Original Article

Royana: Successful Experience in Cloning the Sheep

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Abstract

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Objective: This study describes our experiences in reproductive cloning using two different procedures resulting in birth of the first successfully cloned sheep in Iran and the Middle-East, nick-named "Royana".

Materials and Methods: Abattoir-derived sheep oocytes were enucleated after in vitro maturation for 18-20hrs and then reconstructed by ear-derived sheep somatic cells using two different procedures of renucleation (subzonary, intracytoplasmic), embryo culture (co-culture, sequential medium) and embryo transfer (intra fallopian, intra uterine). Pregnancy status and fetal development were followed regularly and elective cesarean was inducted on day 145 of pregnancy. Histopathological and genetical examinations were performed on either aborted and delivered clones for confirmation different aspects of cloning.

Results: The two procedures were both efficient in producing early and/or advanced cloned embryos, establishing early and/or advanced stages of pregnancy till delivery. Four pregnancies were detected; one were failed at early pregnancy, one aborted on day 90, one was still born and the fourth delivered to a healthy male lamb nick named "Royana".

Conclusion: Many different approaches have been developed for mammalian cloning which all are judged by their ultimate potency for establishment of successful pregnancies terminated to healthy/viable clones. As a preliminary study toward establishment of the technology, this study also successfully examined the competency of two procedures of somatic cell nuclear transfer (SCNT). However, the overall low efficiency of SCNT indicates that many different aspects of the technology remain to be dissolved.

Keywords: Reproductive Cloning, Somatic Cell Nuclear Transfer (SCNT), Mammalian, Sheep

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Introduction

The time is very exciting and promising for biotechnology - the science of using living cells, organisms and their products to make applicable approaches for biotherapy. In the past few years, three great breakthroughs have been achieved in this respect: (I) all the genes that make up the human DNA have been sequenced (1), (II) stem cell technology is the subject of serious attention and active research (2, 3), (III) mammalian cloning has passed its long disappointing period (4). Furthermore, mammalian transgenesis as a method for gene targeting in live stocks holds particular promise for commercial applications (5). On the other hand, despite of long way from curing diseases, the prospect of experiments are exciting and provide a glimmer of hope which indicates that it is worth to contine studies on these technologies (2, 5).

Despite of remarkable and recent rapid achievements, the cloning background turns back to 1891 when Hans

Driesch and others separated the blastomeres at the two-cell stage (6). They produced identical, but dwarfed embryos. Eleven years later, the same experiment with similar results was performed by Hans Spemann in a vertebrate (salamander), using human baby hair, to separate the cells (4, 6). By considering the results of these experiments, Spemann (7) proposed the transfer of nuclei from more advanced developmental stages to zygotes from which the genetic material had been removed as a valuable method for producing cloned mammals. However the unavailability of an efficient handling system hampered the application of the mammalian procedure for almost 80 years (4, 5), Willadsen (8) could produce the first live lambs after transferring nuclei from 8 to 16-cell sheep embryos into enucleated MII oocytes. However, this work was rested by the ever fame work of Wilmut and his co-workers (9) in cloning «Dolly» which named appropriately «the second creation»(10). Since then, somatic cell nuclear transfer (SCNT) efforts has spanned to about 35 different kinds of mammals including live stocks, laboratory animals and even endangered mammals in over 160 laboratories across at least 37 nations by attracted scientists (11). Even though, the numbers of live birth clone are still limited to certain countries.
Application possibilities of cloning in research, industry and agriculture are theoretically almost limitless (4, 6). In fact, the enormous potential applications of somatic cell nuclear transfer can be divided into two general areas: biomedical and agricultural (12, 13). In addition to its practical applications, cloning has become an essential tool for understanding basic laws of biology including the gene expression, reprogramming, differentiation, X-inactivation, as well as many other topics (12, 14-17).
Considering the great potential of farm animal cloning pharmaceutical protein production or cell/xeno-transplantation (18, 19), there is an obvious need for the countries to make them skill forced in the field of nuclear transfer. In this regard, the paper describes successful experience of mammalian cloning for the first time in Iran and the Middle East.

Materials and Methods

Chemicals

All chemicals used in the present study were purchased from the Sigma Chemical Company (St. Louis, MO, USA) and media from Gibco (Life Technologies, Rockville, MD, USA) unless otherwise specified. All media solutions were adjusted at PH 7.4 and osmolarity of 270- 280 mOsmol/kg H_2O.

