Article

Developmental competence of immature and failed/abnormally fertilized human oocytes in nuclear transfer

Sohyun Lee McElroy completed a Veterinary Medicine degree in 2002, and obtained her PhD in 2005 at Seoul National University, Seoul, Korea. During her PhD, she conducted research in the Department of Theriogenology and Biotechnology studying early mammalian embryo development and somatic cell nuclear transfer. She is currently a post-doctoral fellow at Stanford University, Palo Alto, California, USA. Her research interests include the reprogramming potential of human oocytes and germ cell specification and differentiation from human embryonic stem cells.

Dr Sohyun Lee McElroy

Sohyun Lee McElroy[1], Kehkooi Kee[1], Nam Tran[2], Juanito Menses[2], Linda C Giudice[2], Renee A Reijo Pera[1,3]
[1]Institute for Stem Cell Biology and Regenerative Medicine, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, Palo Alto, CA 94304-5542, USA; [2]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California at San Francisco, San Francisco, CA, USA
[3]Correspondence: Tel: +1 650 725 3803; Fax: +1 650 736 2961; e-mail: reneer@stanford.edu

Abstract

Somatic cell nuclear transfer holds great promise for basic studies of reprogramming human somatic cells and for the potential development of novel cell-based therapeutics. The aim of this study was to examine experimental aspects of human nuclear transfer via use of an abundant source of oocytes, those that are routinely discarded from assisted reproduction clinics. The results suggest and reinforce several findings based on the analysis of multiple parameters: first, it was observed that supplementation of commercial culture media with hormones promoted embryo development after parthenogenetic activation. Second, the use of the chemical activation reagent puromycin resulted in significant differences in cleavage rates in oocytes that were failed/abnormally fertilized after intracytoplasmic sperm injection relative to those from IVF ($P < 0.05$). Third, cycloheximide promoted cleavage rates ≥40% in both groups of oocytes; moreover, two blastocysts were produced following cycloheximide treatment. Finally, the use of a subset of oocytes for nuclear transfer resulted in cleaved embryos that expressed green fluorescent protein from a transgene in donor nuclei from human embryonic stem cells. In light of these results, it is suggested that the discarded oocytes can be used to investigate new human nuclear transfer protocols for embryonic stem cell derivation.

Keywords: *abnormal fertilization, human embryonic stem cells, human oocytes, in-vitro maturation, nuclear transfer*

Introduction

Several techniques may ultimately prove useful for the reprogramming of adult somatic cells to a state indistinguishable from that of an embryonic stem cell, including genetic reprogramming, fusion of somatic cells with embryonic stem cells, and somatic cell nuclear transfer (SCNT) (Li, 2002; Cowan *et al*., 2005; Okita *et al*., 2007; Wernig *et al*., 2007). SCNT technology has been used to produce offspring, establish embryonic stem cells, and study epigenetic reprogramming, as mediated by oocytes, in several animal species (Humpherys *et al*., 2002; Li, 2002; Hochedlinger and Jaenisch, 2003; Armstrong *et al*., 2006; Vajta and Gjerris, 2006). In humans, there is hope that SCNT might contribute to the establishment of embryonic stem cell lines of defined genetic compositions for studies of reprogramming, pathogenesis, development and pharmacology, as well as for possible novel therapeutic applications. Nonetheless, there are ethical and practical issues that limit availability of oocytes donated by women of reproductive age, specifically for research. Thus, there is a need to exhaustively explore alternatives, including alternative oocyte sources for human SCNT, such as oocytes that would normally be discarded during routine IVF or intracytoplasmic sperm injection (ICSI). These oocytes are generally categorized as failed/abnormally-fertilized (FA) or immature oocytes, and constitute a significant percentage of oocytes in clinics, as described further below. FA oocytes include failed-fertilized oocytes (FF; no pronuclei observed) and abnormally fertilized oocytes (one or more than two pronuclei).

FA oocytes generally comprise approximately 40% of oocytes following IVF or ICSI (Plachot *et al.*, 2002); these oocytes are collected approximately 16–18 h following IVF or ICSI. Initial studies, with a small number of FF oocytes, have suggested that these oocytes may develop to early embryonic stages after nuclear transfer (NT) with fibroblast donor cells (Lavoir *et al.*, 2005). On the other hand, other studies have indicated that FF oocytes obtained approximately 48 h after retrieval do not cleave after SCNT with either undifferentiated human embryonic stem cells (ESC) or fibroblast-like cells (Stojkovic *et al.*, 2005; Hall *et al.*, 2007). Together, these studies suggest a window of time in which residual developmental potential may remain, though success to date in development to blastocyst stage has not been reported.

It is noted here that, in mice, it was long assumed, in the absence of extensive data and optimization, that fresh oocytes were a requirement for successful fertilization and SCNT (Lanman, 1968; Sakai and Endo, 1988). However, recently, Wakayama and colleagues have found that although embryos obtained following SCNT with mouse FF oocytes were less likely to be useful for reproductive cloning, they were indistinguishable from fresh oocytes in their ability to establish mouse embryonic stem cell lines, once procedures had been optimized (Kishigami *et al.*, 2006; Wakayama *et al.*, 2007). These authors suggest that, if these results could be replicated with human oocytes (via optimization of human SCNT procedures), this method would avoid many of the ethical concerns surrounding fresh oocyte donation. More recently, a second group of researchers extended these results in mice and demonstrated efficient SCNT with mouse post-fertilization zygotes that might be analogous to a sub-class of abnormally-fertilized human oocytes (Egli *et al.*, 2007). Thus, these data, and those from other species, suggest that SCNT techniques and large datasets with human FA oocytes, from multiple laboratories, should be explored to determine the developmental potential of oocytes that would normally be discarded.

Besides oocytes that fertilize abnormally, approximately 20% of retrieved oocytes are classified as immature [termed germinal vesicle (GV) or metaphase I (MI) stage] (Strassburger *et al.*, 2004). Since the first successful fertilization of in-vitro matured oocytes (Edwards *et al.*, 1969), many studies have explored methods to mature oocytes *in vitro* with some notable successes, and many challenges (Trounson *et al.*, 2001; Papanikolaou *et al.*, 2005). Most notably, several case reports document blastocyst development or live birth achieved from oocytes matured *in vitro* from hormone-stimulated cycles (Chian and Tan, 2002; Liu *et al.*, 2003; Otsuki *et al.*, 2006). Additionally, Heindryckx and colleagues demonstrated development of human SCNT embryos, generated from oocytes that were matured *in vitro,* to the morula stage (Heindryckx *et al.*, 2007). These studies strongly suggest that maturation of immature oocytes *in vitro* might be optimized for human SCNT.

