

Embryo development after successful somatic cell nuclear transfer to *in vitro* matured human germinal vesicle oocytes*

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BACKGROUND: Somatic cell nuclear transfer (SCNT) involves the transfer of somatic cell nuclei into enucleated oocytes. Because human *in vivo* matured oocytes are scarcely available, we investigated whether *in vitro* matured (IVM) germinal vesicle (GV) oocytes could also support preimplantation development of human cloned embryos. **METHODS:** Three groups were used for SCNT: *in vitro* matured GV oocytes (IVM oocytes), '*in vivo*' matured oocytes (*in vivo* oocytes) and 'failed fertilized' oocytes after routine-ICSI (FF oocytes). After removal of the chromosome–spindle complex, cumulus cell nuclei were injected, and oocytes were artificially activated and cultured. **RESULTS:** In total 61, 54 and 45 metaphase II oocytes were used for SCNT in the three groups, respectively. Survival and pronuclear rates were 59, 78 and 58% and 61, 64 and 50%, respectively. Of the 22 activated IVM oocytes, 13 cleaved to the 2-cell stage, whereby 2 morulae were formed. For the *in vivo* oocytes, 17 of 27 activated oocytes cleaved to the 2-cell stage and 1 morula was observed. Cleavage to the 2-cell stage in the group of FF oocytes was compromised. **CONCLUSIONS:** To our knowledge, this is the first report describing development of cloned human embryos using IVM oocytes and non-autologous transfer using a conventional method of SCNT.

Keywords: embryo development; GV oocytes; *in vitro* maturation; somatic cell nuclear transfer

Introduction

Although startling progress has been made in the investigation and treatment of human infertility in the last decades, the newly developed *in vitro* fertilization (IVF) techniques do not offer a solution for every infertile patient. For women with ovarian failure or defective oocytes or for men with complete spermatogenic failure (~1–2% of infertile couples), oocyte and sperm donation is currently the only treatment modality. However, this approach lacks the maternal or paternal genetic contribution to the progeny. In order to offer an alternative strategy to those patients who suffer from an absence of gametes, the long-term objective of our research program is the artificial production of gametes through therapeutic cloning.

Embryonic stem (ES) cells retain their pluripotential capacity to produce differentiated cell types of all the major lineages spontaneously in culture (Odorico *et al.*, 2001). The availability of pluripotential ES cells has raised interest in their possible use for transplantation to correct serious degenerative diseases and to replace defective cells for essential function in the body (Wobus and Boheler, 2005). Therapeutic cloning would provide patients with their own genomic identical ES cells (Trounson, 2001a)

herewith avoiding the problem of rejection. The procedure would involve the transfer of cells from the sick or injured patients into mature enucleated oocytes to form blastocysts that can be used to isolate inner cell mass and form ES cells in culture (Trounson, 2001b). In perspective of infertility treatment, the goal is to derive gamete cells *in vitro* from ES cells for sterile patients. It has already been shown that it is possible to differentiate ES cells into different tissue-specific cells (Odorico *et al.*, 2001), even into mouse gametes (Hübner *et al.*, 2003; Toyooka *et al.*, 2003; Geijsen *et al.*, 2004) from embryos of non-cloned origin. ES cell lines have also been produced from somatic nuclear transfer (NT) embryos (Kawase *et al.*, 2000; Wakayama *et al.*, 2001, 2005; Wang *et al.*, 2005). This in itself does not prove that such cells can be used effectively in regenerative medicine; many challenges remain, including the induction and targeting of differentiation, the control of stem cell proliferation, and the dilemma of persistent re-rejection in cases of autoimmune disease.

Human therapeutic cloning is associated with two major obstacles. NT is a multi-stage process that has to be optimized, and therefore requires a large number of human oocytes. Secondly, ES cells derived from human cloned blastocysts have to be successfully differentiated into the wanted cell type either *in vitro* or *in vivo*. Apparently, successful reports on

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the derivation of pluripotent ES cell lines from a cloned human blastocyst by a Korean group were retracted because of fraudulent nature (Investigation Committee of Seoul National University, 2006; Kennedy, 2006). Stojkovic *et al.* (2005) reported low developmental rates of human NT embryos using donor oocytes of four different sources and ES cells as donor nuclei, but only one blastocyst was obtained. Lavoie *et al.*, (2005) have shown that unfertilized oocytes after IVF or ICSI are ineffective as recipients for human somatic cell NT. The reconstructed NT embryos arrested after one or two early cleavage divisions and showed chromosomal abnormalities. Hall *et al.*, (2006) very recently reported poor developmental rates after using FF and fresh oocytes as recipients for human NT. Only one paper reported blastocyst formation after human SCNT using a modified method of SCNT and *in vivo* matured oocytes as recipients donated by volunteers (Lu *et al.*, 2003).

The major bottleneck in the advancement of human NT remains the availability of sufficient donor oocytes to produce ES cells for patients. Instrumental to the NT procedure is the cytoplasm of the oocytes. For laboratory animals or small mammals, good-quality matured oocytes can be recovered *in vivo* from super-ovulated animals, but when it comes to large animals like the sheep or the pig it becomes significantly more expensive, and for cattle or horses it is technically feasible but economically not lucrative.

