A309E Cloning, Transgenesis and Stem Cells

Optimization of RNA concentration for genome editing by CRISPR in rabbit zygotes

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Keywords: CRISPR, rabbit, zona pellucida.

Site-specific genetic modification aiming to delete (knock-out) a gene provides an unequivocal answer to elucidate the function of such particular gene in the whole organism. Site-specific genetic modification has been achieved by homologous recombination, generally in embryonic stem cells, which has made the mouse the most widely used mammalian model. However, the mouse model is not appropriate to study some biological functions or to recapitulate some human diseases. As an example, ZP4, one of the four proteins of the zona pellucida in humans and rabbits, is not present in mice, so its function remains elusive due to the lack of a knock-out model. The use of the newly developed site-specific endonucleases, such as CRISPR, allows site-specific genetic modification in zygotes, being a suitable technique for genetic modification in domestic mammalian species. The aim of this experiment has been to determine the optimal concentration of the two components of the CRISPR system (Cas9 mRNA and gdRNA) for genome editing following microinjection of rabbit zygotes. Capped polyadenylated Cas9 mRNA was produced by *in vitro* transcription from BstBI digested pMJ920 plasmid. A gdRNA was designed against the first exon of rabbit ZP4 gene, cloned into the plasmid px330, amplified by PCR adding T7 promoter and *in vitro* transcribed. Rabbit zygotes were obtained from the oviduct 14 hours after mating. Immediately after collection, zygotes were microinjected into the ooplasm with approximately 10 picoliters of three different combinations of Cas9 capped polyadenylated mRNA and gdRNA: 1) 300 ng/µl Cas9 and 150 ng/µl of gdRNA (300:150), 2) 150 ng/µl Cas9 and 50 ng/µl gdRNA (150:50) and 3) 100 ng/µl Cas9 and 25 ng/µl gdRNA (100:25). Following microinjection, embryos were cultured in TCM199 supplemented with 5% FCS at 38.5ºC in a 5% CO_2, 5% O_2 and 90% N_2 water saturated atmosphere. CRISPR components did not affect preimplantation development, as all embryos surviving microinjection (~90%) developed to the blastocyst stage. At the blastocyst stage, the zona pellucida was removed and blastocysts were individually stored at -20 ºC. Blastocysts were digested in 8 µl of a 100 µg/ml proteinase K buffered solution and 2 µl of the lysate were used to amplify the genomic sequence including the CRISPR target site. PCR products were purified and sequenced to determine genome edition around the target site. All combinations were similarly effective in generating insertion/deletions around the target site: in the groups 300:150 and 150:50 all blastocysts analysed (6/6 in both groups) were edited, whereas in the group 100:25 only one blastocyst out of six was not edited. In conclusion, CRISPR system constitutes an effective means for genome editing in rabbit zygotes and the ooplasm microinjection of 100 ng/µl capped polyadenylated Cas9 mRNA and 25 ng/µl gdRNA achieves high genome editing efficiencies.

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A310E Cloning, Transgenesis and Stem Cells

Superovulation rates and embryo recovery in 12 to 15-month-old genetically modified pigs for biomedical research

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Keywords: transgenesis, pig, superovulation.

