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Comparative analysis of mouse and human preimplantation development following POU5F1 CRISPR/Cas9 targeting reveals interspecies differences

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STUDY QUESTION: What is the role of POU class 5 homeobox 1 (*POU5F1*) in human preimplantation development and how does it compare with the mouse model?

SUMMARY ANSWER: POU5F1 is required for successful development of mouse and human embryos to the blastocyst stage as knockout embryos exhibited a significantly lower blastocyst formation rate, accompanied by lack of inner cell mass (ICM) formation.

WHAT IS KNOWN ALREADY: Clustered regularly interspaced short palindromic repeats—CRISPR associated genes (CRISPR-Cas9) has previously been used to examine the role of *POU5F1* during human preimplantation development. The reported *POU5F1*-targeted blastocysts always retained *POU5F1* expression in at least one cell, because of incomplete CRISPR-Cas9 editing. The question remains of whether the inability to obtain fully edited *POU5F1*-targeted blastocysts in human results from incomplete editing or the actual inability of these embryos to reach the blastocyst stage.

STUDY DESIGN, SIZE, DURATION: The efficiency of CRISPR-Cas9 to induce targeted gene mutations was first optimized in the mouse model. Two CRISPR-Cas9 delivery methods were compared in the B6D2F1 strain: S-phase injection (zygote stage) (n = 135) versus metaphase II-phase (M-phase) injection (oocyte stage) (n = 23). Four control groups were included: non-injected media-control zygotes (n = 43)/oocytes (n = 48); sham-injected zygotes (n = 45)/oocytes (n = 47); Cas9-protein injected zygotes (n = 23); and Cas9 protein and scrambled guide RNA (gRNA)-injected zygotes (n = 27). Immunofluorescence analysis was performed in *Pou5f1*-targeted zygotes (n = 37), media control zygotes (n = 19), and sham-injected zygotes (n = 15). To assess the capacity of Pou5f1-null embryos to develop further in vitro, additional groups of *Pou5f1*-targeted zygotes (n = 29) and media control zygotes (n = 30) were cultured to postimplantation stages (8.5 dpf). Aiming to identify differences in developmental capacity of *Pou5f1*-null embryos attributed to strain variation, zygotes from a second mouse strain—B6CBA (n = 52) were targeted. Overall, the optimized methodology was applied in human oocytes following IVM (metaphase II stage) (n = 101). The control group consisted of intracytoplasmically sperm injected (ICSI) IVM oocytes (n = 33). Immunofluorescence analysis was performed in human CRISPR-injected (n = 10) and media control (n = 9) human embryos.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A gRNA-Cas9 protein mixture targeting exon 2 of *Pou5f1*/*POU5F1* was microinjected in mouse oocytes/zygotes or human IVM oocytes. Reconstructed embryos were cultured for 4 days (mouse) or 6.5 days (human) in sequential culture media. An additional group of mouse-targeted zygotes was cultured to postimplantation stages. Embryonic

development was assessed daily, with detailed scoring at late blastocyst stage. Genomic editing was assessed by immunofluorescence analysis and next-generation sequencing.

MAIN RESULTS AND THE ROLE OF CHANCE: Genomic analysis in mouse revealed very high editing efficiencies with 95% of the S-Phase and 100% of the M-Phase embryos containing genetic modifications, of which 89.47% in the S-Phase and 84.21% in the M-Phase group were fully edited. The developmental capacity was significantly compromised as only 46.88% embryos in the S-Phase and 19.05% in the M-Phase group reached the blastocyst stage, compared to 86.36% in control M-Phase and 90.24% in control S-Phase groups, respectively. Immunofluorescence analysis confirmed the loss of *Pou5f1* expression and downregulation of the primitive marker SRY-Box transcription factor (*Sox17*). Our experiments confirmed the requirement of *Pou5f1* expression for blastocyst development in the second B6CBA strain. Altogether, our data obtained in mouse reveal that *Pou5f1* expression is essential for development to the blastocyst stage. M-Phase injection in human IVM oocytes (n = 101) similarly resulted in 88.37% of the POU5F1-targeted embryos being successfully edited. The developmental capacity of generated embryos was compromised from the eight-cell stage onwards. Only 4.55% of the microinjected embryos reached the late blastocyst stage and the embryos exhibited complete absence of ICM and an irregular trophectoderm cell layer. Loss of *POU5F1* expression resulted in absence of *SOX17* expression, as in mouse. Interestingly, genetic mosaicism was eliminated in a subset of targeted human embryos (9 out of 38), three of which developed into blastocysts.

LIMITATIONS, REASONS FOR CAUTION: One of the major hurdles of CRISPR-Cas9 germline genome editing is the occurrence of mosaicism, which may complicate phenotypic analysis and interpretation of developmental behavior of the injected embryos. Furthermore, in this study, spare IVM human oocytes were used, which may not recapitulate the developmental behavior of in vivo matured oocytes.

WIDER IMPLICATIONS OF THE FINDINGS: Comparison of developmental competency following CRISPR-Cas-mediated gene targeting in mouse and human may be influenced by the selected mouse strain. Gene targeting by CRISPR-Cas9 is subject to variable targeting efficiencies. Therefore, striving to reduce mosaicism can provide novel molecular insights into mouse and human embryogenesis.

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Introduction

Mammalian preimplantation development is directed by cellular mechanisms, which regulate gene expression and maintain the balance between pluripotency and differentiation, thus governing the specification of distinct cell lineages (Boyer *et al.*, 2005; Young, 2011). The mechanisms of lineage specification can, however, vary substantially between species (Niakan and Eggan, 2013). Newly emerged genome editing techniques, such as CRISPR (clustered regularly interspaced, short palindromic repeats)—Cas (CRISPR-associated genes), offer the ability to efficiently perturb gene expression, and functionally investigate embryogenesis-related genes.

One of the key genes regulating early embryonic development is POU class 5 homeobox 1 (*POU5F1*), encoding the homeodomain transcription factor OCT4, and was first identified in mice as an embryonic stem cell (ESC)-specific and germline-specific transcription factor (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Schöler *et al.*, 1990). *POU5F1* belongs to the POU family of transcription factors and consists of three domains: an N-terminal and C-terminal transactivation domain, exhibiting little sequence conservation between the OCT family members and a central, highly conserved, POU (Pit–Oct–Unc) homeodomain (Radzisheuskaya and Silva, 2014; Zeineddine *et al.*, 2014). Regions inside the POU homeodomain are critical for DNA binding and exhibit high affinity for an 8-bp DNA sequence motif (Octamer motif) ATGCAAAT, hence the members of the POU family are known as OCT proteins. In mouse, POU5F1 is a well-known key component of the pluripotency circuitry along with homeobox protein NANOG and SRY (sex-determining region Y)-box 2 (SOX2) (Chew *et al.*, 2005; Rodda *et al.*, 2005). Its reciprocal relationship with Caudal-type homeobox protein 2 (CDX2) is a key determinant of either self-renewal of ESC or their differentiation toward trophectoderm (TE) (Niwa *et al.*, 2005). Additionally, POU5F1 has been shown to work in tandem with SOX2 toward the regulation of expression of a large set of genes involved in self-renewal and pluripotency of ESCs (Chew *et al.*, 2005; Okumura-Nakanishi *et al.*, 2005; Rodda *et al.*, 2005). Evidence of its reprogramming ability was demonstrated by the fact that expression of *Pou5f1* together with *Sox2*, *Klf4*, and *C-myc*, successfully reprogrammed somatic cells back to a pluripotent stem cell state (Takahashi and Yamanaka, 2006). Forced expression of *Pou5f1* can restore pluripotency and rescue *Sox2*-null ESCs from differentiating, hence POU5F1 is often characterized as the genetic "master switch" in the establishment of pluripotency during the life cycle of mammals (Pesce *et al.*, 1999; Masui *et al.*, 2007).

