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## Regulation of murine placentogenesis by the retroviral genes *Syncytin-A*, *Syncytin-B* and *Peg10*



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## ABSTRACT

The murine placenta has a trichorial structure with two multinucleated syncytiotrophoblast (SCT) layers representing a barrier between the maternal and fetal blood system. Genes of endogenous retroviruses and retrotransposon-derived *paternally expressed genes* (*Peg*), remnants of past infections and integrations in the genome, have essential functions in placentogenesis. Previous studies showed that the envelope genes *Syncytin-A* and *Syncytin-B* were essential for cell–cell fusion of the SCT. The goal of this study was to analyze the temporal localization and expression of nine genes throughout placental development from embryonic day (E)8.5 to E18.5 using *in situ*-hybridization and absolute RNA-quantification. These included a comparison of previously characterized genes from the labyrinth *Syncytin-A*, *Syncytin-B*, *Gcm1*, the junctional zone *PL-1*, *PL-2*, *Plf*, *Tpbpa* with two further characterized genes *Peg10* and *Tpbpb*. *Syncytin-A* and *Syncytin-B* RNA localized to SCT-I and SCT-II, respectively. *Peg10* RNA localized to all extraembryonic tissues, specifically to the parietal and sinusoidal TGC of the labyrinth layer, which is in contact with SCT-I and the maternal blood. All three retroviral/retrotransposon-derived genes showed the highest expression at E16.5, but *Peg10* with 188,917.1 molecules/ng cDNA was 208-fold and 106.8-fold higher expressed than *Syncytin-A* and *Syncytin-B*, respectively. *Tpbpb* localized to the junctional zone and showed the highest expression at E16.5 along with *PL-2*, *Plf*, *Tpbpa*, but not *PL-1*, which decreased in expression at E10.5. To investigate a role of *Syncytin-A*, *Syncytin-B* and *Peg10* in cell–cell fusion, we established a cell culture system with fractionated primary trophoblasts from murine placentae. Culturing trophoblasts for up to 72 h partly resembled trophoblast development *in vivo* according to the nine marker genes. Knockdown of *Syncytin-A* demonstrated a functional regulation of cell–cell fusion, where knockdown of *Peg10* showed no involvement in cell fusion. Due to the expression of *Peg10* in TGCs, we propose an essential functional role in the fetal–maternal blood system.

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### 1. Introduction

The mammalian placenta is an organ formed during development by both fetal and maternal tissue. The viability and health of the embryo is dependent on the essential function of the placenta. Apart from the transport of nutrients, waste products and gases, the placenta is also a hormone producing tissue and a barrier against the maternal immune system (Cross et al., 2003; Rawl and Cross, 2008).

Although, both the murine and human placentae are chorio-allantoic and hemochorial they show clear differences. The mouse placenta represents a labyrinthine structure, whereas the human placenta is of a villous type (Georgiades et al., 2002). In addition, the trophoblast portion of the mouse placenta has three layers (trichorial), whereas the human is monochorial. For example, the mouse labyrinth layer where maternal and fetal blood interfaces, is separated by different trophoblast subtypes (Cross et al., 1994). The polar ectoderm forms the extraembryonic ectoderm and ectoplacental cone. The cells of the latter tissues then proliferate and differentiate into four specific trophoblast subtypes of the murine placenta, the spongiotrophoblast (ST), glycogen trophoblast (GlyT), syncytiotrophoblast (SCT) and trophoblast giant cell (TGC) (Cross et al., 1994; Simmons and Cross, 2005). There are

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different ways of TGC differentiation: First, primary TGCs differentiate from mural trophoectoderm, which is not in contact with the inner cell mass; TGCs can also be derived from the extra-embryonic ectoderm, the ectoplacental cone and from ST (Cross et al., 1994; Simmons et al., 2007). Several subtypes of TGCs are known, like the sinusoidal, parietal, canal and spiral artery-associated TGCs, which differ in function and location (Simmons et al., 2007). TGCs are essential for the production of hormones, like members of prolactin and placental lactogens *PL-1* (*Pr13d1*), *PL-2* (*Pr13b1*) and *Plf* (*Pr12c2*) at different developmental time points (Adamson et al., 2002; Faria et al., 1990; Yamaguchi et al., 1994). Progenitors in the ectoplacental cone are characterized by the expression of the marker genes *trophoblast specific protein alpha* (*Tpbpa*) and the rather unknown *trophoblast specific protein beta* (*Tpbpb*) both localized on chromosome 13 and were expressed in STs and GlyTs (Carney et al., 1993; Scott et al., 2000).

The labyrinth layer represents the barrier of maternal and fetal blood systems and is formed by two multinucleated SCT layers, SCT-I and SCT-II and a sinusoidal TGC that line the maternal sinusoids (Hernandez-Verdun, 1974). Fusion of trophoblasts into the SCT layers is one of the most important processes during placentogenesis. Ectopic expression of the transcription factor *Gcm1* (*glial cells missing homolog*) in the labyrinth led to a halt in proliferation and initiated intra-trophoblast cell–cell fusion (Hughes et al., 2004).

Almost 40% of the mouse genome consists of transposable elements (Stocking and Kozak, 2008). Endogenous retroviruses (ERVs) account for ~10% of the murine genome and represent a family of transposable elements, which were derived from exogenous retroviral infection of germ line cells (Gifford et al., 2008; Stoye, 2009). ERVs represent eight different families with a genomic structure similar to exogenous retroviruses: 5'LTR-gag-pol-env-3'LTR. Many of these ERVs have undergone gene inactivating mutations over time, however some ERV genes remained functionally active (Stocking and Kozak, 2008). Following the identification of the human ERV *env* (*envelope*) genes *Syncytin-1* and *Syncytin-2*, two functionally similar, but not orthologous murine ERV *env* genes *Syncytin-A* and *Syncytin-B* were detected. *Syncytin-A* and *Syncytin-B* represent single genes and are located on chromosomes 5qG2 and 14qD1, respectively. Amino acid alignment between *Syncytin-A* and *Syncytin-B* showed a 67% sequence identity (Dupressoir et al., 2005). *Syncytin-A* and *Syncytin-B* were expressed in the SCT-I and SCT-II of the labyrinth layer of murine placentae, respectively and in the brain (Dupressoir et al., 2005; Simmons et al., 2008a). Knockout mice for *Syncytin-A* (*Syncytin-A*<sup>-/-</sup>) resulted in embryonic lethality between E11.5 and E13.5, supporting an essential role for embryogenesis and placentogenesis (Dupressoir et al., 2009). In contrast to *Syncytin-A*<sup>-/-</sup> embryos, *Syncytin-B*<sup>-/-</sup> embryos were viable, however exhibited a reduction in neonate number along with growth restriction (Dupressoir et al., 2011). Importantly, embryos of double knockout mice for *Syncytin-A* and *Syncytin-B* died as early as E9.5 and demonstrated incomplete placental SCT fusions (Dupressoir et al., 2011). Transfection of a trophoblastic stem cell (TS) line with *Syncytin-A* in the presence of FGF4 showed an increase of the cell–cell fusion index after 4–7 days in cell culture (Gong et al., 2007). Blocking the function of *Syncytin-A* either with an antibody to *Syncytin-A* or antisense oligonucleotides decreased cell–cell fusion (Gong et al., 2007).