In vivo matured oocytes are thought to be the best source of mature oocytes from a quality point of view as judged by their ability to develop into blastocysts following fertilization (Dieleman *et al.*, 2002) and their developmental capacity in NT animal experiments (Dieleman *et al.*, 2002; Edwards *et al.*, 2003). *In vitro* matured germinal vesicle (GV) oocytes are an interesting source of cytoplasm recipients in both animal and human NT experiments. Advances in the field of *in vitro* maturation (IVM) have greatly contributed to the possibility of using human IVM oocytes for cloning. The ultimate test of viability of an oocyte is first the development into a blastocyst and then the establishment of a pregnancy. For this reason, *in vitro* embryo production has provided fundamental knowledge to oocyte maturation and therefore the obtainment of viable oocytes for cloning (Miyoshi *et al.*, 2003). The main primary source of oocytes for human NT research may be immature oocytes that are obtained during clinical infertility treatment and are not used.

Several reports indicate that culture systems adequately support nuclear maturation but fail to produce consistently human oocytes with full cytoplasmic competence, thereby resulting in embryos with reduced developmental capacity (Cha and Chian, 1998; Trounson *et al.*, 2001; Combelles *et al.*, 2002). Cytoplasmic maturation is a complex process and encompasses a wide array of metabolic and structural modifications, ensuring the occurrence of normal fertilization, inducing meiotic to mitotic cell cycle progression, and activation of pathways required for genetic and epigenetic programmes of embryonic development (Eppig *et al.*, 1994; Eppig, 1996; Heikinheimo and Gibbons, 1998; Trounson *et al.*, 2001). IVM oocytes are currently commonly used for embryo cloning in cattle, sheep and porcine (Cibelli *et al.*, 1998; Kato *et al.*, 1998; Wells *et al.*, 1999; Betthausen *et al.*, 2000). Still, IVM application

has not yet achieved a comparable performance to conventional IVF/ICSI using *in vivo* matured oocytes in terms of fertilization, cleavage, implantation and pregnancy rates, most probably related to the inferior quality of oocytes matured *in vitro* (Cha and Chian, 1998).

In the present study, we investigated the use of IVM human GV oocytes as recipients for injection with a heterologous donor somatic cell nucleus and studied the preimplantation development of these human NT embryos. Additionally, *in vivo* matured oocytes donated by patients who showed absence of sperm in the testicular biopsy on the day of pick-up, and FF oocytes after ICSI were also used for somatic cell NT (SCNT) experiments as controls.

Materials and Methods

All chemicals and reagents were purchased from Sigma (Sigma-Aldrich, Bornem, Belgium) unless stated otherwise.

Source of oocytes

All oocytes donated for this research were obtained from stimulated patients undergoing ICSI treatment in our Infertility Centre of the Ghent University Hospital between November 2004 and January 2006, after written informed consent as approved by the ethical committee of the hospital. Immature GV oocytes (Groups 1 and 4) were collected from 54 patients undergoing ICSI treatment at our Infertility Centre.

Group 1 ($n = 61$ metaphase II (MII) oocytes) consisted of immature GV oocytes collected from patients who had at least 6 MII oocytes available for their infertility treatment. Oocyte maturity was assessed after denudation with 80 IU/ml hyaluronidase (Type VIII) and mechanical pipetting. Maturity of oocytes was determined as the presence of a first polar body (PB) and mature oocytes were exclusively used for infertility treatment. Immature GV oocytes were matured in TCM199 supplemented with 10 ng/ml epidermal growth factor (EGF), 1 μ g/ml estradiol, 75 mIU/ml follicle stimulating hormone (FSH) Puregon, Organon, The Netherlands), 0.5 IU/ml human chorionic gonadotrophin (hCG, Pregnyl, Organon), 1 mM L-glutamine, 0.3 mM sodium pyruvate, 0.8% human serum albumin (HSA, Belgian Red Cross, Brussels, Belgium) and antibiotics. GV oocytes were cultured individually in drops of 20 μ l in a 6% CO₂ incubator at 37°C. Maturation was evaluated after 24 and 44 h of *in vitro* culture. Oocytes were classified as GV or metaphase I (MI) stage depending on whether a GV was visible or not. Oocytes were classified as MII stage by the presence of a first PB. Group 1 will be further indicated by 'IVM oocytes', used for SCNT.

In vivo matured oocytes donated by 6 patients whose azoospermic partners showed absence of spermatozoa in the testicular biopsy at the day of pick-up constituted Group 2 ($n = 54$ MII oocytes). These MII oocytes were used for NT at various time intervals after oocyte collection, depending on the time of testicular biopsy, the time needed for sperm search and the agreement of patients to donate their oocytes for our research (ranging between 2 h 30 min–7 h 30 min). Group 2 will be further referred to as 'in vivo oocytes', used as recipients for SCNT.