The *Pou5f1*/*POU5F1* expression pattern in mouse and human embryos presents many similarities. In mouse, the maternal *Pou5f1* transcripts ensure protein expression at the zygote stage (Schöler *et al.*, 1989; Rosner *et al.*, 1990; Young Il Yeom *et al.*, 1991; Palmieri *et al.*, 1994). However, maternal *Pou5f1* mRNA levels drop significantly following fertilization and the OCT4 protein is degraded before the end of the two-cell stage (1.5 days post fertilization (dpf)). Mouse

embryonic *Pou5f1* expression is initiated at the eight-cell stage and its expression remains abundant in all cells until the morula stage (2.5 dpf). A proportion of the TE cells retain expression of *Pou5f1* at the early blastocyst stage (3.5 dpf) along with the POU5F1-positive inner cell mass (ICM). Eventually, *Pou5f1* expression gets restricted to the ICM in the late blastocyst (4.5 dpf). Zygotic knockout studies in mice have demonstrated that in POU5F1-deficient mouse models, embryos can reach the blastocyst stage with absent ICM and when embryos were plated in ESC derivation conditions, they failed to generate ICM outgrowths (Nichols *et al.*, 1998; Kim *et al.*, 2002; Foygel *et al.*, 2008; Frum *et al.*, 2013; Wu *et al.*, 2013; Fukuda *et al.*, 2016; Fogarty *et al.*, 2017a).

In human, *POU5F1* expression is initiated at the four to eight cell stage (2.5–3 dpf), consistent with embryonic genome activation (EGA) and remains abundant and uniform in all cells throughout the morula stage (3.5–4 dpf), when the compaction and first lineage specification events occur. *POU5F1* expression remains persistent in the ICM and a subset of TE cells of the blastocyst (5–6 dpf), until it finally becomes restricted to the cells of the ICM in the late blastocyst stage (6.5 dpf) (Niakan and Eggan, 2013). Two distinct layers arise from the ICM: the epiblast, which will give rise to the embryo proper, and the primitive endoderm (PE) or hypoblast, which will form the extraembryonic lineages. At this stage, a POU5F1-positive population can be identified only in the epiblast, consistent with the role of POU5F1 in maintenance of pluripotency in stem cells.

Only recently, with the advent of endonuclease-mediated CRISPR-Cas genome editing technology and its accompanied high editing efficiency, have gene knockout studies become feasible in the human preimplantation embryos. Fogarty and colleagues demonstrated that high *POU5F1* editing efficiency can be achieved in human embryos (Fogarty *et al.*, 2017a). However, following human zygote (S-phase) injection, all blastocysts generated were mosaic, containing at least one cell still expressing POU5F1. Therefore, the question remains whether zygote-stage injection induced the genetic mosaicism and therefore prevented the generation of human *POU5F1*-null blastocysts, or their absence could be explained by the inability of *POU5F1*-null embryos to reach the blastocyst stage.

A strategy to eliminate mosaicism is CRISPR-Cas component delivery, concurrent with intracytoplasmically sperm injected (ICSI) in metaphase II (MII) oocytes, which has been successfully applied by Ma and colleagues for gene correction in human embryos (Ma *et al.*, 2017). Earlier introduction of CRISPR components is expected to facilitate editing prior to the first cell division and eventually reduce or even eliminate mosaicism (Lamas-Toranzo *et al.*, 2019). The cell-endogenous nonhomologous end joining pathway, triggered upon CRISPR-Cas-induced double-strand break (DSB) formation, and responsible for the introduction of indel mutations during DSB repair attempts, has been shown to be active during the early cell cycle phases (Iliakis *et al.*, 2004).

Here we aim to identify the most efficient method of CRISPR-Cas9 introduction yielding the highest editing and mutagenesis efficiencies in the mouse and human germline. Furthermore, we aim to apply the optimized method in order to eliminate mosaicism and reliably further unravel the role of POU5F1 in early development, with a specific focus on interspecies differences and blastocyst formation competency.

Materials and methods

Ethical approval

Mouse animal studies were approved by Animal Ethics Committee of Ghent University Hospital (ECD 18-29). B6D2F1/B6CBAF1 male and female mice were obtained from Charles River Laboratories, Brussels, Belgium. The use of human oocytes, sperm and embryos was approved by Ghent University Hospital Ethical Committee (EC UZG 2018/0908) and the Belgian Federal Commission for medical scientific research on human embryos (FCE-ADV_077_UZ Gent). All used human oocytes, sperm, and embryos used in this study were obtained with signed informed consent.

Follicular stimulation for mouse oocyte and zygote collection

Female B6D2F1 or B6CBAF1 mice (Charles River Laboratories, Belgium) between 6 and 12 weeks old underwent ovarian stimulation, induced by injection (i.p.) of 5 IU pregnant mare's serum gonadotrophin (HOR-272, Boxmeer, Netherlands), followed by 5 IU hCG (CG10-1VL, Sigma-Aldrich, Belgium) 48 h later. Following hCG injection, female mice were allowed natural mating overnight with a fertile male mouse (of the same strain). Zygote collection was carried out 18–19 h after hCG injection. For oocyte collection, MII mouse oocytes were collected 12–14 h following hCG injection. The mice were humanely killed by cervical dislocation; the oocyte/zygote cumulus complexes were removed from the ampulla of the oviduct and were treated in 200 IU/mL hyaluronidase (H3506, Sigma-Aldrich, Belgium) at 37 °C to remove the cumulus cells. Oocytes/zygotes were then washed several times in synthetic oviductal medium enriched with potassium (KSOM) (MR-101, Sigma-Aldrich, Belgium) and transferred to drops of KSOM supplemented with 0.4% bovine serum albumin (BSA) (12657, Millipore, France).

Human oocyte collection

Immature oocytes donated from patients undergoing fertility treatment at the Department for Reproductive Medicine, Ghent University Hospital were subjected to IVM. Oocytes retrieved at prophase I—germinal vesicle stage (GV) was cultured for 24 h in Medicult® media (82214010, Cooper Surgical, Netherlands). Metaphase I (MI) oocytes were cultured for either 3 h or 24 h in Cook Cleavage® medium (G46302, Cook Ireland Ltd, Ireland). Successfully matured oocytes were either used for control ICSI or ICSI-CRISPR.