If oocytes that would normally be discarded are to be used in SCNT, several aspects of the SCNT process must be optimized. For example, human oocyte activation has been accomplished by use of the calcium ionophore A23187 (CI) to generate an initial intracellular calcium transient, followed by a secondary treatment using either puromycin (PUR) or 6-dimethylaminopurine (DMAP) to produce parthenogenotes and activate FF oocytes following ICSI (Nakagawa *et al.*, 2001a; Lu *et al.*, 2006). These sequential treatments inactivate mitosis promoting factor and/or cytostatic factor (CSF), and thus induce meiotic resumption and the first embryonic cell cycle. PUR, and related agents such as cycloheximide (CHX), inhibit protein synthesis, whereas DMAP inhibits non-specific kinase activity (Nakagawa *et al.*, 2001b). Artificial activation of human FF ICSI oocytes has been reported with co-treatment of CI and PUR (Nakagawa *et al.*, 2001a; Lu *et al.*, 2006) or CI treatment (Tesarik and Testart, 1994). However, artificially activated FF oocytes generally arrest early in development (Nakagawa *et al.*, 2001a; Hall *et al.*, 2007). In other species, CHX has been shown to induce parthenogenetic embryo development and a lower frequency of chromosomal abnormalities relative to DMAP (Presicce and Yang, 1994; Alexander *et al.*, 2006).

The techniques used in NT are also important factors that impact on the development of NT embryos. The procedures of enucleation and introduction of the donor nucleus vary depending on species and laboratory (Campbell *et al.*, 2005). Hoechst staining has been used for the invasive enucleation of oocytes, and has been successful in many cases, even though it is known that Hoechst staining and ultraviolet (UV) light may damage oocyte mitochondrial DNA (Dominko *et al.*, 2000). More recently, non-invasive NT techniques that rely on polarized microscopic imaging systems have also been used to visualize the meiotic spindle without DNA staining and UV illumination (Liu *et al.*, 2000). In addition, several methods have been explored for introduction of donor nuclei, including direct injection and fusion methods (Lee *et al.*, 2003; Stojkovic *et al.*, 2005; Hall *et al.*, 2007; Heindryckx *et al.*, 2007). Nonetheless, different enucleation/injection techniques have not yet been explored in human NT.

This study reports the analysis of more than 350 immature and FA human oocytes in order to explore methods for in-vitro maturation, artificial activation and SCNT techniques; donor nuclei from human embryonic stem cells that express green fluorescent protein (GFP) were used.

Materials and methods

Participant consent and approval

Study participants were women who presented for assisted reproduction to the Centre for Reproductive Health at the University of California at San Francisco (UCSF), according to protocols approved by the Committee on Human Research. All oocytes were donated for research to the UCSF IVF Tissue Bank, with written informed consents. Participants consented to different research protocols, depending on preference, with three levels of research participation available: research level 1 indicated that oocytes were approved only for analytical purposes, research level 2 indicated that oocytes were approved for studies that might include establishment of human embryonic stem cell lines, and research level 3 indicated that oocytes were approved for studies of SCNT and establishment of human ESC lines. This resulted in donation of 271 immature oocytes [of which 172 (63.5%) were GV and 99 (36.5%) were MI] and 340 FA oocytes [of which 195 (57.4%) were from ICSI procedures and 145 (42.6%) were from IVF]. Most participants (57%) consented to research at all three levels, yielding more than 350 oocytes for NT analysis in this study. Oocytes were retrieved from women after induction via standard protocols as described (Shen *et al.*, 2006).

Oocyte sources

Following retrieval, oocytes were examined by the clinical embryology team for use in ICSI. A subset of oocytes was deemed immature (GV and MI) approximately 4–6 h after retrieval in order not to interrupt the clinical success. Denuded, immature oocytes were randomly matured *in vitro* in G1.3 plus (VitroLife, Englewood, CO, USA) either without any hormone (G1) or supplemented with hormones (G1 + hormones), 0.075 IU/ml recombinant FSH (rFSH, Gonal-F; Serono Laboratories, Randolph, MA, USA), 0.025 µg/ml recombinant human chorionic gonadotrophin (rHCG, Ovidrel; Serono Laboratories), 1 µg/ml oestradiol (Sigma-Aldrich, St Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Nuclear maturation status was observed at 30 and 48 h post-retrieval and characterized by the extrusion of the first polar body into the perivitelline space and Hoechst staining.

Oocytes that were termed FA were assessed 16–18 h after ICSI or IVF and were characterized by the lack of a visible pronucleus or abnormal number of pronuclei (one or more than two). However, these oocytes were not received by the research group until approximately 24–28 h post-retrieval, so as to avoid compromising clinical success of oocyte donors. All oocytes obtained for research were then used for appropriate protocols including in-vitro maturation, artificial activation or NT purposes, depending on consented research levels.

Artificial activation

Oocytes, at metaphase II (MII) following in-vitro maturation (IVM-MII), were activated using 10 µmol/l CI (Sigma-Aldrich) for 5 min, followed by 10 µg/ml PUR (Sigma-Aldrich) for 4 h at 37°C in 5% CO_2 in humidified air. Oocytes that were classed as FA following ICSI or IVF, were also activated using 10 µmol/l CI for 5 min, and then randomly assigned to one of three activation chemicals: 10 µg/ml PUR, 2 mmol/l DMAP or 10 µg/ml CHX (Sigma-Aldrich) for 4 h at 37°C in 5% CO_2 in humidified air. Activated oocytes were placed in a biphasic culture medium G1.3/G2.3 plus (VitroLife) at 37°C in 6% CO_2, 5% O_2 and 89% N_2. Embryo development was monitored via microscopy on days 1, 3 and 5. Embryonic nuclei were observed by staining with 5 µg/ml Hoechst on days 5–7. In determining cleaved cell number, it was critical that fragments were not scored as cells. Thus, Hoechst staining was relied upon as a guide regarding cell number and fragments. Blastomeres without DNA staining were not included in cleavage cell number.

Donor cell preparation

Two different donor cells were prepared for NT: H9 [National Institutes of Health (NIH) registry designation WA09] human ESC that express human Oct4–GFP (Oct4–ESC), and HSF1 (NIH registry designation UC01) cells that express ubiquitous GFP (GFP–ESC) (Gerrard *et al.*, 2005; K Kee and RA Reijo-Pera, unpublished). Donor cells were cultured in feeder-free conditions in 12-well plates in 5% CO_2 in mouse embryonic fibroblast conditioned medium (20% knockout serum replacement in 80% knockout Dulbecco's modified Eagle's medium, 1 mmol/l L-glutamine, 0.1 mmol/l non-essential amino acids, 0.1 mmol/l β-mercaptoethanol, 4 ng/ml recombinant human basic fibroblast growth factor), as previously described (Clark *et al.*, 2004). At ≥70% confluence, donor cells were treated with collagenase and stored in freezing medium (fetal bovine serum with 10% dimethyl sulfoxide) in liquid nitrogen until use. Donor cells were thawed at 37°C, washed with phosphate-buffered saline, and single cells were resuspended in phosphate-buffered saline with 5% fetal bovine serum by vigorous pipetting.

