

Lentivirus as a Tool for Lineage-Specific Gene Manipulations

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Summary: The trophoctoderm (TE) of blastocysts, the first epithelium established in mammalian development, (1) plays signaling, supportive, and patterning functions during preimplantation development, (2) ensures embryo implantation into the uterine wall, and (3) gives rise to extraembryonic tissues essential for embryo patterning and growth after implantation. We show that mouse TE, itself permissive to lentiviral (LV) infection, represents a robust nonpermeable physical barrier to the virus particles, thereby shielding the cells of the inner cell mass from viral infection. This LV feature will allow modulations of gene expression in a lineage-specific manner, thus having significant applications in mouse functional genetics. *genesis* 45:456–459, 2007.

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Key words: lentivirus vector; preimplantation development; gene delivery; mouse; placenta; trophoblast; tetraploid aggregation

INTRODUCTION

The epithelia and endothelia of multicellular organisms play a crucial role in body compartmentalization, fluid transport, and protection against damaging environmental factors and pathogenic organisms. In many cases, a loss of epithelial or endothelial integrity has been shown to be a cause of diseased state in humans (Mullin *et al.*, 2005; Tucker and Compans, 1993).

In early mouse embryogenesis, the fertilized oocyte goes through three successive rounds of cleavage to form, by 2.5 days post coitum (dpc), an 8-cell embryo which then undergoes a compaction process dependent on transmembrane protein E-cadherin (De Vries *et al.*, 2004). Two subsequent rounds of cell division produce a 32-cell morula, with the outer cells acquiring epithelial characteristics such as the onset of tight junctions. These cells form the trophoctoderm (TE) by around 3.5 dpc, a single-cell epithelial layer surrounding the newly

formed blastocoel and the inner cells mass (ICM). By 4.5 dpc, the ICM gives rise to the epiblast and primitive endoderm (PE), which covers the epiblast and separates it from expanded blastocoel.

Because the TE represents an epithelium, we hypothesized that it might be nonpermeable for virus particles. To address this possibility, the HIV-derived replication-deficient lentivirus LVTHM expressing GFP (Wiznerowicz and Trono, 2003) was produced and incubated with either 4- to 8-cell embryos (1.5–2.5 dpc) in which no onset of compaction was evident or early blastocysts (3.5 dpc) that had already developed a clearly distinguishable blastocoel. Removal of zona pellucida was a prerequisite for successful infection in our experiments, consistent with the previous observation (Pfeifer *et al.*, 2002).

In 4.5 dpc embryos transduced at the early blastocyst stage (3.5 dpc), GFP expression was observed exclusively in the polar TE (pTE), the TE adjacent to the ICM/epiblast, and the mural TE (mTE) but never in the epiblast or PE (Fig. 1a and Supplementary Fig. 1). More than 150 infected blastocysts were examined and none showed a detectable GFP signal outside the TE layer. In contrast, 4.5 dpc embryos transduced at the 4- to 8-cell stage showed GFP expression in both the TE and ICM-derived epiblast and PE (Fig. 1b), suggesting a lack of intrinsic lentivirus (LV) silencing in non-TE lineages. The

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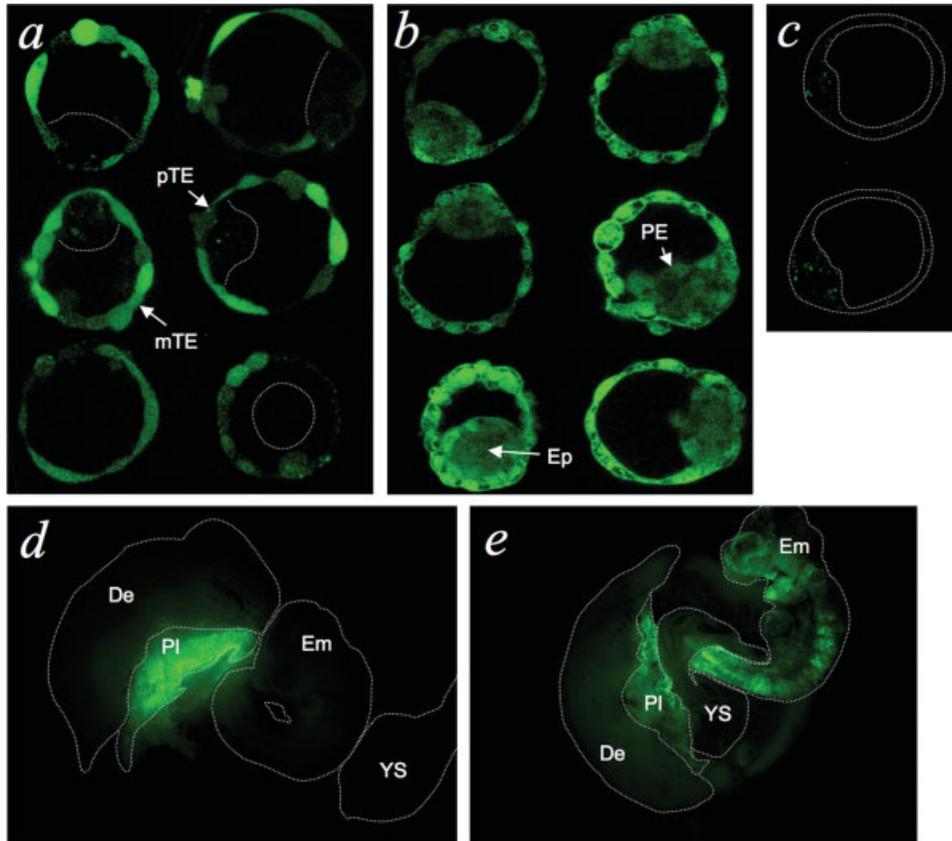


