Published in final edited form as:

Genesis. 2009 July ; 47(7): 433–439. doi:10.1002/dvg.20518.

IN VIVO GENETIC MANIPULATION OF THE RAT TROPHOBLAST CELL LINEAGE USING LENTIVIRAL VECTOR DELIVERY

Dong-Soo Lee, M. A. Karim Rumi, Toshihiro Konno, and Michael J. Soares*

Institute of Maternal-Fetal Biology, Division of Cancer and Developmental Biology, Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160

Abstract

In this report, we have adapted a lentiviral gene delivery technique for genetic modification of the rat trophoblast cell lineage. Blastocysts were incubated with lentiviral particles and transferred into the uteri of pseudopregnant female rats, harvested at various times during gestation, and then analyzed. Two test systems were evaluated: 1) delivery of an enhanced green fluorescent protein (EGFP) gene under the control of constitutive promoters to rat blastocysts; 2) delivery of EGFP short hairpin RNA (shRNA) to rat blastocysts constitutively expressing EGFP. Lentiviral packaged gene constructs were efficiently and specifically delivered to all trophoblast cell lineages. Additionally, lentiviral mediated transfer of shRNAs was an effective strategy for modifying gene expression in trophoblast cell lineages. This technique will permit the in vivo evaluation of 'gain-of-function' and 'loss-of-function' manipulations in the rat trophoblast cell lineage.

INTRODUCTION

Hemochorial placentation involves interactions between extraembryonic tissues and the maternal uterine vasculature. The rat is an exceptional model for investigating uteroplacental interactions, especially those involving invasive trophoblast cells and the uterine spiral arteries. Invasive trophoblast cells of the rat penetrate deep into the uterine mesometrial compartment (Ain *et al.*, 2003; Caluwaerts *et al.*, 2005; Vercruysse *et al.*, 2006), where they are proposed to participate in the remodeling of uterine spiral arteries (Pijnenborg *et al.* 2006). Mouse models are powerful tools for investigating regulatory mechanisms controlling many aspects of hemochorial placentation (Rossant and Cross, 2001). However, the mouse is not a particularly effective model for studying the biology of the invasive trophoblast lineage (Ain *et al.*, 2003; Coan *et al.* 2006). In this species, penetration of trophoblast cells into the uterus is limited to the decidua.

The purpose of this study was to devise a strategy for genetically manipulating the trophoblast lineage of the rat. We have adapted a lentiviral gene delivery approach previously described for the mouse (Georgiades *et al.*, 2007; Okada *et al.*, 2007).

*Correspondence to: Michael J. Soares, Institute of Maternal-Fetal Biology, Division of Cancer and Developmental Biology, Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160; Phone: 913 588-5691; Fax: 913 588-8287; msoares@kumc.edu.

An overview of the trophoblast-specific lentiviral gene delivery strategy presented in this report is shown in Fig. 1. The experimental basis for selective trophoblast infection is directly related to the structure of the blastocyst and the tight epithelium formed by the outer trophoctoderm, which effectively restricts viral particle access to the inner cell mass. Lentiviral delivery of constitutive promoters driving enhanced green fluorescence protein (EGFP) or short hairpin RNA (shRNA) expression were used to demonstrate the feasibility of 'gain-of-function' and 'loss-of-function' approaches for manipulating the trophoblast lineage (Fig. 1).

