



A256E Cloning, transgenesis, and stem cells

## Adipose mesenchymal stem cells: a new tool to restore interesting genotypes by cloning in the rabbit

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**Keywords:** nuclear transfer, multipotent, adipose.

Our objective was to investigate somatic cell nuclear transfer (SCNT) as a tool for restoration of particular genotypes (genome edited mainly) in the New-Zealand rabbit. SCNT efficiency is founded on the capacity of donor cells to be reprogrammed to a totipotent state. Consequently, the less differentiated donor cells are, the more easily they could be reprogrammed by a recipient ooplasm. In rabbit, the lack of functional embryonic stem cells is thus a problem. In Ali/Bas rabbit, V. Zakhartchenko *et al.* (Biol Reprod.84p229. 2011) opened interesting perspectives with the use of bone marrow multipotent cells as donor cells for SCNT. Thus, multipotent mesenchymal stem cells (MSC) could be attractive for our purpose. From this prerequisite but looking for multipotent cells accessible in the least invasive way for the donor rabbit, we tested the ability of adipose-derived mesenchymal stem cells (ASC) to give birth to cloned animals. ASC were easily recovered from abdominal fat under anaesthesia. For this preliminary study, we used 2 different batches of commercial ASC (RBXMD-01001/Cyagen Biosciences, Neu-Isenburg, Germany) chosen for their multipotent state and strong capacity to expand maintaining this state. We used cumulus cells (CC) as “control” of development potential since they have been used widely for SCNT and most rabbit live clones were produced from freshly prepared CC. Nuclear transfer and embryo transfer were performed as described by N. Daniel *et al.* (Methods Mol Biol.1222p15. 2015 and Cold Spring Harb Protoc. 2010). The pregnancies were followed by ultrasound monitoring as described by P. Chavatte-Palmer *et al.* (Theriogenology.69p859. 2008). *In vitro* and *in vivo* embryo developments were compared by Chi-2 or non-parametric Fisher’s exact test and differences were considered significant at  $P < 0.05$ . We first compared 2 ASC lines to make sure that the individual characteristics of each do not influence the developmental competence of SCNT embryos. No significant differences were observed for cleavage, blastocyst, implantation and pregnancy rates, nor for development to term. We then compared ASC versus (vs) CC. ASC showed higher *in vitro* development rates: 88% (492/559) vs 73.5% (180/245) and 46.1% (65/141) vs 32.2% (79/245) for cleavage and blastocyst rates respectively. At mid-gestation, pregnancy rates were not significantly different: 40.1% (9/22) vs 50% (4/8). Term pregnancies were obtained for 1 and 3 recipient females respectively. One clone was born from ASC and 5 from CC. Embryo competence to develop to term was thus significantly lower for ASC 0.4% (1/247) vs 3.6% (5/138). Large Offspring Syndrome was observed for 1 ASC and 2CC clones. Further studies are thus necessary to decrease LOS incidence in rabbit cloning, but our study showed that ASC, which are easily available for multiple cloning sessions, are compatible with full term pregnancy after SCNT.

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### Detection of adult stem cell marker leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) transcripts in bovine oviduct epithelial cells

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**Keywords:** mRNA abundance, oviduct, stem cell.