Oocyte preparation and in vitro maturation

Ovaries were obtained from a local abattoir and maintained in normal saline (0.9%) at 28-32ºC during transport to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles 2-6mm in diameter via a hypodermic needle (1.2 mm internal diameter) attached to 2ml syringe containing 0.5ml aspiration medium (H-TCM 199 with Earle's salts [Gibco, 22340]) supplemented with 10% fetal calf serum [FCS] and 50 IU/ml heparin and then transferred into sterile 10 ml conical tubes (TPP, Switzerland). The tubes were placed into warm bath (38°C) and the follicular material was allowed to settle for 10-15 min. The resulting pellet was transferred into a petri dish (Falcon, 1005) containing 3-4ml diluting/washing medium (H-TCM 199 with Earle's salts [Gibco, 22340] supplemented with 10% FCS and examined for COCs under a stereo microscope. COCs with at least 2-3 compact layers of cumulus cells were selected and washed three times in 200µl of washing droplets and two times in 200µl droplets of maturation medium. Washed COCs were then co-cultured in groups of 5 into 100µL of maturation medium over a previously established monolayer of vero cells (approximately 1×10^5 cells/ml) (20). Maturation medium was comprised of TCM199 (Gibco, 31150) containing 2.5 mM Na pyruvate, 1mM l-glutamine, 100 IU penicillin– 100 µg/ml, 10% (v/v) FCS, 10 µg/ml FSH, 10 µg/ml LH and 1µg/ml estradiol-17β at 38.5ºC, 5% CO_2 and maximum humidity (Labotect C200, Germany).

Collection and culture of ear fibroblasts

Skin biopsies were obtained from the ear of two 7-9 months old Afshari lambs (male and female) with high race purity. The ear tissues were cut into small pieces ($2mm^2$) and then cultured as tissue explants in $25mm^2$ culture flasks (TTP, Switzerland) containing DMEM (Gibco, 11965-092) plus 10% FCS (Gibco, 10270-106) and 1% penicillin/streptomycin (Gibco, 15140-122) at 38.5ºC, 5% CO_2 and humidified air. The fibroblast cells had formed monolayers surrounding the tissue explants after 5-7 days in culture, the explants were removed and the fibroblast cells were cultured to become confluent. Cultured cells were routinely maintained in 30 mm culture dishes (Falcon, 3001) until passage 15 and then stored frozen. For each passage, confluent cells were disaggregated by incubation in a 0.25% trypsin-EDTA (Gibco, 25200-072) solution for 3 min. Two third of the suspension was allocated into two new dishes for further passaging and the remaining part was diluted in 10% dimethyl sulfoxide (Sigma D-5879), 50% FCS and 40% DMEM solution; followed by being aliquoted equally into three cryotubes (Nunc, Denmark) , then stored overnight in 80ºC before being plunged into liquid nitrogen.

Preparation of donor cells

Frozen-thawed adult ear fibroblast cells between passages 10-15 were used as donor cells, which were plated into a 30-mm culture dishes and cultured in DMEM supplemented with 10% fetal bovine serum (FCS) until nearly 70 to 80% confluency. These cells were then subjected to serum starvation (0.5% FCS in DMEM) for 4-6 days. Immediately prior to SCNT, donor cells

were collected after trypsinization and then resuspended in either Hepes-TCM199 (H-TCM199) or Hepes synthetic oviduct fluid (H-SOF1) containing 0.5% FCS (9) as indicated in the procedure I and II.

Procedures
During this study, in vitro production of sheep SCNT embryos and their transfer into the synchronized recipients were performed according to the two different procedures commonly used for mammalian SCNT but with some modifications. Procedure I comprised subzonary insertion of the donor cell for renucleation (9), culture of embryos in medium conditioned by vero cells (20) and transfer of the early stages embryos in to the fallopian tubes of the recipients (21). Procedure II, in contrast, comprised intracytoplasmic insertion of the donor cells for reconstruction (22), prolonged culture of the SCNT embryos in sequential medium till day 8 (23) and transfer of advanced embryos in to the uterine horn (9). Figure 1 represents the schematic pattern of experiments carried out during this study known as procedure I and II.

Procedure I
Media
The base media used during this procedure were TCM 199 and H-TCM 199 + 10% FCS named in this section as base medium: TCM and HTCM respectively. Embryo culture was carried out in TCM with vero cells co-culture.

Enucleation
At 20-22hrs after the onset of maturation (hpm), oocytes were stripped free of the surrounding cumulus cells by gentle pippetting with a narrow-bored micropipette in 300 IU/ml hyaluronidase (Sigma, H-3506) prepared in HTCM. Only oocytes with homogenous cytoplasm and first polar body extruded were selected and incubated in HTCM containing cytochalasin B (CB 7.5 µg/ml; Sigma, C-6762) for 15min before being enucleated in the same medium at 38.5ºC. Enucleation was performed by aspirating a small amount of cytoplasm from directly beneath the first polar body using a beveled 25µm outside diameter glass pipette and an inverted microscope (Olympus equipped with Narishige micro manipulators). The removed cytoplsts were observed in 10 µg/ml Hoechst 33342 (Sigma) under a fluorescent microscope (x100) to determine successful enucleation. Oocytes retaining the karyoplast were discarded. Enucleated oocytes were then returned to the maturation medium at 38.5°C until 26-27hpm.