Group 3 ($n = 45$ MII oocytes) consisted of MII oocytes that showed no signs of fertilization defined as absence of pronuclei and second PB at 16–20 h post injection by ICSI. These 18 patients had at least five normally fertilized oocytes after ICSI. Group 3 will be further referred to as 'FF oocytes', after ICSI used as recipients for SCNT.

As an additional control (Group 4, $n = 29$ MII oocytes) GV oocytes that were *in vitro* matured in the same conditions as Group 1, were injected with sperm donated for research. Embryo culture

was performed in G1/G2 medium (series III, Vitrolife Sweden AB, Kungsbacka, Sweden) until day 5. Group 4 will be further referred to as 'control oocytes', *in vitro* matured GV oocytes fertilized by ICSI with donated sperm.

NT protocol (Fig. 1)

During manipulation, recipient oocytes and donor cells were kept in GMOPS (Vitrolife Sweden AB), and for culture in G1/G2 medium (series III, Vitrolife Sweden AB). After partial zona dissection with a sharp needle (Fig. 1A), creating a tangential slit in the zona in the region above the first PB, the chromosomes were visualized by incubation in 1 $\mu\text{g}/\text{ml}$ Hoechst for 10 min followed by ultraviolet (UV)-irradiation (Fig. 1B). Removal of the spindle-chromosome complex through the slit was carried out in the presence of 1 $\mu\text{g}/\text{ml}$ cytochalasine B using a blunt needle of 15 μm (Fig. 1C). After enucleation, oocytes were kept up to 1.5 h in the incubator before injection of donor nuclei. The somatic cell nuclear donor source consisted of heterologous (IVM and FF oocytes) and autologous (*in vivo* oocytes) cumulus cells that were freshly collected at the day of experiment (Fig. 1D). They were put in GMOPS at room temperature, and 1 h before injection,

polyvinylpyrrolidone (Vitrolife Sweden AB) was added which allowed spreading of the cumulus cells on the bottom of the dish.

Cumulus cell nuclei were injected at 1.5 h after collection with a blunt needle (8 μm) using the conventional ICSI method (Fig. 1E). Artificial activation was done 2 h after injection using incubation in 10 μM ionophore A23187 during 7 min, repeated after a 30 min time interval. After activation, reconstructed oocytes were incubated in medium supplemented with 2 mM 6-dimethylaminopurine for 3 h, and after thorough washing, they were finally cultured in G1.3 medium under mineral oil in 6% CO_2 at 37°C and switched to G2.3. at day 3. The control group was cultured in the same sequential medium after ICSI. Oocytes were checked for pronuclei between 15–20 h after SCNT activation start or ICSI and daily evaluation of development was performed.

Nuclear evaluation of arrested human NT embryos

At day 4, human NT reconstructed embryos arrested at the 2- or 4-cell stage were stained with 10 $\mu\text{g}/\text{ml}$ Hoechst 33258 in GMOPS for 10 min at room temperature. After rinsing in GMOPS embryos were visualised under UV light to screen for nuclear abnormalities.

