Original Article

Development to term of sheep embryos reconstructed after inner cell mass/trophoblast exchange

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ICM EXCHANGE IN SHEEP BLASTOCYSTS

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**Abstract.** Here we report *in vitro* and term development of sheep embryos after the inner cell mass (ICM) from one set of sheep blastocysts were injected into the trophoblast vesicles of another set. We also observed successful *in vitro* development of chimeric blastocysts made from sheep trophoblast vesicles injected with bovine ICM. First, we dissected ICMs from 35 sheep blastocysts using a stainless steel microblade and injected them into 29 re-expanded sheep trophoblastic vesicles. Of the 25 successfully micromanipulated trophoblastic vesicles, 15 (51.7%) re-expanded normally and showed proper ICM integration. The seven most well reconstructed embryos were transferred for development to term. Three ewes receiving manipulated blastocysts were pregnant at day 45 (42.8%), and all delivered normal offspring (singletons, two females and one male, average weight: 3.54 ± 0.358 kg). Next, we monitored *in vitro* development of sheep trophoblasts injected with bovine ICMs. Of 17 injected trophoblastic vesicles, 10 (58.8%) re-expanded after 4 h in culture, and four (40%) exhibited integrated bovine ICM. Our results indicate that ICM/trophoblast exchange is feasible, allowing full term development with satisfactory lambing rate. Therefore, ICM exchange is a promising approach for endangered species conservation.

**Key words:** Blastocysts, Bovine, Inner cell mass exchange, *In vitro* fertilization, Sheep
Global extinction rates are increasing rapidly in what some researchers have called the Sixth Mass Extinction in the Anthropocene [1]. To save species on the brink of extinction, biologists are now considering the application of reproductive technologies [3] in addition to *in situ* conservation efforts [2]. The most suitable reproductive tools for conservation are somatic cell nuclear transfer (SCNT) [4] and embryo production through deriving gametes from embryonic induced pluripotent cells (IPCs) [5].

In theory, both techniques can produce an unlimited number of animals, SCNT through transferring any somatic cell (a drop of blood contains millions of nucleated cells [6]) into enucleated oocytes and IPCs with the potential to generate countless male and female gametes [5]. Currently, neither technology is ready for practical application, but rapid progress suggests that this will change in the near future, especially for IPCs [7]. However, one neglected hurdle for species conservation through artificial reproductive methods is foster mother availability. Endangered species are by definition unlikely to provide sufficient numbers of foster mothers, so such individuals must be found in a domestic species, preferably with a well-characterized reproductive physiology. Unfortunately, interspecies embryos transfer works only within very closely related species and invariably fails between genetically distant ones, owing to early immunological rejection of conceptuses [8]. Classical embryology may provide methods to shelter embryos of different genetic backgrounds from the immune systems of domestic animals. Experiments performed on mice in the 1970s suggest that “hybrid” embryos could be generated through aggregating mouse embryonic cells around genetically different embryos during early development, thus shielding the latter following transfer into foster mothers [9, 10]. These pioneering embryo aggregation studies were later applied to produce sheep/goat chimeras through aggregating blastomeres collected from sheep and goat embryos at the morula stage (2–8 cells) [11, 12]. These hybrid embryos implanted at high rates and developed into normal animals with varying degrees of chimerism [11]. Encouragingly, a sheep impregnated with a sheep/goat embryo successfully delivered a kid that was determined to be genetically goat [12]. A likely explanation for this result is that the ICM consisted entirely of goat embryonic cells and was shielded by sheep trophoblast from uterine immunological attacks. This finding constitutes the first proof of concept that hybrid embryos between genetically different species could implant and develop to term into the womb of a foster mother.
These early chimera studies tended to have smaller scope, such as reconciling different blood groups [13], rather than creating single-genotype animals. Later studies have used embryo aggregation approaches to understand embryonic and extra-embryonic origins of placental failure in cloned mice [14]. These works have revealed that the proportion of embryonic-cell contributions from the two participating species cannot be reliably predicted under these methods. The typical trend, however, is that blastomeres from more advanced embryos contribute preferentially to the ICM, while less advanced embryos provide the trophoblast. Given the unpredictability, the kid born using embryo aggregation was likely a serendipitous outcome. Thus, in this study, our aim was to assemble hybrid embryos with distinct ICM and trophoblast contributions from the two original embryo lines. To this end, we explored the feasibility of ICM/trophoblastic exchange using expanded sheep blastocysts. Our results show that ICM/trophoblast exchange, although unquestionably invasive, can result in the full development (including delivery) of normal lambs at relatively high proportions.

Material and Methods

Ethics statement

All experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) and European Community regulation 86/609. Procedures were also approved by CEISA (Inter-Institutional Ethics Committee for Animal Experimentation) Prot. 79/2013/CEISA Prog. 58. The permit number for this study is CEISA VI, Class 8.1, Prot. 2823.