Nuclear transfer, fusion and activation
For nuclear transfer, successfully enucleated oocytes were removed from the maturation medium and were transferred back into HTCM micro drops with central droplets of serum-starved fibroblast cells in H-TCM199+0.5% FCS. Well-rounded small donor cells (karyoplast) were deposited adjacent to the oocytes through the same slit made during enucleation. For fusion, reconstructed oocytes were first washed in fusion medium (0.3M mannitol, 0.1mM MgSO4), then transferred into a fusion chamber between two platinum wires filled with fusion medium. Cell fusion was induced approximately 27–28hpm by two direct current (DC) electric pulses (1.75 kV/cm for 80µ seconds and 1 second inter delay; Cryologic®, Australia) perpendicularly to the two fusion partners. An alignment current of 800KHZ was applied just before and after the DC pulses to assure better alignment and fusion of the couplet. Fusion or successful reconstruction was examined 1hr after the pulse delivery. Reconstructed oocytes were then washed twice in TCM and cultured under light paraffin oil in TCM microdrops for 1.5 to 2hrs before activation was inducted. The reconstructed embryos were activated by 5-min exposure to 5µM ionomycin (Sigma I-0634), washed thoroughly thereafter in TCM. Further activation was carried out using 6-DMAP (Sigma, D-2629: 2 mM in TCM) for 4hrs before being washed 3 times in TCM and co-cultured in vero-TCM for the next 1or 2 days. Fragmented embryos were discarded and good quality embryos (at stages of 4 to 8 cells) were transferred in micro liters of HTCM to the fallopian tube of synchronized, freshly ovulated ewes using thin, TDT-catheters (CCD-France). Based on availability of good quality embryos, 2, 3 to 8 embryos were transferred for each recipient.

Procedure II
During the second course of studies, completely different approaches were tested based on the following items: 1) improved embryo culture procedure (sequential SOF medium) which could consistently results in 40% blastocyst production in ovine fertilized oocytes, 2) injection of donor cell into the cytoplast i.e.: enucleated oocytes (22) and 3) lazar assisted hatching to overcome zona hardening (24).

Media
The base media used during this course of study was SOF medium in a sequential manner of SOF1&2 for days 1-3 and 3-8 of the in vitro embryo development respectively. SOF1 and HEPES supplemented SOF1 (H-SOF1) were also used during embryo reconstitution.

Enucleation and nuclear transfer
Oocyte preparation, enucleation was similar to procedure I except for media (SOF1 or H-SOF1) and time of enucleation (24-26hpm). Nuclear transfer was carried out immediately by direct insertion of somatic donor cell into the cytoplast through the same slit, made during enucleation. Care was taken to transfer the donor cell with the minimum amount of media. Apparently enucleated-reconstructed oocytes were then cultured for at least 3hrs before fusion in numbered microdrops. Meanwhile, removed karyoplasts were observed in

10µg/ml Hoechst 33342 under the fluorescent microscope (x100) to determine successful enucleation. Simultaneous fusion-activation was only carried out on these oocytes.

Simultaneous fusion-activation procedure

In order to induce better release of donor nucleus into the cytoplast as well as to trigger the activation stimulation, reconstructed oocytes were washed in fusion medium (0.3M mannitol, 0.1mM MgSO4), transferred into a fusion chamber between two platinum wires, filled with fusion medium and two direct current (DC) of electric pulses (1.75 kV/cm for 80µ seconds and 1 second delay; Cryologic®, Australia) were applied. Reconstructed oocytes were then washed twice in SOF1 and were activated by 5-min exposure to 5 µM ionomycin, washed thoroughly thereafter in SOF1. Further activation was carried out using 6-dimethyl aminopurine (6-DMAP: 2 mM in SOF1) for 4 hrs before being cultured in SOF1 for the first 3 days of embryo development. Embryos were scored daily and fragmented embryos were discarded. On day 3rd, good quality embryos were selected and a slit was made in zona by assisted laser hatching (ZILOS, Hamilton-Thorne Research, Beverly, MA). These embryos were further cultured in SOF2 for the next 3 to 4 days before embryo transfer. At this time, 1, 2 to 3 good quality blastocysts (expanding or hatching/hatched blastocysts) were transferred in micro liters of SOF2 to uterine horn of synchronized ewes using thin, TDT-catheters (CCD-France).