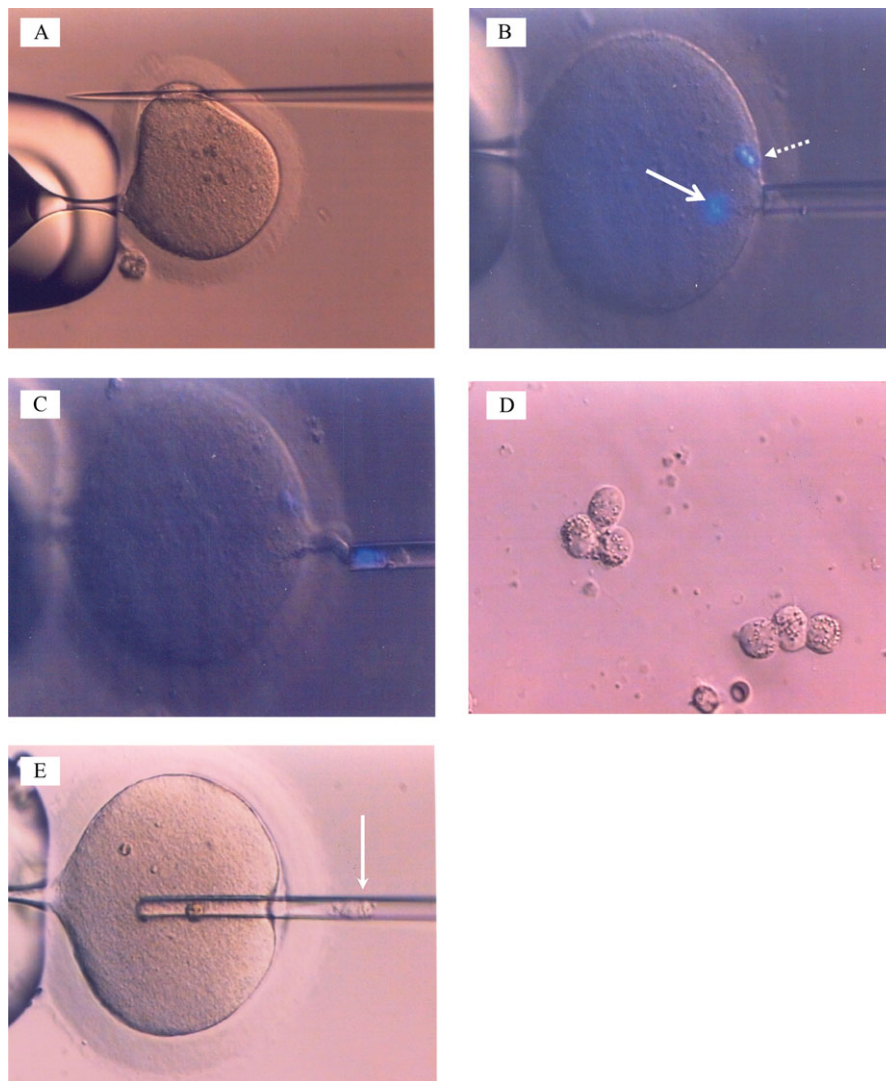


Figure 1: Different steps in the NT protocol: partial zona dissection (A), Hoechst labelling and UV-irradiation: oocyte chromosomes (arrow), chromosomes in first PB (broken arrow) (B), enucleation of oocyte chromosomes (C) cumulus cells as donor cells (D), conventional nuclear injection: nucleus aspirated in injection pipette (arrow) (E)

Table 1: IVM of human GV oocytes 24 and 44 h after start of maturation

No. of GV oocytes	Maturation after 24 h (%)	Maturation after 44 h (%)
116	73 MII (63) 29 MI (25) 14 GV (12)	90 MII (77) 13 MI (12) 13 GV (11)

Statistics

All data were analysed by contingency table analysis followed by chi-square for independence. The level of significance was set at $P < 0.05$.

Results

IVM rate to the MII stage of GV oocytes after 24 and 44 h was 63 and 77%, respectively (Table 1). Only oocytes that reached the MII stage ($n = 90$), revealed by the presence of a first PB were used for NT (IVM oocytes, $n = 61$) and control ICSI (Group 4, $n = 29$) and no distinction was made between oocytes that matured either at 24 or at 44 h.

In vivo matured MII oocytes (*in vivo* oocytes, $n = 54$) were used at various times between oocyte collection and start of the NT experiment: 2h30 (2 patients, 7 oocytes) and 6 h-7h30 (4 patients, 47 oocytes). Unfertilized oocytes after ICSI in Group 3 (FF oocytes, $n = 45$) were used for NT between 24 and 27 h after oocyte collection.

Mature MII oocytes after IVM (IVM oocytes) and FF oocytes had an oolemma of which the fragility rendered conventional nuclear injection extremely difficult for SCNT. Penetrating the oolemma without cytoplasm aspiration broke cytoplasmic membranes of most oocytes. The *in vivo* oocytes and control oocytes behaved like normal MII oocytes during clinical treatment when a minimum time-interval between enucleation and injection of 1 h was used in the *in vivo* oocyte group.

Survival rate and preimplantation development in the three groups after SCNT, and in the control group are shown in Table 2. Significantly more oocytes in the *in vivo* oocyte group survived the SCNT protocol (78 versus 59% in group IVM oocytes and 58% in group FF oocytes). Damage of oocytes mostly occurred after injection of the cumulus cell

nucleus. Significantly more oocytes survived the ICSI procedure in the control group compared to the SCNT groups ($P < 0.05$). Activation rate in the three SCNT groups was 61, 64 and 50%, respectively, mostly showing one large pronucleus without second PB extrusion (see Fig. 2A), significantly lower than the activation rate in the control group ($P < 0.05$) where normal 2 pronuclear (PN) formation after ICSI was observed in 72% of the injected oocytes while 6 (24%) zygotes showed >2 PN. Cleavage to the 2-cell stage was significantly better in the groups of IVM oocytes, *in vivo* oocytes and control group (59, 63 and 72%, respectively) compared to the FF oocytes (8%) where only one of 13 activated oocytes progressed to the 2-cell stage. The majority of the 2-cell SCNT embryos cleaved further but arrested at different stages between the 4-cell and 12-cell stage, while fragmentation in most embryos was limited (see Figs. 2B–D).

Respectively, 2 (9%) and 1 (4%) of the NT embryos reached the compacted morula stage at day 5 in the IVM oocyte and *in vivo* oocyte group. When distinction was made between the time-interval of oocyte collection and the start of use for NT, we noted that *in vivo* oocytes used 2 h 30 min after collection progressed beyond the 2-cell stage much better (83%) than oocytes used after 6 h 30 min–7 h 30 min (43%). Due to the limited number of oocytes, there was no statistical difference between these groups, although only one morula was formed in the group of oocytes used 2 h 30 min after collection. Although early cleavage rates were high in the control group (72 and 64%), most embryos arrested between 3-cell and 9-cell stage while four embryos compacted (16%) and two reached the blastocyst stage (8%).

Nuclear analysis of the human SCNT arrested embryos ($n = 10$) at Day 4 revealed both micro- and multinucleation in the blastomeres (see Fig. 3).