Sperm preparation

Frozen-thawed sperm samples from patients, and B6D2F1 and B6CBA male mice (10–15 weeks) were used for ICSI. Sperm straws were removed from the liquid nitrogen tank and were placed in room temperature for 10 min to allow thawing. For each straw, both ends were cut to allow removal of all sperm into a tube containing gamete buffer (GB) (G46320, Cook Ireland Ltd, Ireland). Thawed sperm was subjected to washing, consisting of centrifugation (300 g, 10 min) followed by the removal of supernatant. Fresh GB was added carefully not to disrupt the pellet. Sperm was allowed to swim up at 37 °C for 1 h.

Following the incubation period, the supernatant containing the high-quality motile sperm was selected and used for ICSI.

Oocyte/zygote microinjection

Mouse oocyte/zygote microinjection was performed using a piezo-driven injection technique. Mouse sperm was decapitated in a drop containing polyvinylpyrrolidone (PVP) (ART-4005-A, Cooper Surgical, Netherlands). The sperm head was aspirated and was placed in a drop containing gRNA-Cas9 ribonucleoprotein (RNP) complexes. An amount equal to the diameter of the egg was aspirated and injected along with the sperm into the MII oocyte. In the S-phase zygote microinjection, two pronuclei (2PN) zygotes were injected only with RNP complexes in similar amounts as previously mentioned. Human sperm was immobilized in a drop containing PVP and was microinjected into MII IVM oocytes in GB.

gRNA sequence and RNP preparation

Mouse CRISPR RNA (crRNA) from Integrated DNA technologies (IDT) (Belgium), (5′-ACCCACCAAAGAGAACG-CCC-3′—mm10 chr17:35,509,207–35,509,229) was resuspended in duplex buffer (1072547, IDT, Belgium) and mixed in a 1:1 molar ratio with tracrRNA (1072532, IDT, Belgium). The mixture was heated to 95 °C for 5 min and allowed to cool at room temperature to allow crRNA-tracrRNA duplex formation. The gRNA mixture was further mixed with Cas9 protein (1072545, IDT, Belgium) in a 1:1 molar ratio. The RNP was diluted with Optimem media (31985-62, Sigma-Aldrich, Belgium) to a final concentration of 25 ng/µL. The human crRNA (5′-ACCCACCAAATAGAACCCCC-3′ – hg38 chr6:31,165,921–31,165,943) was processed in the same way. The crRNA sequences have been adopted from the study Fogarty et al. (2017a) in which that have been thoroughly validated in vitro for high editing efficiencies and negligible off-target effects.

Mouse/human embryo culture

Mouse embryos were cultured in KSOM media supplemented with 4% BSA until the eight-cell stage. Then, the media was replaced with Cook Blasto® until 4.5 dpf. Human embryos were cultured in Cook Cleavage® (G46302, Cook Ireland Ltd, Ireland) followed by a media change to Cook Blasto® (G46296, Cook Ireland Ltd, Ireland) at the eight-cell stage. Culture conditions were set to 37°C, 5% O_2, and 6% CO_2 in all cases.

Mouse/human embryo DNA extraction and genotyping

DNA from mouse and human embryos was extracted using Arcturus picopure™ DNA extraction kit (KIT0103, Life Technologies, Belgium). Single embryos were transferred to a PCR tube and 10 µL of Proteinase K (15 µg/µL) were added. The tube was heated to 65 °C for 4 h and then 95 °C for 10 min to inactivate Proteinase K. Extracted DNA was stored in −20°C until further PCR analysis. Primer pair (5′–3′) 1 (fw: GAACAGTTTGCCAAGCTGCT- rv: CCCCACCTCT GACAGTTCAA) and primer pair 2 (fw: AGGGGAGATTGATAACT GGTGT, rv: ACTAGGTTCAGGGATACTCCTTAG) were used for the amplification of the targeted regions in mouse and human, respectively. The primers have been validated for their target specificity by Fogarty et al. (2017a). Prepared PCR samples were amplified under following PCR conditions: 5 min initial denaturation at 95 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final elongation at 72 °C for 10 min. PCR products were subsequently loaded onto a fragment analyzer and analyzed using the PROsize 3.0 software (Advanced Analytical Technologies, Ankeny, IA, USA). PCR products were subjected to next-generation sequencing (NGS) (Illumina MiSeq) and the results were analyzed by the bioinformatics tool Batch-GE (Boel *et al.*, 2016).

Immunocytochemistry and imaging

Embryos were fixed in 4% paraformaldehyde (P6148, Sigma, Belgium) for 30 min and then washed in PBS (AM9624, Fischer Scientific, Belgium) solution containing 0.1% Triton-X (T8787, Sigma, Belgium) (PBT 0.1%). The cells were further permeabilized in PBS containing 0.5% Triton-X. Finally, the cells were washed in PBS and placed in blocking buffer consisting of PBT 0.1%, Triton X/10%, and fetal bovine serum (10270106, Life Technologies, Belgium) for 1 h. Embryos were incubated overnight at 4 °C with primary antibodies. The antibodies are listed in Supplementary Table SI. After washing three times in blocking buffer, samples were treated with secondary fluorescent antibodies for 1 h at 37 °C, followed by extended washing in the washing/blocking solution. Chromosomes were stained with 20 µg/mL Hoechst-33258 for 30 min. Finally, samples were imaged using a laser scanning confocal microscope (Leica SP8, Leica, Belgium).

Postimplantation

Initial culture was performed in the medium IVC1 containing fetal calf serum, which is important to trigger TE differentiation in giant cells that adhere to the plate. On 6.5 dpc, following confirmation of embryo attachment, the medium was exchanged to IVC2 to support the growth of the emerging egg cylinder. IVC1 and IVC2 media were supplemented with the ovarian steroids progesterone and β-estradiol, which are crucial for implantation in mice and can prolong the implantation time window. The composition of the IVC1 and IVC2 media was prepared as previously described (Bedzhov *et al.*, 2014).

Statistics

All statistical analyses were performed using Graphpad Prism for Mac OS ver. 8.4.0 (455), Graphpad Software Inc, USA. A chi-squared test was performed (significance level was 0.05 p value) to compare the developmental competency among different groups and between the mutational spectrums. A parametric unpaired Student's t-test was used to compare the editing efficiencies, mutagenesis efficiencies, and percentage of attached embryos.