Another retroviral element in the murine genome is the retrotransposon-derived gene *Peg10* (*paternally expressed gene 10*) on chromosome 6. The mouse *Peg10* and its human orthologue *PEG10* are maternally imprinted and both implement a (–1)–frameshift, which results in 2 gag-/pol-like proteins reading frame (RF)1 and RF2 with an identical N-terminus (Lux et al., 2005, 2010; Shigemoto et al., 2001). Analysis of the mouse *Peg10* showed

homologies to long terminal repeat (LTR) retrotransposons, but is not considered an active retrotransposon (Ono et al., 2001, 2003, 2006). *Peg10* was shown to be expressed in mouse placenta (Ono et al., 2001, 2003). Heterozygous knockout *Peg10*<sup>+/-</sup> mice demonstrated small placentas with placental defects at E9.5–10.5 and embryonic growth restriction and early lethality at E10.5 (Ono et al., 2006). For example, analysis of markers for different trophoblast subtypes showed normal TGC differentiation, but absent STs and no development of the labyrinth layer (Ono et al., 2006).

Protein and RNA expression of murine genes important for placentogenesis including *Syncytin-A*, *Syncytin-B*, *Gcm1*, *PL-1*, *PL-2*, *Plf* and *Tpbpa* have been demonstrated throughout developmental stages (Dupressoir et al., 2005; Lee et al., 1988; Lescisin et al., 1988; Simmons et al., 2007, 2008b). However, to date the localization and expression patterns of *Peg10* and *Tpbpb* have only been analyzed in early developmental stages (Ono et al., 2006; Scott et al., 2000). In the present investigation we performed analyses of murine placentae from stage E8.5 to E18.5 for all genes stated above, including *Peg10* and *Tpbpb*, in order to determine a complete development expression pattern by comparing the cellular localization with absolute quantification of gene expression. Therefore, our analyses compared *Peg10* and *Tpbpb* in context with other previously characterized genes. In addition, we established a primary cell culture system of murine trophoblasts fractionated from placentae, which was comparable to our previous cell culture system of human villous trophoblasts (Langbein et al., 2008; Ruebner et al., 2012). Using fractionated murine trophoblasts we analyzed and compared their differentiation *in vitro* along with placental expression of all nine genes from embryonic stages as well as directly addressed a functional role of *Syncytin-A*, *Syncytin-B* and *Peg10* in differentiation and cell–cell fusion.

## 2. Materials and methods

### 2.1. Mouse placenta preparation

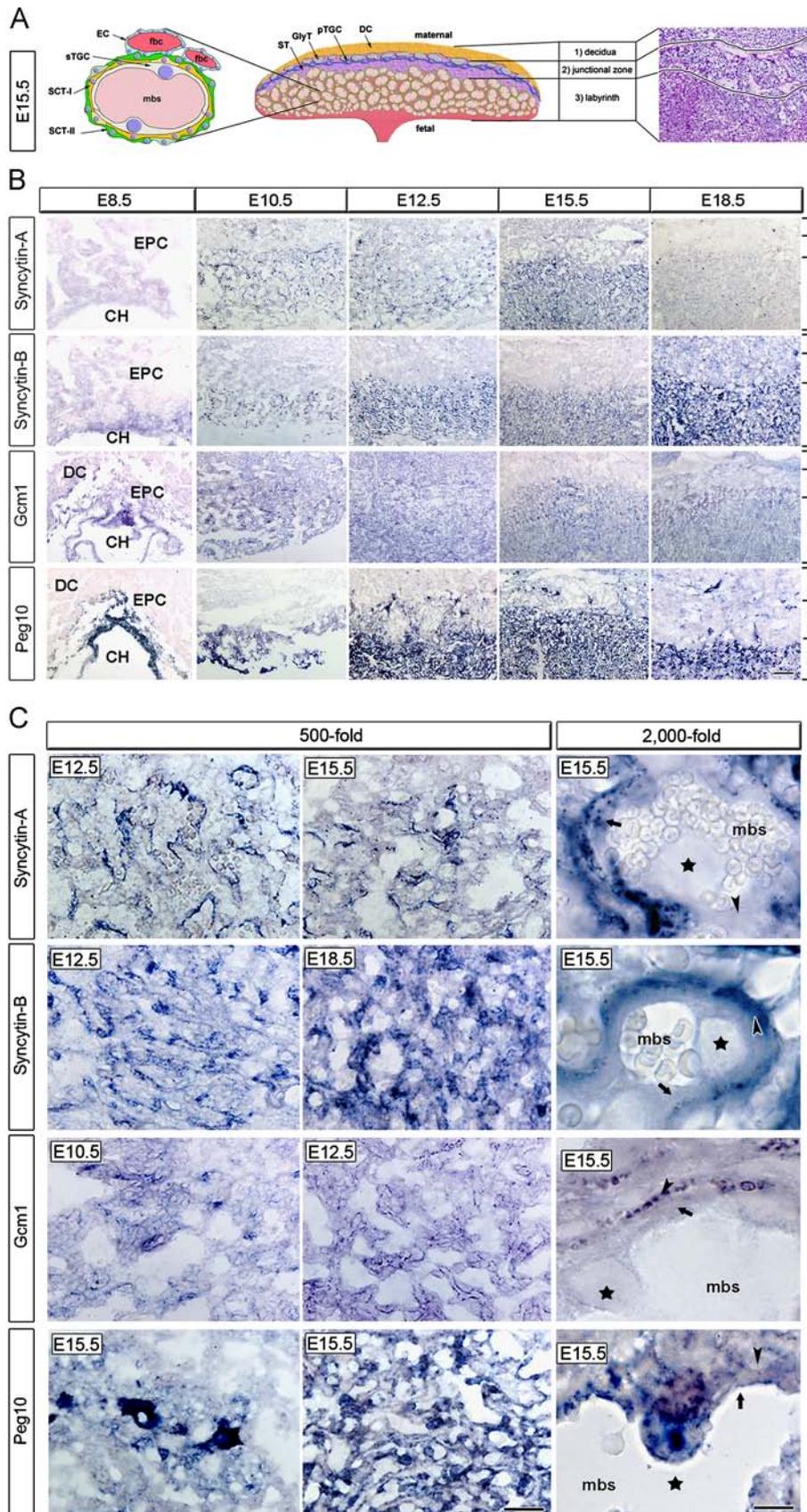
Pregnant wild-type mice of the *C57BL/6J* and *C3H* strains were kindly provided from the groups of Drs. Wegner and Villmann, Institute of Biochemistry of the Friedrich-Alexander-University Erlangen-Nuremberg. The animal experiments were performed in strict accordance with the protocol, which was approved by the Committee on the Ethics of Animal Experiments of the University of Erlangen-Nuremberg (Permit Number: TS-00/12- Biochemistry II). Placentae of E8.5–E18.5 were prepared and snap frozen in liquid nitrogen for RNA-isolation or fixed in 4% PFA for frozen sections. For trophoblast isolation placentae of E14.5 were used.

### 2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from whole snap frozen placentae and cells from culture according to previously published methods (Langbein et al., 2008; Strick et al., 2007). For expression analysis, RNA was pre-treated with DNaseI (Sigma-Aldrich, Germany) and cDNA was generated with High Capacity cDNA Kit (Applied Biosystems (ABI), Germany) in a thermal cycler (ABI2720) for 2 h at 37 °C.

