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Generation of genome-edited mouse epiblast stem cells *via* a detour through ES cell-chimeras

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ABSTRACT

Conventionally, mouse epiblast stem cells (EpiSCs) are derived directly from the epiblast or ectoderm germ layer of the post-implantation embryo. Self-renewing and multipotent EpiSC-like stem cells can also be derived by the conversion of embryonic stem cells (ESCs) via the provision of culture conditions that enable the maintenance of the EpiSCs. Here, we outline an experimental procedure for deriving EpiSCs from post-implantation chimeric embryos that are generated using genome-edited ESCs. This strategy enables the production of EpiSCs where (i) no genetically modified animals or ESCs are available, (ii) the impact of the genetic modification on post-implantation development, which may influence the property of the EpiSCs, is requisite knowledge for using the EpiSC for a specific investigation, and (iii) multiple editing of the genome is desirable to modify the biological attributes of the EpiSCs for studying, for example, the gene network activity on the trajectory of lineage differentiation and tissue morphogenesis.

1. Introduction

Epiblast stem cells (EpiSCs) are self-renewing and multipotent cell lines that are derived from the epiblast or the ectoderm of the mouse embryo (Brons et al., 2007; Tesar et al., 2007). EpiSCs can be isolated from embryos in a window of post-implantation development from the pre-gastrulation (around embryonic day E5.5) to the late gastrulation (E7.75) stage prior to the dismantling of pluripotency (Kojima et al., 2014). Irrespective of the original developmental stage of the epiblast or ectoderm, EpiSCs derived under the same conditions (in the presence of Activin A and Fibroblast Growth Factor-2 (FGF2)) display a similar transcriptome profile. This profile matches most closely with that of the epiblast of the mid-gastrula stage (E7.0) embryo, and the EpiSCs also show strong resemblance to cells in the anterior primitive streak (APS) of the embryo (Kojima et al., 2014). In the embryo, cells localised at this part of the primitive streak are progenitors of the mesoderm and endoderm lineages and they are subjected to strong Nodal (a Transforming growth factor beta (TGFβ) in the same family as Activin A) and FGF (FGF4 and FGF8) signalling activity. It is therefore, likely that EpiSCs *in vitro* have acquired a similar cell state as the *in vivo* counterpart, in response to the signalling activity that mirrors that in the embryo. The EpiSCs, while still maintaining a functional pluripotency gene network, express a wider range and higher levels of gene activity that are associated with lineage specification and differentiation. This suggests these stem cells are at a primed state and poised for exiting pluripotency and initiating differentiation (Nichols and Smith, 2009). In-depth analysis of the transcriptome revealed that the EpiSC cell lines are inherently different from each other regarding the propensity of germ layer differentiation, which may be reflected by the innate heterogeneity of cellular composition of the individual lines. The state of lineage propensity of the self-renewing population can be modulated by changes in the signalling condition: enhanced WNT signalling promotes mesoderm propensity, blocking WNT activity enhances ground state pluripotency, and may also promote ectoderm propensity (Murayama et al., 2015; Sugimoto et al., 2015; Sumi et al., 2013; Tsakiridis et al., 2014). The plasticity of EpiSCs, in the context of lineage propensity, suggests that EpiSCs respond actively to the inductive signalling activity that drives lineage differentiation. This makes the EpiSC a useful experimental model to study the cellular and molecular mechanisms that control the transition from multipotency to differentiation and the

Abbreviations: APS, anterior primitive streak; EpiSC, epiblast stem cell; ESC, embryonic stem cell; FGF, fibroblast growth factor; TGFβ, transforming growth factor beta

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progression of lineage specification and differentiation, as well as for modelling germ layer formation and tissue morphogenesis.

A prerequisite for using EpiSCs as an experimental model for elucidating the molecular control of gastrulation and germ layer differentiation is the ability to experimentally modify the function of genes of interest through genome editing. When mice with the required genetic modification are available, EpiSCs can be derived directly from embryos of the desirable genotype. If only the embryonic stem cells (ESCs) with the appropriate genetic modification are available, they can be used to generate the mice from which embryos could be harvested for isolating EpiSCs. This inevitably requires a work-intensive and protracted process of animal production and breeding. If neither the requisite animal nor the ESC is available, an alternative step would be to create the genetic modification in wild type EpiSCs. The technology for genome editing of EpiSCs, apart from the incorporation of reporter constructs, has yet to be perfected. The low efficiency of single-cell clonal expansion for the selection of editing events poses another significant technical hurdle. At this juncture, the more practical approach is to perform genome editing in the ESCs to generate the necessary genetic modifications at single or multiple genetic loci. The genome-edited ESCs can be cultured through stepwise changes of *in vitro* conditions to convert them into EpiSC-like cells, which thus bypasses the use of embryos from genetically modified animals. The converted cells have been shown to display the gene expression profiles, biochemical properties and morphological features that are comparable to the conventional embryo-derived EpiSCs (Guo et al., 2009). However, it remains to be verified robustly if the converted ESCs, which have skipped the transition through the immediate postimplantaton development *in vivo*, are the true-to-form EpiSCs that possess the phenotypic properties related to the genetic modification.