Discussion

In the present study we have demonstrated that IVM oocytes can be used as recipients for human SCNT. Preimplantation developmental potential of SCNT embryos from IVM oocytes was limited to the morula stage, but this was comparable to the use of *in vivo* matured oocytes for SCNT. IVM

Table 2: Survival and preimplantation development of reconstructed NT oocytes derived from three groups of recipient oocytes: Group 1, IVM oocytes; Group 2, '*in vivo* oocytes' and Group 3, 'FF oocytes' Group 4 are the control IVM GV oocytes fertilized by ICSI with donated sperm, 'control oocytes'

Source of recipients	No. of MII oocytes	No. survived after NT/ICSI (%)	No. of activated oocytes (% of survived)	No. of 2-cell (% of PN)	No. >2 -cell (% of PN)	No. of morulas (% of PN)	No. of blastocysts (% of PN)
Group 1							
IVM oocytes	61	36 (59) ^a	22 (61%) ^a	13 (59) ^a	10 (45) ^a	2 (9)	0
Group 2							
<i>In vivo</i> oocytes	54	42 (78) ^b	27 (64) ^a	17 (63) ^a	14 (52) ^a	1 (4)	0
used after 2 h 30 min	7	6 (86)	6 (100)	5 (83)	5 (83)	1 (17)	0
used after 6 h 30 min–7 h 30 min	47	36 (77)	21 (58)	12 (57)	9 (43)	0	0
Group 3							
FF oocytes	45	26 (58) ^a	13 (50) ^a	1 (8) ^b	0 ^b	0	0
Group 4							
Control oocytes	29	28 (96) ^c	25 (89) ^b	18 (72) ^a	16 (64) ^a	4 (16)	2 (8)

^{a,b,c} $P < 0.05$ at least (χ^2) per column; activation, 2-cell, >2 -cell, morula and blastocyst formation was evaluated 18, 42, 66, 90 and 114 h post start artificial activation, respectively.

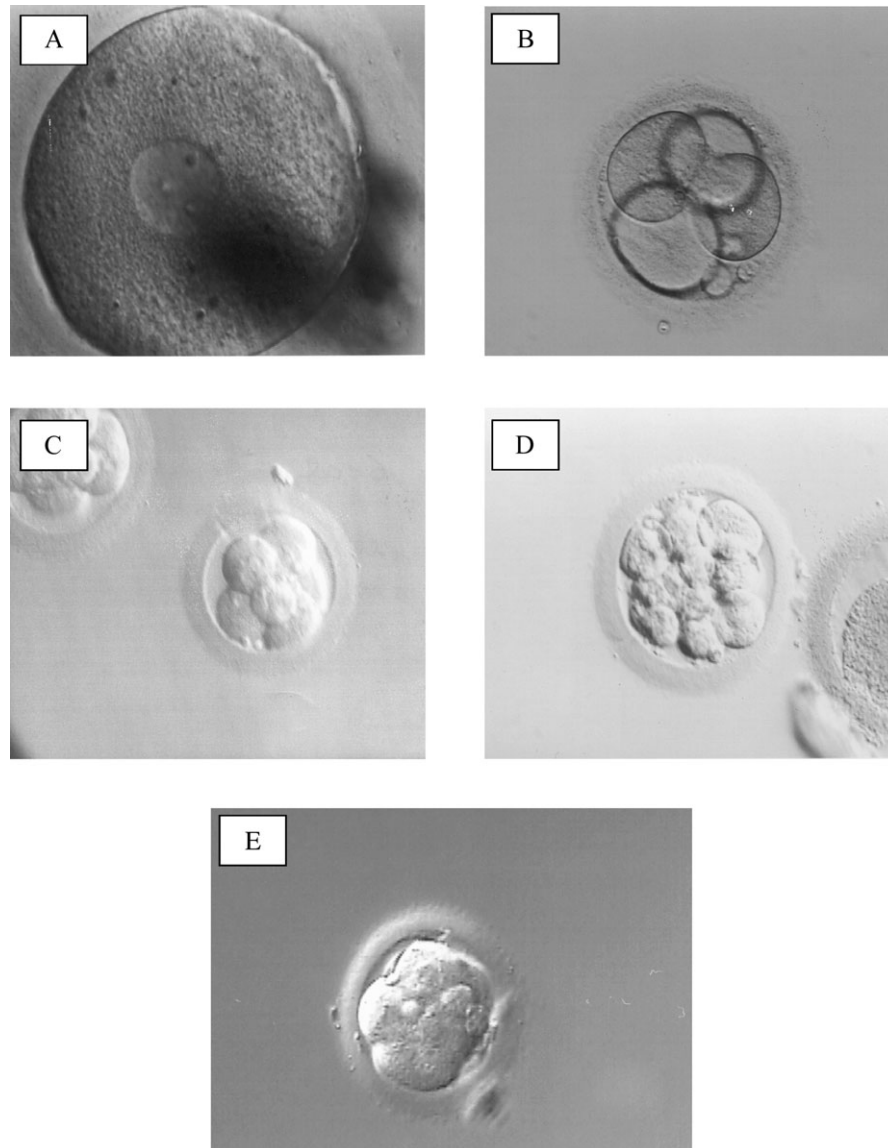


Figure 2: Human NT activated oocyte showing one large pronucleus (A); NT embryos (B–E)