Recipient preparation, embryo transfer and monitoring

Thirty two adult Afshari sheeps in breeding (December-March; 15 ewes for procedureI) or non-breading season (March-June; 17 ewes for procedure II), were selected based on good body condition scoring and previous fecundity. The animals were maintained at the free stall experimental farm of the Royan Institute in Isfahan, Iran. The animals were fed at an alfa-alfa based (at libitum) supplemented with concentrate (twice in 24hrs) and natural light day program. Estrus was synchronized with the insertion of an intra-vaginal progestagen impregnated sponge (40 mg fluorogestone acetate, FGA, Chronogest®, Intervet International, The Netherlands) for either 7 or 14 days in procedure I and II, respectively. 48hrs before sponge removal, ewes received 500IU single injection of pregnant mare serum gonadotrophin (Folligon®, Intervet International, Holland). Single injections of 45 mg prostaglandin (Estrumate®, Shering-Plough, Levallois, France) and an ovulation stimulator (hCG, Chrolon®, Holland) were administered on the morning of day 14th (day of sponge removal) and the day after sponge removal (Day 0 of estrous). Recipients were deprived from food and water 24 and 12hrs before embryo transfer. Embryo(s) were transferred in 20 µL H-TCM199+10% fetal calf serum (FCS) (proc. I) or H-SOF (proc. II) via a TDT-catheter (CCD-France) surgically (25) into the fallopian tube or the top of the uterine horn (through opening previously made by 18 gauge needle) ipsilateral to the ovary bearing at least one functional corpus luteum.

Pregnancy status and fetal development was determined using regular noninvasive monitoring of recipients on days 30-35 of embryo transfer using a high-resolution 7.5 MHz probe. Status of pregnancy was assessed by visualization of pregnancy sac through ultrasound carried out on days 35 and 60 post transfer. From day 140, the ewes were controlled for any sign of changes in body temperature, restlessness, or grazing. From 142-145, daily sonographical exams were carried out to evaluate the precise conditions of placenta, fetus and mother. C-section was inducted by the early signs of placental detachment under local analgesia (lidocaine, Pasture, Iran).

Genetic analysis

Genomic DNA was extracted from liver tissues of dead fetuses or white blood cells from the live born lamb and their corresponding surrogate mothers. Twelve microsatellite markers (as depicted in figure 2) were examined using methods described elsewhere (26). Briefly, microsatellite band patterns were generated following microsatellite variant repeat PCR (MVR-PCR) and size fractionation of reaction products were determined by 12% polyacrylamide gel electrophoresis. Data was confirmed using the ABI373A DNA sequencer (Applied BiosystemsyPerkin–Elmer) and analyzed by GENOTYPER software (Applied BiosystemsyPerkin–Elmer). The resulting microsatellite alleles for the nuclear transfer derived fetuses and the lamb were compared wit those donors the skin biopsies were obtained, and contrasted with those from the recipient ewes that carried the respective pregnancies. To determining the exact number and chromosomal structure of the resulting lambs and fetus in respect to their corresponding donor cells, karyotyping was also done from cultured blood lymphocytes by Gimsa Trypsin Gimsa Banding (GTG banding) method.

Results

In vitro studies

Of 624 ovaries collected during procedure I and II, totally about 4820, 3373 and1441COCs were retrieved, matured in vitro and selected for nuclear transfer, respectively. Table 1 summarizes the results obtained during this study and Table 2 represents the results of preimplantation embryo development in successfully reconstituted, activated oocytes of procedure I and II.

In vivo studies

Table 3 also indicates the details of embryo transfer and the pregnancy out come in procedure I and II.

Procedure I

During the first course of experiments, two pregnancies were observed under ultrasound on day 35 post trans-

fer, resulting in 2.5% pregnancy per embryo transfer. Of these two pregnancies, one was further confirmed on day 60 (Royan-SHE-C1), while the other was lost (Royan-SHE-C3). Upon the removal of the uterine horn for the latter pregnancy, no fetal tissue was observed, suggesting complete loss of pregnancy. The former was continued until day 145 when the emergency cesarean carried out due to severe placental detachment and a very weak fetal heart beat under ultrasound.