Results

Pou5f1 loss compromises mouse pre- and postimplantation development

To target *Pou5f1*, we used a published gRNA sequence (Fig. 1A), shown to exhibit high editing efficiency and no *in-silico*-predicted

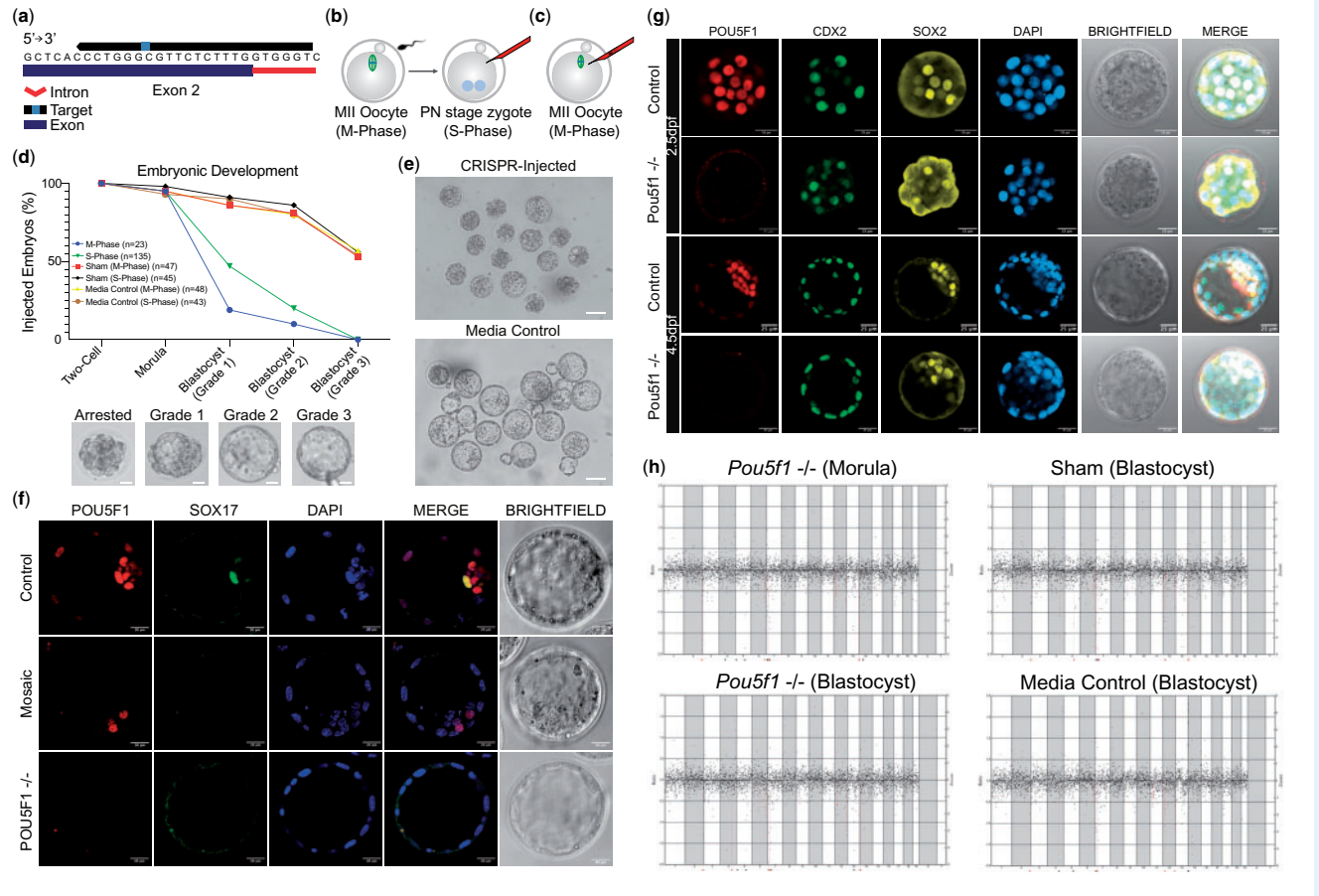

Figure 1. Targeting *Pou5f1* in mouse preimplantation embryos. (**A**) Schematic representation of the mouse POU class 5 homeobox 1 (*Pou5f1*) locus and the single guide RNA (sgRNA) targeted region (**B**) Synthesis Phase (S-Phase) microinjection: Oocytes are fertilized *in vivo*. 19-21h following intra-peritoneal injection (i.p.) of human chorionic gonadotropin (hCG), zygotes are harvested and microinjected with the Clustered regularly interspaced short palindromic repeats—CRISPR-associated genes (CRISPR-Cas9) ribonucleoprotein (RNP) complex. (**C**) Metaphase II (M-Phase) microinjection. Harvested MII-oocytes 14-16h following i.p. of (hCG) are intracytoplasmically sperm injected (ICSI). (**D**) Detailed development kinetics based on morphological criteria of M-Phase (n = 23), S-Phase (n = 135), Sham M-Phase (n = 47), Sham S-Phase (n = 45), Media Control M-Phase (n = 48) and Media Control S-Phase (n = 43) groups along with representative image phenotypes from arrested, Grade 1, Grade 2 and Grade 3 embryos. Grade 1: Small blastocyst with blastocoel equal to or less than half of the embryo volume. Grade 2: Large blastocyst with blastocoel greater than half of the embryo volume or blastocyst with a blastocoel cavity completely filling the embryo Grade3: Expanded and hatching or completely hatched blastocyst. Chi-square test revealed no significant difference between the M-Phase and S-Phase Group ($P = 0.2994$). Both S-Phase and M-Phase CRISPR injected groups presented significant differences with the respective control groups ($P < 0.05$). (**E**) (Top) Brightfield image of CRISPR-microinjected S-Phase embryos on 4.5 days post fertilization (dpf). (Bottom) Brightfield image of Control non-manipulated embryos. (**F**) Immunofluorescence analysis for POU5F1 (Red), SRY-Box transcription factor (SOX17) (Green), and DAPI (Blue) at 4.5dpf. All arrested, Grade 1, Grade 2 and Grade 3, CRISPR-microinjected embryos exhibited complete absence of POU5F1 expression. Only one grade 3 mosaic embryo was detected with reduced POU5F1 expression compared to control. The photos are results of a confocal z-section, scale bar 20 µm. (**G**) Immunofluorescence analysis for POU5F1 (Red), Caudal-type homeobox protein 2 (CDX2) (Green), SRY (sex-determining region Y)-box 2 (SOX2) (Yellow) and DAPI (Blue) at 2.5dpf (morula stage) and 4.5dpf (blastocyst stage) for CRISPR-Cas9 Injected (n = 23) and control embryos (n = 17). All Pou5f1−/− embryos retained expression of trophectoderm marker Cdx2 and lineage specifier Sox2. (**H**) Representative line views following shallow whole genome karyotyping in CRISRP-Cas9 injected (n = 10), sham injected (n = 5) and media control embryos (n = 5). In the CRISPR-Cas9 group, lineviews from an arrested morula and grade 2 blastocysts are presented. In the control group both line views are generated by analyzing grade 3 blastocysts.

off-target effects (Fogarty *et al.*, 2017a,b). The targeted site belongs to the conserved DNA-binding POU homeodomain (exon 2), which is expressed in all transcripts of *Pou5f1* in mouse. Disruption of the POU domain is expected to result in a nonfunctional protein. Preassembled RNPs, consisting of Cas9 nuclease and the gRNA, were initially microinjected into pronuclear (PN) stage (S-phase) zygotes (Fig. 1B). In addition, four control groups were included: non-injected media-control zygotes; sham-injected zygotes; Cas9 protein-injected zygotes;

and zygotes injected with Cas9 protein and a scrambled gRNA, which does not target a specific sequence in the mouse genome (Supplementary Table SII). An additional group of MII oocytes was microinjected with CRISPR-Cas components concurrent with ICSI (Fig. 1C).