The aim of this study was to evaluate the superovulatory response, fertilization rate and quality of embryos recovered from superovulated genetically modified donor pigs. Twenty nine transgenic pigs (Polish Landrace and crossbreed) 12 to 15-month-old with body weight ranging from 120 – 200 kg were used as embryo donors. Pigs with expression one gene CMV-FUT II (n=3) or GAL (n=11) (1TG), and two genes CMV-FUT II x GAL (n=16) (2TG) made generation F2 to F8 of transgenic animals. The pigs were superovulated by intramuscular injection of 1500 IU of PMSG (Folligon, Intervet, Holland), followed 96 hours later by i.m. 1000 IU of hCG (Chorulon, Intervet, Holland). At the onset of oestrus (24 h after hCG administration) the donors were artificially inseminated twice at 12 h intervals with the standard dose of semen of transgenic boar. Presumptive zygotes were collected surgically under general anaesthesia on Day 1 after insemination. Superowulatory response was measured by counting the number of ovulations in each ovary. Each oviduct was flushed with 10 mL of PBS (Sigma Chemical Company, USA) supplemented with 20% fetal calf serum (Sigma Chemical Company, USA) at 30°C. Recovered embryos were morphologically evaluated under stereomicroscope and classified according to IETS standard code. Then zygotes were used for further modifications. The results were analyzed statistically with Chi-square test. There was no significant difference in the proportion of pigs with 1 gene (70%) and 2-genes (81%) that responded to superovulation treatment. There was also no significant difference in the ovary reaction (mean of number of ovulations) of 1TG (9.2) and 2TG (9.3) pigs. On the other hand, there was a significantly higher ($P < 0.01$) embryo yields in 1 TG (65%) than 2TG (48%). There was no significant difference in the number and quality recovered embryos between 1TG (8.8±2.3; code 1, 8.6 ± 2.2) and 2TG pigs (5.4 ± 1.8; code 1, 5.2 ± 1.8). However, 2TG pigs had higher proportion of fertilized ova than 1TG pigs (97.4% and 91.8%, respectively). It was concluded that in our experiment, genetically modified pigs with expression one (CMV-FUT II or GAL) or two genes (CMV-FUTxGAL) had a similar ovarian response and a number of embryos "good" and "excellent" quality. However, superovulatory response of both 1TG and 2TG pigs was low.

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A311E Cloning, Transgenesis and Stem Cells

Effect of the protein kinase inhibitor 6-Dimethylaminopurine on the parthenogenetic activation of mouse oocytes

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Keywords: Cytochalasin B, Latrunculin A, $SrCl_2$.

Parthenogenetic activation is a key step in nuclear transfer procedures, allowing the reconstructed oocytes to initiate embryonic development in the absence of fertilization. To maintain diploidy, extrusion of the second polar body (PB2) must be prevented. In mice, this is typically achieved by adding an inhibitor of actin polymerization, as cytochalasin B (CytoB) or latrunculin A (LatA), to the activation medium, which also contains $SrCl_2$ to induce increases of cytosolic Ca^{2+}. In other mammalian species, the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) is often used to inhibit PB2 extrusion and to further enhance the activation stimulus. The aim of this study was to evaluate the efficacy of 6- DMAP for the activation of mouse oocytes in the presence of $SrCl_2$.
Mature oocytes were collected from B6CBAF1 females at 14 h post-hCG and cultured in KSOM medium for 6 h, to simulate the timing at which nuclear transfer oocytes are usually activated. Activation was performed by 6 h culture in Ca^{2+}-free CZB medium containing 10 mM $SrCl_2$ (Sigma-Aldrich, Madrid, Spain) and either 5 µg/ml CytoB (Sigma-Aldrich, Madrid, Spain), 5 µg/ml LatA (Santa Cruz Biotechnology, Heidelberg, Germany) or 2 mM 6-DMAP (Sigma-Aldrich, Madrid, Spain). In one group the 6-DMAP treatment was reduced to the first 4 h of the $SrCl_2$-induced activation. Activated oocytes were cultured in KSOM medium until the blastocyst stage, and then fixed and stained for Oct4 (Santa Cruz Biotechnology, Heidelberg, Germany) to detect inner cell mass (ICM) cells, and DNA (Hoechst; Fisher Scientific, Madrid, Spain). Data on *in vitro* embryonic development and blastocyst cell numbers were analyzed by Fisher's exact test and Kruskall–Wallis test (GraphPad Prism 5), respectively. A probability value of $P < 0.05$ was considered statistically significant.
Activation rates were similarly high for all treatments (91.8-97%), but the percentage of activated oocytes that extruded PB2 was higher in the 6-DMAP groups (25% for 6 h and 26.7% for 4 h treatments) than in the CytoB (5.2%) and the LatA (6.5%) groups. Accordingly, in vitro development to the blastocyst stage was higher in these last two groups (88.7% CytoB and 93.5% LatA) than in the two 6-DMAP groups (62% for 6 h and 66.7% for 4 h treatments). Blastocyst total cell numbers were higher in the 6 h (76.9 ± 14.9) and 4 h (82.7 ± 12.6) treatments with 6-DMAP than in the treatment with CytoB (64.4 ± 11.9), but the number of ICM cells was similar among all the groups tested.
In conclusion, our results show that 6- DMAP is not a good substitute for inhibitors of actin polymerization in mouse oocyte activation protocols.