The delivered lamb was an immature (crump to tail: 32cm, 3.35kg) which was lost immediately after delivery due to acute apnea. A complicated peritonitis was observed as such the peritoneum was tightly adhered to the uterus at greater curvature- the possible cause of

Table 1: The efficiency of embryo reconstitution (EER) associated with the number of ovine oocytes retrieved, cultured and used during different stages of somatic cell nuclear transfer using adult ovine ear fibroblast cells

Procedure	Ovaries	Retrieved oocytee	In vitro maturation	Selected for nuclear transfer	Enucleated	In vitro matured	Successful enucleated (%)	Lysed during Manipulation	Fused (%)	Activated	Successfully Reconstituted (EER) (%)
I	**286**	**2261**	**1578**	**760**	**626**	**493**	**493 (78)**	**----**	**342 (69.4)**	**342**	**342 (54.6)**
II	**338**	**2556**	**1795**	**681**	**553**	**553**	**389 (70.3)**	**93 (16.8)**	**---**	**247**	**247 (44.7)**
Total	**624**	**4820**	**3373**	**1441**	**1179**	**1046**	**882 (74.8)**	**---**	**---**	**589**	**589 (49.9)**

EER is expressed as a % of enucleated oocyte.
Summaries 19 and 16 replicate in procedure I and II respectively.

Table 2: The results of preimplantation embryo development associated with the number of successfully reconstituted ovine oocytes produced during procedure I and II of this study with adult ovine ear fibroblast cells

Procedure	Successfully reconstituted oocytes	Degenerated-D2 (%)	Fragmentation-D2 (%)	Uncleaved (%)	Cleavage-D2 (%)	*5-8 Cell-D2 (%)	*Compacts-D5 (%)	*Blastocust-D7 (%)
I	**324**	**34 (9.9)**	**139 (40.6)**	**57 (16.6)**	**112 (32.7)**	**80 (71.4)**	**---**	**---**
II	**247**	**27 (10.9)**	**75 (30.6)**	**46 (18.6)**	**99 (40.1)**	**73 (73.7)**	**93 (16.8)**	**18 (18.2)**
Total	**589**	**61 (10.4)**	**214 (36.3)**	**103 (17.5)**	**211 (35.8)**	**153 (72.5)**	**---**	**---**

* Percentage were expressed in relation to number of cleaved day 2 embryos.

Table 3: Pregnancy outcome following transfer of ovine nuclear transferred embryos produced during experimental procedure I and II with ovine adult ear fibroblast cells

Procedure	No. reconstituted embryos	No. embryos transferred	Establishment pregnancy (No. recipients)	% Establishment pregnancy/recipient	% Estad lishment pregnancy/embryos transferred	No. pregnancies lost	Live offspring (% recipient)	Live offspring (% embryos transferred)	No. survived	Cloning efficiency (No. Live offspring/embryo reconstitute)
I	342	80	2 (15)	13.3	2.5	1	1 (6.7)	1 (1.3)	0	0.29
II	247	28*	2 (17)	11.8	7.1	1	1 (5.9)	1 (3.6)	1	0.4
Total	580	108*	4	12.5	3.7	2	2 (6.3)	2 (1.9)	1	0.34

* Consisted of 18 blastocyste and 10 compact embryos.

undetectable running of parturition. Necropsy finding unraveled a retarded pulmonary system with non-structural absolute attelectasia.