Lentiviral packaged gene constructs containing phosphoglycerate kinase or ubiquitin C promoters driving the EGFP reporter were specifically activated in the trophoblast lineage (Figs. 2–4). Transduced blastocysts exhibited trophoctoderm-restricted reporter expression (Fig. 2). Efficient blastocyst transduction required zona pellucida removal and 4.5 h of incubation at a lentiviral particle concentration of approximately 500 ng p24/ml. Extended exposure to acid Tyrode's solution, impure or excessively high viral particle concentrations or prolonged incubations resulted in blastocyst toxicity. The transduced blastocysts were transplanted into d3.5 pseudopregnant rats. Infected embryos implanted and placentation and fetal development proceeded. At d13.5 (Fig. 3) and d18.5 (Fig. 4) of gestation, placental structure and fetal development appeared normal. Our optimized protocol yielded approximately 80% recovery of transduced embryos. All trophoblast lineages derived from embryos transduced with lentiviral constructs containing either the phosphoglycerate kinase or ubiquitin C promoters were positive for EGFP. This included invasive trophoblast cells (Fig. 4b and f), and trophoblast cells within the junctional (Fig. 4c and g) and labyrinth (Fig. 4d and h) zones of the chorioallantoic placenta. There was no evidence of EGFP expression in any non-trophoblast cell types. The intensity of reporter activity was not uniform among the various trophoblast lineages. Trophoblast cells of the junctional zone, especially spongiotrophoblast cells and trophoblast giant cells exhibited the highest levels of EGFP expression. This likely reflects differences in the efficacy of the phosphoglycerate kinase and ubiquitin C promoters in the various trophoblast cell lineages and points to distinct transcriptional environments in the lineages. Similar differences in trophoblast lineage activation have been reported for the Rosa 26 and chicken β -actin promoters (Arroyo *et al.*, 2005; Rosario *et al.*, 2008). Collectively, the data indicate that the rat trophoblast cell lineage can be genetically modified.

We next evaluated the feasibility of disrupting trophoblast gene function with the lentiviral delivery strategy. Initially, experiments were performed to demonstrate that lentiviral transduction of a U6 promoter driven shRNA targeted to EGFP could effectively knock-down EGFP expression in 293 cells (Fig. 5). We subsequently utilized a transgenic strain of rats expressing the EGFP reporter under the control of a chicken β -actin promoter (ch β A-EGFP) and the lentiviral construct containing the human U6 promoter directing the expression of a shRNA targeted to EGFP or a control shRNA. Trophoblast lineages of ch β A-EGFP placentas fluoresce, reflecting their expression of the reporter transgene (Rosario *et al.*, 2008; Fig. 6a and b). Placentas derived from blastocysts transduced with the lentiviral EGFP shRNA construct showed a specific knock-down of EGFP expression in trophoblast cell lineages but not in fetal structures (Fig. 6c and e). The control shRNA did

not affect reporter expression within the ch β A-EGFP placentas (Fig. 6d and e). The results indicate that the activity of endogenous genes can be specifically disrupted in the trophoblast lineage.

In conclusion, we have demonstrated the efficacy of a lentiviral delivery strategy for the in vivo manipulation of the rat trophoblast lineage. Both 'gain-of-function' and 'loss-of-function' genetic modifications can be generated. The approach represents a new experimental tool for investigating the regulation of placentation in the rat.

MATERIALS AND METHODS

Animal and tissue preparation

Holtzman Sprague Dawley (HSD) rats were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). A colony of transgenic rats expressing the enhanced green fluorescence protein (EGFP) driven by a chicken β -actin promoter (ch β A-EGFP; Ikawa *et al.*, 1998; Hasuwa *et al.*, 2002) was established. Animals were housed in an environmentally controlled facility, with lights on from 0600–2000 h, and were allowed free access to food and water. Virgin female HSD rats of 8–10 weeks were cohabited with male HSD or ch β A-EGFP rats. Presence of sperm in the vaginal lavage was designated as day 0.5 of pregnancy. Depending on the experimental design animals were sacrificed on days 4.5, 13.5, or 18.5 of gestation. Placentation sites, including uterus, metrial gland, and placental tissues, were dissected from pregnant animals and frozen in dry ice cooled heptane and stored at -80°C until used for histological analyses. Pseudopregnancy was induced by mating with vasectomized males. Identification of seminal plugs was designated as day 0.5 of pseudopregnancy. Protocols for these procedures have been described (Ain *et al.*, 2006). The procedures for handling and experimentation with rodents were approved by the University of Kansas Medical Center Animal Care and Use Committee.