oocytes fertilized by ICSI showed equal limited development, although blastocyst formation could be obtained. It was demonstrated that the time interval between oocyte collection and start of SCNT is important for further development of SCNT embryos when *in vivo* matured oocytes were used as recipients. *In vivo* matured oocytes showed a significantly higher survival rate than IVM oocytes with the currently used SCNT technology. In our study, the arrested cleavage stage human SCNT embryos showed both micro- and multi-nucleation in many blastomeres. Further we have confirmed that unfertilized oocytes after ICSI are inefficient as an alternative source of recipients for human SCNT.

IVM of oocytes includes two major processes: nuclear maturation involving re-initiation and completion of the first meiotic division, and cytoplasmic maturation that enables fertilization and subsequent embryonic development. Our group has previously shown that IVM GV oocytes can be used as recipients for mouse SCNT, but embryonic development was

compromised compared to that after SCNT with *in vivo* matured oocytes (Heindryckx *et al.*, 2002). Previous studies in the human reported that 80% of immature oocytes develop to MII stage by 48–54 h of culture (Trounson *et al.*, 1994; Russel *et al.*, 1997). However, there is an asynchrony in GV oocytes reaching the MII stage even in women with regular menstrual cycles (Yoon *et al.*, 2001). The overall maturation rate of human GV oocytes to the MII stage in the present study was very efficient after 44 h (77%). The accelerated pace of progression to MII (63%) over 24 h in our IVM medium was in agreement with the study of Combelles *et al.* (2002) who reported a maturation rate of 66.7% after 24 h of denuded human GV oocytes to MII stage. Normal duration of maturation from the GV to the MII stage takes 36 h in the human.

Care should be taken when maturation rates of different reports are compared, because maturation of human GV oocytes is influenced by many factors including: IVM media

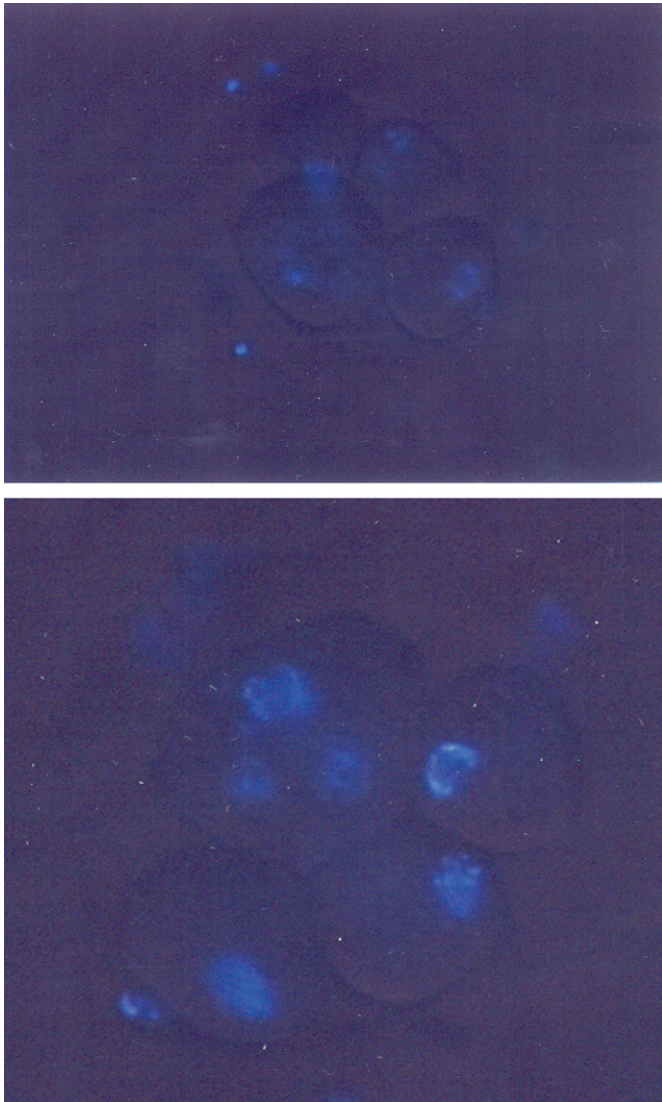


Figure 3: Human NT arrested embryos at Day 4 showing multinucleation in several blastomeres