Here we outline the methodology of isolating embryo-derived EpiSCs from post-implantation chimeras that are generated using genetically modified ESCs. The unique attributes of this procedure are (i) exploiting the versatility and efficacy of the genome editing technique (such as the clustered regularly interspaced short palindromic repeat-associated system, CRISPR-Cas editing) to create the desirable genetic modification in the ESCs that provides a lasting resource of experimental material, (ii) allowing the ESC-derived embryonic cells to partake in the process of embryogenesis through early post-implantation (and may thereby acquire the developmental genetic and epigenetic stamps) to the stage when they are conducive for the isolation of EpiSCs, (iii) fast-tracking the EpiSC production using chimeric embryos that are constituted predominantly by the edited ESCs, and (iv) enabling an independent assessment of the phenotype of the ESC-derived chimera to inform on the expected properties of the genetically modified EpiSCs in the context of lineage potency and differentiation.

2. The core protocol: Isolating EpiSCs from ESC-derived chimeric embryos

2.1. Generation of chimeric embryos from ESCs

2.1.1. Experimental strategy

ESCs are used to generate chimeric post-implantation embryos from which EpiSCs can be isolated from the epiblast / ectoderm through *in vitro* derivation. ESCs are introduced by micro-injection into early pre-implantation embryo to enable the descendants of ESCs to contribute to the chimera. Previous work has shown that a significant contribution of ESC-derived tissue in the embryo proper can be achieved when ESCs are introduced into host embryos at the eight cell stage (Kraus et al., 2010) or 4-cell stage tetraploid embryos generated by electrofusion of the blastomeres of 2-cell embryos (Murayama et al., 2015). In our hands, chimeras can be generated more efficiently when ESCs are introduced into zona-intact 8-cell stage diploid recipient embryos (Fossat et al., 2015), rather than into zona-free tetraploid recipient embryos produced by embryo aggregation or into tetraploid blastocysts by micro-injection (Nagy et al., 2003).

2.1.2. Procedure

Eight- cell diploid embryos are collected from superovulated ARC/s mice at E2.5, by flushing the uterus with M2 medium (Sigma, Cat# M7167). The embryos are transferred into a 100μl drop of KSOM medium (Merck Millipore, Cat# MR-020-5F) under oil (Sigma, Cat# M8410-500 ML, to prevent evaporation), and placed at 37°C, 5% CO_2. Batches of 10-12 embryos that have reached 8-cell stage are transferred into a 100 μL drop of M2 media containing ESCs, which is covered by mineral oil. Using Eppendorf transferman NK micro-manipulators (Eppendorf, Cat# 5178) fitted to an Olympus IX70 microscope, the cell transfer pipette is manoeuvred to position its tip next to a cluster of ESCs, and between ten to fourteen cells are drawn in a minimal amount of medium into the pipette. Using the micro-manipulators controlling the holding pipette, a recipient embryo is held against the polished tip of the pipette. Under 40x or higher magnification, the transfer pipette containing the ESCs is pushed through the zona with a sharp jab and the ESCs are deposited gently amongst the blastomeres of the embryo. Following the micro-injection, the recipient embryos are transferred into KSOM and incubated at 37°C and 5% CO_2 in air. Embryos are cultured overnight or until they develop into blastocysts. Embryos that are already hatching from the zona at the end of culture are excluded. Between ten to twelve recipient embryos are transferred surgically into the uterus of E2.5 pseudopregnant ARC/s mice (Nagy et al., 2003). Embryos are harvested four days after uterine transfer (at 6.5 days of pregnancy) for the derivation of EpiSCs.

2.2. Dissection of the epiblast from the chimeric embryo

2.2.1. Procedure

Pseudopregnant mice carrying the chimeric embryos are sacrificed by cervical dislocation. Decidua are dissected from the uterus and placed in PB1 medium (Rivera-Pérez et al., 2010). Embryos are carefully exposed from the decidua by inserting the closed tips of Dumont #5 CO forceps (F.S.T (Fine Science Tools), Cat# 11295-20) into the base of the deciduum and opening the forceps to tear the half-deciduum apart to reveal the embryo. Alternatively, the dissection of the deciduum to expose the embryo can be accomplished by cutting the deciduum into halves with iridectomy scissors (Chenoweth and Tesar, 2010; Strum and Tam, 1993). The embryo is then gently extracted from the deciduum by teasing away the surrounding tissues using fine forceps. The Reichert's membrane is removed by first pinning down the extraembryonic region with one set of forceps. Then, using another set of forceps, a nick in Reichert's membrane is made at a point next to the embryonic/extraembryonic region of the embryo, and grasped by these forceps. By moving the two forceps apart, the Reicherts membrane is torn open and retracted around the embryo (Strum and Tam, 1993). The now membrane-free embryonic region is cut away from the extraembryonic region by transecting with the forceps. The embryonic fragment is transferred into a 100 μL drop of TrypLE™ Select (1X) (Thermo Fisher Scientific, Cat# 12563-011) and allowed to stand for 1 min. The embryo is then gently triturated by repetitive pipetting to separate the endoderm and mesoderm from the epiblast. The isolated epiblast is rinsed successively through 3 drops of EpiSC basal medium (20% Knock-Out™ serum replacer (Thermo Fisher Scientific, Cat# 10828-028),