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Placenta-specific gene manipulation in rabbits

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ABSTRACT

Lentiviral gene constructs can be efficiently and specifically delivered to trophoblast cell lineages in rodents. *In vivo* genetic manipulation of trophoblast cell lines enables functional and developmental studies in the placenta. In this report we show that genetic modification can be produced in the extraembryonic tissues of rabbits by lentiviral gene constructs. When 8–16 cell stage embryos were injected with lentiviral particles, strong reporter gene expression resulted in the rabbit placenta. The expression pattern displayed some mosaicism. A strikingly high degree of mosaic GFP expression was detected in some parts of the yolk sac, which is a hypoblast-derived tissue. Whereas expression of the reporter gene construct was detected in placentas and yolk sacs, fetuses never expressed the transgene. As rabbits are an ideal model for functional studies in the placenta, our method would open new possibilities in rabbit biotechnology and placentation studies.

1. Introduction

In mammals, trophectoderm cells are one of the first cell types to be specified in blastocyst-stage embryos. Trophectoderm cells surround the inner cell mass (ICM), and they have an important role in the initiation of implantation; however, a subset of these cells persist as trophectodermal stem cells. ICM cells and trophectoderm cells express different molecular markers. Trophectoderm cells differentiate and produce the trophoblastic cells of the mature placenta, which is an essential organ for the mother-fetus dialogue and connection (Rossant and Cross, 2001). These cells also facilitate nutrient, gas and waste exchange to ensure proper fetal development. ICM cells give rise to the fetus and some extra-embryonic cells. Trophoblast formation is a dynamic and finely regulated process, where various abnormalities can lead to the loss of pregnancy or to its complications (Norwitz et al., 2001). Currently, more than a hundred mutant mouse lines exist with manifested defects in placental development.

During rabbit embryo implantation trophoblast cells adhere and fuse to the apical surface of uterine epithelial cells to create large knob-like structures (Enders and Schlafke, 1971). The rabbit placenta is a discoid, hemochorial placenta. Its hemodichorial structure with two cellular layers of chorion between the maternal and the fetal blood is more similar to human placenta than that of rodents which develops as a hemotrichorial placenta (Fischer et al., 2012). Hemodynamic changes in the rabbit during pregnancy are comparable with those in humans. Additionally, the relative size of the rabbit placenta compared to that of other laboratory animals makes it easier to monitor fetal and placental growth using modern imaging equipments (Chavatte-Palmer et al., 2008), therefore the rabbit is an ideal animal model for studying human placental diseases.

Placental defects can be easily investigated using trophoblast-specific gene transfer by lentiviral transduction of embryos. The first placenta-specific gene manipulation was performed in rhesus monkeys: blastocysts were injected with a lentiviral vector containing GFP as a reporter gene (Wolfgang et al., 2001). Similar experiments were implemented in mice, where selective lentiviral transduction of the trophectoderm and its derivatives were performed (Tolkunova Elena, 2007). Later complementation of placental defects and embryonic lethality by trophoblast-specific gene transfer in mice by lentivirus vectors was reported in mice (Okada et al., 2007). Ets2-, Mapk14- and Mapk1-deficient mice were rescued from embryonic lethality. Placenta-specific downregulation of GFP was performed using shRNA constructs in rats. Lentiviral-mediated transfer of shRNAs efficiently moderated GFP expression (Lee et al., 2009). Placenta-specific gene activation and inactivation using the Cre/loxP system were first described by Morioka et al. (Morioka et al., 2009); transient inducible placental expression by tetracycline was performed in 2012 (Fan et al., 2012). These steps enabled a wide range of genetic modifications with trophoblast-specific origin using lentiviral systems. Noninvasive monitoring of placenta-specific gene expression by lentiviruses was also technically resolved

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(Fan et al., 2011).

As a result of the above technological innovations, gene function studies in the rodent placenta by lentivirus-specific gene transfer has become common in the last decade. Furin, CUL3, FOSL1, sFLT1, sENG, Ets2, Gli2, Gli3 and Shadoo gene functions in the placenta were investigated respectively (Kubota et al., 2015; Kumasawa et al., 2011; Odiatis and Georgiades, 2010; Pan et al., 2015; Passet et al., 2012; Zhang et al., 2015; Zhou et al., 2013).

Transgenic rabbits expressing GFP as a reporter gene using lentiviral technology have been already reported. SIV-derived lentiviral constructs were used to efficiently produce transgenic rabbit founders. Mosaic expression was found in all founders, and the germline transmission of the transgene was limited (Hiripi et al., 2010).

A recent review emphasized the importance of recognizing the pros and cons of various rodent and non-rodent animal models used to understand mechanisms involved in fetal programming with a particular focus on placentation, which is critical for translating animal findings to humans (Chavatte-Palmer et al., 2016). Even though the rabbit would be a better model of studying placental gene function, trophectoderm-specific gene manipulation has not been reported. Here we report for the first time the trophectoderm-specific gene manipulation in rabbits using HIV-derived lentiviral constructs. GFP as a reporter gene was expressed in the placenta and also in the yolk sac. Lentiviral constructs were transfected into 8–16 cell stage rabbit embryos, unlike in other species where blastocysts were used.