Nuclear transfer

Protocols for NT included invasive NT that relied on Hoechst staining of oocyte nuclei followed by direct injection of donor cells, and non-invasive NT that used polarized light to detect the spindle followed by electric fusion to introduce the donor nucleus.

For invasive NT, all oocytes were incubated in 5 µg/ml Hoechst in G-MOPS plus (VitroLife) for 5 min, followed by incubation in 7.5 µg/ml cytochalasin B (Sigma-Aldrich) for 5–15 min. A bevelled (13–15 µm inner diameter) pipette was inserted through the zona pellucida, and the nucleus (metaphase plate, pronuclei or inseminated sperm head) was aspirated into the pipette. Enucleation was confirmed by brief excitation by UV light. Each donor cell membrane was disrupted by bevelled injection pipette (13–15 µm inner diameter) and injected directly into the oocyte cytoplasm. Nuclear transferred embryos were incubated in G1.3 plus (VitroLife) at 37°C in a humidified incubator for 2 h before chemical activation. Nuclear transferred embryos were activated by exposure to 10 µmol/l CI for 5 min, followed by 4 h incubation in 2 mmol/l DMAP. Nuclear transferred embryos were cultured for 5–7 days *in vitro*, and observed for development, as described above. Again it should be noted that, in determining cleaved cell number, care was taken to ensure that fragments were not scored as cells. Hoechst staining was relied upon as a guide regarding cell number and fragments; blastomeres without DNA staining were not included in cleavage cell number.

For non-invasive NT, the spindle imaging system (Oosight™ Imaging System; Cambridge Medical Instrumentation, Inc., Woburn, MA, USA) was used to visualize the meiotic spindle of recipient oocytes as an alternative to Hoechst staining, and electric fusion was adopted as an alternative to direct injection of donor cells. Oocytes were incubated in 7.5 µg/ml cytochalasin B (Sigma-Aldrich) for 5–15 min. The metaphase spindle was visualized based on its high birefringence in the Oosight System, as described elsewhere (Liu *et al.*, 2000). Then, a bevelled (13–15 µm inner diameter) pipette was inserted through the zona pellucida and the metaphase spindle was aspirated into the pipette. Single donor cells were injected into the perivitelline space, and fused in fusion medium consisting of 280 mmol/l mannitol, 0.1 mmol/l $MgSO_4$, 0.5 mmol/l HEPES, and 0.05% bovine serum albumin with two consecutive electrofusion pulses of 1.8 V/cm of 15 µs duration, using a BTX Electro-Cell Manipulator 2001 (BTX, Inc. San Diego, CA, USA). Nuclear transferred embryos were incubated in G1.3 plus (VitroLife) at 37°C in a humidified incubator for 2 h before chemical activation. Fused nuclear transferred embryos were activated, cultured, and observed via nuclear staining as described above.

Genomic DNA extraction and amplification by polymerase chain reaction

Genomic DNA was isolated from individual embryos by incubation in lysis buffer at 95°C for 5 min. Genomic DNA

from donor human embryonic stem cells was isolated via the PicoPure DNA extraction system (Arcturus, Inc., Mountain View, CA, USA). Polymerase chain reaction (PCR) primers were designed to detect the presence of the GFP transgene (GFP forward: 5-CCTGAAGTTCATCTGCACCA-3; GFP reverse: 5-GCTCCTGGACGTAGCCTTC-3). PCR reactions were carried out in a 30 μl volume containing template DNA from each cleaved embryo, 5 μmol/l of each primer, 2 mmol/l dNTPs, 1.25 units HotStarTaq Plus DNA polymerase and buffer (Qiagen, Valencia, CA, USA). PCR amplification was as follows: 95°C, 5 min, one cycle; followed by 94°C, 30 s; 50°C, 30 s; 72°C, 1 min, 40 cycles; 72°C, 10 min; one cycle. Nested PCR was performed with 2 μl of first PCR product using the same conditions. Amplicons were analysed by gel electrophoresis, stained with ethidium bromide and visualized by UV excitation.

Statistical analysis

For a given comparison, data corresponding to that comparison were entered into a two-by-two contingency table. Fisher's exact test was used to generate a *P*-value indicating the probability of obtaining the configuration of the data within the table by chance. Significant differences between treatments were noted when the comparison-wise *P*-value was less than 0.05.

Results

Hormone supplementation and in-vitro maturation of oocytes

This study focused on optimizing several factors in human NT. First, the potential of immature oocytes for NT was examined, via in-vitro maturation in G1 medium, with and without supplementation with the hormones FSH, HCG and oestradiol (**Table 1**). It was noted that in G1 alone, 63% of GV oocytes demonstrated nuclear maturation, as characterized by the extrusion of the first polar body into the perivitelline space within 30 h post-retrieval. An additional 7.4% GV oocytes matured within 30–48 h post-retrieval in the absence of hormone supplementation, whereas the remainder (29.6%) did not demonstrate maturation. In G1 medium supplemented with hormones, fewer GV oocytes (48.6%) demonstrated maturation compared with G1 alone, although differences were not significant. When maturation of MI oocytes was examined, it was observed that, within 30 h after retrieval, approximately 82.8% of MI oocytes demonstrated nuclear maturation in G1 alone and 83.3% demonstrated nuclear maturation in G1 supplemented with hormones. These results clearly indicate that there was no significant increase in nuclear maturation as a function of hormonal supplementation.

In contrast to observations with nuclear maturation, when the developmental competence of IVM-MII oocytes matured from GV or MI in G1 with and without hormone supplementation was examined (**Table 2**), it was observed that IVM-MII oocytes that were matured in G1 with hormone supplementation were more likely to produce parthenogenetic embryos than those in G1 medium alone (50.0 versus 25.0%). Furthermore, none of the embryos that underwent cleavage divisions developed beyond the 4-cell stage in G1, whereas 5 out of 10 (50%) developed beyond the 4-cell stage in G1 + hormones medium (**Figure 1A**).

