachi R: **Cloning of male mice from adult tail tip cells**. *Nat Genet* 1999, **22:**127-128.
8. Mountford PS, Smith AG: **Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis**. *Trends Genet* 1995, **11:**179-184.
9. Munsie M, Peura TP, Michalska A, Trounson AO, Mountford PS: **Novel method for demonstrating nuclear contribution in mouse nuclear transfer**. *Reprod Fert Dev* 1998, **10:**633-637.
10. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM: **Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells**. *Nat Biotech* 1998, **16:**642-646.
11. Ogura A, Inoue K, Ogonuki N, Noguchi A, Takano K, Nagano R, *et al.*: **Production of male cloned mice from fresh, cultured and cryopreserved immature Sertoli cells**. *Biol Reprod* 2000, **62:**1579-1584.
12. Klug MG, Soonpaa MH, Koh GY, Field LJ: **Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts**. *J Clin Invest* 1996, **98:**216-224.
13. McDonald JW, Liu X-Z, Qu Y, Liu S, Mickey SK, Turetsky D, *et al.*: **Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord**. *Nat Med* 1999, **5:**1410-1412.
14. Potocnik AJ, Kohler H, Eichmann K: **Hemato-lymphoid *in vivo* reconstitution potential of subpopulations derived from *in vitro* differentiated embryonic stem cells**. *Proc Natl Acad Sci USA* 1997, **94:**10295-10300.
15. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Matrin F: **Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice**. *Diabetes* 2000, **49:**157-162.