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Subfertility and zona pellucida alterations in ZP4 KO rabbits produced by CRISPR

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Keywords: CRISPR, zona pellucida, rabbit.

Mammalian zona pellucida, the glycoprotein layer that surrounds oocytes and embryos up to the blastocyst stage, may be composed by 4 different glycoproteins. One of these proteins, ZP4, is present in the zona pellucida of rabbits, cattle and women, among others, but it is absent in the only species where Knock-out (KO) models were readily available: the laboratory mouse. For this reason, the function of ZP4 remains elusive. CRISPR technology greatly simplifies the generation of KO models in livestock species such as rabbits. In this experiment, we have generated ZP4 KO rabbits, i.e. rabbits lacking ZP4 protein, by CRISPR technology and have compared their reproductive performance to that of heterozygous (Hz) and wild type (wt) rabbits. Delivery rates following natural breeding with males of proven fertility were analysed in 5 animals per experimental group (wt, Hz and KO). Pregnancy was clearly impaired in KO animals, with only one female producing a litter of 4 pups, resulting in a significant reduction in litter size compared to wt or Hz groups (pups delivered: wt 9.2±0.6; Hz 10.6±0.5; KO 0.8±0.8; mean±s.e.m., Kruskal-Wallis ($P<0.05$)). Aiming to elucidate the possible causes of subfertility, ovulation and cleavage rates were assessed following natural mating. Surprisingly, neither ovulation (oocytes ovulated: wt 11.7±1; Hz 15±2.9; KO 13.3±2.9), nor cleavage rates (% of cleavage: wt 81.7±0.1; Hz 95.5±0.1; KO 87.3±0.1) showed significant differences between groups. However, clear morphological differences were noted on the zona pellucida from oocytes ovulated by KO rabbits compared to those produced by Hz or wt rabbits. Zona pellucida thickness was significantly reduced in KO compared to Hz or wt (thickness μm: wt 15.2±1.5; Hz 15.3±1.4; KO 10.9±0.7, ANOVA ($p<0.05$)). Besides, KO rabbits produced irregular zonae pellucida, i.e. not perfectly spherical as in Hz of wt animals, and noticeable less elastic and easier to deform. These results suggest that the impaired fertility in ZP4 KO rabbits is not due to reduced ovulation or cleavage, and that ZP4 may act as a crosslinker of other ZP proteins, conferring mechanical properties to the zona pellucida which are important for embryo survival. This study is supported by the projects AGL2014-58739-R and RYC-2012-10193 (to PBA), AGL2015-70159-P (to MA) and AGL2015-65572-C2-1-R (to PGR and PL). ILT and NFB are supported by FPI grants.

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Does scriptaid-dependent epigenomic modulation of peripheral blood-derived fibroblast-like cells affect the *ex vivo* developmental abilities of caprine-porcine nuclear-transferred embryos to reach blastocyst stage?

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Keywords: SCPT-dependent epigenomic modulation, adult goat peripheral blood-derived fibroblast-like cell, inter-species (caprine-porcine) NT embryo.

The present research was carried out to ascertain whether inter-family and inter-genus (caprine→porcine) nuclear-transferred (NT) embryos can acquire and retain the competences to complete their extracorporeal development to the blastocyst stage. To generate inter-species (caprine→porcine) cloned embryos, enucleated *in vitro*-matured pig oocytes were subzonally microinjected and subsequently electrofused with adult goat peripheral blood-derived fibroblast-like cells (AGPB-FLCs) that either had been epigenetically transformed by exposure to 350 nM scriptaid (SCPT) during their 24-h contact inhibition (Group I) or had not been exposed to SCPT (Group II). Efficiently electroactivated caprine→porcine nuclear-ooplasmic hybrids were cultured to the morula and blastocyst stages for 7 to 8 days. Among 231 inter-species NT embryos assigned to Group I, 172 (74.5%)[a] underwent cleavage divisions. The percentages of embryos that progressed to the morula and blastocyst stages were 65/231 (28.1%)[a] and 26/231 (11.3%)[a], respectively. In Group II, out of 217 hybrid NT embryos, 147 (67.7%)[a] were able to divide *ex vivo* ([a,a] $P \geq 0.05$; χ^2 test), but 36 (16.6%)[b] and 0 (0.0%)[b] developed to the morula and blastocyst stages, respectively ([a,b] $P < 0.01$; χ^2 test). Summing up, inter-species (caprine→porcine) NT embryos that had been reconstructed with porcine enucleated oocytes and SCPT-treated AGPB-FLC nuclei exhibited developmental capabilities to reach the blastocyst stage. In contrast, their counterparts originating from porcine enucleated oocytes and SCPT-untreated AGPB-FLC nuclei were not developmentally competent to progress to the blastocyst stage. Additionally, due to desirable enhancement of donor cell nuclear reprogrammability, the strategy of SCPT-mediated epigenomic modulation of AGPB-FLCs resulted in not only remarkable improving morula formation rate of hybrid (caprine→porcine) NT embryos, but also acquiring and maintaining capacities to complete the *in vitro* development to the blastocyst stage.
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Effect of Estradiol and Progesterone on ovine Amniotic Epithelial Cells

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Keywords: oAEC, estradiol, progesterone.