### 2.3. Absolute quantitative real time PCR

For absolute quantitative real time PCR (qPCR) cDNA fragments of *Syncytin-A*, *Syncytin-B*, *Gcm1*, *Peg10*, *PL-1*, *PL-2*, *Plf*, *Tpbpa* and *Tpbpb* were amplified with specific primers (suppl. Table 1) and cloned into the pSC-A-vector (Stratagene, Germany).



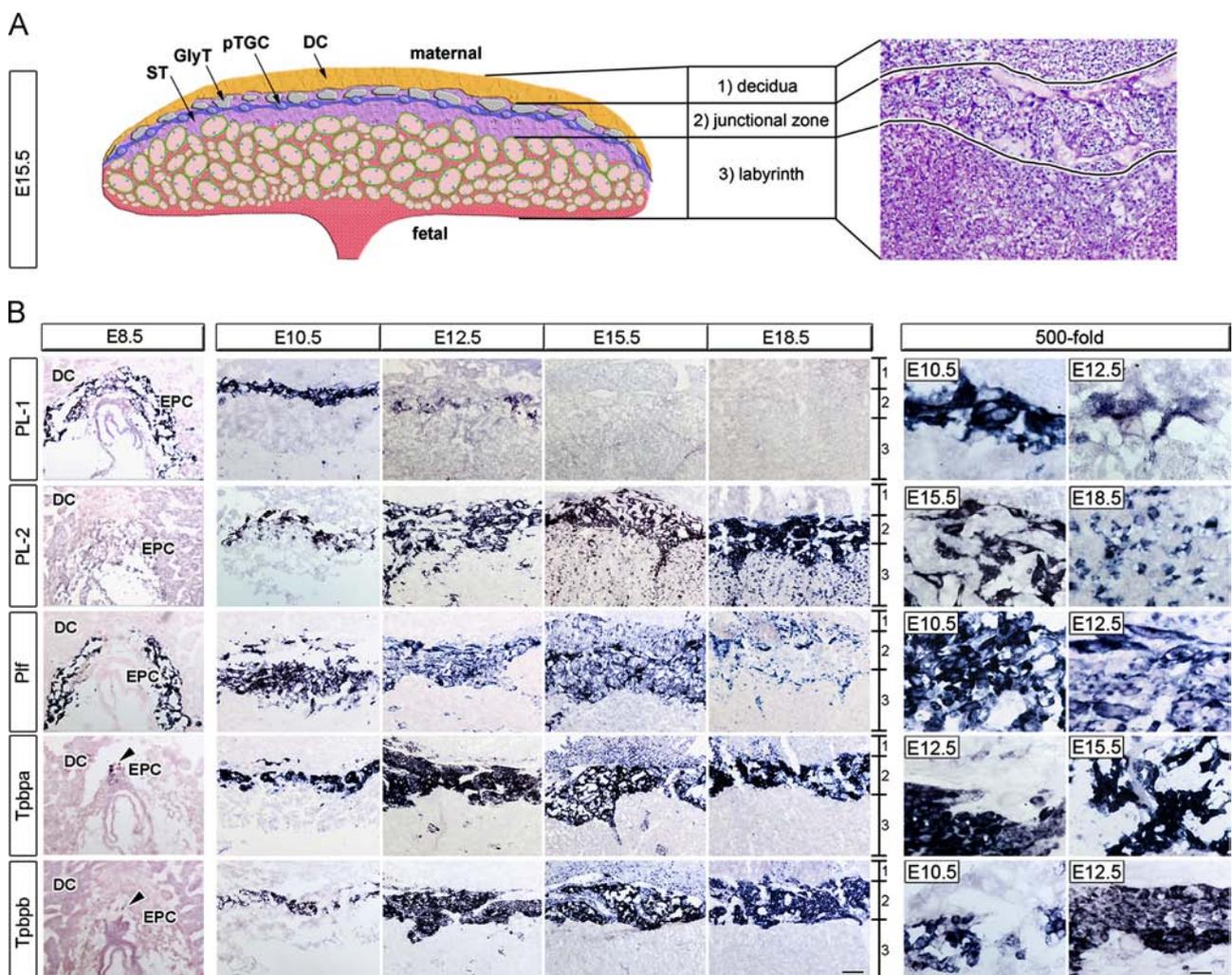
**Fig. 1.** Localization of marker genes in the murine placenta by ISH: (A) center schematic drawing shows an overview of the structure of the murine placenta at E15.5 with decidua (1), junctional zone (2) and labyrinth (3) correlated with a hematoxylin/eosin stained tissue section (right). DC, decidua cells; pTGC, parietal trophoblast giant cells; GlyT, glycogen trophoblasts; ST, spongiotrophoblasts. Top left schematic drawing shows that within the labyrinth the maternal blood space (mbs) is surrounded by a sinusoidal trophoblast giant cell (sTGC) and two layers of syncytiotrophoblasts (SCT-I and SCT-II). Adjacent to SCT-II are endothelial cells (EC) of the fetal blood capillaries (fbc). (B) *In situ* hybridization for *Syncytin-A*, *Syncytin-B*, *Gcm1* and *Peg10* at E8.5, E10.5, E12.5, E15.5 and E18.5. Panels at different stages represents a 100-fold magnification with the decidua (1), junctional region with TGCs, GlyTs, and STs (2), and the labyrinth layer (3) (scale bar=400  $\mu$ m). (C) 500-fold and 2000-fold magnifications. Scale bar 500-fold=80  $\mu$ m, scale bar 2000-fold=20  $\mu$ m. DC=decidual cells, EPC=ectoplacental cone, CH=chorion, and mbs=maternal blood space.

To quantitate all genes from placentae and fractionated primary trophoblasts qPCR based on SYBR-green technology with specific primers and 40 ng cDNA/well were used with an ABI7300 (suppl. Table 1). 18SrRNA amplification was used for normalization of each sample with 1 ng cDNA/well according to previously published methods (Ruebner et al., 2010). Known copy numbers of the cloned genes were used to generate a standard curve with the cycle threshold (CT) value against the log of the amount standard. The standard curves of all genes were used to calculate the absolute copy number of gene expression for each sample in molecules (mol) per ng cDNA (suppl. Table 2).