Developmental competence of failed/abnormally-fertilized oocytes following artificial activation

Oocytes that were classed as FA were mature already and had been obtained following ICSI or IVF. FA oocytes were used to test whether cleavage rates were associated with the mode of insemination and subsequent artificial activation. Cleavage rates, across all groups, varied from approximately 15.0 to 50.0% (**Table 3**). With PUR post-activation treatment, FA-ICSI oocytes had a significantly higher cleavage rate compared with FA-IVF oocytes (50.0% versus 15.0%; $P < 0.05$). CHX treatment resulted in similar cleavage rates of 40% in FA-ICSI and 43.5% in FA-IVF oocytes (**Table 3**). Among cleaved embryos, six from FA-ICSI oocytes and 12 from FA-IVF oocytes developed beyond the 4-cell stage (**Figure 1B**). Indeed, in two cases, blastocysts were produced following CHX activation (one each from the FA-ICSI and FA-IVF groups). The blastocyst derived from FA-ICSI contained approximately 100 cells, whereas that from FA-IVF was highly abnormal and had only 10 cells, confirmed by Hoechst staining (**Figure 2A, B**). The blastocyst from FA-ICSI was seeded onto mouse fibroblast feeder cells after removal of the zona pellucida in an attempt to generate a human ESC line, but it did not attach to the feeder cells.

Developmental competence of in-vitro matured or failed/abnormally-fertilized oocytes following nuclear transfer

The potential for use in NT of oocytes matured *in vitro* or those that were FA by either invasive or non-invasive techniques was next examined. It was observed that there were no significant differences in the parameters of enucleation, survival and cleavage rates among the different oocyte sources (those that had been matured *in vitro* to MII or those that were classified as FA) or in association with different donor cells (OCT4–ESC, GFP–ESC) with invasive NT (**Table 4**). Indeed, among the cleaved NT embryos obtained, two from IVM-MII, four from FA-ICSI oocytes, and one from an FA-IVF oocyte developed beyond the 4-cell stage (**Figure 1C**). However, none of these embryos expressed GFP as detected by fluorescence microscopy, even though the presence of the GFP genomic DNA was confirmed by PCR analysis in a cleaved NT embryo (**Figure 3**).

Next, whether the use of the non-invasive technique (with the Oosight™ SpindleView system and electrofusion) might prove superior to the invasive techniques was examined. As shown in **Table 5**, IVM oocytes arrested at GV stage (IVM-GV) had significantly lower enucleation rates ($P < 0.05$) compared with oocytes at other stages, presumably due to the fragility of the GV ooplasm. However, no significant differences were observed in fusion (21.4–50.0%) and cleavage rates (20.0–50.0%) among the different oocyte sources (IVM-MI versus IVM-MII versus FA-ICSI versus FA-IVF) (**Table 5**). Among the cleaved NT embryos derived, three from IVM-MII oocytes and one from an FA-IVF oocyte developed beyond the 4-cell stage (**Figure 1D**). Among the cleaved NT embryos, one embryo from IVM-MI, two from IVM-MII, and one from an FA-IVF oocyte expressed GFP as assessed by fluorescence microscopy (**Figure 2C, D**). It was also

Table 1. Nuclear maturation after in-vitro maturation of immature oocytes.

Culture medium	*Oocyte stage*	*Nuclear maturation rate*[a] *30 h*	*48 h*
G1	GV (27)	17 (63.0)	2 (7.4)
	MI (29)	24 (82.8)	–
G1 + hormones	GV (35)	17 (48.6)	–
	MI (24)	20 (83.3)	–

Values are *n* or *n* (%).
[a]Maturation time was calculated from oocyte retrieval.
G1 = G1.3 plus medium; G1 + hormones = G1.3 plus medium supplemented with hormones: 0.075 IU/ml recombinant FSH, 0.025 μg/ml recombinant human chorionic gonadotrophin and 1 μg/ml oestradiol; GV = germinal vesicle stage; MI = metaphase I stage.

Table 2. Developmental competence of in-vitro-matured oocytes following parthenogenetic activation.

Group	*MII oocytes*	*Cleaved embryos*
G1	16	4 (25.0)
G1 + hormones	20	10 (50.0)

Values are *n* or *n* (%).
G1 = G1.3 plus medium; G1 + hormones = G1.3 plus medium supplemented with hormones: 0.075 IU/ml recombinant FSH, 0.025 μg/ml recombinant human chorionic gonadotrophin, and 1 μg/ml oestradiol; MII = metaphase II.

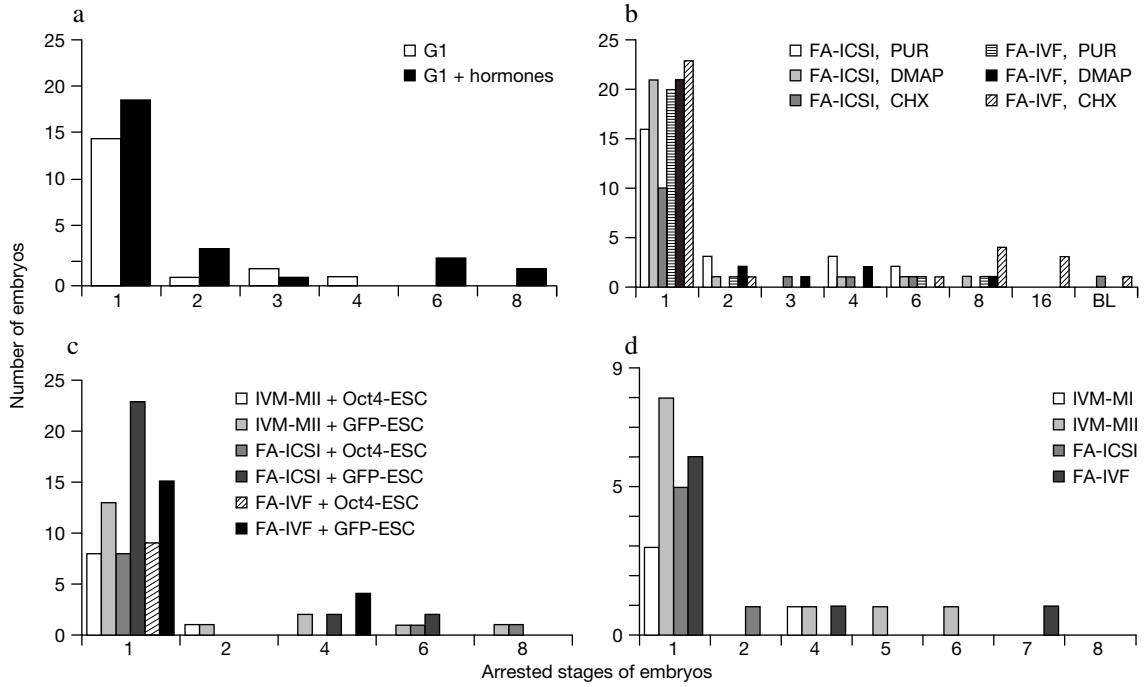

Figure 1. Embryo development following activation and nuclear transfer. (**a**) Arrested stages of human embryo development observed following parthenogenetic activation of oocytes matured *in vitro*. (**b**) Arrested stages of human embryo development observed following activation of oocytes that failed/abnormally fertilized (FA-ICSI or FA-IVF oocytes). Arrested stages of human embryo development following nuclear transfer using in-vitro matured or FA oocytes and either invasive (**c**) or non-invasive (**d**) techniques. G1 = G1.3 plus medium; G1 + hormones = G1.3 plus medium supplemented with hormones. CHX = cycloheximide; DMAP = 6-dimethylaminopurine; ESC = embryonic stem cell; GFP = green fluorescent protein; IVM = in-vitro maturation; M = metaphase; PUR = puromycin; Oct4–ESC = H9 human embryonic stem cells expressing human Oct4–green fluorescent protein.