In vitro production of sheep embryos

All reagents were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise indicated. Following the collection of ovaries from adult sheep (mixed breeds) at slaughter, oocytes were aspirated for microscopic evaluation. Only those surrounded by at least two layers of granulosa cells and exhibiting evenly granulated cytoplasm were selected for in vitro maturation. Oocytes were matured in bicarbonate-buffered TCM-199 (275 mOsm; Gibco, Grand, Island, NY) containing 2 mM glutamine, 100 µM cysteamine, 0.3 mM sodium pyruvate, 10% fetal bovine serum (FBS; Gibco), 5 µg/ml follicle
stimulating hormone (FSH) (Ovagen, ICP, Auckland, New Zealand), 5 µg/ml luteinizing hormone (LH), and 1 µg/ml estradiol. Maturation conditions were a humidified atmosphere of 5% CO₂ at 39°C for 24 h [15].

*In vitro* fertilization (IVF) medium was bicarbonate-buffered synthetic oviduct fluid (SOF) supplemented with 20% (v:v) heat-inactivated estrus sheep serum, 2.9 mM calcium lactate, and 16 µM isoproterenol. Frozen-thawed semen from rams of proven fertility was used for IVF. Fertilization was performed in 50 µl drops of SOF containing 1 × 10⁶ sperm/ml, under a humidified, 5% CO₂ atmosphere at 39°C for 18 h. Presumptive zygotes were transferred into 20 µl drops of SOF enriched with 1% (v:v) basal medium eagle (BME)-essential amino acids, 1% (v:v) minimum essential medium (MEM)-nonessential amino acids (Gibco), 1 mM glutamine, and 8 mg/ml fatty acid-free BSA (SOFaaBSA). Zygotes were cultured in a humidified atmosphere of 5% CO₂, 7% O₂, and 88% N₂ at 39°C; the medium was renewed on days 3 and 5 of culturing.

*In vitro production of bovine embryos*

Ovaries of Holstein cows were collected immediately after slaughter and transported to the laboratory in Dulbecco’s PBS with antibiotics. Oocytes were aspirated and selected based on morphology (uniform, non-expanded, non-atretic cumulus cells). They were then washed and matured for 20–24 h in TCM199 supplemented with 10% fetal calf serum (FCS), gonadotropins (FSH and LH, 0.05 IU each; Pergovet, Serono, Italy), ITS (insulin, transferrin, sodium selenite), Long-IGFI (100 ng/ml), and Long-EGF (50 ng/ml).

Post-maturation oocytes were fertilized with frozen-thawed bull semen. Thawed semen was separated on a Percoll gradient and suspended in Hepes-buffered SOF medium supplemented with fatty acid-free BSA (6 mg/ml), modified Eagle medium (MEM) amino acids, 1 mg/ml heparin, 20 mM penicillamine, 1 nM epinephrine, and 10 nM hypotaurine.

Zygotes were cultured in SOF medium supplemented with Eagle medium amino acid and BSA (4 mg/ml) until day 8. Medium was partially changed on days 4 and 6. Initial embryonic cleavage per
group was assessed 30 h post-fertilization [16]. Cleavage rate, compaction (on day 6), and blastocyst rate (on days 7, 8) were recorded. Early blastocysts were transferred into 10 ml conical tubes filled with pre-conditioned culture medium, placed into portable incubators (Biotherm INC-RB1, Cryologic, Australia), and shipped to Teramo through express courier.

*Embryo manipulation*

Expanded IVP grade one (Fig. 1 shows grade one; a, and poor quality; b, day 7 sheep blastocysts) sheep blastocysts at day 7 of culture (n = 35, produced in four replicates) were used. Before manipulation, trophoblast opposite of the ICM was patched with a large-bore pipette containing a concentrated solution of Hoechst 33342 (100 µg/ml in Hepes buffered TCM 199) for 10 min (Fig. 1c, d). Embryos were held with a holding pipette for dissecting ICM (Fig. 2a) with a stainless steel micro-blade (AB Technologies, USA). The ICM clump was carefully cleared from residual trophoblastic cells, then transferred along with trophoblastic vesicles to the incubator for culturing (see “In vitro production of bovine embryos”). Bacterial-grade Petri dishes were used for ICMs to avoid adhesion. For 15 min, ICMs were incubated in red fluorescent cell linker for membrane labeling (PKH26, Cell Tracker 236, Sigma), then injected into trophoblastic vesicles with a large (45 µl) pipette (Fig. 2b-c). The aim was to maintain original ICM location through releasing the cells on the opposite side of the Hoechst spot on trophoblast. After 4 h, reconstructed blastocysts were incubated and monitored for re-expansion. Figure 3 (a, b) examples of successful (a) and unsuccessful (b) integration of the injected ICM.