Oviduct epithelial cells (OEC) are cultured *in vitro* to study their function with the aim of improving conditions for *in vitro* embryo production. However, primary culture of OEC is hampered by their limited lifespan and low passage capacity. This study aimed to determine whether the OEC population includes adult stem cells of the type identified in other tissues. Stem cells have the capacity to both self-renew and differentiate into cell types of ecto-, endo or mesodermal lineages. The leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is considered an adult stem cell marker in the epithelia of many tissues. In a first step to identify adult stem cells in oviduct epithelium, we investigated the presence of *LGR5* in OEC. Oviducts from cows obtained from a commercial slaughterhouse were dissected post-mortem and classified into four estrous cycle stages based on ovarian morphology: immediate post-ovulatory, mid-luteal, late-luteal, and pre-ovulatory (n = 3/stage). The OEC from each oviductal segment (infundibulum, ampulla, and isthmus) from ipsilateral and contralateral (relative to the CL or pre-ovulatory follicle) oviducts were collected separately using a mechanical technique for OEC collection. Briefly, the OEC from infundibulum and ampulla were scraped with a thin blade, and the sections of isthmus were gently squeezed with forceps. Relative *LGR5* level was analyzed by RT-qPCR, and normalized using the geometric mean of the two best reference genes (GAPDH encoding glyceraldehyde dehydrogenase and RLP15 encoding ribosomal protein L15). Student's t-test was used to evaluate differences in *LGR5* levels between ipsilateral and contralateral oviducts and ANOVA with post-hoc Tukey-Kramer test to assess differences in OEC *LGR5* levels between the three oviductal segments and four estrous cycle stages (JMP software; SAS Institute Cary, NC). No differences in *LGR5* levels were observed between ipsi- and contralateral oviducts ( $P > 0.05$ ). However, when compared to ampulla, *LGR5* was detected  $156.4 \pm 121.3$  times more in the isthmus, while  $0.51 \pm 0.22$  lower in the infundibulum ( $P < 0.05$ ). *LGR5* levels were estrous cycle stage independent ( $P > 0.05$ ) with the exception of the isthmus in which  $2.75 \pm 1.15$  and  $1.73 \pm 0.85$  times higher *LGR5* levels were detected during the pre-ovulatory stage when compared to the mid- and late-luteal stages, respectively ( $P < 0.05$ ). In short, *LGR5* mRNA was detected in all oviductal segments throughout the estrous cycle with highest levels in pre-ovulatory isthmus. The detection of *LGR5* transcripts suggests the presence of adult stem cells among bovine OEC. Experiments are ongoing to confirm the presence and location of LGR5 protein and to isolate LGR5 positive cells for future improvement of OEC cultures.

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## Early microinjection of bovine zygotes reduces mosaicism rates following CRISPR-mediated genome edition

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**Keywords:** CRISPR, mosaicism, IVF.

Direct gene ablation (knock-out, KO) can be achieved by CRISPR microinjection at the zygote stage. KO generation mediated by CRISPR is based on the generation of insertion-deletions (indels) at the coding region of a gene that can disrupt its open reading frame leading to a truncated protein. Gene disruption occurs only when the indel is not multiple of three, therefore not all randomly generated indels produce a KO. Direct KO generation requires all alleles (indels) harbored by an individual to be frame-disrupting. In this sense, a reduction in the number of indels generated in a given individual increases the chances of direct KO generation. Ideally, CRISPR should generate 2 indels, one on each parental chromosome, however, if the edition occurs after DNA replication, mosaicism (i.e. the presence of more than two indels on the same embryo) arises. The objective of this study has been to develop a protocol based on early microinjection of bovine oocytes to reduce mosaicism rate following CRISPR edition. A preliminary study was conducted to evaluate the shortest gamete co-incubation time compatible with normal developmental rates. Fertilization rates were dramatically reduced at 8 or 9 h co-incubation times, but 10 h yielded similar cleavage rates than the conventional 20 h *in vitro* fertilization ( $83.8 \pm 5.0$  vs.  $90.9 \pm 5.5$  for 10 or 20 h groups, respectively, ANOVA ( $P > 0.05$ )). Next, we evaluated DNA replication timing by 5-Ethynyl-2'-deoxyuridine (EdU) incorporation from 10 to 20 hours post-insemination (hpi), observing that most zygotes began their S-phase well before 20 hpi (zygotes in S-phase (%):  $39.0 \pm 5.61$ ;  $78.0 \pm 7.54$ ;  $92.5 \pm 4.13$   $77.5 \pm 4.48$ ;  $56.1 \pm 6.41$ ;  $37.5 \pm 5.09$ , at 10, 12, 14, 16, 18 and 20 hpi respectively; mean  $\pm$  s.e.m., 3 replicates). Finally, we performed CRISPR microinjection (300 ng/ $\mu$ l Cas9 mRNA and 100 ng/ $\mu$ l sgRNA) at 10 or 20 hpi, targeting an intronic region where indels do not exert any detrimental effect on development. Genome edition rates of blastocysts derived from microinjected zygotes was similar in both microinjected groups (85.7% -6/7- for 20 hpi vs. 92.3% -12/13- for 10 hpi). However, clonal sequencing of the edited embryos (10 clones sequenced per embryo) revealed a significantly lower incidence of mosaicism in blastocysts from 10 hpi group (100% -6/6- for 20 hpi vs. 33.3% -4/12- for 10 hpi. Fisher's exact test ( $P < 0.05$ )). In conclusion, an earlier delivery of CRISPR components reduces mosaicism rates and achieves similar developmental and genome edition rates.