Lentiviral vectors and viral particle production

Gene delivery was performed via lentiviral transduction. Lentiviral constructs and packaging systems used in the experimentation were obtained from Addgene (Cambridge, MA) and are illustrated in Fig. 1. The constructs included: pLKO.3G (Addgene plasmid 14748), a construct containing a phosphoglycerate kinase promoter driving expression of EGFP; FG12 (Addgene plasmid 14884), a construct containing the ubiquitin C promoter driving EGFP (Qin *et al.*, 2003); pLKO.1 puro GFP siRNA (pLKO.1-shEGFP; Addgene plasmid 12273), a construct containing the human U6 promoter directing the expression of an shRNA targeted to EGFP (Orimo *et al.*, 2005); pLKO.1-shSCR (Addgene 1864), a construct containing the human U6 promoter directing the expression of an shRNA targeted to no known mammalian gene (Sarbasov *et al.*, 2005). Lentivirus was produced following transient transfection of a transducing vector, third generation packaging system plasmids (pMDLg/pRRE, Addgene plasmid 12251; pRSV-Rev, Addgene plasmid 12253; Dull *et al.*, 1998), and a VSVG envelope plasmid (pMD2.G, Addgene plasmid 12259) into 293FT cells (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen) in Opti-MEM I (Invitrogen). Thereafter, cells were maintained in DMEM with high glucose and 5–10% fetal bovine serum. Culture supernatants containing lentiviral particles were harvested every 24 h for two

to three days, centrifuged to remove cell debris, filter sterilized, concentrated by ultracentrifugation, and stored at 4°C or at -80° until used. Lentiviral vector titers were determined by measurement of p24 gag antigen by ELISA (Advanced Bioscience Laboratories, Kensington, MD).

Transduction of blastocysts and in vivo transplantation

Rat embryos were collected by flushing uteri with M2 medium (Millipore, Temecula, CA) at gestation d4.5. Recovered blastocysts were washed in microdrops containing KSOM medium (Millipore). Zona pellucidae were removed with acid Tyrode's solution (Sigma, St. Louis, MO) and incubated with concentrated lentiviral particles (~ 500 ng of p24/ml) for 4.5 h. Transduced blastocysts were transferred to uteri of d3.5 pseudopregnant rats for subsequent evaluation of gene transfer activities in developing embryonic and extraembryonic structures.

Histological examination

Histological analyses were performed on 10-µm tissue sections prepared with the aid of a cryostat. The cryosections were exposed to formaldehyde vapor (Jockusch *et al.*, 2003) and either stained with hematoxylin and eosin or examined for fluorescence. All histological and fluorescence images of cryosections were inspected and captured using a Leica MZFLIII stereomicroscope (Leica Microsystems GmbH, Welzlar, Germany) or a Leica DMI 4000 microscope (Leica); both equipped with Leica CCD cameras (Leica).

Western blot analysis

GFP was examined in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) and placental lysates by western blot analysis. HEK 293 cells were co-transduced with a lentiviral vector expressing EGFP (FG12) and another lentiviral vector expressing a control shRNA (pLKO.1-shSCR) or an shRNA to EGFP (pLKO.1-shEGFP). Forty-eight h after transduction, images were captured and then cells were washed with phosphate buffered saline, pH 7.4 (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.2, 1% Triton X-100 or 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin) processed for western blotting and detection of GFP. Gestation d18.5 placental lysates derived from blastocysts transduced with control (pLKO.1-shSCR) or EGFP (pLKO.1-shEGFP) shRNAs were prepared by extraction with RIPA buffer. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, CA). GFP was detected with an anti-GFP rabbit polyclonal antibody (AB3080, 1: 500 dilution, Chemicon International, Temecula, CA). β-Actin was detected with a mouse monoclonal antibody (A1978, Clone AC15; 1:5000 dilution, Sigma-Aldrich, St. Louis, MO) and used as a control for loading accuracy and protein integrity.

ACKNOWLEDGEMENTS

We appreciate the advice of Dr. Masahito Ikawa of Osaka University, Osaka, Japan and Marina Gertsenstein of Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada. The chβA-EGFP transgenic rat line was generously provided by Dr. Masaru Okabe of Osaka University, Osaka, Japan. We would also like to

acknowledge Christophe Benoist, Diane Mathis, David Baltimore, David M. Sabatini, and Robert A. Weinberg for providing lentiviral constructs used in the experimentation.