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A312E Cloning, Transgenesis and Stem Cells

Trichostatin A-mediated epigenetic transformation of bone marrow-derived mesenchymal stem cells or blood-derived fibroblast-like cells brings about an abundance of nuclear-transferred pig embryos at the morula and blastocyst stages

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Keywords: TSA-mediated epigenomic modulation, nuclear donor cell, porcine cloned embryo.

The current research was aimed at a comparative exploration of the influence of either epigenomically modulated adult bone marrow-retrieved mesenchymal stem cells (ABM-MSCs) or adult peripheral blood-derived fibroblast-like cells (APB-FLCs) that provided a source of nuclear donor cells (NDCs) on the extracorporeal development of porcine cloned embryos. Before use for somatic cell cloning (SCC), clonal ABM-MSC lines (derived from bone marrow aspirates recovered from the iliac crests of a postnatal female piglet) or clonal APB-FLC lines (derived from blood samples collected from external jugular vein of a postnatal female piglet) were epigenetically transformed by exposure to 50 nM trichostatin A (TSA) during 24-h contact inhibition. Gilt/sow oocytes that had acquired meiotic maturity status under *in vitro* culture conditions were utilised as nuclear recipient cells for SCC. Cumulus-oocyte complexes (COCs) were matured *ex vivo* for 20 to 22 h in TC 199 medium supplemented with 10% FBS, 10% porcine follicular fluid, 5 ng/mL recombinant human basic fibroblast growth factor, 10 ng/mL recombinant human epidermal growth factor, 0.6 mM *L*-cysteine, 1 mM dibutyryl cyclic adenosine monophosphate (bucladesine), 0.1 IU/mL human menopausal gonadotropin (hMG) and 5 mIU/mL porcine follicle-stimulating hormone (pFSH). The COCs were subsequently cultured for an additional 22 to 24 h in the maturation medium lacking bucladesine, hMG and pFSH. The oocytes that had been enucleated underwent insertion of TSA-treated ABM-MSCs (Group I) or APB-FLCs (Group II) into their perivitelline spaces. The ooplasts were then electrofused with NDCs and simultaneously activated by applying two consecutive DC pulses of 1.2 kV/cm for 60 µs. The electroactivated nuclear-ooplasmic hybrids were exposed to 5 µg/mL cytochalasin B for 2 h, followed by *in vitro* culture to morula/blastocyst stages in BSA- and FBS-enriched NCSU-23 medium for 6 to 7 days. In Groups I and II, 156/168 (92.9%)^{a} and 164/185 (88.6%)^{a} oocytes were successfully fused/activated and intended to be cultured, respectively (^{a,a} $P \geq 0.05$; χ^2 test). Groups I and II yielded proportions of 149/156 (95.5%)^{A} and 135/164 (82.3%)^{B} for cleaved embryos, respectively (^{A,B} $P < 0.001$; χ^2 test). The rates of cloned embryos that reached the morula and blastocyst stages were 134/156 (85.9%)^{A} and 97/156 (62.2%)^{A} or 117/164 (71.3%)^{B} and 65/164 (39.6%)^{B} in Groups I or II, respectively (^{A,B} $P < 0.001$; χ^2 test). Cumulatively, the competences of the cell nuclei that had been inherited from TSA-exposed ABM-MSCs to support both cleavage divisions and *ex vivo* development of nuclear-transferred pig embryos to morula and blastocyst stages were remarkably higher than the competences of those that had been inherited from TSA-exposed APB-FLCs.

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A313E Cloning, Transgenesis and Stem Cells

Extracorporeal development of porcine somatic cell nuclear transfer (SCNT)-derived embryos is biased by scriptaid-induced epigenetic modulation of adult cutaneous fibroblast cells

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Keywords: adult cutaneous fibroblast cell, scriptaid, cloned pig embryo.

