Parthenogenesis and Somatic cell nuclear transfer in sheep oocytes using Polscope

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Parthenogenesis and Somatic cell nuclear transfer (SCNT) techniques, offer a unique approach to manipulate the genetic composition of derived human embryonic stem cells – an essential step if the full opportunities for disease modeling, drug discovery or individualized stem cell therapy are to be realized. The present study describes the use of sheep oocytes to acquire expertise and establish methods to reconstruct embryos for obtaining blastocysts before venturing into human SCNT where the oocytes are a very precious starting material. Maturation of sheep eggs in vitro for 20-24 hr resulted in 65% metaphase II (MII) eggs which were either parthenogenetically activated using calcium ionomycin or ethanol or subjected to SCNT using cumulus cell as somatic cell. Sixteen blastocysts were produced by parthenogenetic activation of 350 eggs whereas reconstructed embryos, after SCNT carried out in 139 eggs, progressed only up to morula stage. The procedure of parthenogenesis and SCNT will be useful to generate autologous ES cells using human eggs.

Keywords: Embryonic stem cell, Parthenogenesis, Polscope, SCNT, Sheep oocyte

The immune system is bound to reject transplanted embryonic stem cells as 'foreign', when used to treat various cellular degenerative diseases in future. This rejection could be avoided by the use of immunosuppressive drugs which are known to have serious side-effects. Alternate approaches involve use of homologous recombination techniques that allows the host immune system to recognize the embryonic stem (ES) cells as 'self' by replacing the MHC genes in ES cells with the host MHC genes. Elimination of MHC class I and II gene loci could also be done, though this would be technically challenging and would be clinically problematic because cells lacking MHC class I surface expression are targeted by NK cells. One possible alternative is to establish a bank of MHC-compatible hESC lines. Taylor et al. reported that a reasonable HLA match for around 85% of the UK population could be achieved with around 150 lines. However, the number would greatly increase for India which has a varied and multi-racial population. Another possible way to overcome immune rejection is to use transgenes that can suppress the immune system such as fas-ligand into ES cells. It has also been suggested that removal of certain cell surface molecules like B7 antigens or CD40 ligands that are immunologically reactive from ES cells prior to transplantation could suppress immune rejections. Recently, it has been possible to transfet mouse as well as human skin fibroblasts with four genes termed ‘Yamanaka factors’ and revert them back to embryonic state. Thus there are several methods by which nuclear reprogramming of adult differentiated somatic cells is carried out. However, the best and most extensively studied option is the generation of autologous ES cell lines by therapeutic cloning also known as somatic cell nuclear transfer (SCNT) and parthenogenesis. During SCNT, patient-specific ES cell lines are derived from blastocysts produced by fusion of a donor cell from patient into an enucleated donor oocyte whereas during parthenogenesis cell lines are derived by inducing an egg to form a blastocyst without undergoing fertilization. Both SCNT and parthenogenesis will yield autologus ES cell lines that will help to avoid immunological rejection during cell based therapies in future.

The concept of nuclear transfer dates back to 1938 when a German scientist Spemann suggested the importance of nucleus and cytoplasm to control early embryonic development. The technology was first demonstrated in amphibians as they have large sized eggs and rapid rates of development. Since then, live offspring using SCNT have been born in several animal species like mouse, pig, sheep, cow and...
recently also in rabbit. These success stories reveal the extraordinary capacity of oocyte cytoplasm to reprogram an adult somatic cell to embryonic state and the ability of the reprogrammed cell to give rise to a whole individual. However, reproductive cloning has not yet been achieved in higher primate species including man.

Therapeutic cloning differs from reproductive cloning since the ultimate aim is not to create clones but derive ES cell lines from the cloned blastocyst for medical therapeutics including cell based therapies, gene therapy, creation of in vitro disease ES cell lines etc. The success rate of reproductive cloning is around 0.2% but for deriving nuclear transfer ES cell lines the efficacy is about 20%. Recently Mitalipov et al. successfully carried out therapeutic cloning in nonhuman primates using adult differentiated somatic cell despite earlier reports that this may not be possible in primates. Parthenogenesis has also been successfully carried out to derive monkey and human embryonic stem cell lines.

Since human eggs are precious, animal oocytes are used to establish the sophisticated procedures of parthenogenesis and therapeutic cloning that has been earlier reported in inter-species SCNT, and successful derivation of hES cell line using somatic cell of adult human fibroblast into enucleated rabbit oocyte. Recently, Human Fertilization and Embryology Authority (HEFA) have granted permission to UK based researchers to carry out inter species SCNT. Besides the availability of eggs to carry out SCNT, enucleation of recipient oocytes is a critical step during SCNT and is usually performed by aspirating a portion of cytoplasm underlying the first polar body. Spindle viewing and enucleation is evaluated under UV light after Hoechst 33342 staining which is deleterious to the egg since it is associated with a loss of membrane integrity, decreased methionine incorporation, altered protein synthesis and decreased viability. Recently, success was reported in nonhuman primate SCNT using Polscope - a non-invasive technique for spindle viewing and enucleation of oocytes. Use of Polscope during SCNT has also been reported in buffalo and in a preliminary study from our lab using human eggs.