2. Materials and methods

2.1. Animals

New Zealand White female rabbits at the age of 18–22 weeks were used in this study. Animals were kept under standard light-dark cycle (06.00–18.00 h) at 19 °C with food and water available ad libitum and caged separately. This study was carried out in strict accordance with the recommendations and rules in the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes. The protocol was approved by the Animal Care and Ethics Committee of the NAIC-Agricultural Biotechnology Institute and the Pest County's governmental office (permission number: PEI/001/329-4/2013). The method used for euthanasia: concussion under anesthesia (ketamine/xylazine). All efforts were made to minimize suffering.

2.2. Lentiviral vector construction

Lentiviral sequences are coded on three plasmid constructs. The envelope plasmid construct encodes the viral envelope protein pseudotyped with VSV-G (vesicular stomatitis virus G-protein). The packaging plasmid construct encodes all enzymatic and structural elements required for the assembly of retroviral vectors. The transfer plasmid construct carries the transgene of interest with LTR sequences. The transgene was an GFP reporter gene under the control of EF1 (Ryu et al., 2007) or PGK promoter. The transfer construct contains a central polypurine tract for increased nuclear transport, WPRE (Woodchuck hepatitis virus post-transcriptional responsive element) for prolonged mRNA half-life. Lentiviral particles were produced as published previously (Kvell et al., 2005). Viral particles were re-suspended in DMEM. Titrations of particles were performed with HeLa cells. Following concentration by ultracentrifugation, titers reached 10^8 TU/ml.

2.3. Embryo collection and transfer, micromanipulation

Sexually mature New Zealand White rabbits were superovulated as published (Hiripi et al., 2003). 8–16 cell stage embryos were recovered from the oviducts of the donor does 44–46 h after insemination. Microinjection of the lentiviral particles into the perivitelline space of the embryos was described earlier [24]. Approximately 200–500 pl of vector was injected in a single injection into the perivitelline space of the 8–16 cell stage embryos. Embryos were kept in RDH medium (Jin et al., 2000) at 38.5C°, 5% CO2 and 85% relative humidity for a short period of time (1–2 h) before embryo transfer. Recipient does received 84 mg GnRH analogue intramuscularly in 12-h asynchrony with the donor rabbits. Six to ten 8–16 cell stage embryos were transferred to each oviduct of the recipient rabbits. Pregnancies were terminated at the age of 14.5 days when fetuses and extraembryonic tissues were analyzed. Embryos for *in vitro* data were cultured in RDH medium at 38.5 C°, 5% CO2 and 85% relative humidity until blastocyst-stage and analyzed by fluorescent microscopy.

2.4. Purification of genomic DNA and transgene detection

Genomic DNA was purified from extraembryonic tissues and fetuses by a simplified DNA isolation method (Laird et al., 1991). Samples were screened for transgene integration by transgene-specific PCR using the following oligonucleotide primers. GFP1 5′ATCACATGAAGCAGCACGACTT3′; GFP2 5′GTCACGAAGCCGAAGTAGATCA3′. The PCR reactions were carried out with REDTaq ReadyMix (Sigma-Aldrich, USA) using 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The resulting 448 bp fragment was analyzed on 1,5% agarose gel.

2.5. Expression analysis by RT-PCR

500 ng total RNA was extracted from the placenta and yolk sac of 14.5 days pc. rabbit embryos by using the RNeasyH Plus Mini Kit that includes a DNase digestion step (Qiagen GmbH, Germany). The first strand of cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Life Technologies, USA). Subsequently, cDNA was diluted 1/10 and PCR-amplified with gene-specific GFP primer pairs as described above. The PCR reactions were carried out with REDTaq ReadyMix (Sigma-Aldrich, USA) using 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Amplified products were resolved on 1,5% agarose gels.

2.6. GFP detection and in vivo fluorescence imaging

Wet tissues were analyzed using a GFSP-5 headset (Biological Laboratory Equipment, Maintenance and Service Ltd., Budapest). Fluorescent photos of embryos, fetuses, placentas and yolk sacs were taken with Olympus BH2 research microscope, or Olympus SZH Stereo Zoom microscope using the MAA-03/B light source (Biological Laboratory Equipment, Maintenance and Service Ltd., Budapest).

3. Results

3.1. GFP expression restricted to trophectoderm cells in vitro

As a pilot experiment, 22 eight-cell-stage embryos were microinjected with a lentiviral construct harboring the GFP transgene under the EF1 promoter. The embryos were cultured until blastocyst-stage, and GFP expression was detected by *in vivo* fluorescent imaging. Interestingly, only trophectoderm cells expressed the transgene in nine embryos. Transgene expression was strong but showed a mosaic pattern: not all the trophectoderm cells expressed the transgene (Fig. 1). This experiment was repeated using the same lentiviral construct with the promoter replaced by PGK, where 17 embryos were injected. Seven embryos expressed the transgene in the trophectoderm cells (data not shown). Pilot experiments were designed to assess the effectiveness of this method and to test the possible promoter effect. Following the 3R principles for animal research we predetermined the minimal but essential number of experiments for placenta specific gene transfer.