The current study was conducted to examine the *ex vivo* developmental competences of cloned pig embryos reconstructed with oocytes receiving the cell nuclei of adult dermal fibroblast cells that had been epigenetically transformed by treatment with new-generation non-specific inhibitor of histone deacetylases, known as scriptaid (6-(1,3-dioxo-1*H*,3*H*-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide). Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 h in Tissue Culture Medium 199 (TCM 199). The maturation medium was enriched with 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU/mL equine chorionic gonadotropin (eCG), 10 IU/mL human chorionic gonadotropin (hCG), 10% foetal bovine serum (FBS), 10% porcine follicular fluid, 10 ng/mL recombinant human epidermal growth factor, 5 ng/mL recombinant human basic fibroblast growth factor and 1.2 mM *L*-cysteine. Afterwards, the COCs were cultured for an additional 22 to 24 h in the db-cAMP- and eCG+hCG-deprived TC 199 medium. Prior to use for SCNT, the permanent fibroblast cell lines (between passages 1 and 3) that had been established from the primary cultures derived from ear skin biopsies of a prepubertal boar were exposed to 350 nM scriptaid during 24-h contact inhibition. Reconstruction of enucleated *in vitro*-matured oocytes was accomplished by their electrofusion with epigenetically modulated fibroblast cells. Simultaneous fusion and electrical activation of reconstituted oocytes were triggered using two consecutive DC pulses of 1.2 kV/cm for 60 µs. Immediately after fusion/activation, nuclear-transferred oocytes (clonal cybrids) were incubated in North Carolina State University-23 (NCSU-23) medium supplemented with 5 µg/mL cytochalasin B for 2 h, followed by *in vitro* culture to morula and blastocyst stages in NCSU-23/BSA/FBS medium for 144 to 168 h. The rates of dividing embryos (187/252; 74.2%^{A}), morulae (145/252; 57.5%^{A}) and blastocysts (76/252; 30.2%^{C}) that originated from nuclear-transferred oocytes reconstituted with adult cutaneous fibroblast cells undergoing scriptaid treatment were significantly higher than in the scriptaid-unexposed group (133/229; 58.1%^{B}, 101/229; 44.1%^{B} and 42/229; 18.3%^{D}, respectively) [^{A,B} $P < 0.001$; ^{C,D} $P < 0.01$; χ^2 test]. Altogether, the improvements in not only cleavage activity of porcine cloned embryos, but also their morula/blastocyst yields seem to arise from enhanced abilities for promotion of faithful and complete epigenetic reprogramming of scriptaid-treated adult dermal fibroblast cell nuclei in a cytoplasm of reconstituted oocytes.

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A314E Cloning, Transgenesis and Stem Cells

Adult peripheral blood-derived fibroblast-like cells provide a source of nuclear donor cells that is much less susceptible to promote the *in vitro* development of porcine cloned embryos than adult bone marrow-derived mesenchymal stem cells

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Keywords: adult peripheral blood-derived fibroblast-like cell, adult bone marrow-derived mesenchymal stem cell, porcine nuclear-transferred embryo.

The objective of the current study was to ascertain the impact of not only adult peripheral blood-derived fibroblast-like cells (APB-FLCs), but also adult bone marrow-derived mesenchymal stem cells (ABM-MSCs) on the *ex vivo* developmental capabilities of nuclear-transferred pig embryos generated using either type of genomic DNA donor cell. Oocytes that had attained the meiotic maturity status under extracorporeal conditions were utilised as a source of genomic DNA recipient cells for the purposes of somatic cell nuclear transfer (SCNT). Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 to 22 h in Tissue Culture Medium 199 that was enriched with 10% foetal bovine serum (FBS), 10% porcine follicular fluid, 5 ng/mL recombinant human basic fibroblast growth factor, 10 ng/mL recombinant human epidermal growth factor, 0.6 mM *L*-cysteine, 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 0.1 IU/mL human menopausal gonadotropin (hMG) and 5 mIU/mL porcine follicle-stimulating hormone (pFSH). The COCs were subsequently cultured for a further 22 to 24 h in the fresh maturation medium depleted of db-cAMP, hMG and pFSH. To form the ooplast-nuclear donor cell complexes, the previously enucleated oocytes were subjected to microinjection of contact-inhibited/trypsinised APB-FLCs (Group I) or ABM-MSCs (Group II) under their zonae pellucidae. The ooplasts then underwent simultaneous fusion and electrical activation. The electroactivated nuclear-ooplasmic hybrids (clonal cybrids) were treated with 5 μg/mL cytochalasin B for 2 h, followed by *in vitro* culture to morula and blastocyst stages in 0.4% bovine serum albumin- and 10% FBS-supplemented North Carolina State University-23 medium for 6 to 7 days. A total of 293 and 234 enucleated oocytes that were electrically fused with either APB-FLCs or ABM-MSCs were simultaneously activated in Groups I and II, respectively. In Groups I and II, 172/196 (87.8%)^{a} and 161/174 (92.5%)^{a} oocytes were efficiently electrofused/electroactivated and classified for *in vitro* culture, respectively (^{a,a} $P \geq 0.05$; χ^2 test). Out of 172 and 161 cultured SCNT-derived embryos assigned into Groups I and II, 106 (61.6%)^{A} and 147 (91.3%)^{B} exhibited cleavage activities, respectively (^{A,B} $P < 0.001$; χ^2 test). The percentages of embryos that completed their development to the morula and blastocyst stages were 85/172 (49.4%)^{A} and 41/172 (23.8%)^{A} or 126/161 (78.3%)^{B} and 68/161 (42.2%)^{B} in Groups I or II, respectively (^{A,B} $P < 0.001$; χ^2 test). In conclusion, porcine SCNT-derived embryos reconstructed with APB-FLCs were characterized by significantly lower competences to undergo the cleavage divisions and to reach the morula/blastocyst stages as compared to those reconstructed with ABM-MSCs.