Following culture until 4.5 dpf, DNA from single embryos was subjected to NGS. NGS analysis revealed very high editing efficiencies (% edited embryos). In the S-Phase group, 95% of the embryos (n = 19 out of 20) were CRISPR-edited compared to 100% (n = 19 out of 19) in the M-Phase Group (Supplementary Fig. S1A). From the edited embryos, the mutagenesis efficiency (% edited alleles) was 98.50% for the S-Phase group and 97.53% for the M-Phase group (Supplementary Fig. S1D); 89.47% of the embryos (17 out of 19) in the S-Phase group and 84.21% (16 out of 19) in the M-Phase group were non-mosaic, containing only edited alleles (no WT) (Supplementary Fig. S1B; Tables SIII and SIV). In the non-mosaic group, 70.59% (12 out of 17) in the S-Phase and 75% (12 out of 16) carried frameshift mutations (Supplementary Fig. S1C; Tables SIII and SIV). For both the M-Phase and S-Phase groups, a small number of edited embryos contained up to four different mutant alleles, showcasing that editing following the DNA replication in the S-phase did also occur (Supplementary Fig. S1E; Tables SIII and SIV). A stereotypic pattern of the induced indels was observed, with a 28 bp deletion present in 20.5% of the NGS analyzed embryos (Supplementary Table SV).

Successful interruption of *Pou5f1* expression in CRISPR-Cas microinjected mouse embryos revealed that POU5F1 is required for efficient development to the blastocyst stage. Only 19.05% of M-Phase and 46.88% of S-Phase CRISPR microinjected embryos reached the blastocyst stage (grade 1), in contrast to the developmental rate of control groups, which exceeded 86% (Fig. 1D; Supplementary Table SVI). The majority of CRISPR microinjected embryos exhibited functional defects. Blastocysts had no apparent ICM, accompanied by failure of the blastocoel to expand and hatch (Fig. 1E). No statistically significant difference ($P > 0.05$, Chi-squared test) was observed in the developmental rates between the control groups, suggesting that neither CRISPR component microinjection nor the mechanical procedure itself, had adverse effects on embryo development (Supplementary Table SII).

To investigate if developmental arrest is associated with unwanted large chromosomal deletions mediated by CRISPR-Cas9 genome editing or the microinjection procedure itself, we performed shallow whole genome sequencing for molecular karyotyping in CRISPR-injected (n = 10), sham (n = 5), and media control (n = 5). All embryos appeared normal and representative line views from each group are shown (Fig. 1H).

A total number of 71 mouse embryos were immunostained for POU5F1 and SOX17 (Fig. 1F; Supplementary Table SVII). As it has been previously shown that *Pou5f1*-null mouse blastocysts lack the expression of PE marker *Sox17*, the absence of SOX17 was used as a secondary marker to identify *Pou5f1*-null embryos (Frum *et al.*, 2013; Le Bin *et al.*, 2014). Immunofluorescence analysis of CRISPR-injected embryos, arrested in grade 1 and grade 2 blastocysts, confirmed the complete loss of POU5F1 in 97.29% of the embryos (36 out of 37), with the exception of one embryo being mosaic and formed a grade 2 blastocyst (Fig. 1F; Supplementary Table SVII). Expression of PE marker *Sox17* was absent in all POU5F1-depleted embryos (Fig. 1F).

To further examine if the TE lineage is affected and also to provide a more extensive molecular characterization, we immunostained for the TE marker CDX2 and ICM lineage specifier SOX2, CRISPR-Cas9 microinjected (n = 23) and control embryos (n = 17) (Fig. 1G), in two distinct developmental stages, morula (initiation of *Sox2* expression) and blastocyst (restriction of *Cdx2* in the TE). All embryos retained positive expression of CDX2 and SOX2 following successful targeting.

To assess if *Pou5f1*-null blastocysts retained the capacity to further develop in vitro, we applied a protocol supporting the development of the mouse embryo beyond the blastocyst stage, extending the in vitro culture period to 8.5 dpf, which corresponds to 6.5 dpc in vivo (Bedzhov *et al.*, 2014). S-Phase CRISPR-injected zygotes (n = 29) and control (n = 30) embryos were cultured in vitro until 8.5 dpf. Extended embryo culture revealed that *Pou5f1*-null embryos showed outgrowths containing only trophoblast giant cells (Fig. 2A). Immunofluorescence analysis of these outgrowths, confirmed the absence of POU5F1 and CDX2 expression, but identified positive expression of GATA binding protein 3 (GATA3) (Fig. 2B, Supplementary Fig. S2). Although 69% (20 out of 29) of CRISPR-injected embryos were attached on 5.5 dpf compared to 26.7% (8 out of 30) in the control (Supplementary Fig. S1F), by 6.5dpf, embryo attachment was 100% for both the CRISPR injected and control groups.

Influence of mouse strain background on the developmental effect of Pou5f1 targeted embryos

Next, we hypothesized that mouse strain variation could influence the induced *Pou5f1*-null phenotype, complicating the direct comparison of mouse and human interspecies differences. We directly applied our *Pou5f1*-targeting methodology optimized in the B6D2F1 mouse strain also in the B6CBAF1 mouse strain, a cross between female C57BL/6J and male CBA/J, which from now on will be referred to as the B6CBA strain (Lamas-Toranzo *et al.*, 2019). In total, n = 52 S-phase B6CBA zygotes were microinjected with CRISPR components. Fifty 4.5 dpf embryos were analyzed revealing an editing efficiency of 100% (50 out of 50 embryos edited) and mutagenesis efficiency 92.61% (% alleles edited) (Fig. 3A, B). Seventy-two percent (36 out 50) of the CRISPR-injected embryos exhibited only mutant alleles (no WT) (Supplementary Table SVIII). Interestingly, blastocyst development rates across grades 1–3 were significantly higher in the B6CBA group compared to B6D2 ($P = 0.0001$), as 83% of the embryos reached the blastocyst stage (grades 1–3), compared to 46.9% for the B6D2F1 strain (S-Phase). Overall, B6CBA development rates were similar to the control group ($P = 0.2671$) (Fig. 3C; Supplementary Table SVI). Our results suggest that strain background has a significant effect on the onset of developmental arrest and overall developmental potential in mouse embryos following *Pou5f1* targeting.