In the second part of the experiment, ICM/trophoblastic exchange was performed between sheep (n = 21) and bovine blastocysts (n = 19). Red-fluorescent labelled bovine ICMs were injected into sheep trophoblasts (n = 17), and monitored for the following 24–36 h.

*Embryo transfer*

Successfully re-expanded blastocysts were surgically transferred into previously synchronized ewes (Crono-Gest sponges for 14 days, followed by 200 UI of PMSG at sponge removal), 7 days after the
start of estrus. Two embryos were transferred per ewe. Pregnancy status was determined through echography (Aloka, 7.5-Mhz high-resolution linear probe) at 40 and 60 days post-transfer. Resultant pregnancies were allowed to develop to term.

Statistical analysis

A chi-square test was used to evaluate implantation rates. Blastocyst developmental rates were compared using a one-way analysis of variance in SPSS Base 9.0 (1999 Applications Guide, SPSS, Chicago, IL).

Results

Microsurgery procedures were relatively invasive (Fig. 2a–c) and slightly reduced embryo viability, as suggested from lower (but not significant) in vivo implantation rates (42.8% manipulated vs. 52% control, Table 1). Following injection and beginning 6 h post-reconstruction, 25 blastocysts re-expanded, with 15 (75%) exhibiting normal morphology (ICM in close association with trophoblast). Note that despite our efforts, injected ICMs wound up attached far from the original location at a different portion of the trophoblast (Fig. 3b), thus, all reconstructed blastocysts experienced perturbation of original structure. Re-expanded blastocysts with normal morphology were kept in vitro for two additional days and behaved identically to un-manipulated, control blastocysts (data not shown).

Seven high-quality manipulated blastocysts were transferred for development to term. As a control, 21 in vitro produced (IVP) embryos were also transferred. Three ewes receiving manipulated blastocysts were pregnant at first scanning. Their implantation rate was lower (but not significantly so) than control IVF embryos (42.8% vs. 52.3%, P = 0.66), but all delivered normal offspring (singletons, two females and one male, average weight: 3.54 ± 0.358 kg). Placentae were spontaneously delivered. Weight at birth was comparable to the breed (Sarda) standard. Likewise, growth rate over one year overlapped with Sarda breed standards (Table 3).
No gross placental abnormalities were detected; morphometric parameters were within the breed standard (mean placental weight $437 \pm 39$ g; cotyledon diameter $3.8 \pm 0.8$ cm, [17]). Lamb growth rates were normal (Table 3).

We then monitored the *in vitro* development and structure of sheep trophoblastic vesicles containing bovine ICMs (Table 2). After 4 h of culture, 10 of 17 (58.8%) injected sheep trophoblastic vesicles re-expanded, and four (40%) exhibited clear bovine ICM integration. *In vitro* development of chimerical sheep/bovine blastocysts proceeded normally over the ensuing 2 days of culture. Subsequently, ICMs began to degenerate in a manner similar to un-manipulated bovine and sheep blastocysts cultured as controls.

**Discussion**

We demonstrated here that the ICM and trophoblast could be switched between sheep blastocysts without compromising the ability to implant and develop into normal lambs. Although we systematically failed to inject ICM into the same location as indigenous ICM, normal morphology was regained quickly in culture. Based on a lower implantation rate (48% pregnancy rate *vs.* 52% in control embryos), the manipulation slightly compromised embryo viability both *in vitro* and *in vivo*. However, we believe any developmental failure was largely related to technical skill and not biology, because we observed progressive improvement in implantation rates as we gained experience.

We obtained far higher frequencies of to-term development than a similar mouse study [18], where only one pup was produced over 164 transferred blastocyst. The probable reason for this difference is that polarization is instrumental to intramural implantation in mice [19, 20]. Thus, the ICM/trophoblast exchange affected mouse blastocysts more than sheep embryos, where implantation occurs via focal apposition of extraembryonic tissues to specialized endometrial areas (caruncle) far later in development [17, 21]. Despite aggressive micromanipulation procedures and total alteration of blastocyst structure, we were pleasantly surprised by the relatively high proportion of embryos brought to term.
As our ultimate goal was to apply the present procedure for conservation, we also designed a procedure to produce sheep/bovine chimeric blastocysts. Many of our reconstructed blastocysts successfully integrated bovine ICM and developed normally in vitro. Re-integration frequency of sheep ICM into bovine trophoblast was lower than the frequency achieved in sheep embryos (40% vs. 75%), but our low sample size precluded any definitive conclusions regarding effectiveness. However, we lower bovine ICM integration rates were probably due to experimental artifacts, such as leaving too many trophoblast cells when injecting bovine ICM, thus preventing appropriate adhesion. Although we cannot completely exclude the possibility of incompatibilities between the two cell lines, we find this explanation more unlikely. Yet another possible reason for the difference may lie in the 24-h shipping time for bovine blastocysts; conditions during transport were sub-optimal for culturing, reducing developmental capacity. Because we did not transfer these embryos into recipient ewes, we could only observe their behavior in vitro. We found that embryos were normal under all criteria until day 9 of culture, when signs of apoptosis and degeneration began.

