NANOG is Required for Proper Hypoblast Development in Ovine

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By the late blastocyst stage, mammalian embryos have experienced two lineage differentiation events. The first one leads to the formation of the trophectoderm (TE) and the inner cell mass (ICM), and the second differentiates ICM cells into epiblast and hypoblast. The TE and the hypoblast will form the extraembryonic membranes, while the epiblast will give rise to the embryo proper. NANOG is a key transcription factor involved in the second lineage specification. Its role has been extensively studied in mice, where *Nanog* KO embryos arrest between E5.5 and 7.5, not being able to develop the epiblast and failing to express the hypoblast marker SOX17. In bovine, *NANOG* ablation reduces Day (D) 8 blastocyst cell number, but does not impair ICM differentiation. However, the role of NANOG during post-hatching development is unknown in any ungulate species. The objective of this study was to analyze the role of *NANOG* in ovine post-hatching embryo development through CRISPR-mediated gene ablation (knock-out, KO). *In vitro* matured oocytes were microinjected with Cas9 mRNA and a sgRNA against *NANOG* (C+G), or with Cas9 alone as microinjection control (C). Microinjected oocytes were fertilized and cultured *in vitro* up to D8 (in SOF medium) or D12 (in N2B27 medium from D6/7), when pictures were taken and embryos were fixed and immunostained to detect SOX2 and NANOG (epiblast markers), and SOX17 (hypoblast marker). Embryo genotyping in C+G group was performed by deep sequencing. Blastocyst rate was similar between the group containing *NANOG* KO embryos (C+G) and the control group (C) (34.6±5.8% vs. 33.2±6.5%; mean ± s.e.m; 6 replicates; t-test, $p>0.05$). In C+G group, 13/18 (72.2%) blastocysts were KO and lacked NANOG protein. No significant differences were detected in the number of TE (negative to NANOG, SOX2 and SOX17) or total cells between KO and WT D8 blastocysts (TE: 188±31.35 vs. 225.6±66.3; total: 219.4±32.9 vs. 331±62.3; mean±s.e.m; t-test, $p>0.05$). However, the number of ICM cells (positive to NANOG, SOX2 and/or SOX17) was significantly lower in KO blastocysts (44.7±7.5 vs. 116.8±33; mean±s.e.m.; t-test, $p < 0.05$). Within the ICM, no significant differences were detected in the number of SOX2+ cells between KO and WT blastocysts (SOX2+: 23.1±4.9 vs. 14.2±2.9; mean±s.e.m; t-test, $p>0.05$). However, SOX17+ cell number was significantly lower in KO blastocysts (28.15±7.9 vs. 94.9±21.5; mean±s.e.m; t-test, $p < 0.05$). Post-hatching embryo survival at D12 was similar between C+G and C groups (80.4±1.3% vs. 88.6±1%; mean±s.e.m; 3 replicates; t-test, $p>0.05$). In C+G group, 22/36 (61.1%) embryos were *NANOG* KO. No differences were detected in embryo area (0.36±0.06 vs. 0.37±0.03 mm^2; mean±s.e.m; Mann-Whitney test, $p>0.05$), in the number of embryos showing surviving epiblast cells (17/22 [77.3%] vs. 46/62 [74.2%]; Chi-square test, $p>0.05$) or in SOX2+ cell number (15.8±4.2 vs. 21.1±3.3; Mann-Whitney test, $p>0.05$) between KO and WT embryos at D12. However, hypoblast migration, measured as the percentage of inner embryo surface covered by SOX17+ cells, was significantly reduced in KO embryos (34.9±6.9% vs. 51±3.5%; Mann-Whitney test, $p>0.05$). In conclusion, *NANOG* is dispensable for ovine blastocyst formation, epiblast development and hypoblast differentiation, but its ablation impairs significantly hypoblast proliferation and migration at post-hatching stages.

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