Targeting POU5F1 in human preimplantation embryos

Donated GV or MI oocytes, which are normally discarded from patients undergoing ICSI/IVF treatment, were cultured in vitro to allow maturation to the MII stage (M-Phase). Concurrent with ICSI, the oocytes were microinjected with CRISPR-Cas components targeting *POU5F1* exon 2, similar to the strategy used for mouse embryos. The

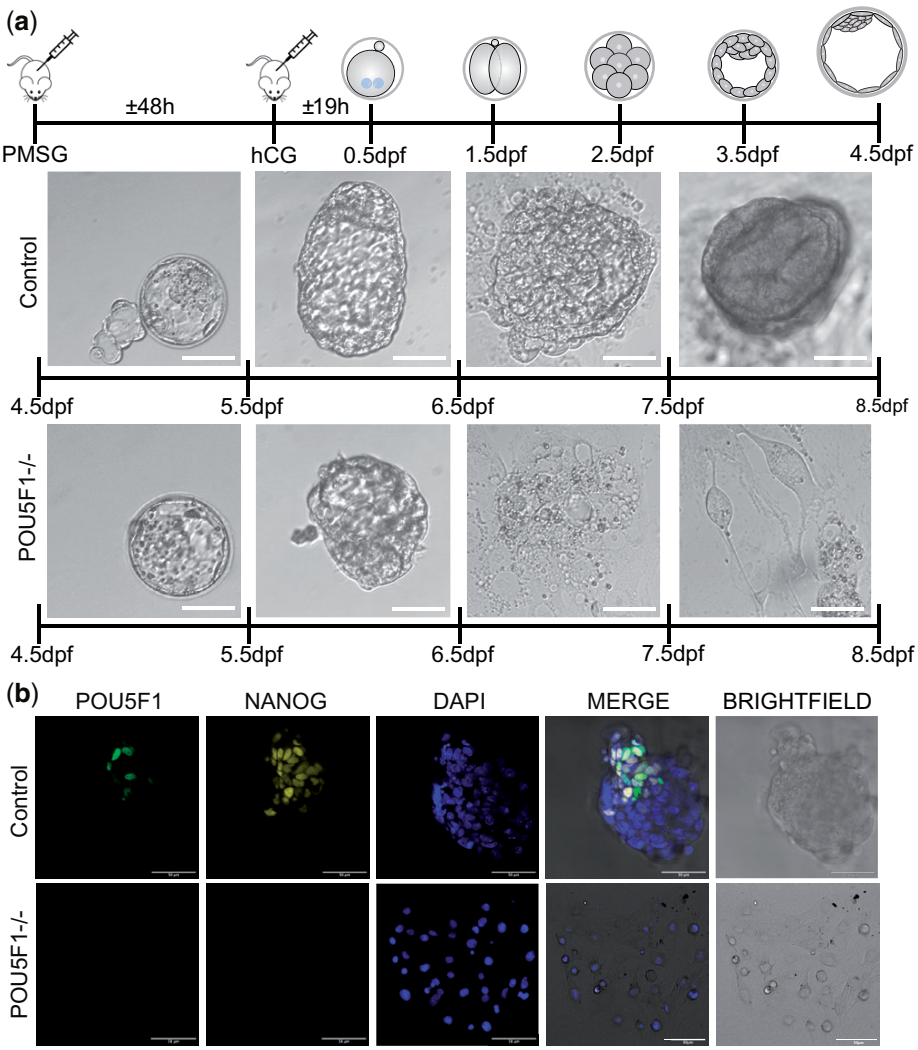

Figure 2. Evaluation of post-implantation developmental potential in *Pou5f1*-null and control mouse embryos. (**A**) *Pou5f1*-null embryos, when plated, outgrew trophoblast giant cells, confirming the *in vitro* requirement of POU5F1 in self-renewal of post-implantation epiblast. Control embryos successfully developed and formed an egg cylinder with an efficiency of 10–15%. (**B**) Immunofluorescence analysis for POU5F1 (green), homeobox protein NANOG (yellow) and DAPI (blue) nuclear staining following extended mouse embryo culture (8.5dpf) of *POU5F1*-null and Control embryos. The photos are results of a confocal z-section, scale bar 50 μm.

gRNA has been previously described as exhibiting high on-target mutagenesis efficiency without generating detectable levels of off-target mutations (Fogarty *et al.*, 2017a,b). ICSI-CRISPR was performed in n = 101 MII oocytes resulting from IVM. The control group consisted of ICSI-injected IVM oocytes (n = 33). Following embryo culture until 6.5 dpf, NGS was performed on 43 embryos and the calculated editing efficiency was 88.37%, as 38 out of 43 embryos were successfully edited. The average mutagenesis efficiency was 72.83%. More specifically, 76.31% (29 out of 38) of the edited generated embryos displayed mosaic editing patterns, containing both CRISPR-edited and WT alleles. The remaining nine embryos exhibited only CRISPR-edited alleles (no WT) (Supplementary Table SIX). Three out of these nine *POU5F1*-null embryos reached the blastocyst stage with absent ICM and irregular TE cells (Grade 3CC–4CC) (Fig. 4B). The overall developmental capacity of *POU5F1*-targeted embryos was significantly compromised beginning from the eight-cell stage, coincident with EGA. Only 12.12% of the embryos reached the eight-cell stage compared with 33.33% of the ICSI-control group (Fig. 4A, Supplementary Table SX). Furthermore, only 4.55% of the microinjected embryos reached the late blastocyst stage compared to 23.81% in the control group (Fig. 4A). All 6.5 dpf edited blastocysts exhibited complete absence of the ICM, displaying an irregular TE cell layer, with a portion of the cells being grainy (Fig. 4B). Furthermore, generated blastocysts had retarded ability to expand and hatch. Overall, no adverse effects in development related to culture conditions or microinjection procedure were observed. Immunofluorescence analysis in the edited blastocysts