composition (hormone/growth factor/serum supplementation) and culture environment, denuded versus cumulus-enclosed GV oocytes, GV oocytes collected from stimulated versus unstimulated cycles, patient population (Prins *et al.*, 1987; Cha and Chian, 1998; Goud *et al.*, 1998; Trounson *et al.*, 1998; Anderiesz *et al.*, 2000; Combelles *et al.*, 2002). Removal of cumulus cells and supplementation of the IVM medium with gonadotrophins as in our study have both been reported to accelerate meiotic maturation *in vitro* (Gomez *et al.*, 1993; Cha and Chian, 1998; Goud *et al.*, 1998; Trounson *et al.*, 2001). Son *et al.* (2005) compared the developmental capacity of oocytes according to the IVM time (day of oocyte aspiration, day 1 and day 2) required to reach MII stage in an IVM cycle. Oocytes that matured late *in vitro* (day 2) had a significantly higher blockage of cleavage at the PN stage compared with oocytes matured *in vivo* and on day 1. Blastocyst formation rates were significantly lower in oocytes that matured on day 2. The authors speculated that various sizes of follicles were presented in ovaries of patients undergoing IVM cycles and oocytes collected from smaller

follicles may show slower maturation and incomplete developmental competence. This study was in agreement with data from Chian and Tan (2002) who compared developmental potential between GV oocytes matured either during 24 and 48 h in two different IVM media. Embryos reached the blastocyst stage at a higher rate in both media if GV-stage oocytes had matured within 24 h. Miyoshi *et al.* (2002) reported that MII porcine oocytes for NT experiments recovered from maturation medium at 24 h of culture would already have acquired or initiated cytoplasmic maturation *in vivo*. Porcine oocytes are commonly matured *in vitro* for 40–44 h before manipulation. Oocytes that matured after 24 h produced cloned porcine embryos with better developmental rates than oocytes that matured later. Inducing arrest of meiotic resumption of recipient porcine oocytes at the GV stage by dbcAMP did not improve reconstructed embryo NT developmental rates (Miyoshi *et al.*, 2002). Due to the low number of available human oocytes per day, we did not make a distinction between GV oocytes matured either at 24 or 44 h, but this will be performed in the future.

Studies in pigs and sheep have shown that GV matured cytoplasm is less capable of supporting NT development compared to *in vivo* matured cytoplasm (Wells *et al.*, 1997; Onishi *et al.*, 2000). In a previous study we also showed that IVM GV oocytes showed a hampered developmental potential to the blastocyst stage when used as recipients for mouse NT (Heindryckx *et al.*, 2002). A serial transfer of PN to an *in vivo* matured zygotic cytoplasm could overcome this. In the present study, control IVM oocytes fertilized by ICSI showed decreased developmental potential, in particular in the final stages. Twelve of 16 control embryos arrested at the stage at which embryo genome activation takes place, comparable with the NT group. This reflects the inferior quality of the IVM ooplasm and that removing oocytes from their follicular environment prior to luteinisation yields oocytes with compromised developmental potential. The blastocyst rate obtained in our IVM control group after ICSI was comparable to that reported by Chian and Tan (2002). These authors observed 0 and 12.9% blastocyst formation after ICSI of naked IVM GV oocytes derived from stimulated cycles that had been matured in two different IVM media. The efficiency of blastocyst formation was more efficient after ICSI on IVM cumulus-enclosed GV oocytes that were obtained from patients primed with only hCG and were matured for either one or two days (50 and 11%, respectively) (Son *et al.*, 2005). Still, there is now general consensus that IVM human oocytes do not have the same developmental capacity as *in vivo* matured ones and it has to be stressed that most studies evaluating the cytoplasmic and nuclear development after IVM have all been performed with immature oocytes from stimulated cycles (Lanzendorf, 2006). These studies showed desynchronization of nuclear and cytoplasmic maturation, as well as high rates of meiotic spindle, chromosome, and nuclear abnormalities. In this perspective and in view of the low blastocyst formation obtained in our control group, our future research should focus on ways to improve preimplantation development after the use of naked IVM oocytes from stimulated cycles, which form a primary source of oocytes for our SCNT experiments.

Still, intriguing is the fact that no difference could be observed in development between *in vitro* versus *in vivo* matured oocytes as recipients in our human NT experiments, which could be indicative of the sub-optimal use of the 'aged' *in vivo* matured oocytes or the lack of a key-technology factor to make human SCNT preimplantation development efficient.