LITERATURE CITED

- Ain R, Canham LN, Soares MJ. Gestational stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Dev Biol.* 2003; 260:176–190. [PubMed: 12885563]
- Ain, R.; Konno, T.; Canham, LN.; Soares, MJ. Phenotypic analysis of the placenta in the rat. In: Soares, MJ.; Hunt, JS., editors. *Placenta and Trophoblast: Methods and Protocols*. Vol. 1. Totowa, New Jersey: Humana Press; 2006. p. 295–313.
- Arroyo JA, Konno T, Khalili DC, Soares MJ. A simple in vivo approach to investigate invasive trophoblast cells. *Int J Dev Biol.* 2005; 49:977–980. [PubMed: 16281175]
- Caluwaerts S, Vercruysse L, Luyten C, Pijnenborg R. Endovascular trophoblast invasion and associated structural changes in uterine spiral arteries of the pregnant rat. *Placenta.* 2005; 26:574–584. [PubMed: 15993707]
- Coan PM, Conroy N, Burton GJ, Ferguson-Smith AC. Origin and characteristics of glycogen cells in the developing murine placenta. *Dev Dyn.* 2006; 235:3280–3294. [PubMed: 17039549]
- Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third generation lentivirus vector with a conditional packaging system. *J Virol.* 1998; 72:8463–8471. [PubMed: 9765382]
- Georgiades P, Cox B, Gertsenstein M, Chawengsaksophak K, Rossant J. Trophoblast-specific gene manipulation using lentivirus-based vectors. *BioTechniques.* 2007; 42:317–325. [PubMed: 17390538]
- Hasuwa H, Kaseda K, Einarsdottir T, Okabe M. Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Letters.* 2002; 532:227–230. [PubMed: 12459495]
- Ikawa M, Yamada S, Nakanishi T, Okabe M. ‘Green mice’ and their potential usage in biological research. *FEBS Letters.* 1998; 430:83–87. [PubMed: 9678599]
- Jockusch H, Voigt S, Eberhard D. Localization of GFP in frozen sections from unfixed mouse tissues: immobilization of a highly soluble marker protein by formaldehyde vapor. *J Histochem Cytochem.* 2003; 51:401–404. [PubMed: 12588968]
- Konno T, Rempel LA, Arroyo JA, Soares MJ. Pregnancy in the Brown Norway rat: a model for investigating the genetics of placentation. *Biol Reprod.* 2007; 76:709–718. [PubMed: 17202390]
- Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Ohhora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M. Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer. *Nat Biotechnol.* 2007; 25:233–237. [PubMed: 17220877]
- Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell.* 2005; 121:335–348. [PubMed: 15882617]
- Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta.* 2006; 27:939–958. [PubMed: 16490251]
- Qin XF, An DS, Chen IS, Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci USA.* 2003; 100:183–188. [PubMed: 12518064]
- Rosario GX, Konno T, Soares MJ. Maternal hypoxia-activated endovascular trophoblast cell invasion. *Dev Biol.* 2008; 314:362–375. [PubMed: 18199431]
- Rossant J, Cross JC. Placental development: lessons from mouse mutants. *Nat Rev Genet.* 2001; 2:538–548. [PubMed: 11433360]
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science.* 2005; 307:1098–1101. [PubMed: 15718470]
- Vercruysse L, Caluwaerts S, Luyten C, Pijnenborg R. Interstitial trophoblast invasion in the decidua and mesometrial triangle during the last third of pregnancy in the rat. *Placenta.* 2006; 27:22–33. [PubMed: 16310034]

Fig. 1. Schematic representation of trophoblast-specific lentiviral gene delivery

Lentiviral gene constructs are delivered to zona pellucida-free blastocysts, which are then transferred to pseudopregnant recipients and placentation sites analyzed during various stages of gestation. **Panel a)** Delivery of an EGFP expressing lentiviral vector to wild-type blastocysts. **Panel b)** Delivery of an EGFP shRNA lentiviral vector to transgenic blastocysts expressing EGFP driven by a chicken β -actin promoter (ch β A-EGFP). Note that these strategies permit generation of 'gain-of-function' and 'loss-of-function' manipulations within the trophoblast lineage. **Panel c)** Lentiviral constructs containing phosphoglycerate kinase (PGK, pLKO.3G, top) or ubiquitin C (Ubi C, FG12, bottom) promoters driving enhanced green fluorescence protein (EGFP). **Panel d)** Lentiviral constructs containing the human U6 promoter driving the expression of an shRNA for EGFP (shEGFP, pLKO.1-shEGFP, top) or an shRNA with a control sequence (shSCR, pLKO.1-shSCR, bottom).