This study was designed to clarify Estradiol (E2) and Progesterone (P4) steroid effects on ovine Amniotic Epithelial Cells (oAECs) that has a conserved plasticity and highly self-renewable capacity (Parolini et al., Stem Cells, 26(2), 300–311, 2008; Barboni et al., Stem Cell Rev Rep, 10:725–741, 2014). Based on their conserved immunomodulatory properties, oAECs are suitable for allo and xeno-transplantation (Barboni et al., Cell Transplant, 21(11), 2377–2395, 2012; Muttini et al., Res Vet Sci, 94(1),158–169, 2013). To date, no information is present on the effects of prolonged steroid exposition on AECs. oAECs were cultured as previously reported (Barboni et al., Cell Transplant. 21(11), 2377–2395, 2012) and treated with 12.5µM and 25µM of E2 or P4 (Sigma-Aldrich, Milan, Italy), alone and in both combinations, for three passages. Untreated cells were marked control (CTR). At 70% confluency, cells were detached for doubling time (DT) evaluation. Cells at fourth passage were differentiated for 21 days in osteogenic media (DM) (Mattioli et al., Cell Biol Int 36(1):7-19, 2012) without steroid. Alizarin Red and Alcian-Blue (Sigma-Aldrich, Milano, Italy) stainings were performed. RNA and cDNA were obtained as previously reported (Barboni et al., Cell Transplant. 21(11), 2377–2395, 2012). Real Time for *NANOG*, *SOX2* ,*OCT4* stemness genes expression were performed by SensiFast SYBR (Bioline, Aurogene, Rome, Italy) using specific primers (Mattioli et al., Cell Biol Int. 36(1):7-19, 2012).The protocol was: 5 min at 95°C, 30 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec. Comparative Ct $2^{-\Delta\Delta C(t)}$ normalization to *GAPDH* was applied. IHC analyses were carried out for Cytokeratin 8 and αSMA expression as previously reported (Barboni et al. PLoS ONE 7(2): e30974, 2012). Data expressed as mean (±SD), compared by one-way ANOVA followed by Tukey's test (GraphPad Prism 5). Significant values for $P < 0.05$. Steroids treated ovine AECs proliferate with significant differences between concentrations. While P4 treated cells showed cuboidal shape and Cytokeratin expression until third passage, CTR and E2 treated cells showed a rapid downregulation of Cytokeratin and increased αSMA expression. oAECs with E2+P4 showed both cell type morphology. Steroids modified stemness genes based on the concentration. 12.5 µM E2, 25µM P4 and 25µM of both E2+P4 treatments maintained higher *OCT4*, *NANOG* and *SOX2* expressions in treated cells despite their progressive downregulation in the CTR. Moreover, compared to CTR, after Alizarin staining, steroid pretreated cells suffered morphological changes under DM acquiring Alcian Blue-positive chondrogenic-like morphology. AECs stemness properties and plasticity can be modified by prolonged steroidal treatment. These data improve our knowledge, opening new prospective on oAEC use in stem cell-based therapy.
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Intra-family and inter-genus (caprine-bovine) cloned embryos do not fail to complete their *in vitro* development to blastocyst stage

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Keywords: intra-family and inter-genus (caprine-bovine) cloned embryo, intra-species (caprine) cloned embryo, *ex vivo* developmental capacity.

The current study was undertaken to comparatively analyze the *ex vivo* developmental outcomes of inter-species (caprine→bovine) nuclear transfer (NT)-derived embryos (Group I) and intra-species (caprine) NT-derived embryos (Group II). In Group I, to create inter-species clonal cytoplasmic hybrids (cybrids), enucleated extracorporeally matured heifer/cow oocytes were reconstituted with the cell nuclei of adult goat peripheral blood-retrieved fibroblast-like cells (AGPB-FLCs) that had undergone the *in vitro* synchronization of mitotic cycle at the G1/G0 phases by contact inhibition. In Group II, to produce intra-species clonal cybrids, enucleated metaphase II-stage doe oocytes were reconstituted with the cell nuclei of contact-inhibited AGPB-FLCs. The inter- or intra-species clonal cybrids that had been successfully electrofused and then were subjected to calcium ionomycin- and 6-dimethylaminopurine (6-DMAP)-mediated activation were classified for *in vitro* culture. In Group I, from among 212 cultured inter-species NT-derived embryos, 168 (79.2%)[a] were cleaved. The proportions of embryos that developed to morula and blastocyst stages were 69/212 (32.5%)[a] and 41/212 (19.3%)[a], respectively. In Group II, out of 203 cultured intra-species NT-derived embryos, 172 (84.7%)[a] were able to divide, but 75 (36.9%)[a] and 48 (23.6%)[a] reached the morula and blastocyst stages, respectively ([a,a] $P \geq 0.05$; χ^2 test). To summarize, the *ex vivo* developmental capacities of inter-species (caprine→bovine) cloned embryos to progress to the morula and blastocyst stages did not differ considerably from those indicated among intra-species (caprine) cloned embryos. This seems to result from close taxonomic distance and phylogenetic consanguinity between donor specimens of somatic cells (*Capra aegagrus hircus*) and donor specimens of nuclear recipient oocytes (*Bos primigenius taurus*). Such symptomatic relationships undoubtedly encompass intra-family (*Bovidae*) and inter-genus (*Capra-Bos*) model of inter-species cloning of domestic goats by somatic cell nuclear transfer (SCNT).
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