The Development and Ultrastructure of Intergeneric Nuclear Transfer Embryos Using Ovine Ooplasm

by

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Abstract

This thesis encompasses work that aimed to further understand genomic reprogramming, an event crucial in obtaining development in cloned embryos produced by somatic cell nuclear transfer (SCNT). An increasing number of different mammalian species have been cloned using nuclear transfer technology since Dolly the cloned sheep was first successfully produced. However, the biological mechanisms involved in the process of nuclear reprogramming are yet to be fully described. At the centre of this study was an intergeneric SCNT model, which was implemented to determine whether reprogramming factors are conserved across genera. The interaction between donor nucleus and recipient ooplasm was characterised with regard to developmental potential, timing of genome activation, nucleolus formation, and expression of significant proteins.

In initial studies, fusion parameters of the intergeneric SCNT procedure were optimised for the ovine cytoplast and porcine donor granulosa cell. Cell fusion and lysis percentages were determined over a range of electrical pulse voltage, duration and repetition. The optimal electrofusion settings were a single DC pulse of 1.5 kV/cm for 20 μsec following a 2 sec 400 kHz alignment pulse. In addition, it was demonstrated that ovine oocytes were sensitive to electric stimulation to the extreme that oocyte activation would occur no matter how low the voltage. The practical significance was that it would not be possible to implement a fusion before activation protocol.

The ability of the ooplasm of one species to replicate chromosomes and support early embryo cleavage was determined in a preliminary experiment where intergeneric embryos were produced by SCNT using bovine and ovine foetal fibroblasts, and ovine ooplasm. After their construction, the embryos were allowed to develop for 7 days in vitro and the developmental stage determined by Hoechst staining and nuclei counting. In addition, chromosome spreads of the ovine and

bovine somatic foetal fibroblast cell lines used in SCNT, as well as the intra- and intergeneric SCNT embryos were prepared to determine whether the ovine ooplasm was replicating the chromosomes according to the karyotype of the donor nucleus. The somatic cells were karyotyped with 54 and 60 chromosomes counted for ovine and bovine cells respectively. Bovine-ovine embryos were characterised as having a bovine karyotype as distinct from an ovine karyotype, due to the presence of only two metacentric chromosomes as compared with six that are found in the latter. These preliminary results indicated that bovine nuclei obtained from foetal fibroblast cells could initiate early pre-implantation embryo development with the support of ovine oocyte cytoplasm. The development of a proportion (33%) of ovine-ovine intrageneric SCNT embryos beyond the 16-cell stage indicated that an extensive characterisation of an intergeneric model could be performed satisfactorily.

It was hypothesised that the ovine ooplasm would possess the ability to direct in vitro preimplantation embryo development after nuclear transfer using donor nuclei from a different genus, as has been demonstrated in studies using bovine and rabbit ooplasm. In this study, intergeneric SCNT embryos were constructed by the separate fusion of porcine and bovine cells with ovine cytoplasts (bovine-ovine and porcine-ovine respectively), cultured in vitro and the developmental characteristics compared with ovine-ovine SCNT embryos as well as ovine in vitro produced (IVP) embryos. These four groups of embryos were sampled to determine embryo cell numbers at 24, 36, 48, 72, 96, 120 and 168 h post-activation to compare development over time. Despite cleaving normally and undergoing the first three cleavage divisions at a rate comparable with ovine-ovine SCNT embryos, a major block in development occurred in the intergeneric embryos at the 8-16 cell stage. Consequently, no blastocyst formation was obtained as observed for the IVP and ovine-ovine SCNT controls. These results indicate that unlike the rabbit and bovine ooplasm, the ovine ooplasm is not suitable for intergeneric reprogramming of somatic nuclei from another genus, at least of porcine or bovine origin.

To determine the effect of a less differentiated donor nucleus on intergeneric developmental potential, embryonic cell nuclear transfer (ECNT) was conducted in a separate experiment by fusing pluripotent bovine and ovine donor cells (obtained from day-4 preimplantation embryos) to ovine cytoplasts. After 7 days of culture, the cell number of embryos was determined by Hoechst staining and fluorescent observation. Despite observing a single bovine-ovine blastocyst (4.8%), the developmental block remained at the 8-16 cell stage of development. This outcome indicates that a less differentiated nucleus does not increase intergeneric developmental capability.

It is well documented that the ooplasm supplies a large amount of mRNA and protein to the newly formed embryo, crucial for normal development leading up to the major activation of the embryonic genome. However, the interaction between the ooplasm as compared with the donor nucleus in SCNT embryos during this developmental period is poorly understood. This intergeneric SCNT model provided an opportunity to determine the role of the ooplasm on nucleolus formation, which is a marker for genome activation. Ultrastructural evidence was obtained that indicates the ovine ooplasm directs the initial assembly of the nucleolus independent of the species of the nuclear donor. Intergeneric porcine-ovine SCNT and intrageneric ovine-ovine SCNT embryos were constructed and the nucleolus ultrastructure and nucleolus associated rRNA synthesis examined in 1-, 2-, 4-, early 8-, late 8- and 16-cell embryos using transmission electron microscopy (TEM) and light microscopical autoradiography. Intergeneric porcine-ovine SCNT embryos exhibited nucleolar precursor bodies (NPBs) of an ovine (ruminant) ultrastructure, but no active rRNA producing fibrillogranular nucleoli at any of the stages. Unusually, cytoplasmic organelles were located inside the nucleus of two porcine-ovine SCNT embryos. The ovine-ovine SCNT embryos, on the other hand, revealed fibrillogranular nucleoli in 16-cell embryos. In parallel, autoradiographic labelling over the nucleoplasm and, in particular, the nulcleoli was detected. Bovine-ovine SCNT embryos at the 8-

cell stage were examined for nucleolar morphology and exhibited ruminant-type NPBs as well as structures that appeared to comprise of broken down fibrillar material, perhaps formerly of nucleolar origin from the donor cell. These observations indicate that factors within the ovine ooplasm are playing a role in the initial assembly of the embryonic nucleolus in intrageneric SCNT embryos.

To further characterise nucleolus formation, immunocytochemical localisation by confocal microscopy of nucleolin, fibrillarin and RNA polymerase, three key proteins involved in processing rRNA transcripts, was performed on early 8-, late 8- and 16-cell embryos for ovine-ovine and porcine-ovine SCNT embryos. Nucleolin was localised throughout the nucleoplasm for all developmental stages examined in porcine-ovine and ovine-ovine SCNT embryos and, in particular, intensity around the presumptive nucleolar compartments in the later developmental stages. Fibrillarin and RNA polymerase I, on the other hand, were not detected in any ovine-ovine or porcine-ovine SCNT embryos or ovine IVP controls, although both proteins were detected in control bovine IVP blastocysts. This result indicates that the antifibrillarin and anti-RNA polymerase I were not compatible with the ovine form of these respective proteins. As nucleolin is not present in porcine in vivo embryos before the major activation of the embryonic genome, its presence in porcine-ovine SCNT embryo nucleus indicates that nucleolin is derived from the abundant protein and mRNA stored in the ovine ooplasm.

The intergeneric SCNT model established in this thesis demonstrates that the ovine ooplasm lacks the ability to support embryonic development beyond the 16-cell stage. The TEM and autoradiographical studies, in combination with the protein immunocytochemistry study, confirmed that these embryos are unable to undergo the major activation of the embryonic genome, and that the ooplasm influences the initial nucleolar assembly in these embryos.