This study was supported by the Polish Ministry of Science and Higher Education as the statutory activity No. 02-011.1.

A315E Cloning, Transgenesis and Stem Cells

Epigenomically transformed peripheral blood-derived fibroblast-like cells can be successfully utilised as a novel type of nuclear donor cells for generation of cloned pig embryos

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Keywords: pig, epigenomically transformed APB-FLC, NT embryo.

The present study was undertaken to assess the *in vitro* developmental outcome of porcine nuclear-transferred (NT) embryos created using adult peripheral blood-derived fibroblast-like cells (APB-FLCs) that had been epigenetically modified by exposure to non-selective inhibitor of histone deacetylases (HDACs), designated as trichostatin A (TSA; [*R*-(*E*,*E*)]-7-[4-(dimethylamino)phenyl]-*N*-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide). Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 h in TC 199 medium enriched with 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU/mL equine chorionic gonadotropin (eCG), 10 IU/mL human chorionic gonadotropin (hCG), 10% porcine follicular fluid, 10 ng/mL recombinant human epidermal growth factor, 5 ng/mL recombinant human basic fibroblast growth factor and 0.6 mM *L*-cysteine. Afterwards, the COCs were cultured for 22 to 24 h in the db-cAMP- and eCG+hCG-free medium. Before their use for somatic cell cloning, the adherent fibroblast-like cell lines (between passages 1 and 5) that had been established from the primary cultures originating from blood samples, collected with the aid of peripheral venipuncture and intravascular cannulation via external jugular venous catheterisation of postnatal female piglet, were treated with 50 nM TSA during 24-h serum starvation. Reconstruction of enucleated metaphase II-stage oocytes was achieved by their electrofusion with epigenomically transformed APB-FLCs that was evoked by two successive DC pulses of 1.2 kV/cm for 60 µs. The same DC pulses that triggered the fusion of ooplast-nuclear donor cell couplets were simultaneously applied to induce activation of reconstituted oocytes (clonal cybrids). These latter were subsequently incubated in NCSU-23 medium supplemented with 5 µg/mL cytochalasin B for 1.5 to 2 h, followed by *in vitro* culture to morula and blastocyst stages in NCSU-23/BSA/FBS medium for 6 to 7 days. The percentages of cleaved embryos (208/257; 80.9%[A]), morulae (175/257; 68.1%[A]) and blastocysts (96/257; 37.4%[C]) developing from NT embryos that were reconstructed with APB-FLCs undergoing TSA treatment were significantly higher as compared to the TSA-unexposed group (159/243; 65.4%[B], 126/243; 51.9%[B] and 62/243; 25.5%[D], respectively) [[A,B] $P < 0.001$; [C,D] $P < 0.01$; χ^2 test]. Collectively, the enhancements in both cleavage rate of porcine cloned embryos and their morula/blastocyst formation rates appear to result from increased functional abilities for proper onset and progression of epigenetic remodelling and reprogramming of TSA-treated APB-FLC nuclei in a cytoplasm of clonal cybrids.