Fig. 2. Rat trophectoderm-specific lentiviral gene delivery

Rat blastocysts were incubated with lentiviral constructs expressing EGFP under the control of phosphoglycerate kinase (**panels a–c**) or ubiquitin C (**panels d–f**) promoters. Images were captured under phase (**panels a and d**) or fluorescence (**panels b and e**) microscopy. Merged phase and fluorescence images are shown in **panels c and f**. Note that EGFP expression was restricted to trophectoderm of the blastocysts. The abbreviation ICM indicates the location of the inner cell mass.

Fig. 3. Trophoblast-specific lentiviral gene delivery assessed at gestation d13.5

Rat blastocysts were incubated with lentiviral constructs expressing EGFP under the control of phosphoglycerate kinase (**panels a–e**) or ubiquitin C (**panels f–j**) promoters. Transduced blastocysts were transferred into uteri of pseudopregnant rats and harvested at gestation d13.5. Fetal-placental units were observed: bright field (**panels a and f**) and fluorescence (**panels b and g**). Merged bright field and fluorescence images are also shown (**panel c** represents a merge of **panels a and b**; **panel h** represents a merge of **panels f and g**).

Hematoxylin and eosin stained (**panels d and i**) and fluorescence (**panels e and j**) images of

cross-sections from gestation d13.5 placentation sites. Note that EGFP expression was restricted to trophoblast derivatives of the placenta. Abbreviations: JZ, junctional zone; LZ, labyrinth zone. The location of endovascular invasive trophoblast cells are indicated by the presence of arrowheads (**panels e and j**).

Fig. 4. Trophoblast-specific lentiviral gene delivery assessed at gestation d18.5

Rat blastocysts were incubated with lentiviral constructs expressing EGFP under the control of phosphoglycerate kinase (**panels a–d**) or ubiquitin C (**panels e–h**) promoters. Transduced blastocysts were transferred into uteri of pseudopregnant rats and harvested at gestation d18.5. Cryosections were prepared and examined following hematoxylin and eosin staining (**panels a and e**) or by fluorescence (**panels b, c, d, f, g, and h**). Higher magnification fluorescence images from the metrial gland (**blue boxes, panels b and f**), junctional zone (**green boxes, panels c and g**), and labyrinth zone (**orange boxes, panels d and h**). Note

that EGFP expression was restricted to trophoblast derivatives of the placenta.
Abbreviations: JZ, junctional zone; LZ, labyrinth zone. The locations of invasive trophoblast cells are bracketed by the dashed lines (**panels b and f**).

Fig. 5. Lentiviral knock-down of EGFP expression in HEK 293 cells

HEK 293 cells were co-transduced with a lentiviral vector expressing EGFP (FG12) and another lentiviral vector expressing a control shRNA (pLKO.1-shSCR, **panels a and c and lane 1 in panel e**) or an shRNA to EGFP (pLKO.1-shEGFP, **panels b and d and lane 2 in panel e**). Forty-eight h after transduction, images were recorded (**panels a–d**) and then cells were washed with PBS, lysed in RIPA buffer, processed for western blotting, and detection of GFP (**panel e**). **Panels a and b** are fluorescence images and **panels c and d** are the same

fields shown in **panels a and b**, respectively, but were captured by phase contrast microscopy. Note that the shRNA targeted to EGFP effectively inhibited expression of GFP.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 6. Lentiviral knock-down of EGFP expression in chicken β -actin promoter driven EGFP (ch β A-EGFP) transgenic rat trophoblast using EGFP shRNA
 ch β A-EGFP transgenic rat blastocysts constitutively expressing EGFP were incubated with lentiviral constructs expressing shRNAs specific to EGFP or control sequences. Fluorescence images from cryosections of gestation d18.5 placentation sites. **Panel a)** no manipulation; **Panel b)** blastocyst transfer without exposure to virus; **Panel c)** blastocyst transfer following transduction with virus delivering an shRNA targeting EGFP (pLKO.1-shEGFP); **Panel d)** blastocyst transfer following transduction with virus delivering a control

shRNA (pLKO.1-shSCR). **Panel e**) Western blot analysis of GFP from placental lysates derived from blastocysts transduced with a control shRNA (pLKO.1-shSCR, **lane 1**) or an shRNA targeting EGFP (pLKO.1-shEGFP, **lane 2**). Note the trophoblast-specific knockdown of EGFP in placentas derived from ch β A-EGFP blastocysts receiving EGFP shRNA.