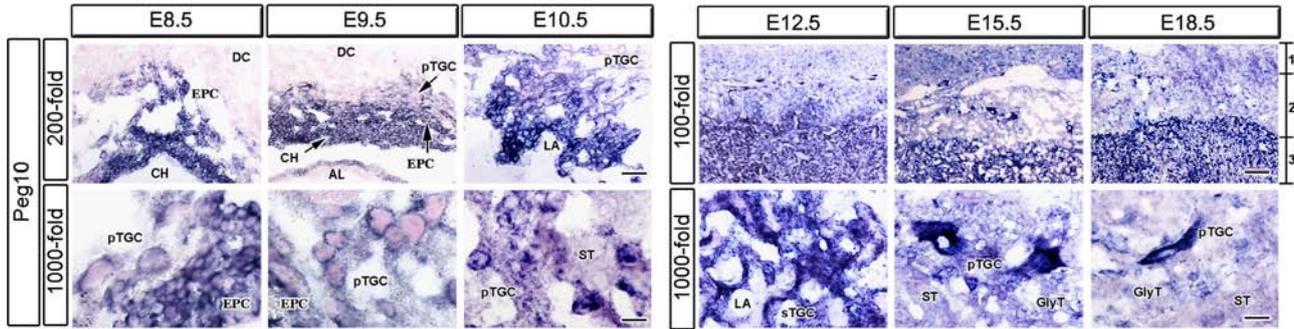
2.4. *In situ hybridization (ISH)*

Digoxigenin (DIG)-labeled antisense and sense RNA probes were generated for *Syncytin-A*, *Syncytin-B*, *Gcm1*, *Peg10*, *PL-1*, *PL-2*, *Plf*, *Tpbpa*, and *Tpbpb*. Plasmids were linearized with restriction enzymes and RNA probes were transcribed and DIG-labeled with a RNA-Labeling Kit (Roche, Germany). All probes were then pre-treated with DNaseI (Sigma-Aldrich). Placentae at E8.5 till E18.5 were collected in PBS, fixed overnight in 4% PFA at 4 °C, washed in PBS, incubated for 24 h in 30% sucrose and embedded in Tissue-

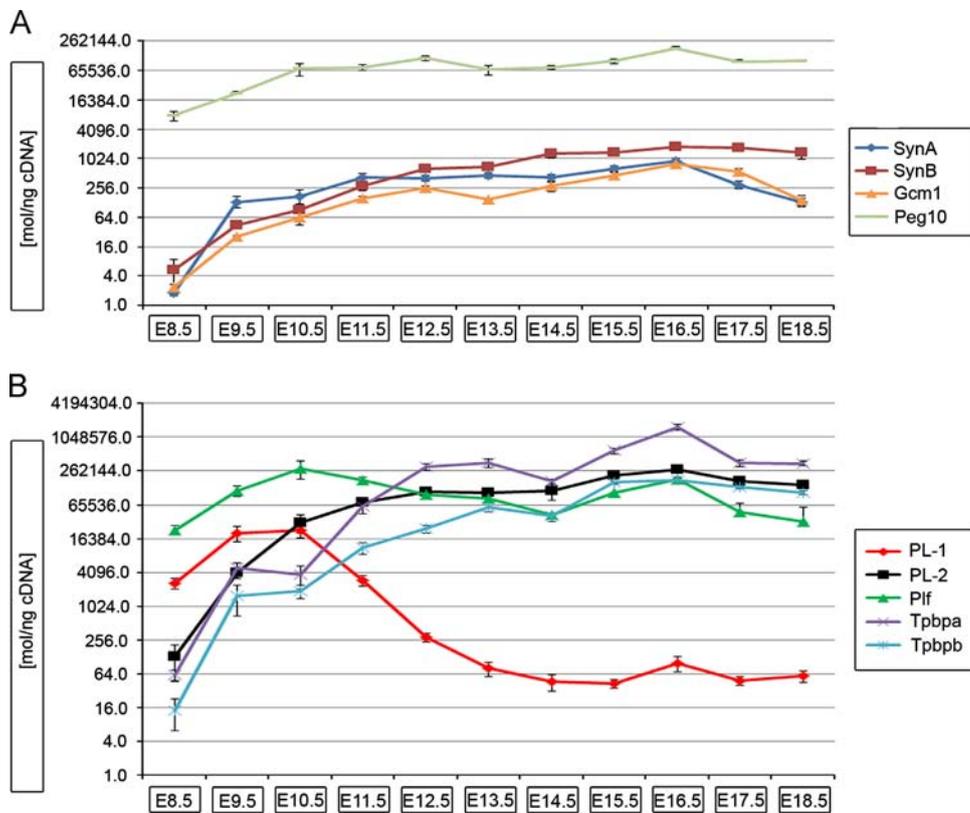
Tek (Sakura, Netherlands). Frozen tissue sections (12 μm) were incubated with Proteinase K (50 μg/ml; Roche), washed in PBS and fixed with 4% PFA and then incubated with a prehybridization buffer (50% formamide, 50 μg/ml heparin, 50 μg/ml yeast RNA, 2% Blocking Powder (Roche), 0.1% Tween, 5 × SSC, 5 mM EDTA) for 2 h at 68 °C. Following the prehybridization step, tissue sections were then incubated in a hybridization buffer (50% formamide, 0.3 M NaCl, 50 mM Tris-HCl pH8.0, 10% Dextran Sulfate, 1 × Denhardt's, 0.5 mg/ml yeast RNA) with the linearized DIG-labeled RNA probes at 68 °C overnight. Three post-hybridization washes followed including wash buffer 1 (50% formamide, 0.1% Tween20, 1 × SSC, 1 × PBS) at 68 °C for 1 h, wash buffer 2 (0.2 × SSC, 1 × PBS) for 15 min at RT and then with a 1 × PBS wash for 15 min at RT. Tissue sections were blocked with a Blocking solution (0.5% Blocking Powder (Roche), 10% FCS, 0.1% Tween20, 1 × PBS) for 1 h at RT and incubated with the Anti-Digoxigenin-AP, Fab-Fragments (1:200; Roche) in blocking buffer (0.5% Blocking Powder (Roche) in 1 × PBS) for 2 h at RT. Slides were washed two times in wash buffer 3 (1 × PBS, 0.1% Tween20) for 30 min and two times with 1 × PBS for 15 min. A final wash was performed with NTMTw (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl<sub>2</sub>, 0.1% Tween20) for 5 min and incubated with NBT/BCIP (Roche) in NTMTw with 2 M Levamisol (Sigma-Aldrich) overnight at RT. The



**Fig. 2.** Localization of marker genes in the murine placenta by ISH: (A) schematic drawing shows an overview of the structure of the murine placenta at E15.5 with decidua (1), junctional zone (2) and labyrinth (3) correlated with a hematoxylin/eosin stained tissue section (right). DC, decidua cells; pTGC, parietal trophoblast giant cells; GlyT, glycogen trophoblasts; ST, spongiotrophoblasts. (B) *In situ* hybridization for *PL-1*, *PL-2*, *Plf*, *Tpbpa*, and *Tpbpb* at E8.5, E10.5, E12.5, E15.5 and E18.5. Panels at different stages represents a 100-fold magnification with the decidua (1), junctional region with TGCs, GlyTs, and STs (2), and the labyrinth layer (3) (scale bar=400 μm). Right panels show 500-fold magnification. Scale bar 500-fold=80 μm. DC=decidual cells, EPC=ectoplacental cone, and TGC=trophoblast giant cells.



**Fig. 3.** Localization of the placental marker gene *Peg10* at stages E8.5, E9.5, E10.5, E12.5, E15.5 and E18.5 by ISH. ISH experiments for early and later mouse placental stages. Panels at E8.5 till 10.5 represent a 200-fold and 1000-fold magnification and E12.5 till E18.5 a 100-fold and 1000-fold magnification with the decida (1), junctional region with TGCs, GlyTs, and STs (2), and the labyrinth layer (3). DC=decidual cells, EPC=ectoplacental cone, CH=chorion, pTGC=parietal trophoblast giant cells, sTGC=sinusoidal trophoblast giant cells (scale bar 100-fold=400  $\mu$ m; scale bar 200-fold=200  $\mu$ m; scale bar 1000-fold=40  $\mu$ m).