The present study was carried out to standardize the methodology and study embryonic development after SCNT and parthenogenesis using sheep eggs, using Polscope.

Materials and Methods

All the chemicals and media were purchased from Sigma (St Louis, MO, USA) and disposable plasticware were from Nunc (Roskilde, Denmark) unless otherwise mentioned. Animal experiments were conducted after obtaining permission from Institute Animal Ethics Committee of NIRRH.

In vitro maturation of oocytes

Sheep (Ovis aries) ovaries, collected from a local slaughterhouse, were transported to laboratory in 0.9% saline at 30°C within 2 hr in an insulated flask. Ovaries were washed thoroughly in pre-warmed normal saline supplemented with (50 μg/ml) gentamycin 2-3 times, once in 70% ethanol and 3-4 times again in normal saline prior to being kept in a 90 mm searching dish containing oocyte aspiration medium (OAM) viz. HEPES modified TCM-199 supplemented with 2 mM L-glutamine, 3 mg/ml bovine serum albumin (BSA) and 50 μg/ml gentamycin. The surface follicles (2-5 mm diameter) were punctured with sterile 18 gauge needle. The dish was screened for oocytes under stereo zoom microscope attached with warm stage (Olympus SZX7, Japan). Oocyte maturation and embryo culture media were pre-equilibrated in a humidified atmosphere of 5% CO2 in air at 38.5°C for at least 2-3 hr before use.

Parthenogenesis

In vitro matured oocytes were removed from the maturation medium dish and cumulus was removed by repeated pipetting in hyaluronidase solution.
(1 mg/ml). Mature MII oocytes with first polar body were activated by two different activation protocols viz. 5 μM calcium ionomycin for 5 min and 7% ethanol for 7 min. After activation the oocytes were incubated for 3 hr with 2 mM 6-dimethylaminopurine (DMAP) in Modified Charles Rosenkrans 2 (mCR2) medium and supplemented with essential amino acid (1X), non-essential amino acid (1X) and 0.6% BSA. At the end of incubation, 6-DMAP was removed by several washes and then 10-15 oocytes were cultured in fresh 100 μl drops of mCR2 medium at 38.5°C. Oocytes were observed after 24 hr for cleavage and media change was given every 48 hr with mCR2 supplemented with 10% FBS, up to blastocyst stage. Growth arrested embryos were stained with 5 μg/ml of Hoechst 33342 for 15 min at 38.5°C and viewed under UV light.

Somatic cell nuclear transfer

Preparation of somatic cells for SCNT—Cumulus cells were isolated from in vitro matured MII oocytes by repeated pipetting in washing medium and were kept at room temperature until further use. The cells were resuspended in a drop of 10% PVP prior to use. Preparation of recipient cytoplast—After in vitro maturation, the oocytes were removed from the OMM and cumulus was removed by repeated pipetting in hyaluronidase solution. The completely denuded oocytes with evenly granular cytoplasm showing presence of polar body were selected and examined for the presence of spindle using Polscope (CRI, USA). Briefly, the oocytes were placed in OAM in a glass bottom dish (WillCo Wells BV WG PLEIN 287, Netherlands), the spindle was viewed and images were captured using Oosight™ imaging system (CRI, USA). Later the oocytes with spindle were placed in OAM drops containing 5 μg/ml cytochalasin B for 15 min at 38.5°C prior to further processing.

Somatic cell nuclear transfer—18 μm enucleation pipette was used to extract the spindle along with a minimal amount of cytoplasm. Spindle extraction procedure was performed live under the Polscope and completed within fraction of minute. Enucleated cytoplasts were washed 3-4 times in OAM and placed in a fresh OAM drop. Individual somatic cells in 10 % PVP drop (see above) were picked up with a 9 μm injecting needle (Humagen, USA) and injected into the enucleated cytoplast. SCNT reconstructed oocytes were allowed sufficient time to heal at injection site before removal from manipulation chamber and were later activated by treatment with 5 μM calcium ionomycin in OAM medium for 5 min. After three washes, the SCNT reconstructed oocytes were incubated for 3 hr at 38.5°C in 5% CO₂ incubator with 2 mM 6-DMAP in mCR2 medium supplemented with essential amino acids (1X), non-essential amino acids (1X), and 0.6% BSA. At the end of incubation, 6-DMAP was washed off and a group of 10-15 oocytes was cultured in 100 μl drops of mCR2 medium at 38.5°C in 5% CO₂ incubator. Oocytes were observed after 24 hr for cleavage. Medium was replaced with mCR2 supplemented with 10% FBS, after every 48 hr interval up to blastocyst stage. Growth arrested embryos were stained with 5 μg/ml of Hoechst 33342 for 15 min at 38.5°C and viewed under UV light.