Table 3. Developmental competence of failed/abnormally fertilized oocytes following artificial activation.

Oocyte source	*Activation*	*Total oocytes*	*Cleaved embryos*[a]	*Blastocysts*[b]
FA-ICSI	PUR	16	8 (50.0)[c]	0 (0.0)
	DMAP	21	4 (19.0)	0 (0.0)
	CHX	10	4 (40.0)	1 (25.0)
FA-IVF	PUR	20	3 (15.0)[d]	0 (0.0)
	DMAP	21	6 (28.6)	0 (0.0)
	CHX	23	10 (43.5)	1 (10.0)

Values are *n* or *n* (%).
[a]Percentage of total activated oocytes; [b]percentage of total cleaved embryos.
[c,d]Values with different superscript letters are significantly different ($P < 0.05$).
CHX = cycloheximide; DMAP = 6-dimethylaminopurine; FA-ICSI = failed/abnormally fertilized oocytes derived from intracytoplasmic sperm injection; FA-IVF = failed/abnormally fertilized oocytes derived from IVF; PUR = puromycin.

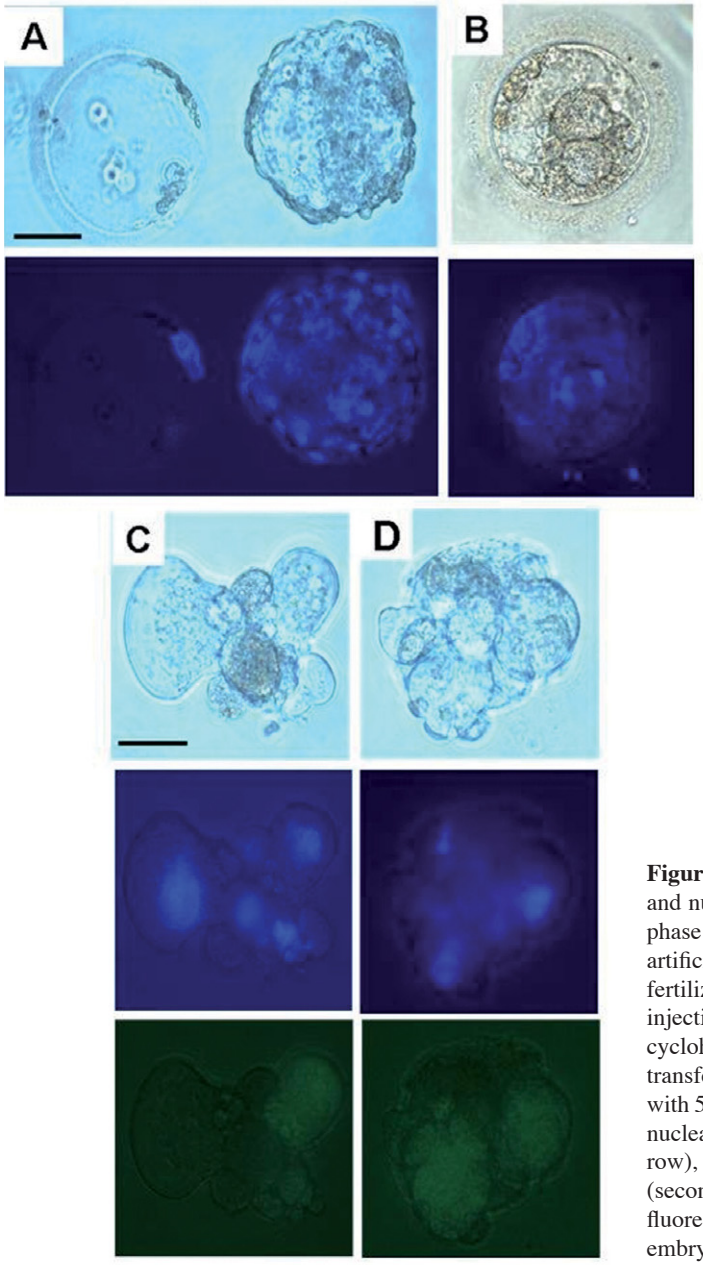

Figure 2. Embryo development following artificial activation and nuclear transfer. (**A**) Phase contrast and merged images of phase contrast and Hoechst staining of blastocyst produced by artificial activation with cycloheximide treatment of a failed-fertilized (FF) oocyte, following intracytoplasmic sperm injection. (**B**) Blastocyst produced by artificial activation with cycloheximide treatment of a FF oocyte following IVF. Nuclear transfer embryo from an in-vitro-matured oocyte (metaphase II) with 5 cells (**C**) and from a FF oocyte with 7 cells (**D**) following nuclear transfer by non-invasive techniques. Phase contrast (first row), merged images of phase contrast and Hoechst staining (second row), and merged images of phase contrast and green fluorescent protein fluorescence (third row) of nuclear transfer embryos. Scale bar = 30 μm.

Table 4. Developmental competence of in-vitro-matured or failed/abnormally fertilized oocytes following invasive nuclear transfer.

Oocyte source	*Donor cells*	*Total oocytes*	*Enucleated*[a]	*Survived after injection*[a]	*Cleaved*[b]
IVM-MII	Oct4–ESC	11	8 (72.7)	7 (63.6)	1 (14.3)
	GFP–ESC	16	13 (81.3)	13 (81.3)	5 (38.5)
FA-ICSI	Oct4–ESC	11	8 (72.7)	8 (72.7)	2 (25.0)
	GFP–ESC	38	28 (73.7)	26 (68.4)	5 (19.2)
FA-IVF	Oct4–ESC	14	9 (64.3)	9 (64.3)	0 (0.0)
	GFP–ESC	16	15 (93.8)	15 (93.8)	5 (33.3)

Values are *n* or *n* (%).
[a]Percentage of total oocytes; [b]percentage of total survived oocytes after injection.
FA-ICSI = failed/abnormally fertilized oocytes derived from intracytoplasmic sperm injection; FA-IVF = failed/abnormally fertilized oocytes derived from IVF; GFP–ESC = HSF1 human embryonic stem cells expressing ubiquitous green fluorescent protein; IVM-MII = metaphase II oocytes after in-vitro maturation; Oct4–ESC = H9 human embryonic stem cells expressing human Oct4–green fluorescent protein.