**Fig. 4.** Gene expression of placental marker genes during murine development from E8.5 to E18.5. Histogram represents the mean expression from 4 to 6 placentae in molecules per ng cDNA (mol/ng cDNA)  $\pm$  SEM for each day of gestation from E8.5 to E18.5 of *Synctin-A*, *Synctin-B*, *Gcm1* and *Peg10* (A), and *PL-1*, *PL-2*, *Plf*, *Tpbpa* and *Tpbpb* on a logarithmic scale (B). All values are listed in supplemental Table 3.

reaction was stopped with KBTBw (50 mM Tris pH 7.5, 10 mM KCl, 1% Tween, 150 mM NaCl). Tissue sections were counterstained with nuclear fast red (Sigma-Aldrich) and mounted in Mowiol (Roth, Germany).

### 2.5. Fractionation and culturing of mouse placental trophoblast

Placentae from mice at E14.5 were harvested and removed from the uterine wall and the embryo. After removal of the maternal decida, placentae were cut in pieces and enzymatically digested with a final concentration of 0.27 mg/ml Trypsin (Invitrogen, Germany), 0.6 U/ml Dispase (Roche) and 0.14 mg/ml DNaseI (Roche) per gram tissue at 37 °C. After 30 min a further digestion with 0.07 mg/ml DNaseI (Roche) and 0.28 mg/ml Trypsin (Invitrogen) was performed

and further incubated for 60 min at 37 °C on a MACS-rotator (Miltenyi Biotec, Germany). Tissue digestion was monitored microscopically to ensure a complete digestion for the enrichment of single cells. To obtain single cells the cell suspension was drawn through sterilized Pasteur pipettes and then loaded and separated on a Percoll-step gradient (Easycoll, Biochrom, Germany) with densities of 1.08, 1.06, 1.05, 1.04, 1.028 and 1.005 g/ml. Density gradient centrifugation was carried out at 720g for 30 min. The cellular fraction between 1.05 and 1.06 g/ml density was recovered and washed in DMEM (Zuckermann and Head, 1986). Using FACS analysis (FACS Canto II, BD Biosciences) fractionated cell populations were characterized for cellular viability and specific antibody staining. Propidium iodide (PI) staining showed 87.5% live cells after fractionation (PI-negative) (suppl. Fig. 1). A mean of 86.8% of the live cells were

positive for cytokeratin 7 (CK7) using a CK7-conjugated PE antibody (Santa Cruz Biotechnology, Germany) representing trophoblasts. The cell population also consisted of a mean of 6.75% CD45-APC (eBiosciences, Germany) positive leukocytes and 0.42% CD34 positive cells demonstrating a minimal amount of placental endothelial and hematopoietic stem cells (eBiosciences, Germany) (suppl. Fig. 1). We attribute the remaining 6.03% CK7 negative cells to erythrocytes and fibroblasts. For cell culture 30,000 viable cells were seeded per cm<sup>2</sup> culture dish with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal calf serum, 20 mM Hepes, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and non-essential amino acids and cultured in a humidified 5% CO<sub>2</sub> environment at 37 °C. Following 6 h after seeding, the media was changed to remove any non-adherent cells and debris and thereafter changed every 24 h.

### 2.6. siRNA mediated knockdown of Syncytin-A, Syncytin-B and Peg10

Sixty nM siRNA specific for *Syncytin-A*, *Syncytin-B*, *Peg10* (Silencer<sup>®</sup> select pre-designed, Ambion, Life technologies) were transfected directly after trophoblast fractionation in suspension with HiPerFect (Qiagen, Germany) according to manufactures protocols and Strick et al. (2007) and cultured in DMEM as described. 60 nM siRNA was determined as the most efficient concentration to achieve maximum inhibition but still representing the highest cell viability, as tested with Trypan blue. The transfection media was removed after 15 h and the cells were harvested after a total of 24 h. Mock siRNA (Alexa488 scrambled

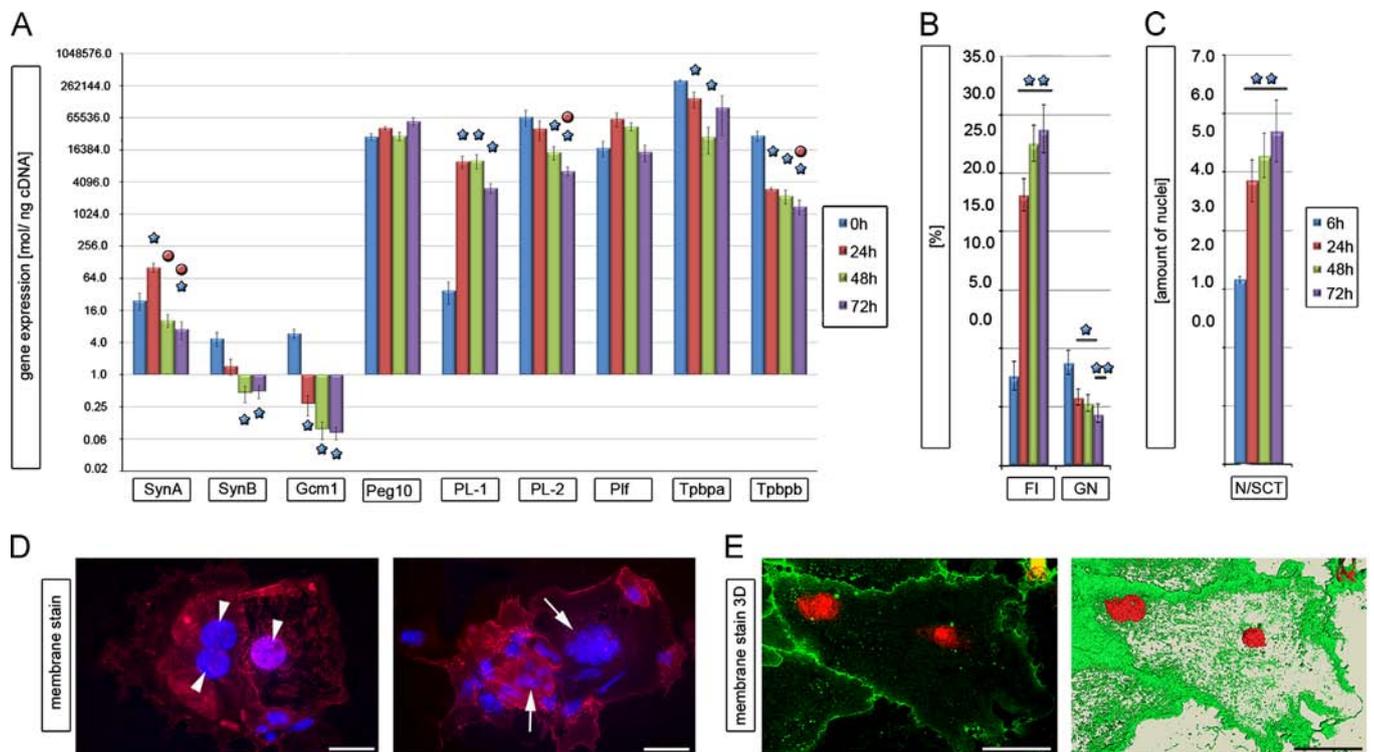
negative siRNA, Qiagen) was used as a control, demonstrating > 80% transfection efficiency by microscopy.