Results

In vitro maturation of sheep eggs and spindle viewing

Almost 60-75% of grade A and B oocytes with 2-3 layers of compact cumulus exhibit maturation after 24 hr incubation as evident by expanded, loosely attached cumulus clear cytoplasm and release of first polar body (Fig. 1-D and E). All eggs with vacuolated cytoplasm or dark appearing cytoplasm were excluded from the study. The spindle of the oocytes was easily visualized using Polscope (Fig. 1-F). In most of the oocytes the position of the spindle was located adjacent to the polar body within the range of 20°.

Parthenogenesis

Results of 2 different activation protocols on ovine oocytes are shown in Table 1 and their development in vitro is shown in Figs 2 and 3. As evident from the results, parthenogenetic activation with 5 μM ionomycin for 5 min is more effective as compared to the 7% ethanol for 7 min. Hoechst staining of the parthenotes confirmed the nuclear division (Fig. 3). The blastocysts developed after 9-10 days in culture and showed the typical morphology with a healthy inner cell mass. As evident the growing embryos were accompanied with thinning of zona, increase in size and became more transparent in appearance (Fig. 2C). Natural hatching of the inner cell mass was observed in couple of parthenote blastocysts on Day 10-11 (Fig. 2D). Based on these results, activation post reconstruction of SCNT embryos was carried out using 5 μM ionomycin for 5 min followed by treatment with 2 mM 6-DMAP for 2-3 hr before embryo culture.
Somatic cell nuclear transfer

Once the eggs with spindle were sorted under Polscope from a maturation droplet, it took anywhere from 10 to 15 min for enucleation of 10 eggs and another 10 min to transfer the somatic cell. Of the 139 eggs, 5 were damaged during the procedure as evident by sudden gushing out of the ooplasm. Otherwise the procedure was well standardized, the removed spindle was clearly visualized in the enucleation needle and complete enucleation of the oocyte was confirmed under Polscope (Fig. 4).

After 48 hr of incubation in mCR2 medium, cleavage was observed in 62 out of 139 oocytes (Table 2) whereas the remaining failed to cleave and degenerated in due course of time. Majority showed equal cleavage and easily crossed 6-8 cell stage but the number of morula obtained decreased dramatically. Nuclear division was confirmed by Hoechst staining (Fig. 5). None of the reconstructed embryo developed past morula stage.

**Discussion**

During cloning egg cytoplasm acts as a host for dedifferentiation of the somatic cell nuclei and basically it modifies the expression program of somatic cell nucleus from adult to embryonic state. It has been over 10 years since first cloned animals were generated by SCNT, including DOLLY\(^{22}\) and mouse\(^{23}\), from adult somatic cells. Besides reproductive cloning, therapeutic cloning and the subsequent derivation of ES cell lines have several associated advantages viz. it is an ideal method to generate autologous ES cell lines for regenerative medicine, to create novel disease specific ES cell
Fig. 2—Parthenote embryos at different stages of development: (A) 4-cell embryo; (B) parthenotes growing on cumulus bed; (C) fully expanded blastocyst with thin zona and transparent appearance; (D) hatched blastocyst with empty zona (→)

Fig. 3—Hoechst staining of parthenotes to ensure nuclear division post activation (A & B); 2-cell embryo under bright field and after Hoechst staining; (C & D) Morula under bright field and after Hoechst staining
lines, to elucidate complex interaction during chromatin remodeling, to study the interaction of cytoplasm and nucleus, to study nuclear reprogramming including DNA methylation and gene expression during development, for production of transgenic animals with 100% efficiency, and for the preservation of genetic resources – e.g. when gametes may not be available for reproduction24.

The present study reports procedures for parthenogenesis and SCNT using sheep oocytes. In agreement with the available literature25, it was found that ionomycin activated oocytes were more likely to form blastocysts as compared to ethanol activated group (Table 1) – perhaps because of different method of calcium mobilization25 – although the quality of blastocysts formed in two groups was similar. Both calcium ionomycin and ethanol simulate fertilization events by inducing elevation of calcium by release from intracellular stores. It is known that ionomycin induces smaller and thus less cytotoxic rise of intracellular calcium– whereas ethanol induces extracellular as well as intracellular release of calcium25. Thus calcium ionomycin activation for post reconstruction of oocytes during SCNT was used in this study.

Artificial stimulus to activate egg during parthenogenesis – should induce periodic small rise in free intracellular calcium – but most of the protocols cause monotonic rise in free calcium26. Hence, it has been suggested that a combination of electric and chemical stimulation27 could improve activation protocol and thus the efficacy of obtaining parthenote blastocysts. This needs to be carefully evaluated post reconstruction of SCNT oocytes.

