Placental gene editing via trophectoderm-specific Tat-Cre/loxP recombination

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ABSTRACT

The ways in which placental defects affect embryonic development are largely overlooked because of the lack of a trophoblast-specific approach for conditional gene ablation. To tackle this, we have established a simple, fast and efficient method for trophoblast-specific Cre/loxP recombination. We used the natural permeability barrier in mouse blastocysts in combination with off-the-shelf Tat-Cre recombinase to achieve editing of conditional alleles in the trophoblast lineage. This direct approach enables gene function analysis during implantation and placentation in mice, thereby crucially helping to broaden our understanding of human reproduction and development.

KEY WORDS: Blastocyst, Trophoderm, Placenta, Trophoblast, Cre/loxP, Conditional knockout

INTRODUCTION

The trophectoderm (TE) defines the hollow-shaped architecture of the blastocyst, mediates implantation and gives rise to the placentotonic tissues supporting foetal development to term. During the early stages of pregnancy, the crucial process that mediates the connection between the conceptus and the maternal tissues is the implantation of the blastocyst into the uterine wall. Importantly, although implantation defects have been shown to account for 49% of pregnancy failures in human-assisted reproduction (Koot et al., 2012), the mechanisms of blastocyst attachment and trophoblast invasion, as well as the factors governing discontinuation of pregnancy at implantation, are poorly understood because of the lack of suitable mouse models. Moreover, in-depth analysis of 103 embryonic lethal and subviable knockout mouse lines from the DMDD (deciphering the mechanisms of developmental disorders) program identified that 68% of these mice exhibited placental abnormalities (Mohun et al., 2013; Perez-García et al., 2018). Among those, the E9.5-E14.5 lethality was predominantly associated with severe placental dysfunction. These placental defects are coupled to and can indirectly cause malfunctions in the foetal brain, heart and vasculature (Perez-García et al., 2018). This underlines the largely overlooked significance of trophoblast factors governing discontinuation of pregnancy at implantation, are poorly understood because of the lack of suitable mouse models. Moreover, in-depth analysis of 103 embryonic lethal and subviable knockout mouse lines from the DMDD (deciphering the mechanisms of developmental disorders) program identified that 68% of these mice exhibited placental abnormalities (Mohun et al., 2013; Perez-García et al., 2018). Among those, the E9.5-E14.5 lethality was predominantly associated with severe placental dysfunction. These placental defects are coupled to and can indirectly cause malfunctions in the foetal brain, heart and vasculature (Perez-García et al., 2018). This underlines the largely overlooked significance of trophoblast defects as contributors of abnormal embryonic development and the pressing need for an experimental approach for generating placenta-specific loss-of-function alleles.

The most common conditional knockout system relies on loxP sites that flank critical exon(s) of the target locus, in combination with Cre recombinase expressed under the control of a tissue-specific promoter. The loxP-flanked region is excised only in cells that express the recombinase, generating tissue-specific loss of function (Kim et al., 2018). For example, Zp3-Cre enables gene deletion during oocyte maturation, resulting in the deletion of maternal transcripts in the zygote (de Vries et al., 2000); Sox2-Cre drives Cre activity in the epiblast, deleting genes in the embryo proper (Hayashi et al., 2002); and Trt-Cre recombines conditional alleles in the visceral endoderm (VE) (Kumar et al., 2015).

Although the Cre/loxP system is a powerful tool for studying mouse development, a Cre-expressing mouse line suitable for TE-specific gene deletion is currently not available, thus preventing the direct study of placental effects. This is a major gap in the gene editing toolbox, so, to tackle this, we have established a simple and efficient method for Cre/loxP recombination in the TE without affecting the inner cell mass (ICM). The recombinated allele is inherited in the trophoblast lineage, enabling placenta-specific Cre/loxP gene editing.

RESULTS

Achieving excision of a loxP-flanked genomic region in all cells of the trophoblast lineage requires a Cre recombination event in the earliest trophoblast progenitors – the TE cells of the pre-implantation embryo. However, establishing a Cre line that can mediate recombination of conditional alleles only in the trophoblast lineage without later affecting the embryo proper is hindered by the lack of suitable target genes with exclusive TE activity for controlling the Cre expression. For example, although Cdx2, Eomes and cytokeratin 8 are expressed only in the TE at the blastocyst stage (Kemler et al., 1981; Russ et al., 2000; Strumpf et al., 2005), later on Cdx2 is expressed in the gut (Saad et al., 2011), Eomes in the primitive streak and brain (Arnold et al., 2008; Costello et al., 2011), and cytokeratin 8 in various epithelial tissues (Baribault et al., 1994; Odaka et al., 1994; Odaka et al., 2013). To bypass the requirement of transgenic Cre expression, we used a recombinant cell-permeant Cre protein (Tat-Cre), where Cre is fused to a nuclear localisation sequence, a translocation peptide sequence derived from the HIV-Tat protein and a N-terminal histidine tag (Peitz et al., 2002) (Fig. 1A).