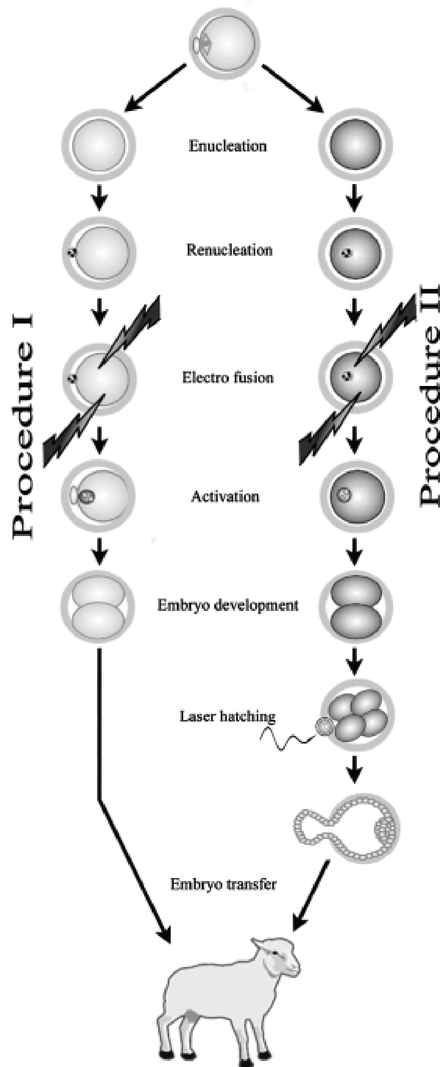

Fig 1: Schematic representation of experimental designs:
Procedure I: enucleated oocytes were reconstituted at 26-28hrs post maturation (hpm) by subzonal insertion of donor cells, followed by immediate fusion and activation on 2 hours post fusion using ionomycin + 6-DMAP. Reconstructed embryos (2 to 8 cells with no fragmentation), obtained as a result of co-culture over Vero cells, were transferred surgically to the fallopian tube of the synchronous ewes.
Procedure II: apparently enucleated oocytes were immediately renucleated by intracytoplamic donor cell insertion and rested in incubator for 3 to 4 h while enucleation was confirmed. Reconstituted oocytes were then imposed to electrical pulses followed by further activation using ionomycin and 6-DMAP. Activated embryos were cultured in SOF1 for 3 days. At this time, embryos were subjected to laser hatching before being cultured in SOF2. On day 7 to 8, blastocyst(s) were transferred surgically to the uterine horn of the synchronous ewes.
Procedure II

Two confirmed pregnancies were detected at day 35, resulting in 7.1% pregnancy per embryo transfer. Of two pregnancies, one continued till day 95 (Royan-SHE-C4) and then aborted to a 13cm (crump to tail) morphologically normal female fetus having well formed heart, lung, visceral apparatuses and urogenital system. However, a mild purulative vaginal muca was observed just before abortion. The second pregnancy (Royan-SHE-C2 nick named Royana) was continued till day 145 under precise monitoring when ultrasound revealed a fully matured healthy fetus within a slightly detaching placenta. Elective cesarean was preceded by two IM injections of dexametazon (Daropakhsh, Iran). After delivery, the lamb immediately started breathing. The lamb was dried off, oxygen therapy was provided via a face mask, and the lamb was positioned in sternal recumbency. Blood gas and electrode values were monitored as if it was in normal range. Despite of some major arrhythmia in heart beat and respiratory distress, the general status was improved at the next hours when Royana received his first colostrums via stomach tube. One hundred and fifty days after, when drafting the manuscript, Royana was a ealthy lamb fostering by his mother in a pasture.

Microsatellite analysis

The results of microsatellite analysis clearly revealed that all three lambs obtained during this study were genetically identical to their donors. Figure 2 shows the molecular profiling and DNA fingerprinting of Royana and his respective somatic donor, which are identical and different from his foster mother. Karyotyping analysis also revealed a normal diploid chromosome number (2n=54) in cloned lambs, their foster mother and relative donors.

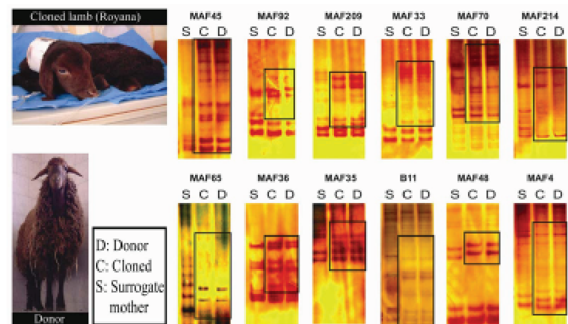

Fig 2: Microsatellite analysis of Royana, his 18 months donor and Royana's surrogate mother, confirming that Royana is genetically identical to his donor.

Histopathological analysis

The tissues were prepared from both the viscera of each aborted lambs, as well as the placenta of the 3 lambs. On histopathological exam, marginal mild necrosis at the apical lobe of lung, liver and kidney were detected in still birth lamb (Royan-SHE-C1) whereas the aborted fetus (Royan-SHE-C4) showed no obvious sign of

abnormality. The placenta of both the aborted fetus and the still birth lamb had clear cellular apoptosis and premature atrophy, while the signs was modest for the live lamb.

Discussion

The cloning technique is an extension of research that has been ongoing for over 50 years using nuclei derived from embryonic and fetal cells as well as completely differentiated adult's cells (6). The ability to obtain a fully term mammal via somatic cell reprogramming was our basic start point toward establishment genetically modified animals and more importantly therapeutic cloning. Accordingly, during 2004-2006 a series of interrelated experiments were carried out to defining the most suitable conditions for in vitro culture of live stocks embryos (mainly cattle and sheep) as an essential prerequisite for embryo reconstitution studies. A series of preliminary studies were then undertaken to establishment the best settings for oocyte enucleation, renucleation and parthenogenetic activation, donor cells preparation and cell cycle adjustment, recipient endometrial preparation and embryo transfer. Since the cattle and sheep are the two major live stocks, the cloning efforts were concentrated mainly to cattle and then the sheep. Despite of initial major successful achievements in the field of bovine cloning, the project shifted to the sheep, concerning financial problems in cattle.