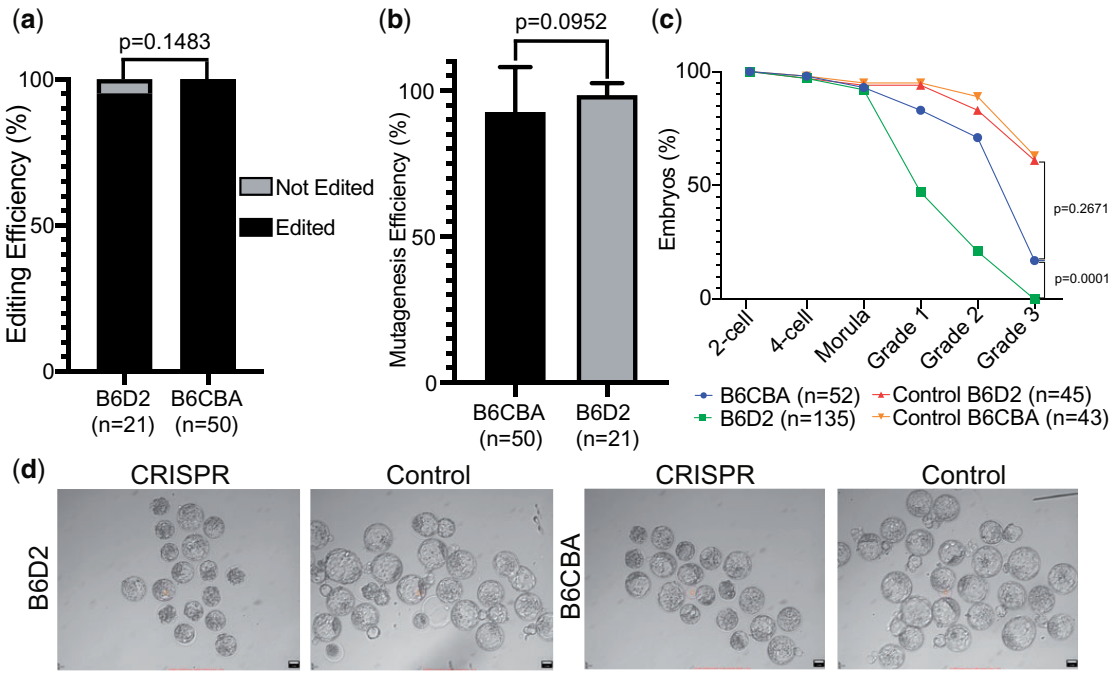

Figure 3. Identification of mouse interstrain differences following *Pou5f1* targeting. (**A**) Editing efficiency comparison of the S-phase microinjected group between B6D2 (left) and B6CBA (right) strain, assessed by next-generation sequencing (NGS). A parametric unpaired Student's *t*-test revealed no significant difference ($P = 0.1483$). (**B**) Mutagenesis efficiency estimated by Batch-GE bioinformatic analysis following NGS in the B6D2 (n = 21) versus the B6CBA (n = 52) group. A parametric unpaired Student's *t*-test revealed no significant difference between the two groups ($P = 0.0952$). (**C**) Development kinetics based on morphological criteria of B6CBA (n = 52), B6D2 (n = 135), Sham S-Phase (n = 45), Media Control S-Phase (n = 43). Blastocyst development rates across grade 1–3 significantly differed between the B6D2 and B6CBA strain ($P = 0.0001$). Comparison of blastocyst development rates between the B6CBA and control group revealed no significant differences ($P = 0.2671$). Details on the development rates are provided in Supplementary Table SVI. (**D**) Representative phenotypes of 4.5 dpf CRISPR-microinjected or control embryos in the B6D2 and B6CBA strain.

confirmed successful interruption of the *POU5F1* locus. In total, immunofluorescence analysis was performed in 10 CRISPR-injected embryos, from which eight were arrested at the morula stage and displayed complete absence of POU5F1 expression. Only two embryos reached the blastocyst stage, which retained positive expression of POU5F1 in one or two cells respectively (Fig. 4C).

Discussion

CRISPR-Cas9-mediated genome editing has proven to be an invaluable tool for the functional study of genes involved in pre- and postimplantation development owing to its ability to edit the genome with very high efficiency. In this study, CRISPR-Cas9 was applied in both the mouse and human germline, targeting *Pou5f1*/*POU5F1*, with the aim to elucidate the biological relevance in human embryo development, which is centered around the regulation of pluripotency and blastocyst formation and further compare it with mouse toward the identification of interspecies differences. Although *Pou5f1*/*POU5F1* is consistently expressed during EGA in both species, suggesting conserved roles during preimplantation development, a major species difference has been identified. We demonstrated that POU5F1 has an earlier role in human, as developmental arrest was observed earlier compared to mouse, indicating distinct mechanisms of lineage specification between the species with implications on the role in the blastocyst formation. During the stages of methodology optimization, it was hypothesized that earlier introduction of CRISPR-Cas9 components, prior to DNA replication occurring during the S-phase of the zygotic cell cycle, would increase editing efficiencies and minimize mosaicism. However, when comparing S-phase versus M-phase stage CRISPR-Cas9 introduction in mouse, we noted similar mutagenesis efficiencies and mosaicism rates in both delivery methods. This is, however, in contrast to earlier findings in human, where earlier administration in the M-phase significantly improved editing rates compared to S-phase administration, at least when performing gene correction (Ma *et al.*, 2017). The explanation for these findings is the high mutagenesis efficiencies (97.53% for the M-Phase and 98.50% for the S-Phase), which left very narrow margins for the identification of significant improvements. Additional comparisons between M- and S-phase injections using less-efficient CRISPR-Cas components directed to different target sites, both in mouse and human, could provide further clarification. Another possibility for the higher effectiveness of the earlier CRISPR-Cas9 introduction in human

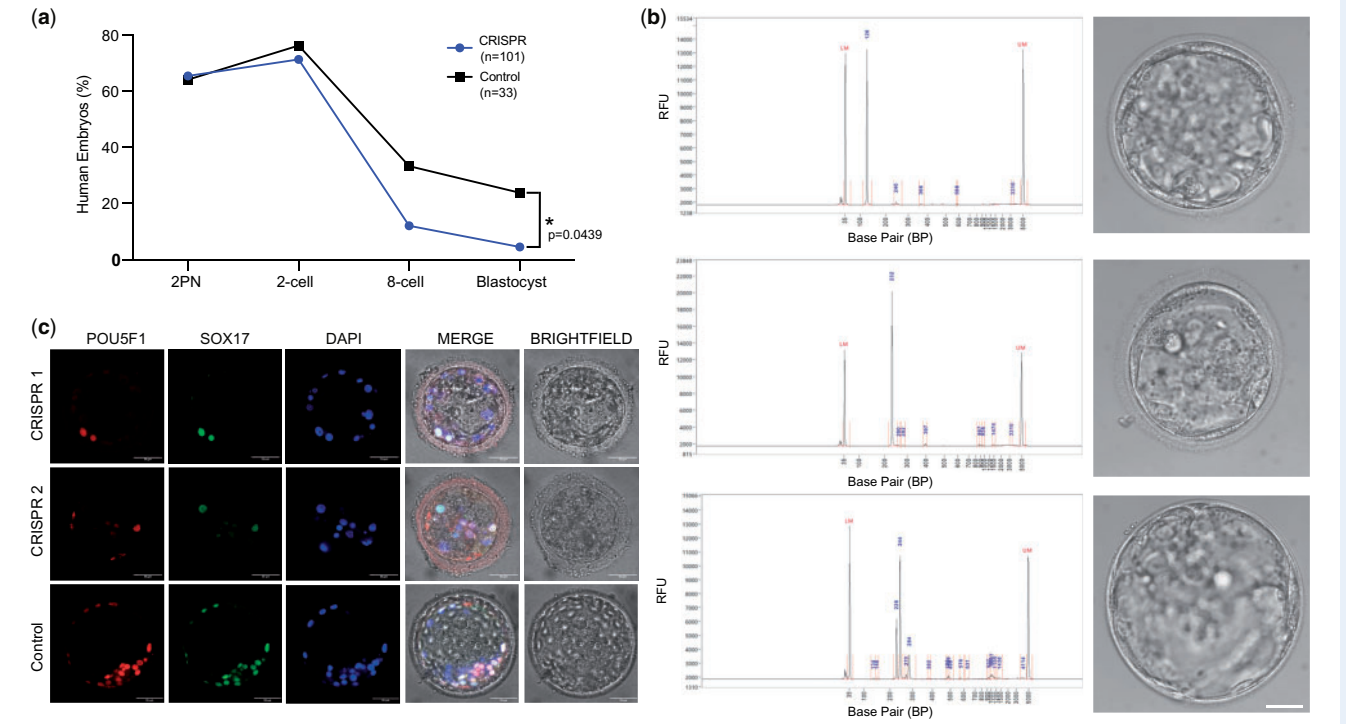

Figure 4. Developmental potential of human embryos following CRISPR-Cas9-mediated genome editing. (**A**) Developmental comparison of ICSI-CRISPR-generated human embryos against control embryos. *POU5F1* edited embryos exhibited increased developmental arrest starting from eight-cell stage. Chi-squared test ($P = 0.0439$) revealed a significant difference between the developmental rates in CRISPR-ICSI ($n = 101$) and control embryos ($n = 33$). (**B**) Brightfield images of the generated *POU5F1*-null human blastocysts and the corresponding fragment analysis results, confirming the editing of the targeted locus. (**C**) Immunofluorescence analysis for POU5F1 (Red), SOX17 (Green), and DAPI (Blue) at 6.5 dpf.