FIG. 1. Selective lentiviral (LV) transduction of the trophectoderm (TE) and its derivatives. Confocal images of 4.5 dpc blastocysts infected with a GFP-expressing LV (LVTHM), at early blastocysts (3.5 dpc) (a) or at 4- to 8-cell stage (1.5–2.5 dpc) (b). Noninfected 4.5 dpc blastocysts are shown as a negative control (c). For postimplantation analysis, preimplantation embryos were infected with LVTHM at 3.5 dpc (d) or 1.5–2.5 dpc (e), transferred to pseudopregnant foster females at 3.5 dpc, dissected at 10.5 dpc, and analyzed under fluorescent stereomicroscope. Note that, in blastocyst-infected embryos, GFP is detectable exclusively in the pTE-derived placenta (PI, cross-cut) and not in epiblast (Ep)- and primitive endoderm (PE)-derived embryo proper (Em) and visceral yolk sac (YS), respectively. The TG cells, descendants of mTE, located on the surface of the Reichert membrane were frequently GFP positive (data not shown). De, decidua. Dotted lines in (a) and (c) depict the location of the epiblast/PE/TE cells within the blastocysts.

absence of bias toward LV silencing in a particular embryonic or extraembryonic lineage, including epiblast and PE, has been previously reported (Pfeifer *et al.*, 2002).

After implantation, the pTE continues proliferation to form the extraembryonic ectoderm and ectoplacental cone. These cells will eventually give rise to the spongiotrophoblast and labyrinth compartments of the placenta. mTE cells cease proliferation and become polyploid trophoblast giant (TG) cells located on the outer surface of the Reichert membrane. To trace the fate of GFP posi-

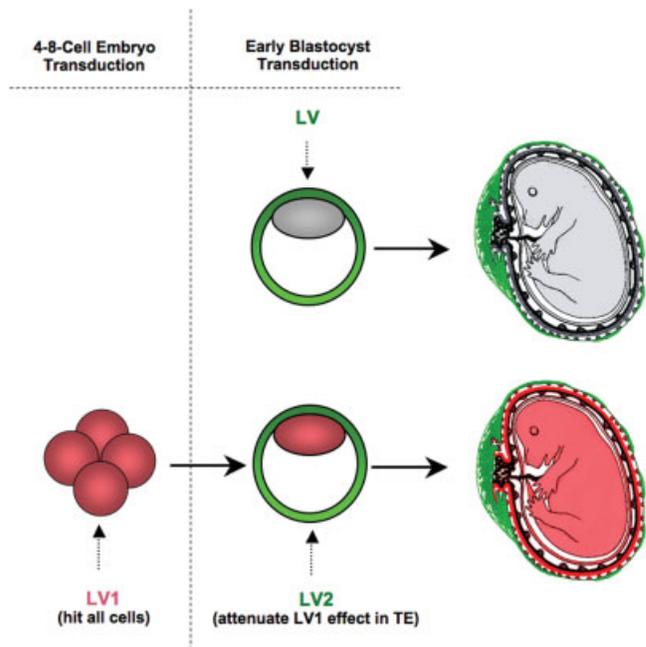


FIG. 2. Scheme illustrating an implementation of the observed inability of replication-deficient LVs to infect cells beyond the TE layer. A simple one-step transduction enables the targeting of LV-encoded DNAs or interfering RNAs specifically to the TE and placenta, leaving ICM- and PE-derived tissues unaffected (upper row). A two-step approach can be used to perform a reciprocal manipulation, consisting of targeting only the ICM and its derivatives (lower row). LV-infected cells are depicted in color, green or pink, and non-infected cells are gray. See text for further details.