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A316E Cloning, Transgenesis and Stem Cells

Generation of monogenetic twin embryos and progeny by modified bisection of zona-perforated pig blastocysts

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Keywords: porcine hatching blastocyst, modified bisection, monogenetic twin offspring.

Microsurgical splitting of post-compaction morulae and blastocysts is the standard method of experimental embryo duplication and subsequent production of genetically identical twin offspring in several species of mammals, with the exception of pig. In this method, embryos are incised vertically through the meridional or equatorial median plane into two equivalent embryo halves. Disadvantage of the conventional embryo bisection technique that is related to high incidence of cell loses among manually micro-dissected embryos was eliminated by development of the modified approach to bisection of bovine and rabbit blastocysts (Skrzyszowska *et al.*, 1997; Theriogenology). This was achieved by the accomplishment of one-point drilling in their zonae pellucidae and assisted induction of their specific hatching process with a figure-of-eight pattern through zona perforation. At last, two parts of the hatching blastocyst (the first inside and the second one outside the zona pellucida) were split into demi-embryos via performing the vertical midline incision downstream of the zona perforation and across a thin cellular cross-bridge connecting both embryonic compartments. The purpose of the study was to use this modified method of embryo microdissection for generation of monozygotic twin piglets. A total of 541 embryos at the expanding/expanded blastocyst stages that had been recovered from uterine horns of 15 hormonally stimulated donor sows were subjected to the microsurgical puncturing of zonae pellucidae and selected to be extracorporeally incubated for 20 to 22 h. After the *ex utero* incubation had passed, 208 zona-punctured blastocysts that progressed to hatch according to a specific figure-8 pattern were manually bisected with the aid of a glass needle. As a result, 416 blastocyst halves were obtained. All the half-embryos (both zona-free and those remaining inside their zonae pellucidae) were together intended to be surgically transferred into uterine horns of 38 recipient sows. Additionally, 333 zona-perforated isogenic blastocysts that did not undergo the hatching process were transferred into reproductive tracts of the same recipients. The number of transferred demi-blastocysts and non-hatching zona-drilled blastocysts per recipient ranged from 8 to 14 and from 7 to 11, respectively. Twenty three to 28 days after embryo transfer, ultrasound examinations of recipient females were performed in order to confirm pregnancy. Pregnancies were detected in 7 foster mothers, from among which 5 sows farrowed, delivering the litters that, in total, included 24 piglets (22 live and 2 stillborn). The genomic DNA isolated from blood samples that had been collected from 20 specimens out of 22 piglets (2 piglets died within 3 weeks after parturition) was analysed to detect and profile the consanguinity or inbreeding extent. In summary, the molecular genetic analysis has confirmed the production of 2 pairs of monogenetic twin piglets (brothers and sisters) that have been selected to undertake and apply preclinical biomedical research.

A317E Cloning, Transgenesis and Stem Cells

Culture optimization to obtain mouse embryonic stem cell lines from single blastomeres

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Keywords: KSR, N2B27, 2i.

The derivation efficiency of mouse embryonic stem cell (mESC) lines is determined by several factors such as the genetic background of the embryos, the culture medium or the presence of molecules that modify the activity of signalling pathways (Czechanski *et* al., Nat Protoc, 9: 559-74, 2014). The aim of this study was to determine the role of these factors in the derivation of mESC from single blastomeres.

Blastomeres were isolated from 8-cell embryos from 129S2xC57BL and B6CBAF1 permissive strains and from the non-permissive CBA strain, seeded in microdrops with a monolayer of feeder cells and cultured at 37ºC and 5% CO_2. Two DMEM-based derivation media were used, a serum-free medium supplemented with N2B27 (Gibco, Madrid, Spain) and a defined medium containing KnockOut Serum Replacement (Life Technologies, Barcelona, Spain), both supplemented with 0.1 mg/ml adrenocorticotropic hormone (ACTH; ProspecBio, East Brunswick, USA). For each strain and derivation medium, a group of blastomeres was cultured with 2i (Ying *et* al., Nature, 453: 519-523, 2008) a combination of inhibitors consisting of 1 µM of the MAPK inhibitor PD0325901 and 3 µM of the GSK3β inhibitor CHIR 99021 (Axon Medchem, Groningen, Netherlands). After a week, outgrowths were subcultured and maintained for 5 more weeks in 4-well plates in the same culture conditions except for the absence of ACTH. To verify the stemness of the putative mESC lines an immunofluorescence analysis was performed to detect pluripotency markers Oct4 (Santa Cruz, Heidelberg, Germany) and Sox2 (Merck Millipore, Madrid, Spain) and, after culturing the cells under differentiation conditions for 10 days, the differentiation potential was assessed using markers Tuj1 (BioLegend, San Diego, USA), αSMA (Sigma, Madrid, Spain) and AFP (R&D Systems, Minneapolis, USA). Results were statistically analyzed with a Fisher exact test. A minimum of 145 blastomeres were analysed per group with at least 3 replicates.