Stojkovic *et al.* (2005), recently reported on the use of donated oocytes for human NT experiments from patients undergoing treatment, using undifferentiated donor cells of ES cell origin. They reported one embryo developing to the blastocyst stage after heterologous donor NT with their current protocol. Only oocytes obtained by follicle reduction before insemination possessed developmental potential, resulting in one blastocyst. When *in vivo* matured oocytes after failed fertilization or failed insemination were used, cleavage was not achieved. They speculated that these oocytes were too old, 46 h and 7 h after recovery, respectively to use for NT. IVM oocytes collected from small follicles during routine cystectomy also failed to cleave after NT, but only 4 oocytes were used. Donated *in vivo* matured oocytes in our experiment were used 2 h 30 min at earliest for SCNT, while the majority was used 6 h 30 min–7 h 30 min after oocyte collection. Developmental potential tended to be higher when oocytes were used shortly after oocyte collection. Stojkovic *et al.*, (2005) reported the potential benefit of early enucleation of recipient oocytes, preferable immediately after collection, when oocytes are in the prometaphase II. When *in vivo* matured oocytes were donated in our study, they were used for SCNT at least 2 h 30 min after collection, so well beyond the prometaphase II stage. It would be interesting to verify whether the use of accurate timing of IVM of GV oocytes to the prometaphase II stage can influence the developmental potential of these recipient oocytes. Lavoit *et al.*, (2005) reported satisfying survival rates (77%) after their NT protocol with FF oocytes, using a piezo injector for nuclear injection, but when the original number of oocytes that were used for NT is taken into account, the total survival rate of their NT procedure is 58%. In our study, survival rate was comparable (58%) using FF oocytes as recipients, but significantly lower than using *in vivo* oocytes (78%) due to the fragility of the oolemma and the invasive method of conventional nucleus injection by aspiration instead of piezo-injection. The efficiency of the NT procedure reported by Hall *et al.* (2006) was 13 and 75% of the FF oocytes and *in vivo* oocytes, respectively, successfully reconstructed by NT. Early development using these FF oocytes was limited (Lavoit *et al.*, 2005), as in our study, with few embryos progressing beyond the PN stage. Simerly *et al.* (2004) and Hall *et al.* (2006) further demonstrated the inability of FF oocytes to serve as recipients for NT in non-human primates. In the latter study, assessment of the spindle-pole protein, NuMA, revealed aberrant expressions and abnormal tetrapolar spindles in these FF oocytes. In our study, the arrested cleavage stage human SCNT embryos showed both micro- and multinucleation in many blastomeres, which could be indicative for abnormal spindle formation. Using a modified method of human SCNT, Lu *et al.* (2003) could obtain blastocyst formation using *in vivo* matured

oocytes as recipients. These authors circumvented the use of Hoechst staining and UV-irradiation during enucleation by removal of the female PN after artificial activation. This is an important finding suggesting that human SCNT requires an adapted protocol in comparison with animal SCNT experiments. Unfortunately, this paper is not referred to when going through the literature of human SCNT.

Major obstacles which remain to be investigated in the human SCNT technology process includes: enucleation of the recipient oocyte, type of donor cell, transfer of donor nucleus: electrofusion/piezo injection/conventional nuclear injection, artificial activation and the appropriate culture media. However, the scarce availability of human oocytes for research makes it difficult to change parameters in one experiment. In contrast with mouse oocytes, the spindle with the chromosomes lying on the metaphase plate is not visible with the currently used microscope technology in mature human oocytes. One can also aspirate a small part of cytoplasm close to the first PB, where the spindle normally resides, but confirmation of successful enucleation is still warranted by staining. In our study, enucleation was performed by aspiration of the chromosome-spindle complex after Hoechst labelling and under UV irradiation. Using this procedure, very little cytoplasm surrounding the spindle was removed. Although the use of excitable fluorochromes and UV light are commonly used for complete enucleation, they also pose the risk of damaging the maternal cytoplasm. Smith (1993) has shown that exposure to UV irradiation for periods as short as 60 s causes alterations to both membrane and intracellular components of bovine oocytes matured *in vitro*, decreases methionine incorporation and alters protein synthesis patterns in bovine oocytes. The high damage rate after conventional nucleus injection in our study might be attributed to the loss of integrity at the oolemma level induced by Hoechst staining and UV irradiation. Interestingly, IVM control oocytes fertilized by ICSI showed a high survival rate after microinjection, suggesting that the SCNT protocol is responsible for the high damage in the SCNT groups, rather than the IVM origin of the oocytes. Preimplantation development can also negatively be influenced by application of Hoechst and irradiation as demonstrated by Dominko *et al.*, (2000).

Different activating agents will also have to be investigated for cloning technology. The work we have done about assisted oocyte activation (Heindryckx *et al.*, 2005) will be useful for this matter, and further research on other activating agents will be undertaken. Finally, appropriate culture media will have to be tested for supporting the development of human cloned embryos. As shown in our study in mice, cloned embryos require other medium support compared to normally fertilized embryos (Heindryckx *et al.*, 2001, 2006). These findings were later confirmed in other studies on mouse (Chung *et al.*, 2002; Gao *et al.*, 2003a, b; Gao and Latham, 2004) who reported that cloned mouse embryos prefer somatic cell-like culture conditions, which differ from those, required by normally fertilized or parthenogenetic control embryos.

In our study, many reconstructed human SCNT embryos arrested around the 4 to 8-cell stage, the stage at which embryonic genome activation takes place. In mice the 2-cell block,

stage of embryonic genome activation, could be overcome by the appropriate choice of culture media for normally fertilized derived embryos (Chatot *et al.*, 1989, 1990), and even for mouse SCNT embryos (Heindryckx *et al.*, 2001, 2006). Further research has to reveal whether culture conditions alone can improve human SCNT development.

Conclusion

In conclusion, we have shown for the first time that IVM oocytes can be used as recipients for human SCNT, although preimplantation development was limited to the morula stage. Question remains whether the inferior quality of the ooplasm of IVM oocytes is responsible for blastocyst formation failure or the incomplete reprogramming of the donor somatic nucleus, or whether still a key factor in the human NT technology is missing as shown by the equally compromised developmental potential of the *in vivo* matured oocytes as recipients.

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