### 2.7. Staining of fused cells and calculation of the fusion index

After 6, 24, 48 and 72 h untransfected cells and after 24 h transfected cells were stained with the membrane stain wheat germ agglutinin conjugated with Alexa Fluor 594 and the nuclear stain Hoechst 33342 (H33342) (all Life technologies) according to previously published methods (Ruebner et al., 2010; Strick et al., 2007). The fusion index (FI) was calculated according to Langbein et al. (2008) and Ruebner et al. (2010, 2012). For confocal microscopy (Leica SP5X), cells were stained with wheat germ agglutinin conjugated with Alexa Fluor 488 and nuclei were counter stained for 15 min with DRAQ5 (Biostatus Lim., UK) according to manufacturer's instructions. Alexa Fluor 488 stained membranes were detected with an Argon laser and DRAQ5 stained nuclei detected with a Helium/Neon laser at 633 nm. 3-D imaging processing was performed using the computer program AMIRA (Burlington, MA, USA).

### 2.8. Immunoblot

Peg10 protein was analyzed in lysates from trophoblast cultures transfected with mock siRNA and siRNA-Peg10 using immunoblots. Fifteen micrograms of cell lysates were immunoblotted and then hybridized with Peg10-specific antibody (1:500, Antibodies-Online) and GAPDH (1:1000; Santa Cruz) according previously published methods (Ruebner et al., 2010).



**Fig. 5.** Gene expression and cell fusion of cultured primary trophoblasts over 72 h: (A) histogram represents the mean expression of placental marker genes from different trophoblast cultures after 0, 24, 48, and 72 h in culture in molecules per ng cDNA (mol/ng cDNA) ± SEM for each gene on a logarithmic scale. Statistical significance ( $P < 0.05$ ) are indicated for each time point relating to earlier time points. Blue stars show the significance to 0 h and the red circle to 24 h. All values are listed in supplemental Table 4. (B) Histogram represents the cell fusion index (FI) and giant nuclei (GN) in percentage of cells in culture after 6, 24, 48 and 72 h. (C) Histogram show nuclei per SCT (N/SCT) in total numbers after 6, 24, 48, and 72 h. Single star indicates a significant change to 6 h ( $P < 0.05$ ) and double star a high significant change to 6 h ( $P < 0.005$ ). All values are listed in supplemental Table 5. (D) Trophoblast cultures demonstrating examples of cell fusions and giant nuclei corresponding to Fig. 5B. Cells were stained with cell membrane stain (wheat germ agglutinin 594=red) and counterstained with H33342 to identify nuclei (blue). Arrowheads indicate giant cells and arrows indicate cell fusions. Scale bar=50 µm. (E) Confocal image composite of 17 z-sections (left) showing a multinucleated cell with three nuclei in a single membrane. Cells were stained with cell membrane stain (wheat germ agglutinin 488=green) and counterstained with DRAQ5 to identify nuclei (red). The adjacent right figure represents a three dimensional model showing the representative stains of the confocal image.

2.9. Statistical analysis

All data were expressed as a mean ± SEM. Differences were assessed using the independent-sample *t*-test and Mann–Whitney *U*-test (PASW 18.0, SPSS, Inc.). A *P*-value of less than 0.05 was considered statistically significant.

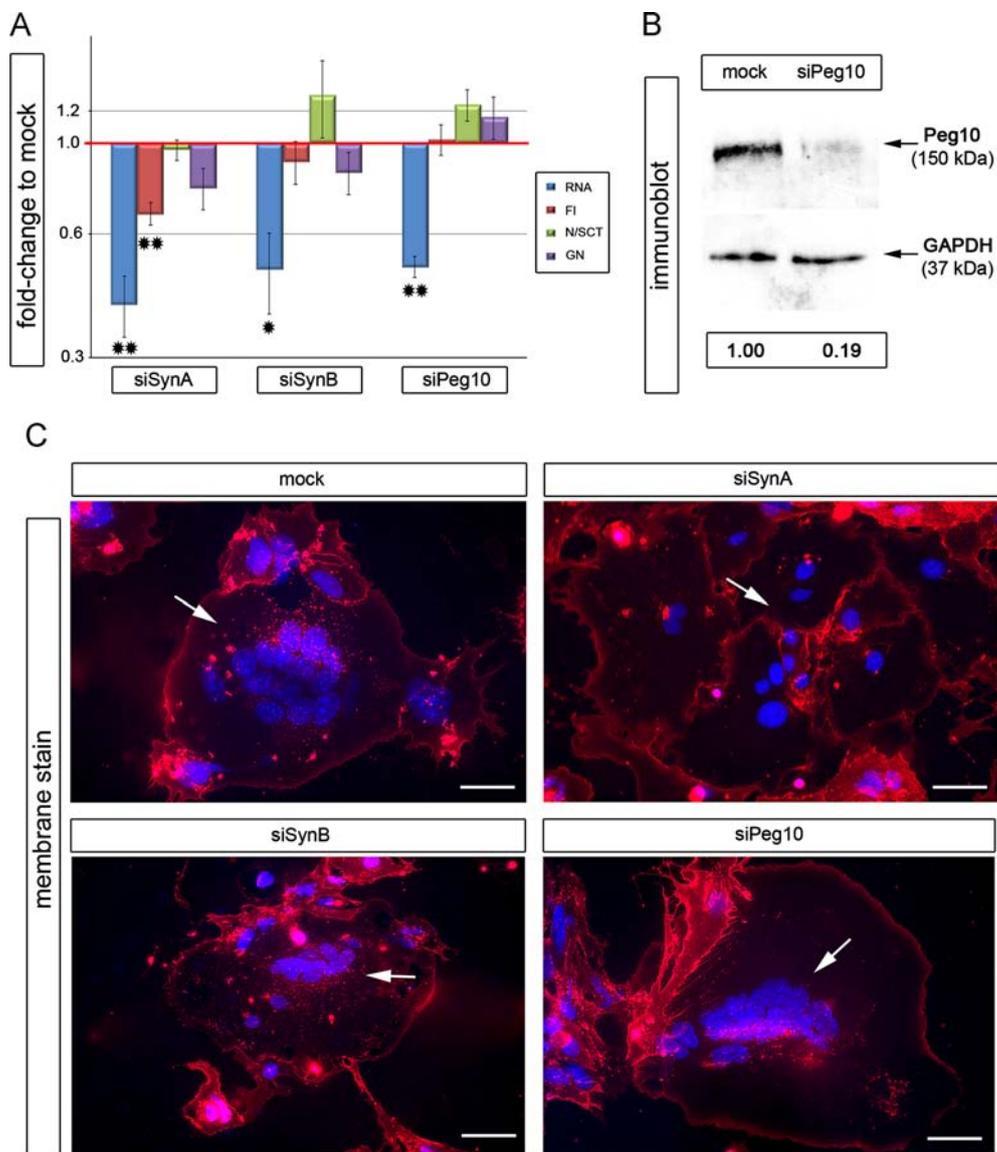
3. Results

3.1. Expression of marker genes in murine placenta from embryonic day E8.5 to E18.5

The murine placenta is characterized by different trophoblast subtypes. Figs. 1A and 2A represents a schematic model of the murine placenta showing three separate tissue layers and their

different cell types. In order to gain new information regarding the expression and localization of the nine genes: *Syncytin-A*, *Syncytin-B*, *Gcm1*, *PL-1*, *PL-2*, *Plf*, *Tpbpa* and especially the so far uncharacterized *Peg10* and *Tpbpb* in the murine placenta, we performed ISH with specific antisense oligonucleotides on fresh frozen tissues from developmental stages E8.5, E9.5 (*Peg10*), E10.5, E12.5, E15.5 and E18.5 (Figs. 1, 2B, and 3). In parallel we performed absolute qPCR of these nine genes from 11 different embryonic stages from E8.5 to E18.5 to quantitate the expression pattern in mol/ng cDNA and correlated the results obtained from the ISH (Fig. 4).