mESC derivation rates from single blastomeres were low in both media for all strains (0-5.9%). Addition of 2i significantly increased mESC derivation efficiencies from blastomeres of 129S2xC57BL and B6CBAF1 strains, but only when defined medium was used (23.9% and 22.9%, respectively). In serum-free medium, derivation rates remained low despite the addition of 2i (0.7%-1.6%). Although results for the CBA strain are still preliminary, derivation efficiency did not improve in this strain by the addition of 2i to defined medium (2.2%).

In conclusion, serum-free medium does not allow an efficient mESC derivation from single blastomeres. The only combination to efficiently obtain mESC is to culture blastomeres from permissive strains in defined medium supplemented with 2i.

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A318E Cloning, Transgenesis and Stem Cells

Efficient derivation of embryonic stem cells from mouse B6CBAF1 blastocysts

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Genetic background is one of the variables influencing the derivation efficiency of mouse embryonic stem cells (mESC). Accordingly, mouse strains can be classified as permissive or non-permissive for mESC derivation (Ohtsuka *et* al., Development, 142: 431-437, 2015). This study aimed to determine the behaviour of B6CBAF1 blastocysts for mESC derivation, using 129S2xC57BL and CBA embryos as controls of permissive and non-permissive strains, respectively. Embryos were collected at the 2-cell stage and cultured in KSOM medium at 37ºC and 5% CO_2 until the blastocyst stage. Blastocysts were denuded using acidic Tyrode's solution, seeded on a monolayer of human foreskin fibroblasts feeder cells and cultured at 37ºC and 5% CO_2 with two DMEM-based derivation media: a serum-free medium supplemented with N2B27 (Gibco, Madrid, Spain) or a defined medium containing KnockOut Serum Replacement (Life Technologies, Barcelona, Spain). For each strain and medium, one group was cultured with 2i, consisting of 1 µM of the MEK inhibitor PD0325901 and 3 µM of the GSK3B inhibitor CHIR99021 (Axon Medchem, Groningen, Netherlands). Putative mESC were weekly subcultured and the medium was changed every other day. After 6 passages, the stemness of mESC lines was proved by immunofluorescence with the pluripotency markers Oct4 (Santa Cruz, Heidelberg, Germany) and Sox2 (Merck Millipore, Madrid, Spain) and, after culturing the cells under differentiation conditions for 10 days, the differentiation potential was assessed with the markers Tuj1 (BioLegend, San Diego, USA), αSMA (Sigma, Madrid, Spain) and AFP (R&D Systems, Minneapolis, USA). Results were statistically analysed with a Fisher exact test. A minimum of 30 embryos were analysed per group with at least 3 replicates.
The defined medium allowed a high derivation rate from 129SvxC57BL (74.3%) and B6CBAF1 (77.4%) embryos, but a statistically significant lower rate (46.7%) from CBA embryos. The addition of 2i significantly improved the derivation from CBA embryos (87.1%), resulting in equivalent derivation rates for all strains tested (75.9%-87.1%). In the serum-free medium, derivation rates were low for the three strains (3.1%-9.4%), although the addition of 2i treatment increased the derivation rates for all strains tested (82.4%-96.9%).
Our results confirm that the 2i treatment compensate for the differences in derivation rates due to the genetic background (Czechanski *et* al., Nat Protoc, 9: 559-74, 2014), allowing an efficient derivation of mESC lines in both serum-free and defined media. Moreover, our results indicate that B6CBAF1 embryos behave as 129S2xC57BL/6 when deriving mESC from whole blastocysts, and differ from CBA, demonstrating that B6CBAF1 should be considered a permissive strain.

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