In the experimental procedure I, the reconstructed oocytes were co-cultured in TCM199 with Vero cells (20). Despite of using co-culture system to overcome developmental block, the developmental rate was retarded. Therefore, it was decided to transfer the reconstructed embryos into fallopian tube as early as possible and to transfer higher numbers of embryos to increase the chance of achieving an established pregnancy. Although this procedure was undertaken to overcome in vitro deficiencies, two pregnancies (2.5% per embryos transferred and 13.3% per recipients) were achieved. One of the pregnancies successfully continued to term, even though, it was reported that embedding embryos in agar chips were necessary to protect the embryos from immunocompetent cells present in the oviductal lumen (21). Experimental procedure II was mainly designed to overcome retarded embryo development in procedure I. Thus, sequential semi-defined embryo culture medium, SOF 1 & 2 (23), in which cloned embryos could developed as a reasonably acceptable rate (18.2% blastocyst development for SCNT) were established (Table 2). Although it was suggested that in vitro culture environment exerted significant effects on embryo development (23, 27), embryo culture conditions had not been optimized for cloned embryos and the effects of culture conditions on the successful initiation of clonal development had not been examined (27). Therefore it is proposed that the block to development observed in procedure I may be caused by adverse culture conditions, such as imbalances in the concentrations of certain constituents (27, 28).

During the procedure II of this study, the technique for embryo reconstitution was also changed from that conventionally insertion of donor cell into the perivitelline space (9) to more recently applied method known as intracytoplasmic donor cell injection (22). This method may have the advantage of overcoming the low fusion efficiency reported in the former procedure. On the other hand, the possibility of proper expulsion of donor cell into the recipient cytoplasm and possible damages to nucleus should be considered (29). The technique of intracytoplasmic cell injection was first accredited to Wakayama et al (22) which led to successfully cloned, two generation of mice. This technique was then applied for SCNT production of the other animals such as pig and cow with results comparable to the routine technique of sub zonal donor cell insertion (30, 31). It is of note that Wakayama was the first who applied piezo-actuated microinjection system due to very high sensititivity of mouse oocytes to the enucleation-renucleation processes (22). Other researchers also proposed piezo-actuated microinjection for improving the survival rate after intracytoplamic injection (32). Therefore, it is likely that the excessive lysis observed during reconstruction of oocytes in procedure II (16.8%) may be contributed to the method of intracytoplasmic renucleation which causes excess pressure into the cytoplasts. However, it has been reported that this modified procedure requires prolonged manipulation of donor cells for nucleus isolation which is not only labor intensive but also can reduce the overall cloning efficiency due to the damages to the isolated nucleus (29, 33). The later technique was further improved by conducting whole-cell injection bypassing both the fusion and nucleus isolation process, thus simplifying the cloning procedure. This technique was used successfully for producing panda-rabbit interspecies cloned embryos (33). Our experience also confirms that this technique is a simple, less labor-intensive method of cloning.

Despite of acceptable rate of embryo production in the procedure II (table 2), unlike naturally fertilized embryos, the reconstructed embryos were collapsed and degenerated during blastocyst expansion and prior to hatching. It was believed that this phenomenon might be related to spontaneous progressive hardening of the zona pellucida (34). Therefore, laser assisted hatching was performed on 8-16 cell embryos which drastically improved hatching ability of blastocysts as compared to non assisted embryos (24). In our experience, the second procedure as modified in our hand may provide a simple cloning procedure for labs starting cloning.

Limited overall success rates in terms of live born offspring (0.34% per reconstructed embryos), achieved during this study, is not a new surprising phenomenon. Published data reveal that on average only about 0-3% of the reconstructed embryos (number of live offspring as a percentage of the number of nuclear transfer embryos) resulted in live offspring, irrespective of the species, the donor cell type or technique (35). Considering that unsuccessful cloning experiments is unlikely to be pub-

lished, the actual cloning success rate will be substantially lower. Even though we were on the way of mastering this technology, overall efficiency of cloning is still the main milestone despite broad application of the technology.

Conclusion

Although our study was not a comparative study, but our experience may reveal that procedure II may provide a simple method for future cloning. We are hopping to use this procedure for production of transgenic live stock.