After activation with calcium ionomycin, the reconstructed eggs were treated with 6-DMAP since diploidy is essential for maximum and successful parthenogenetic development. It is a kinase inhibitor that promotes a diploid pronuclei formation without the extrusion of second polar body26.

![Images of somatic cell nuclear transfer](image_url)

Table 2—Development of embryos reconstructed by nuclear transfer

<table>
<thead>
<tr>
<th>Total no. of oocytes</th>
<th>Total cleavage</th>
<th>Irregular cleavage</th>
<th>2-4 cell embryos</th>
<th>6-8 cell embryos</th>
<th>Morula</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>139</td>
<td>62 (44.60)</td>
<td>6 (4.32)</td>
<td>56 (40.29)</td>
<td>52 (36.69)</td>
<td>25 (17.99)</td>
<td>0</td>
</tr>
</tbody>
</table>

Values in parenthesis are in %
In contrast to parthenogenesis where the activated oocyte developed into blastocysts, none of the reconstructed embryos after SCNT developed post morula stage during the culture period in the present study. There could be several reasons for this. Culture system is the most important factor affecting efficacy of SCNT. SCNT not only involves exchange of cytoplast and donor nuclei factors but also reprogramming of somatic cell cycle. MII oocyte cytoplasm has the ability to support 1-3 embryonic cell cycles of newly introduced nuclear DNA in the absence of embryonic transcription or during the time nuclear reprogramming is occurring28 – thus its quality may affect gene expression during nuclear reprogramming. Failure to obtain blastocysts post SCNT in the present study could also be because of quality of oocytes matured in vitro – good cytoplasmic maturation is crucial for effective and successful SCNT. Moreover, the requirements of SCNT and IVF embryos in vitro are different. The early cloned embryo is extremely vulnerable due to the incomplete nuclear reprogramming of gene expression till 8 cell stage – since the gene expression is intermediate between donor cell and normal embryo – leading to a physiologically and metabolically dysfunctional state – this needs to be kept in mind while evolving better culture conditions. If nuclear reprogramming is incomplete immediately following oocyte activation – it has been reported that presence of glucose in the medium during early stage is beneficial in mice29. One needs to ensure that the overall physiology and metabolism of cloned embryo operates efficiently – to maintain viability and to support nuclear reprogramming.

Another important issue is whether the donor cell is reprogrammed efficiently or not. Severe effects of culture medium occur during the first few cell cycles post reconstruction of the embryos. Nature of donor cell also contributes towards the success of SCNT. It has been reported earlier30 that efficacy of embryo formation from fetal fibroblasts (34%) is much higher as compared to using cumulus cell (14%) or oviductal cells (20%). Another factor playing a role may be the method of nuclear transfer. Intracytoplasmic micromanipulation injection (IMI) of donor cell is successful – but the efficacy depends on the skill of...
individual. Electrofusion is considered a better way to reconstruct embryo post SCNT as compared to micromanipulation (63.1 vs. 3.3%). In the present study we carried out injection of somatic cell— and it will be worth while to study in future whether we get better results by electrofusion of somatic cell. Comparing with IVF where a sperm head pierces into the egg cytoplasm and undergoes fertilization, tightly packed sperm DNA is exposed to egg cytoplasm and decondenses after fertilization in the zygote. During SCNT a somatic cell with loosely packed DNA is placed into the cytoplasm - we still do not know whether this environment is conducive enough for the somatic cell. Perhaps it is because of this reasons electrofusion yields better results.

Successful embryo culture to blastocyst stage by parthenogenesis – indicates that reprogramming of the cumulus cell was insufficient in the reconstructed oocytes during SCNT. A compromised developmental ability after SCNT compared to parthenogenetic derived embryos may be associated with several factors based on published literature viz.(i) developmental error may occur during reprogramming of the embryo (ii) cell cycle or cellular events of donor cells may be incompatible to recipient cytoplasm for synchronization post reconstruction or (iii) integrity of donor cell membrane may be damaged by serum starvation – resulting in damaged chromosomes or damaged architecture of cytoskeleton – this affects nuclei formation in the reconstructed embryo. In the present study either of the first two factors may be having a role since the somatic cell was not serum starved prior to use. The cumulus cells used for transfer may not be apt since in mice Wakayama et al.23 reported that only cells of size 10-12 μm yielded results whereas cumulus cells of size 8-9 or 13-15 μm hardly supported embryo development beyond 8 cell stage. Such selective strategy for cumulus cell was not used in the present study. It will be worthwhile to carry out and compare SCNT results using fetal and adult fibroblast and study the efficacy of obtaining blastocyst in vitro. More research is essential to evaluate various factors affecting developmental competency of the cloned embryo.

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