The TE is the first epithelium formed during mouse embryogenesis, and it is crucial for proper blastocyst formation and function. Tight junctions between the TE cells seal the epithelial monolayer and establish a permeability barrier (White et al., 2018). Therefore, we hypothesised that directly exposing E3.5 blastocysts to Tat-Cre would result in an uptake of the recombinant protein by the TE cells only. The Tat-Cre can translocate into the TE cells via the exposed apical cell membrane surface, but the tight junctions should block paracellular transport into the blastocoel cavity and the ICM. Subsequently, the nuclear translocation of the Tat-Cre will mediate the recombination of loxP-flanked genomic sequences in a TE-specific manner. Furthermore, although the Tat-Cre protein has a limited half-life, the pulse of recombinase activity can generate a permanent genetic modification in the TE that is inherited in all placental tissues after implantation.
To test this hypothesis, first we verified the formation of tight junctions in E3.5 blastocysts. We stained the embryos for the tight junction component Zo-1 in combination with trophodermal (Cdx2) and basolateral membrane (β1 integrin) markers. Zo-1 was detectable in the apical part of the lateral cell-cell contacts, indicating a zipper-like sealing of the TE (Fig. 1B).

Next, we considered a mouse strain with an integrated conditional allele that provides clear readout of Cre recombination events in live embryos. We took advantage of the mT/mG reporter mouse that harbours a membrane-targeted tandem dimer Tomato (mT) expression cassette flanked by loxP sites, followed by membrane-targeted GFP (mG) (Muzumdar et al., 2007). The mT/mG construct is integrated in the Rosa26 locus, enabling uniform red (mT) expression in all tissues of the embryo. Upon Cre/loxP recombination, the mT cassette is excised and de novo mG expression is initiated, thus switching the red fluorescence to green.

We isolated E3.5 mT/mG embryos and removed the enclosing zona pellucida by a brief incubation with Tyrode’s solution to directly expose the TE. The zona-free blastocysts were incubated for 2 h in KSOM medium containing increasing concentrations of Tat-Cre: 0.75 µM, 1.5 µM and 3 µM. Afterwards, the embryos were thoroughly washed by sequential transfer into three wells of a 96-well plate filled with fresh medium. The Tat-Cre treated and control (untreated) embryos were cultured overnight and the next day were examined for mT and mG fluorescence (Fig. 1C,D). We found that the lowest Tat-Cre concentration resulted in mosaic expression of mG in the TE, whereas in the 1.5 µM- and 3 µM-Tat-Cre (n=10) and 3 µM-Tat-Cre (n=10). The right panels provide representative images of ESC (n=5 for each Tat-Cre dose) and TSC (n=5 for each Tat-Cre dose) lines isolated from control and Tat-Cre-treated blastocysts. The ESCs were stained for Tom (red) and the pluripotency marker Oct4 (white). The TSCs were stained for GFP (green), Tom (red) and the trophoblast marker Cdx2 (white). Scale bars: 20 µm.
To confirm that only the TE and not the ICM exhibited Tat-Cre/loxP recombination, we derived trophoblast stem cells (TSC) and embryonic stem cells (ESC) from control and Tat-Cre-treated blastocysts. In accordance with the expression pattern in the TE, the TSCs derived from 0.75 µM Tat-Cre-treated embryos contained mT- as well as mG-positive colonies, whereas the TSCs derived from 1.5 µM and 3 µM Tat-Cre treatments were only mG positive. We never observed mG expression in ESCs established from the Tat-Cre treated embryos, confirming the TE-specific recombination of the mT/mG locus (Fig. 1D).

Finally, we traced the inheritance of the recombined allele in the early post-implantation trophoblast and placenta. We treated E3.5 mT/mG blastocysts for 2 h with 1.5 µM Tat-Cre, and, after washing, control (untreated) and Tat-Cre treated embryos were transferred into pseudopregnant females for subsequent isolation at E6.5 or E10.5 (Fig. 2A). Compared with control embryos, there were no obvious alterations in the implantation capacity and the post-implantation recovery rate of the Tat-Cre-treated embryos that could suggest toxicity or any other disadvantage mediated by the short-term Tat-Cre exposure (Fig. 2B and Fig. S1B). At E6.5 the Tat-Cre treated embryos consisted of mT-positive VE and EPI tissues, and mG-positive trophoblast tissues (the extra-embryonic ectoderm and ectoplacental cone; Fig. 2C). At E10.5, the foetus, umbilical cord and the yolk sack expressed mT, and only the placenta was mG positive, confirming the trophoblast-specific excision of the mT cassette (Fig. 2D).