As expected, ISH at E8.5 showed a lower expression of *Syncytin-A*, *Syncytin-B* and *Gcm1* in the chorion, representing the placental precursor cells of the labyrinth. The expression of these three genes increased further in the following developmental stages (> E10.5) specifically localizing to the labyrinth (Fig. 1B).



**Fig. 6.** Knockdown of *Syncytin-A*, *Syncytin-B* and *Peg10* genes in primary trophoblasts using specific siRNAs: (A) histogram represents the mean RNA expression, fusion index (FI), nuclei per SCT (N/SCT) and number of giant nuclei (GN) of *Syncytin-A*, *Syncytin-B* or *Peg10* siRNA transfected cells after 24 h in fold change compared to mock transfected cells (Y-axis) for each transfection set to 1.0. Double star indicates a high significant change ( $P < 0.005$ ). All values are listed in supplemental Table 6. (B) Immunoblot of siRNA-Peg10 transfected trophoblasts was performed with a Peg10-antibody at 48 h post transfection. A mock siRNA Alexa488 scrambled negative siRNA was used as a transfection control. The fold change for Peg10 protein (150 kDa) normalized to GAPDH (37 kDa) is shown below. (C) Trophoblast cultures demonstrating examples of cell fusions and giant nuclei corresponding to Fig. 4A. Cells were stained with cell membrane stain (wheat germ agglutinin 594=red) and counterstained with H333342 to identify nuclei (blue). Arrows indicate cell fusions. Scale bar=50 μm.

In contrast to *Syncytin-A* where expression decreased at E18.5, expression of *Syncytin-B* remained high till E18.5 (Fig. 1B). Higher magnification ( $500\times$ – $2000\times$ ) demonstrated gene expression around the maternal blood vessels in the SCT layers, where *Syncytin-A* localized to SCT-I and *Syncytin-B* to SCT-II (Fig. 1C). ISH results of *Peg10* also showed an increase of expression from E8.5 till E18.5 (Figs. 1B and 3). At E8.5 and E9.5, TGCs and all other placental precursor cells were positive for *Peg10* in the chorion and ectoplacental cone (EPC). In the following developmental stages all extra-embryonic tissues were positive for *Peg10* with specific expression at the pTGCs, GlyT, ST and especially in the labyrinth layer (Figs. 1 and 3). However, in relation to the pTGCs expression of *Peg10* was weaker in the ST and GlyT (Figs. 1B and 3). Importantly, we interpreted the localization of *Peg10* in the labyrinth layer specific to the nuclei and cytosol of sinusoidal TGCs encompassing the maternal blood vessels (Figs. 1C and 3). In all developmental stages we did not observe any expression stemming from the maternal decidua.

In corroboration with ISH, qPCR of *Syncytin-A*, *Syncytin-B* and *Gcm1* demonstrated an increase of gene expression from E8.5 to E16.5. *Syncytin-A* represented a higher expression than *Syncytin-B* and *Gcm1* stemming from the developing labyrinth layer at early stages (E9.5, E10.5 and E11.5) (Fig. 4A, suppl. Table 3). At E16.5 *Syncytin-A* showed the highest expression level (908.1 mol/ng cDNA) and then decreased continuously to 131.7 mol/ng cDNA at E18.5. *Syncytin-B* expression levels increased gradually during early stages and reached, like *Syncytin-A*, the highest level at E16.5 (1769.5 mol/ng cDNA), but remained high till E18.5. Between E14.5 and E18.5 *Syncytin-B* was 2- to 10-fold higher expressed than *Syncytin-A* (Fig. 4A, supplemental Table 3). The qPCR of the transcription factor *Gcm1* resulted in a similar expression pattern like *Syncytin-A* with maximum levels at E16.5 (783.1 mol/ng cDNA) and then decreasing to 143.6 mol/ng cDNA at E18.5 (Fig. 4A, supplemental Table 3). *Gcm1* showed lower expression at all stages than *Syncytin-B* and partly *Syncytin-A*, except for E8.5, E17.5 and E18.5.

At the earliest E8.5 stage measured, *Peg10* expression was high (7766.5 mol/ng cDNA), increased further to 188,917.1 mol/ng cDNA at E16.5 and remained at high expression levels till E18.5 (Fig. 4A, supplemental Table 3). *Peg10* was established as the highest expressed gene in the labyrinth layer according to our qPCR and ISH results and had in general 648-fold higher expression levels compared to *Syncytin-A* and 332-fold higher levels than *Syncytin-B*.

Placental lactogenes and prolactin showed different expression patterns during placental development. *PL-1*, *PL-2*, *Plf*, *Tpbpa* and *Tpbpb* demonstrated high expression levels at early development stages, but showed changes in different cell types (Figs. 2B and 4B). For example, *PL-1* was detected in parietal TGCs, but absent from the labyrinth layer (Fig. 2B). In contrast to all other genes, *PL-1* expression reached the expression peak at an early stage of E10.5 (23,114.9 mol/ng cDNA). *PL-2* expression was localized to parietal TGCs, but was also observed in the STs (Fig. 2B). At E16.5 *PL-2* showed maximum expression levels (279,885.3 mol/ng cDNA) and strong expression in parietal TGCs, STs and additionally in sinusoidal TGCs until term (Figs. 2B and 4B, supplemental Table 3). In addition to *Tpbpa*, which is considered as a marker protein of STs and GlyTs, *Tpbpb* was also expressed in the STs and GlyTs, but both genes were not detected in TGCs and labyrinth cells (Fig. 2B). *Tpbpa* and *Tpbpb* expression levels increased continuously from E8.5 to the maximum level at E16.5 with 1,604,372.8 mol/ng cDNA for *Tpbpa* and 180,457.6 mol/ng cDNA for *Tpbpb* (supplemental Table 3). *Tpbpa* expression was in general 5-fold higher compared to *Tpbpb* throughout placental development (Figs. 2B and 4B, supplemental Table 3).

### 3.2. Expression of placental marker genes in primary fractionated trophoblast cultures

In order to fractionate, cultivate and characterize primary murine trophoblasts for the same nine genes described above, we chose the middle developmental stage of E14.5, when all nine genes except *PL-1* were still increasing in expression according to our qPCR results (Fig. 4). In addition, the E14.5 developmental stage was previously used for trophoblast fractionation and culturing (Zuckermann and Head, 1986). After fractionation and culturing of primary placental cells for 0, 24, 48 and 72 h the expression pattern of all nine genes was evaluated by qPCR ( $n=4$ ) (Fig. 5A, supplemental Table 4). QPCR analyses at the earliest time point of culturing at 0 h showed expression of all nine genes.