might be the presence of distinct molecular repair mechanisms present between the species. The cell repair machinery in mouse and human has not been fully elucidated and we only assume that it is similar due to the evolutionary distance between the two species. Nevertheless, due to the extremely high potency of the CRISPR-Cas9 components used, targeting mouse *Pou5f1* has been achieved with very high efficiently and disruption of the *Pou5f1* locus resulted in significantly compromised development toward the blastocyst stage in the B6D2F1 strain. Immunofluorescence analysis confirmed the loss of POU5F1 and also the loss of expression of the PE marker SOX17 due to the cell-autonomous requirement for fibroblast growth factor 4 (FGF4) and mitogen-activated protein kinase (MAPK) signaling (Frum *et al.*, 2013; Le Bin *et al.*, 2014). CRISPR-Cas9 genome editing may lead to the introduction of frameshift mutations in the open reading frame of the target gene, which truncates the coding sequence, and the corresponding transcript is targeted for degradation by nonsense-mediated mRNA decay, prior to reaching the cell translational machinery. In this regard, no end product protein is detected by immunofluorescence analysis, confirming the successful interruption of the locus by immunostaining. Additionally, in mouse we showed that the expression of the TE marker *Cdx2* remains unaffected, pointing to a functional TE lineage and explaining the formation of the blastocyst during the preimplantation stages. Differentiation of the TE lineage in mouse is initiated during transition from the 8- to 16-cell morula stage, when the inner and outer cells are formed. Components downstream of the Hippo signaling pathway interact with the transcription factor TEAD4 and initiate transcription of the TE lineage specifier *Cdx2* (Niwa *et al.*, 2005; Yagi *et al.*, 2007). The expression of *Pou5f1* during EGA, and its reciprocal inhibition with Cdx2, it is believed to act as a barrier, destined to keep the two-cell populations (TE versus ICM) in distinct developmental programs. Expression of the pluripotency circuitry member Sox2 also remained unaffected until 4.5 dpc.

Use of the postimplantation model offered an additional method to evaluate the capacity of *Pou5f1*-null embryos to further develop (Bedzhov *et al.*, 2014). Plated mouse embryos gave rise only to characteristic giant TE cells indicating that ablation of POU5F1 resulted in loss of pluripotency in the ICM and restricted differentiation towards the TE lineage. Surprisingly, plated *Pou5f1*-null embryos attached 1 day earlier. These findings are in line with previous data obtained from stem cells studies in which *Pou5f1* RNA interference induced TE differentiation in mouse ESC (Velkey and O'Shea, 2003). Furthermore, in a study by Niwa and colleagues, repression of *Pou5f1* in mouse ESCs, induced loss of pluripotency and differentiation to TE (Niwa *et al.*, 2005). Immunostaining of the resulting outgrowths confirmed absence of the CDX2-positive population identified in the control group, although the formation of TE giant cells, which stained positive for

GATA3, following TE differentiation remained unaffected, suggesting that *Pou5f1* expression is required for successful TE lineage segregation.

The observed difference in the developmental competency and phenotype of 4.5 dpf *Pou5f1*-targeted mouse embryos compared to previous studies led us to investigate possible effects related to strain differences (Nichols *et al.*, 1998; Fogarty *et al.*, 2017a,b). The development competency of the injected B6CBA strain was similar to the control B6CBA group, in line with a previous report in Pou5f1-null embryos (Nichols et al., 1998).

The significance of these results lies in the fact that strain selection is important when performing functional developmental studies and ideally more than one strain should be examined in every scenario. The presence of interstrain differences points towards the presence of alternative mechanisms orchestrating developmental processes, worthy of investigation.

We next proceeded to evaluate the requirement for POU5F1 in human embryo development. M-Phase microinjection was applied in donated human oocytes obtained by IVM. One caveat of using oocytes for CRISPR/ICSI is that it requires holding a license to create human embryos for research. In some countries it is only permitted to utilize spare zygotes or embryos donated for research. The legislation in Belgium allows the use of human embryo creation for research purposes.

POU5F1 deletion had a stronger impact in human embryo development, as developmental arrest was observed earlier, starting from the eight-cell stage following EGA, compared to late morula stage in mouse. These data suggest that *POU5F1* expression is required earlier in human development than in mice, as has been previously suggested, indicating that signaling pathways responsible for early lineage specification are divergent between species (Fogarty *et al.*, 2017a,b).

NGS analysis identified for the first-time complete *POU5F1* knockout human embryos, which are able to reach the blastocyst stage (6.5 dpf). This contrasts with previous reports from Fogarty and colleagues, where only mosaic embryos were able to reach the blastocyst stage. Hence, the previous inability to generate complete *POU5F1* knockout embryos can be attributed to inefficient editing related to late CRISPR-Cas9 component introduction. The induced *POU5F1*-null blastocyst phenotype in our experiments was characterized by absence of ICM and a TE composed of very few cells, along with inability of the edited blastocyst to expand and hatch. This is supported by findings in bovine studies, demonstrating that POU5F1 is required for the blastocyst expansion (Daigneault *et al.*, 2018). In the future it would be informative to examine if these blastocysts can further develop to postimplantation stages. Recent studies in human embryos revealed that CRISPR-Cas9 mediated gene editing can induce large genomic deletions and, in some cases, frequent loss of the targeted chromosome, indicating that germline gene editing would pose a substantial risk for aneuploidy and other adverse genetic consequences (Zuccaro *et al.*, 2020).

Overall, we showcased that CRISPR-Cas9 is a very effective tool in developmental studies. We efficiently perturbed POU5F1 gene expression in mouse revealing the requirement of POU5F1 in mouse pre-and post-implantation development and further identified an interstrain difference in the developmental capacity of mouse embryos. In human, we provided data indicating that mosaicism can be efficiently eliminated and we report the novel finding of the generation of POU5F1-null embryos that are able to reach the blastocyst stage.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Authors' roles

P.S. conceived the project, designed the study, performed the experiments, performed data analysis and wrote the manuscript. A.B, G.C, M.P, B.B, R.R.G, M.T performed data analysis. B.H., P.C., S.M.C.S.L., D.S, B.M, F.V.N, P.D.S. conceived the project and interpreted the data. All authors reviewed the manuscript and approved the final version.

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Conflict of interest

The authors declare no competing interests.

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