**DISCUSSION**

Although a large set of conditional knockout mouse models are available from public repositories such as The Jackson Laboratory, functional trophoblast-specific analysis are hindered by the lack of suitable Cre-expressing mouse line. Here, we used the natural permeability barrier of the TE in combination with commercially available Tat-Cre recombinase to achieve trophoblast-specific Tat-Cre/loxP recombination. This is a simple, fast and efficient method that relies on an off-the-shelf recombinant protein that has...
previously been successfully used in embryonic stem cells, early cleavage embryos and neurons (Haupt et al., 2007; Kim et al., 2009; Peitz et al., 2002; Ryder et al., 2014). The short exposure of embryos to Tat-Cre does not affect their developmental capacity and, at the same time, circumvents the need for transgenic Cre and potential ‘leaky’ Cre expression in other tissues. In addition, crossing conditional loxP and Cre mouse lines generates offspring in a Mendelian ratio, where only a subset of all embryos has the desired genotype. Using homozygous loxP mouse lines in combination with Tat-Cre mediated recombination enables the conversion of all embryos into conditional TE knockouts, reducing the breeding time and number of experimental animals.

The Tat-Cre-mediated recombination also circumvents undesirable safety challenges that can be encountered using lentiviral delivery of transgenes in the TE (Georgiades et al., 2007). As it targets only the trophoblast lineage, the Tat-Cre approach also has an advantage over the tetraploid complementation assay, where donor ESC can compensate for the endogenous epiblast cells, but still both the TE and the primitive endoderm are host derived (Nagy et al., 1993). Thus, any genetic modification in the host embryo is maintained not only in the trophoblast, but also in the primitive endoderm lineage.

Taken together, the TE-specific Tat-Cre/loxP recombination provides a direct approach for analysing gene functions in the trophoblast lineage that can aid in deciphering the processes of implantation and placentation in mice, which will directly impact our understanding of human reproduction and development.

MATERIALS AND METHODS

Mice

The mice used in this study were 6 weeks to 12 weeks old. The animals were maintained under a 14-h light/10-h dark cycle with free access to food and water. Female mice were housed in groups of up to four per cage, and male stud mice were housed individually. Both mT/mG and wild-type B6C3F1 mouse lines were used in this study. To obtain mT/mG embryos, homozygous mT/mG stud males were crossed with B6C3F1 females. Wild-type embryos were obtained from B6C3F1 inter-crosses. Animal experiments and husbandry were performed according to the German Animals Welfare guidelines and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (State Agency for Nature, Environment and Consumer Protection of North Rhine-Westphalia).

Embryo isolation and Tat-Cre treatment

E3.5 blastocysts were isolated by flushing uteri with M2 medium (Sigma). Zona pellucida was removed by brief exposure to acidic Tyrode’s solution (Sigma). The zona-free blastocysts were incubated with 0.75 µM, 1.5 µM or 3 µM of Tat-Cre (Millipore) in pre-warmed KSOM medium (Millipore) for 2 h at 37°C in 5% CO2 atmosphere in air. After washing, the embryos were cultured in KSOM medium overnight at 37°C in 5% CO2 and examined for mTom and mGFP expression on the next day. For tracing the post-supplemented with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 U/ml penicillin-streptomycin, 0.1 mM 2-mercaptoethanol, 25 ng/ml FgH4 and 1 µg/ml heparin. After 4 days of culture, the blastocyst outgrowths were dissociated using trypsin and transferred into fresh wells where ESC or TSC colonies emerged.

Immunofluorescence staining and imaging

E3.5 blastocysts, ESCs or TSCs were fixed with 4% PFA for 10 min and permeabilised for 3 min using a permeabilisation buffer of 0.1 M glycine and 0.3% Triton X-100 in PBS. After that, the samples were incubated overnight at 4°C with primary antibodies in 10% FCS/PBS. On the next day, the samples were washed with 5% FCS/PBS and incubated with the secondary antibodies and DAPI overnight. After washing, the samples were mounted for imaging. The embryos were placed in droplets of PBS covered with mineral oil (Millipore) on glass-bottomed petri dishes (Gremer). Confocal microscopy was performed using Leica SP5 and Zeiss LSM780 systems. The images were processed using ImageJ software (Fiji).

Primary antibodies used were as follows: mouse anti-Cdx2 (1:300, Biogenex, MU392A-5UC), mouse anti-Oct4 (1:300, Santa Cruz Biotechnology, sc5279), goat anti-GFP (1:300, R&D, AF4240-SP), rabbit anti-RFP (1:300, Rockland Immunocytologicals, 600-401-379), rabbit anti-Zo1 (1:500, Invitrogen, 61-7300) and FITC-conjugated hamster anti-b1 integrin (CD-29 FITC; 1:200, Thermo Fisher, 561796). Secondary antibodies used were Alexa 488 donkey Anti-goat (1:300, Thermo Fisher, A-11055), Alexa 594 donkey anti-rabbit (1:300, Thermo Fisher, A-21207) and Alexa 647 donkey anti-mouse (1:300, Thermo Fisher, A-31571).

Images of live E3.5, E6.5 and E10.5 embryos were taken using Leica SP5, Leica DMi6000 B and Leica Thunder systems, respectively. The images were processed using ImageJ software (Fiji).

Competing interests

The authors declare no competing or financial interests.

Author contributions


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