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References

1. Venter JC, Adams MD, Myers EW. The sequence of the human genome. Science 2001; 16: 1304-1351
2. Gordon K, Snodgrass HR. Human embryonic stem cells: the future is now. Nat Med 1999; 5: 151-152
3. Pompe S, Bader M, Tannert C. Stem-cell research: the state of the art. EMBO reports 2005; 6: 297-300
4. Vajta G, Gjerris M. Science and technology of farm animal cloning: State of the art. Anim Reprod Sci 2006; 92: 211-230
5. Colman A. Somatic cell nuclear transfer in mammals: progress and applications. Cloning 2000; 4: 185
6. Campbell KHA. Background to nuclear transfer and its applications in agriculture and human therapeutic medicine. J Anat 2002; 200: 267-275
7. Spemann H. Embryonic development and induction. New York: Hafner Publishing Co 1983.
Willadsen S. M. Nuclear transplantation in sheep embryos. Nature 1986; 320: 63-65
8. Willadsen S. Cloning of sheep and cow embryos.Genome1989; 30: 15-18
9. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385: 810-813
10. Wilmut I, Keith Campbell KH, Tudge C. The second creation. Headline Books 2000; pp 362 ISBN 0747275300
11. Oback B, Wells DN. Cloning cattle: the methods in the madness. Adv exp med biol 2007; 591: 30-57
12. Wolf DP, Mitalipov S, Norgren RB. Nuclear transfer technology in mammalian cloning. Arch Med Res 2001; 32: 609-613
13. Niemann H, Rath D, Wrenzycki C. Advances in biotechnology: New tools in future pig production for agriculture and biomedicine. Reprod Domest Anim 2003; 38: 82-89
14. Han Y M, Kang Y K, Koo DB, Lee KK. Nuclear reprogramming of cloned embryos produced in vitro. Theriogenology 2003; 59: 33-44
15. Eggan K, Akutsu H, Hochedlinger K, Rideout W 3rd, Yanagimachi R, Jaenisch R. X-chromosome inactivation in cloned mouse embryos. Science 2000; 290: 1578-1581
16. Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E et al.. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr Biol 2003; 13: 1116-1121
17. Dean W, Santos F, Reik W. Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. Semin Cell Dev Biol 2003; 14: 93-100
18. Niemann H, Kues WA. Transgenic livestock: premises and promises. Anim Reprod Sci 2000; 277: 60-61
19. Anderson GB, Seidel GE. Cloning for profit. Science 1998; 280: 1400-1401
20. Moulavi F, Hosseini SM, Ashtiani SK, Shahverdi A, Nasr-Esfahani MH. Can Vero cell co-culture improve in-vitro maturation of bovine oocytes? Reprod Bio Med Online 2006; 13: 404-411
21. Loi P, Boyazoglu S, Fulka J, Cappai P, Feil R, Moor RM, et al. Embryo cloning by nuclear transfer: experiences in sheep. Livest Produ Sci 1999; 60: 281-264
22. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394: 369-374
23. Tervit HR, Whittingham DG, Rowson LE. Successful culture of sheep and cattle ova. J Reprod Fertil 1972; 30: 493-497
24. Ali J, Rahbar S, Burjaq H, Sultan AM, Al Flamerzi M, Shahata MA. Routine laser assisted hatching results in significantly increased clinical pregnancies. J Assist Reprod Genet 2003; 20: 1771-181
25. Boundy T, Clarkson MJ, Winter AC. Embryo transfer in sheep under practice conditions. Vet Rec 1985; 117: 379-381
26. Buitkamp J, Ammer H, Geldermann H. DNA fingerprinting in domestic animals. Electrophoresis 1991; 12: 169-74
27. Chung YG, Mann MR, Bartolomei MS, Latham KE. Nuclear-cytoplasmic «tug of war» during cloning: effects of somatic cell nuclei on culture medium preferences of preimplantation cloned mouse embryos. Biol Reprod 2002; 66: 1178-1184
28. Lawitts JA, Biggers JD. Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol Reprod 1991; 45: 245-251
29. Lee JW, Wu SC, Tian XC, Barber M, Hoagland T, Riesen J, et al. Production of cloned pigs by whole-cell intracytoplasmic microinjection. Biol Reprod 2003; 69: 995-1001
30. Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, et al. Pig cloning by microinjection of fetal fibroblast nuclei. Science 2000; 289: 1188-1190
31. Kazufumi G, Michihiro T, Shoji O, Yoshihiko N. Production of a nuclear transferred calf by the intracytoplasmic injection of donor cells. J Reprod Dev 1997; 43: 257-260
32. Kuhholzer B, Prather RS. Advances in livestock nuclear transfer. Proc Soc Exp Biol Med 2000; 224: 240-245
33. Jiang MX, Yang CX, Zhang LS, Zheng YL, Liu SZ, Sun QY, et al. The effect of chemical enucleation combined with whole cell intracytoplasmic injection on panda-rabbit interspecies nuclear transfer. Zygote 2004; 12: 315-320
34. De Felici and Siracusa. Spontaneous hardening of the zona pellucida of mouse oocytes during in vitro culture. Mol Reprod Dev 2005; 6: 107-113
35. Paterson L, Gene expression and development, Roslin Institute. Complied data by Lesley Paterson, scientific secretary to Ian Wilmut at www. roslin. ac. uk