tive cells after implantation, embryos infected with LVTHM at different stages of the preimplantation period were retransferred into pseudopregnant foster females, dissected together with the extraembryonic membranes and placenta at 10.5 dpc, briefly fixed, and examined under fluorescent stereomicroscope. Consistent with the results described earlier, GFP expression of blastocyst stage-transduced embryos was detected exclusively in TE derivatives, i.e. TG cells on the Reichert membrane (data not shown) and in the labyrinth/spongiotrophoblast of the placenta (Fig. 1d). A total of 40 blastocysts transferred following transduction, of which 25 (62%) showed GFP expression at 10.5 dpc, which was confined exclusively to the placenta or Reichert membrane, or both. The placenta at this developmental stage comprises cells of both trophoblast and embryonic origins. However, the lack of GFP expression in the embryo proper and allantois suggests that the GFP-positive cells are derived exclusively from the infected TE. Consistent with blastocyst analysis, embryos infected at the 4- to 8-cell stage showed a widespread distribution of GFP-positive cells, including the embryo proper (Fig. 1e). Again, the results suggest a lack of intrinsic LV silencing in ICM-derived tissues.

Before the retransfer, 3.5 dpc blastocysts were incubated with the LV for 2–4 h (see Materials and Methods), whereas 4- to 8-cell embryos were infected overnight and cultured for another day before the retransfer. To rule out the contribution of different infection/incubation periods to the distinct GFP patterns, we performed overnight infection of 3.5 dpc blastocysts and cultured them for another day. We observed a stable maintenance of GFP expression in the TE and besides, its absence in the ICM-derived epliblast and PE of 5.5 dpc blastocysts (Supplementary Fig. 2).

Next, we analyzed embryos and placentas near the term of the pregnancy (18.5 dpc), following retransfer of LVTHM-infected 3.5 dpc blastocysts. First, we found that LV-driven GFP expression is stably maintained in placenta until 18.5 dpc, whereas embryos proper and ICM-derived extraembryonic membranes (not shown) were strictly negative (Supplementary Fig. 3a). Perhaps most important, we found that integrated LV was readily detectable in the placenta but absent in the genomic DNA of embryos proper (Supplementary Fig. 3b). These results suggest that the absence of detectable GFP in the embryos proper is not due to an epigenetic silencing of the LV but due to the absence of integrated LV, originating from the inability of the LV to infect the ICM at the blastocyst stage.

In this article, we confirm the previously reported possibility of stable transduction of preimplantation embryos with LVs (Pfeifer *et al.*, 2002). More importantly, we provide compelling evidence that the TE, as can be anticipated from its epithelial nature, can serve as a robust natural barrier to LV particles, thereby protecting ICM cells against infection. On the other hand, further dissemination of LV from the infected TE or its derivatives could not occur, owing to the replication-

deficient nature of this virus. To note, lentivirus-based delivery of a transgene to the placenta of Rhesus monkey has also been reported (Wolfgang *et al.*, 2001). More recently, there was a report showing the use of lentiviruses for trophoblast-specific rescue of several mouse placental defects (Okada *et al.*, 2007). Intriguingly, the observed inability to traverse the TE is unlikely to be limited solely to LVs but a property shared by other mammalian viruses. For instance, we have found that this is also fully valid for adenovirus 5 (Supplementary Fig. 4), thus extending the range of laboratory and clinical tools for applications outlined later.

Molecular and cellular TE components that constitute the nonpermeable barrier for virus particles are to be defined. However it is plausible that the E-cadherin-mediated or tight junction or both types of intercellular contacts contribute to this barrier (De Vries *et al.*, 2004). Supporting this notion, E-cadherin has been shown to be essential for the maintenance of airway epithelium integrity and protection against adenovirus infection (Man *et al.*, 2000).

Our finding holds a substantial promise with regard to lineage-specific gene manipulation in the laboratory mouse and possibly to gene therapy in the human placenta. For instance, the virus-based approach should provide a significantly less time- and effort-consuming alternative to tetraploid aggregation, the method commonly used to distinguish the embryonic versus extraembryonic functions of genes during mouse development (Nagy *et al.*, 1990; Tanaka *et al.*, 2001). As illustrated in Figure 2 (upper part), when cDNA-encoding or interfering RNA (such as small hairpin RNA, shRNA)-encoding replication-deficient LVs are applied to blastocysts, they can be used to constitutively express or downregulate a desired gene product in the TE and trophoblast of the placenta, respectively. In addition, the approach can be adapted to manipulate gene expression selectively in the ICM and its derivatives (Fig. 2, bottom part). In a transgenic scenario, embryos can be first transduced between the 4- and 8-cell stage to integrate cDNA into all cells. In the second round, once the embryos have reached the early blastocyst stage, the expression of introduced cDNA can be attenuated in the TE with LV encoding a complementary shRNA. This will additionally target the endogenous RNA, which, if desired, can be avoided by tagging the introduced cDNA with a neutral DNA sequence and designing shRNA against the tag. In the scenario aimed at gene silencing in the ICM or ICM-derived lineages, a shRNA-LV is introduced into all cells of embryo in the first round, and a cDNA-LV—in the second transduction rounds (Fig. 2, bottom). Again, the shRNA will interfere only with the endogenous RNA if the introduced cDNA deviates from the endogenous RNA in the shRNA-complementary sequence, for example in the third positions of nucleotide triplets. This virus-based lineage-specific approach can be further adapted to more specific needs, such as temporal control of gene expression, if combined with the Tet-system (Wiznerowicz and Trono, 2003).