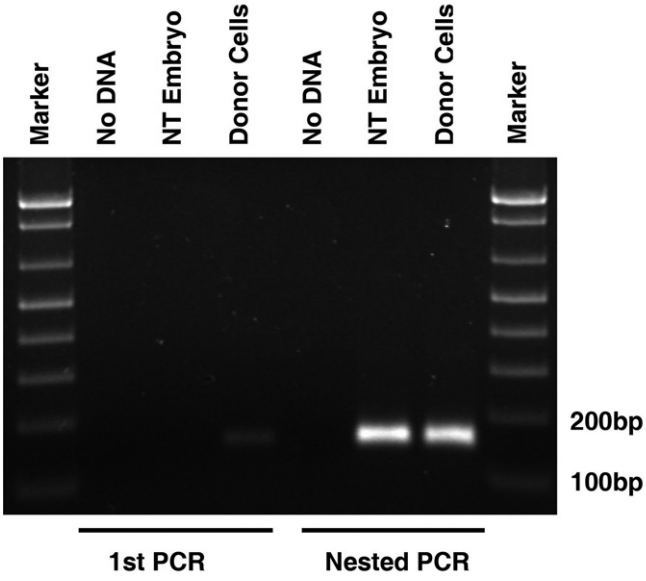

Figure 3. Polymerase chain reaction (PCR)-based analysis demonstrating the presence of the green fluorescent protein (*GFP*) gene in a cleaved nuclear transfer (NT) embryo reconstructed from GFP-positive human embryonic stem cell donor cells. Genomic DNA was extracted from a single embryo via nested PCR, as described in the Materials and methods. The GFP transgene was detected in one embryo.

Table 5. Developmental competence of in-vitro-matured or failed/abnormally fertilized oocytes following non-invasive nuclear transfer.

Oocyte source	*Donor cells*	*Total*	*Enucleated*[a]	*Fused*[a]	*Cleaved*[b]
IVM-GV	GFP–ESC	14	2 (14.3)[c]	0 (0.0)	–
IVM-MI		14	10 (71.4)[d]	3 (21.4)	1 (33.3)
IVM-MII		25	17 (68.0)[d]	8 (32.0)	4 (50.0)
FA-ICSI		18	12 (66.7)[d]	5 (27.8)	1 (20.0)
FA-IVF		12	12 (100.0)[d]	6 (50.0)	2 (33.3)

Values are *n* or *n* (%).
[a]Percentage of total oocytes; [b]percentage of total fused oocytes. [c,d]Within columns, values with different superscripts are significantly different ($P < 0.05$).
FA-ICSI = failed/abnormally fertilized oocytes derived from intracytoplasmic sperm injection; FA-IVF = failed/abnormally fertilized oocytes derived from IVF; GFP–ESC: HSF1 human embryonic stem cells expressing ubiquitous green fluorescent protein; IVM-GV = germinal vesicle stage oocytes after in-vitro maturation; IVM-MI = metaphase I oocytes after in-vitro maturation; IVM-MII = metaphase II oocytes after in-vitro maturation.

noted that although there were low fusion rates in non-invasive NT (electrofusion) compared with the survival rates in invasive NT (direct injection), the fusion rates were similar to previous reports (Hall *et al.*, 2007).

Discussion

There is great interest in developing methods to derive patient-specific human ESC lines that might be used for basic studies of human genetics and development, as well as for potential novel therapeutic applications. In this study, several parameters of developmental competence were examined in a sample of oocytes that would normally have been discarded. Several of the observations made suggest such oocytes could be used for human NT for ESC derivation, but that the methods need further optimization. This is of particular interest given the common assumption that fresh oocytes recruited from donors for research may overcome the low nuclear transfer embryo development encountered; yet, to date, no successful derivation of human ESC following SCNT using human oocytes, from any source, has been reported. The results presented here demonstrate at least residual developmental competence of oocytes that failed to fertilize or abnormally fertilized in assisted reproduction treatments, and extend previous findings to the use of immature oocytes. Several considerations might improve human NT as described below.

First, although it is well-documented that success in fertilization requires synchronized maturation of both the nucleus and cytoplasm of oocytes, only nuclear maturation can be observed microscopically. This is an obvious caveat in nuclear transfer experiments, since cytoplasmic maturation determines embryo development following nuclear transfer. In this study, nuclear maturation was directly observed by Hoechst staining, and cytoplasmic maturation was assessed, indirectly, by embryo development after parthenogenetic activation or nuclear transfer. It was observed that the majority of nuclear maturation occurred within the first 30 h post-retrieval. Moreover, the results with immature MI oocytes in two groups (with and without hormonal supplementation of the culture medium) were similar to those obtained by others with IVM medium (78.6%) or tissue culture medium (TCM)-199 (70.8%) with hormone supplementation (Chian and Tan, 2002). However, it is not clear why fewer GV oocytes matured in G1 medium with hormone-supplement relative to G1 medium alone, IVM medium or TCM-199. The higher cleavage rate of oocytes matured in hormone-supplemented medium after activation suggests that gonadotrophins have a potential role in oocyte maturation and embryo development, as also suggested in previous studies (Patsoula *et al.*, 2003). Nonetheless, there were no blastocysts from activation of IVM oocytes; this might be due to the lack of cytoplasmic maturation and/or inefficient activation. In order to use in-vitro matured oocytes for NT, further optimization of culture conditions for maturation, as well as activation protocols, preferably based on natural oocyte conditions (Telfer and McLaughlin, 2007) is required.

Second, CHX has been used in many animal parthenogenesis and SCNT experiments and has been shown to prolong the first embryonic cell cycle, which may result in fewer chromosomal abnormalities in SCNT embryos compared with DMAP treatment (Alexander *et al.*, 2006; Bhak *et al.*, 2006). In this study, delayed cleavage in the CHX treatment group compared with other activation approaches was not observed. Interestingly, however, blastocysts were produced only in the CHX treatment group. The embryo development data of activated FA oocytes suggested that a few FA oocytes may still have development competence depending on activation protocols. Since CHX had higher cleavage rates in activation of both FA-ICSI and FA-IVF oocytes, it will be interesting to compare the nuclear transfer embryo development by using CHX and DMAP in the future.

Third, no significant difference in cleavage rates or embryonic development derived from either oocyte source was observed. Interestingly, several cleaved NT embryos were achieved from FA oocytes in our study. There are a number of possible reasons why improved embryo development from FA oocytes was obtained relative to previous studies (Lavoir *et al.*, 2005; Stojkovic *et al.*, 2005; Hall *et al.*, 2007; Heindryckx *et al.*, 2007), including the reduction in the window of time from retrieval to collection of oocytes and differences in nuclear transfer techniques. The importance of the time interval between oocyte collection and start of SCNT has already been suggested in a previous study with in-vivo matured oocytes (Heindryckx *et al.*, 2007). Nonetheless, the majority of cleaved embryos did not develop beyond the 4-cell stage, a critical stage of embryo genome activation. Thus, in order to improve the reprogramming potential of FA oocytes, serial NT (Ono *et al.*, 2001) or treatment of donor cells or nuclear transfer embryos with DNA methylation inhibitors (Enright *et al.*, 2003; Kishigami *et al.*, 2006) could be considered in the future.