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### **The use of a novel microfluidic culture device and predictive metabolic profiling as a means to improve murine embryo developmental competence *in vitro***

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**Keywords:** microfluidics, metabolism, embryo quality.

Successful embryo development *in vitro* is directly dependent on the provision of an optimal culture environment that supports coordinated embryonic cell division, metabolism, and genetic and epigenetic development. A number of attempts have been made over recent years to use microfluidic devices in IVF as a means to control the culture environment and so improve embryo developmental competence (quality) *in vitro*. In this study we have designed, engineered and tested a novel microfluidic device for the *in vitro* production of murine embryos from the 1 cell zygote stage to the blastocyst stage. Soft lithography was used to prepare microfluidic devices in polydimethylsiloxane (PDMS). The microfluidic device consists of a 400 nL circular chamber (radius 750 mm) where 10 embryos can be loaded, kept in static culture for the full period of culture and visualized by optical and fluorescent microscopy. A series of 2 experiments were conducted to evaluate the efficacy of our microfluidic device for mouse embryo culture. Cryopreserved, IVF-derived, mouse embryos of strain C57BL/6N, provided by MRC Harwell, UK were cultured in KSOM media. Microfluidic culture was used in conjunction with non-invasive analysis of glucose (G), pyruvate (P) and lactate (L) metabolism in spent zygote culture media as a means to improve embryo quality. In both experimental series, data from microfluidic cultures were compared to equivalent end point analyses of control embryos grown in conventional microdrop cultures under oil. Experiment 1: 2 cell embryos were thawed and cultured in groups of 8-10 in microfluidic devices (n = 46) or 10µl control (n = 32) drops for 3 days at 37°C under 5%CO<sub>2</sub>/5%O<sub>2</sub>/N<sub>2</sub> balance. Embryos were removed to individual culture drops for 24h for analysis of energy substrate turnover using the method of Guerif et al. (PLOS ONE, 2013) followed by transfer to fibronectin-coated dishes for assessment of attachment and outgrowth according to the method of Hannan et al. (Endocrinology, 152 (12), p4948-4956, 2011). Blastocyst grade, hatching, attachment, outgrowth rates, and pyruvate and glucose consumption were assessed and were similar between device and control groups (P > 0.05). However, lactate output was significantly reduced following device culture vs controls (4.1 ± 0.8 vs 1.4 ± 0.3 pmol/embryo/hr, P = <0.0001). GPL metabolism did not predict embryo attachment or outgrowth in either culture environment. Experiment 2: 1 cell zygotes were cultured individually overnight for analysis of GPL metabolism and assigned to culture groups based on pyruvate consumption, with 1 device and 1 microdrop group per tertile per culture with 10 embryos per group (total n = 60 device and n = 60 control). Following group culture, individual blastocyst pyruvate consumption was reduced (5.4 ± 2.2 vs 12 ± 1.5 pmol/embryo/hr, P = < 0.0001). Pyruvate consumption tertile was unaffected by device culture. Device culture was non-toxic and did not affect embryo development. However, blastocyst pyruvate consumption and lactate output were reduced compared to controls. This may suggest microfluidic culture can be utilised to achieve a controlled, moderate metabolic phenotype, reducing variation between embryo metabolism.