MATERIALS AND METHODS

Preparation of Lentivirus Stock

The LVTHM (20 µg), pMD2G (5 µg), and packaging pCMV-dR8.74psPAX2 (5 µg) plasmids were cotransfected into 293T cells by calcium-phosphate method. Produced lentivirus was concentrated from supernatant by ultracentrifugation method, resuspended in KSOM, frozen in aliquots at -80°C , and titered using 293T cells as described previously (Wiznerowicz and Trono, 2003) (<http://tronolab.epfl.ch/>).

Transduction and GFP Visualization

Females of C57Bl6 or FVB mouse strains were superovulated and mated with congenic males. The 4-cell (1.5 dpc) and 8-cell (2.5 dpc) embryos were flushed from oviducts and early blastocysts (3.5 dpc) were flushed from uteri, respectively. To remove zona pellucida, embryos were incubated briefly in acidic tyrode solution, washed several times in M2 media drops, and incubated overnight with concentrated LVTHM (0.1×10^7 to 1×10^7 TU/ml) in KSOM drops under paraffin oil (37°C , 5% CO_2). Following the infection, the embryos were transferred into fresh KSOM and cultured, when appropriate, until 4.5 dpc, then fixed in 4% paraformaldehyde for 5–10 min at room temperature, and examined on the Leica PS2 confocal microscope, using GFP filter setting. For postimplantation embryo analysis, 4–8 cell embryos were also incubated with LVTHM overnight then transferred to into the uteri of pseudopregnant 2.5-dpc NMRI foster females when reached 3.5 dpc. However, 3.5 dpc blastocyst were incubated with the virus only for 2–4 h before being transferred into the foster uteri in the afternoon of the same day. This shortened incubation was sufficient for an efficient transduction, as revealed later. Embryos were dissected together with extra-embryonic membranes and placenta at 10.5 dpc (foster mother plug timing), fixed with 4% paraformaldehyde for 30 min, and examined under Leica MZ 16 FA fluores-

cent binocular microscope equipped with GFP optical filter set.

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LITERATURE CITED

- De Vries WN, Evsikov AV, Haac BE, Fancher KS, Holbrook AE, Kemler R, Solter D, Knowles BB. 2004. Maternal β -catenin and E-cadherin in mouse development. *Development* 131:4435–4445.
- Man Y, Hart VJ, Ring CJ, Sanjar S, West MR. 2000. Loss of epithelial integrity resulting from E-cadherin dysfunction predisposes airway epithelial cells to adenoviral infection. *Am J Respir Cell Mol Biol* 23:610–617.
- Mullin JM, Agostino N, Rendon-Huerta E, Thornton JJ. 2005. Keynote review: Epithelial and endothelial barriers in human disease. *Drug Discov Today* 10:395–408.
- Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E, Markkula M, Rossant J. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110:815–821.
- Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-Hora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M. 2007. Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer. *Nat Biotechnol* 25:233–237.
- Pfeifer A, Ikawa M, Dayn Y, Verma IM. 2002. Transgenesis by lentiviral vectors: Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci USA* 99:2140–2145.
- Tanaka M, Hadjantonakis AK, Nagy A. 2001. Aggregation chimeras. Combining ES cells, diploid and tetraploid embryos. *Methods Mol Biol* 158:135–154.
- Tucker SP, Compans RW. 1993. Virus infection of polarized epithelial cells. *Adv Virus Res* 42:187–247.
- Wiznerowicz M, Trono D. 2003. Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 77:8957–8961.
- Wolfgang MJ, Eisele SG, Browne MA, Schotzko ML, Garthwaite MA, Durning M, Ramezani A, Hawley RG, Thomson JA, Golos TG. 2001. Rhesus monkey placental transgene expression after lentiviral gene transfer into preimplantation embryos. *Proc Natl Acad Sci USA* 98:10728–10732.