Fourth, in this study, IVM-MII oocytes showed relatively higher cleavage rates compared with IVM-GV, IVM-MI or FA oocytes. In a recent study, morula-stage NT embryos were produced from IVM-MII oocytes, which were comparable to the in-vivo matured oocytes (Heindryckx *et al.*, 2007). In the present experiments, the later oocyte collection after retrieval, and the unsynchronized cell cycle stage of the human ESC that were used as donor cells, may have had a negative impact on development after NT compared with the previous study (Heindryckx *et al.*, 2007). Nonetheless, both experiments and previous ICSI with IVM-MII oocytes (Chian and Tan, 2002) demonstrated the potential of in-vitro matured oocytes as recipients for human NT. However, further studies of the cell stages of oocytes and donor cells, and NT techniques should be carried out to optimize human NT.

Finally, it is noted that when GFP-positive human ESC were used as donor cells, it was expected that GFP expression during embryo development following nuclear transfer would be observed. However, when the invasive NT technique (Hoechst staining and donor cell direct injection) was used, GFP fluorescence from any cleaved NT embryos was not observed, even though the genomic DNA for GFP was detectable by PCR. On the other hand, when the non-invasive NT technique (polarization imaging and electric fusion of donor cell) was used, GFP fluorescence was observed in four out of eight cleaved NT embryos. However, it is noted that GFP expression was observed only in a subset of blastomeres, as has been described previously in other species that demonstrated mosaic expression (Park *et al.*, 2002). This may represent the demise of some blastomeres of the embryos or an inherent lack of transcriptional activation in some embryonic blastomeres (Park *et al.*, 2002; Armstrong *et al.*, 2006). This observation

merits increased scrutiny in a larger sample set to determine the frequency with which transcription/translation occurs, and to begin the process of optimizing the derivation of human ESC lines from single, or few, blastomeres.

However, there are still several issues that should be addressed. In the present experiments, cleaved embryos following activation and NT showed asymmetric cleavage, with different size blastomeres and high fragmentation rates. Similar observations have been reported in previous NT reports with IVM (Heindryckx *et al*., 2007) and FF oocytes (Hall *et al*., 2007). To understand early reprogramming and cell cleavage after NT, spindle formation and chromosome segregation during cell division of NT embryos should be analysed in more detail. Moreover, it has been shown that approximately 29% of anucleated (no pronucleus; 0PN) oocytes from IVF (Van Blerkom *et al*., 2004) and 86% of 0PN oocytes from ICSI (Nasr-Esfahani *et al*., 2007) contained sperm nuclear material that can form paternal spindle components and potentially induce delayed cleavage and multi- and micronucleation of blastomeres. In order to elucidate mechanisms of limited human NT embryo development, spindle formation, blastomere ploidy and sperm contribution of cleaved embryos from activation or NT should be analysed during the first cell cleavage.

In conclusion, the present study documents early embryo development following artificial activation or nuclear transfer using oocytes that were matured *in vitro* and those that failed to fertilize or abnormally fertilized in assisted reproduction treatments. The results indicate many challenges, as most embryos arrested with just a few blastomeres, though there were a few successful cases where more advanced developmental stages were reached and GFP expression from donor cells was observed by modification of NT techniques. In spite of the caveats discussed above, it is suspected that the residual potential of oocytes that would normally be discarded is greater than previously thought and may be used to optimize the protocol of human NT. Furthermore, with improvements in NT techniques and embryo culture conditions to the blastocyst stage, and optimization of methods to produce human ESC lines, discarded oocytes may provide a feasible and abundant resource in human NT for human ESC derivation. Thus, at a minimum, tools are available to enable examination of the early programmes of development, relative to those of embryos derived via successful IVF/ICSI, including the ability to examine growth, gene expression, and genetic regulation of early programmes, and these may potentially be able to optimize for ESC line derivation (Dobson *et al*., 2004; Armstrong *et al*., 2006).

Acknowledgements

The study participants who kindly donated oocytes for research are acknowledged. The dedication to this project and technical support of Ms Marty Flores is gratefully acknowledged. The authors also gratefully acknowledge those who manage the UCSF IVF Gamete and Embryo Bank for their expertise, as well as Dr Shehua Shen for experimental advice, Ha Nam Nyugen for assistance with microscopy, and members of the Reijo Pera and Guidice laboratories for helpful comments and input. Dr Wei Cui, Roslin Institute is thanked for providing the Oct4–GFP construct. This work was supported by a Lalor Post-doctoral Research Fellowship (to SLM) and funding from the UCSF School of Medicine, Stanford University Institute for Stem Cell Biology and Regenerative Medicine and, the California Institute for Regenerative Medicine (CIRM) and the California Tobacco-Related Disease Research Program (TRDRP; to RARP).

References

Alexander B, Coppola G, Di Berardino D *et al*. 2006 The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. *Molecular Reproduction and Development* **73**, 20–30.

Armstrong L, Lako M, Dean W *et al*. 2006 Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. *Stem Cells* **24**, 805–814.

Bhak JS, Lee SL, Ock SA *et al*. 2006 Developmental rate and ploidy of embryos produced by nuclear transfer with different activation treatments in cattle. *Animal Reproduction Science* **92**, 37–49.

Campbell KH, Alberio R, Choi I *et al*. 2005 Cloning: eight years after dolly. *Reproduction in Domestic Animals* **40**, 256–268.

Chian RC, Tan SL 2002 Maturational and developmental competence of cumulus-free immature human oocytes derived from stimulated and intracytoplasmic sperm injection cycles. *Reproductive BioMedicine Online* **5**, 125–132.

Clark AT, Bodnar MS, Fox MS *et al*. 2004 Spontaneous differentiation of germ cells from human embryonic stem cells *in vitro*. *Human Molecular Genetics* **13**, 727–739.

Cowan C, Atienza J, Melton D *et al*. 2005 Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science* **309**, 1369–1373.

Dobson A, Raja R, Abeyta M *et al*. 2004 The unique transcriptome through day 3 of human preimplantation development. *Human Molecular Genetics* **13**, 1461–1470.

Dominko T, Chan A, Simerly C *et al*. 2000 Dynamic imaging of the metaphase II spindle and maternal chromosomes in bovine oocytes: implications for enucleation efficiency verification, avoidance of parthenogenesis, and successful embryogenesis. *Biology of Reproduction* **62**, 150–154.