Obtained lambs were healthy, with a normal birth weight and growth comparable to control IVF lambs. Likewise, placentae were delivered regularly, exhibiting no gross-morphology or morphometric abnormalities. This outcome indicates that once blastocysts are reorganized, development in extraembryonic tissue and the fetus proper follows a normal course. Future efforts can raise survival rate through improving the precision of manipulation methods required for embryo reconstruction, for instance by switching to piezo-steppers or laser assistance.

In this study, our primary goal was to demonstrate that ICM/trophoblastic exchange was compatible with normal development. Therefore, we did not focus on the resulting chimerism in the offspring. Indeed, the embryos were all derived from the same white breed (Sarda), so overt chimerism was not detectable through variation in patches of wool color.

In conclusion, we demonstrated that ICM/trophoblast exchange is compatible with full term development in a large animal model, the sheep. This ICM aggregation approach has also been used
successfully to provide functional placenta in cloned mice [22]. The final proof of concept necessary for applying this procedure to conservation is to produce hybrid embryos between sheep and an unrelated, wild animal (e.g., deer) that results in the safe delivery of a genetically pure endangered species from a sheep foster mother.

**Acknowledgments**

This research was funded by MIUR/CNR, Program FIRB GA B81J12002520001 (GenHome), and the H2020 Twinning action “EraofArt,” GA 698165. The authors are participating in the COST action CA16119. JF Jr is supported with funding from RO 0716.
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**Fig. 1.** a) High-quality blastocyst. b) Low-quality blastocysts. c) Hoechst 33342 staining of ad-embryonic pole. d) Integration of inner cell mass (ICM) at 90° relative to the Hoechst 33342 mark (out of focus in the center). Scale bar = 100 µm.
Fig. 2.  a) Dissecting the ICM. b) ICM injection. c) Injected trophoblastic vesicle. d) Re-expanded blastocyst. Arrows indicate ICM.
Fig. 3.  a) ICM integration into injected trophoblastic vesicle. b) Failed integration of ICM into trophoblastic vesicle.
Table 1  
*In vitro and in vivo* development of reconstructed sheep blastocysts following inner cell mass (ICM)/trophoblast exchange

|                        | Injected trophoblasts (with ICMs) | Expanded (% over total injected trophoblasts) | ICM integration (% over total injected trophoblasts) | Transferred | Pregnancies on day 30 | Offspring |
|------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------------------------|-------------|-----------------------|-----------|
| Manipulated blastocyst | 35                                | 29 (82.8%)                                     | 25 (86.2%)                                          | 15 (51.7%)  | 7 (4 ewes)           | 3 (42.8%) |
| Control blastocysts    | 21                                | –                                              | –                                                   | –           | 21 (11 ewes)          | 11 (52.3%) * |

* No significant differences (P = 0.66) were found between groups.

Table 2  
Development *in vitro* of sheep trophoblast vesicles injected with bovine ICMs

| Number of manipulated bovine blastocysts | Number of sheep trophoblastic vesicles injected with bovine ICMs | Number and percentage of re-expanded trophoblasts | Number and percentage of ICM integration into trophoblasts |
|-----------------------------------------|---------------------------------------------------------------|--------------------------------------------------|-----------------------------------------------------------|
| 19                                      | 17                                                          | 10 (58.8%)                                       | 4 (40%)                                                   |
Table 3  Birth weight and growth rate of Sarda lambs delivered following ICM/trophoblast exchange compared with control

|                     | Weight at birth (kg) | Weight at 30 days (kg) | Weight at 90 days (kg) | Weight at 180 days (kg) | Weight at 350 days (kg) |
|---------------------|----------------------|------------------------|------------------------|-------------------------|-------------------------|
| Female offspring # 1| 3.3                  | 8.5                    | 15.6                   | 26.7                    | 32.1                    |
| Female offspring # 2| 3.5                  | 7.9                    | 14.0                   | 24.2                    | 30.1                    |
| Average weight of control singleton female lambs | 3.6 | 8.8 | 16.0 | 27.8 | 32.5 |
| Male offspring      | 3.7                  | 8.8                    | 16.7                   | 33.8                    | 43.9                    |
| Average weight of control singleton male lambs | 3.9 | 10.4 | 17.5 | 35.3 | 44.6 |