Edwards RG, Bavister BD, Steptoe PC 1969 Early stages of fertilization *in vitro* of human oocytes matured *in vitro*. *Nature* **221**, 632–635.

Egli D, Rosains J, Birkhoff G *et al*. 2007 Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. *Nature* **447**, 679–685.

Enright BP, Kubota C, Yang X *et al*. 2003 Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin a or 5-aza-2-deoxycytidine. *Biology of Reproduction* **69**, 896–901.

Gerrard L, Zhao D, Clark AJ *et al*. 2005 Stably transfected human embryonic stem cell clones express Oct4-specific green fluorescent protein and maintain self-renewal and pluripotency. *Stem Cells* **23**, 124–133.

Hall VJ, Compton D, Stojkovic P *et al*. 2007 Developmental competence of human *in vitro* aged oocytes as host cells for nuclear transfer. *Human Reproduction* **22**, 52–62.

Heindryckx B, De Sutter P, Gerris J *et al*. 2007 Embryo development after successful somatic cell nuclear transfer to *in vitro* matured human germinal vesicle oocytes. *Human Reproduction* **22**, 1982–1990.

Hochedlinger K, Jaenisch R 2003 Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. *New England Journal of Medicine* **349**, 275–286.

Humpherys D, Eggan K, Akutsu H *et al*. 2002 Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proceedings of the National Academy of Sciences USA* **99**, 12889–12894.

Kishigami S, Mizutani E, Ohta H *et al*. 2006 Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. *Biochemical and*

Biophysical Research Communications **340**, 183–189.
Lanman JT 1968 Delays during reproduction and their effects on the embryo and fetus. 2. Aging of eggs. *New England Journal of Medicine* **278**, 1047–1054.
Lavoir M, Weier J, Conaghan J *et al*. 2005 Poor development of human nuclear transfer embryos using failed fertilized oocytes. *Reproductive BioMedicine Online* **11**, 740–744.
Lee JW, Wu SC, Tian XC *et al*. 2003 Production of cloned pigs by whole-cell intracytoplasmic microinjection. *Biology of Reproduction* **69**, 995–1001.
Li E 2002 Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews Genetics* **3**, 662– 673.
Liu J, Lu G, Qian Y *et al*. 2003 Pregnancies and births achieved from *in vitro* matured oocytes retrieved from poor responders undergoing stimulation in *in vitro* fertilization cycles. *Fertility and Sterility* **80**, 447–449.
Liu L, Oldenbourg R, Trimarchi JR *et al*. 2000 A reliable, noninvasive technique for spindle imaging and enucleation of mammalian oocytes. *Nature Biotechnology* **18**, 223–225.
Lu Q, Zhao Y, Gao X *et al*. 2006 Combination of calcium ionophore A23187 with puromycin salvages human unfertilized oocytes after ICSI. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **126**, 72–76.
Nakagawa K, Yamano S, Moride N *et al*. 2001a Effect of activation with Ca ionophore A23187 and puromycin on the development of human oocytes that failed to fertilize after intracytoplasmic sperm injection. *Fertility and Sterility* **76**, 148–152.
Nakagawa K, Yamano S, Nakasaka H *et al*. 2001b A combination of calcium ionophore and puromycin effectively produces human parthenogenones with one haploid pronucleus. *Zygote* **9**, 83–88.
Nasr-Esfahani MH, Razavi S, Mardani M *et al*. 2007 Effects of failed oocyte activation and sperm protamine deficiency on fertilization post-ICSI. *Reproductive BioMedicine Online* **14**, 422–429.
Okita K, Ichisaka T, Yamanaka S 2007 Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313–317.
Ono Y, Shimozawa N, Ito M *et al*. 2001 Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biology of Reproduction* **64**, 44–50.
Otsuki J, Momma Y, Takahashi K *et al*. 2006 Timed IVM followed by ICSI in a patient with immature ovarian oocytes. *Reproductive BioMedicine Online* **13**, 101–103.
Papanikolaou EG, Platteau P, Albano C *et al*. 2005 Immature oocyte in-vitro maturation: clinical aspects. *Reproductive BioMedicine Online* **10**, 587–592.
Park KW, Lai L, Cheong HT *et al*. 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.
Patsoula E, Loutradis D, Drakakis P *et al*. 2003 Messenger RNA expression for the follicle-stimulating hormone receptor and luteinizing hormone receptor in human oocytes and preimplantation-stage embryos. *Fertility and Sterility* **9**, 1187–1193.
Plachot M, Belaisch-Allart J, Mayenga JM *et al*. 2002 Outcome of conventional IVF and ICSI on sibling oocytes in mild male factor infertility. *Human Reproduction* **17**, 362–369.
Presicce GA, Yang X 1994 Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 h and activated with combined ethanol and cycloheximide treatment. *Molecular Reproduction and Development* **37**, 61–68.
Sakai N, Endo A 1988 Effects of delayed mating on preimplantation embryos in spontaneously ovulated mice. *Gamete Research* **19**, 381–385.
Shen S, Rosen MP, Dobson AT *et al*. 2006 Day 2 transfer improves pregnancy outcome in *in vitro* fertilization cycles with few available embryos. *Fertility and Sterility* **86**, 44–50.
Stojkovic M, Stojkovic P, Leary C *et al*. 2005 Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes. *Reproductive BioMedicine Online* **11**, 226–231.
Strassburger D, Friedler S, Raziel A *et al*. 2004 The outcome of ICSI of immature MI oocytes and rescued *in vitro* matured MII oocytes. *Human Reproduction* **19**, 1587–1590.
Telfer EE, McLaughlin M 2007 Natural history of the mammalian oocyte. *Reproductive BioMedicine Online* **15**, 288–295.
Tesarik J, Testart J 1994 Treatment of sperm-injected human oocytes with Ca^{2+} ionophore supports the development of Ca^{2+} oscillations. *Biology of Reproduction* 51, 385–391.
Trounson A, Anderiesz C, Jones G 2001 Maturation of human oocytes *in vitro* and their developmental competence. *Reproduction* **121**, 51–75.
Vajta G, Gjerris M 2006 Science and technology of farm animal cloning: State of the art. *Animal Reproduction Science* **92**, 211–230.
Van Blerkom J, Davis P, Alexander S 2004 Occurrence of maternal and paternal spindles in unfertilized human oocytes: Possible relationship to nucleation defects after silent fertilization. *Reproductive BioMedicine Online* **8**, 454–459.
Wakayama S, Suetsugu R, Thuan NV *et al*. 2007 Establishment of mouse embryonic stem cell lines from somatic cell nuclei by nuclear transfer into aged, fertilization-failure mouse oocytes. *Current Biology* **17**, R120–R121.
Wernig M, Meissner A, Foreman R *et al*. 2007 *In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**, 318–324.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 29 August 2007; refereed 12 October 2007; accepted 30 November 2007.