*Tpbpa* and *Tpbpb* were specific to STs and GlyT, whereas *PL-1*, *PL-2* and *Plf* were specific to TGCs and showed relatively high gene expression. *Syncytin-A*, *Syncytin-B* and *Gcm1* specific for SCTs were relatively lowly expressed. In contrast, *Peg10* showed high expression levels starting from 0 h till 72 h of culturing (Fig. 5A and supplemental Table 4). After a 24 h culturing of cells *Syncytin-A* expression increased to maximum levels of 101.6 mol/ng cDNA (4.1-fold higher than at 0 h) and decreased at 48 and 72 h. *Syncytin-B* and *Gcm1* expression levels decreased after 0 h in culture. *PL-1* (259.62-fold) and *Plf* (3.48-fold) increased in their expression after 24 h culturing compared to 0 h. *Tpbpa* and *Tpbpb* exhibited the highest expression levels at 0 h with comparable expression levels to the qPCR of placenta at E12.5–E14.5 (supplemental Tables 3 and 4). Culturing fractionated primary trophoblasts for 48 and 72 h we observed that except for *Peg10*, *Plf* and *Tpbpa* all other genes exhibited a statistically significant decrease of expression, supporting that trophoblast differentiation mainly occurred within 24 h of cell culture (Fig. 5A, supplemental Table 4).

The establishment of a murine trophoblast cell culture system also presented the possibility to investigate functional SCT differentiation. Multinucleated SCTs were identified by staining cellular membranes with wheat germ agglutinin and nuclei with H33342 following the culturing of primary cells at 6, 24, 48 and 72 h (Fig. 5D). Multinucleated SCTs were also studied using confocal microscopy where greater than 15 z-sections confirmed a single membrane housing multiple nuclei (Fig. 5E). Furthermore, we analyzed the FI, number of nuclei per SCT (N/SCT) and giant nuclei (GN) (Fig. 5B–D). After 6 h 7.67% of primary cells were fused with a mean of 3.16 nuclei per SCT. After 24 h the fusion index (FI) significantly increased to 23.15% with a mean of 4.85 nuclei per SCT and further rose after 48 h (FI: 27.57%, 5.28 nuclei/SCT) and 72 h (FI: 28.8%, 5.7 nuclei/SCT). Regarding TGCs, we considered all nuclei as giant nuclei, which were 3-fold larger in size compared to normal nuclei (Ruebner et al., 2012). Results showed that the number of giant nuclei were highest at 6 h with 8% and then significantly decreased to 4.47% at 72 h (Fig. 5B–D, supplemental Table 5).

Taken together, we interpret the above findings that the fractionated placental cells contained all cell types specific to each placental layer and that developmental programming occurred early within 24 h along with differentiation to SCT.

### 3.3. siRNA mediated knockdown of *Syncytin-A*, *Syncytin-B* and *Peg10* gene expression

Knockdown of *Syncytin-A* and *Syncytin-B* gene expression in fractionated primary trophoblasts was performed with specific siRNA's directed against *Syncytin-A* and *Syncytin-B* to test for their functional roles in cell–cell fusion (Fig. 6). In addition, siRNA mediated knockdown of *Peg10* in primary trophoblasts was also performed to determine a possible functional role of *Peg10* in SCT or TGC development (Fig. 6).

*Syncytin-A*, *Syncytin-B* and *Peg10* all showed similar gene expression levels and cell fusion indexes compared to untransfected cells following transfection of fractionated primary cells with a scrambled siRNA control after 24 h. In contrast, siRNA-*Syncytin-A* transfected cells at 24 h showed a statistically significant reduction of both the gene expression (0.41-fold,  $p=0.002$ ) and the fusion index (0.67-fold,  $p=0.001$ ) compared to mock transfected cells, indicating a functional role of *Syncytin-A* with cell–cell fusion (Fig. 6A, supplemental Table 6). In contrast, the amount of nuclei per SCT did not change between transfected mock and siRNA-*Syncytin-A* cells (0.95-fold). Although, the number of giant nuclei decreased from 8.03% to 6.25% (0.78 fold) after siRNA-*Syncytin-A* transfection this was not statistically significant (Fig. 6A and C, supplemental Table 6). After transfection of trophoblasts with siRNA-*Syncytin-B*, *Syncytin-B* gene expression was significantly reduced (0.49-fold,  $P=0.032$ ) compared to mock transfected cells (Fig. 6A, supplemental Table 6). In parallel a non-significant decrease of the cell fusion index (0.9-fold) and TGCs (0.85-fold) was found (Fig. 6C).

Transfection of fractionated primary cells with siRNA-*Peg10* statistically significantly inhibited *Peg10* RNA expression (0.5-fold,  $p=0.002$ ) at 24 h and *Peg10* protein expression at 48 h to a 19% level of expression (Fig. 6A and B, supplemental Table 6). Although, the expression of *Peg10* was significantly reduced, *Peg10* could not be correlated significantly with cell–cell fusion, the amount of nuclei per SCT or TGC development (Fig. 6A and C, supplemental Table 6).

#### 4. Discussion

This present report demonstrated the absolute expression and localization of nine marker genes in the murine placenta during development from E8.5 to E18.5. Particularly, the labyrinth layer of the trichorial placenta is separated in three trophoblast subtypes: Mononuclear TGC—lining the maternal blood spaces followed by two SCTs (Hernandez-Verdun, 1974; Simmons and Cross, 2005). Inactivation of *Gcm1*, one of the main transcription factors in the labyrinth layer showed defects in development of SCTs (Anson-Cartwright et al., 2000). The expression level of *Gcm1* as determined in our investigation showed a similar expression to *Syncytin-A* and *Syncytin-B*. Studies with *Gcm1* knockout mice and trophoblast stem cells showed that *Gcm1* was involved in binding and subsequently the regulation of *Syncytin-B*, but not of *Syncytin-A* (Simmons et al., 2008a). In addition, it was also shown that *Gcm1* was expressed in SCT-II, like *Syncytin-B* (Simmons et al., 2008a). In this investigation the absolute expression of the two *env* genes *Syncytin-A*, *Syncytin-B* and *Gcm1* showed maximum levels at E16.5. However, *Syncytin-B* showed ~2 to 2.2-fold higher expression than *Syncytin-A* and *Gcm1*, respectively. *In situ* hybridization indicated the localization of the three genes in the multinucleated SCTs, which was demonstrated previously; *Syncytin-A* in SCT-I, *Syncytin-B* and *Gcm1* in SCT-II (Simmons et al., 2008a). *Syncytin-A*<sup>-/-</sup> and *Syncytin-B*<sup>-/-</sup> mice showed defects in the labyrinth by reason of incomplete cell fusion in their respective SCTs resulted in growth restriction of the placenta, diminished perfusion and a disturbed transport of nutrients and waste products. Regarding the embryo, an abnormal phenotype was noted showing growth and development restriction ending in intrauterine lethality (Dupressoir et al., 2009, 2011).

Our absolute qPCR results of placental marker genes demonstrated a high expression of TGC markers *PL-1*, *PL-2* and *Plf* as well as for the ST and GlyT markers *Tpbpa* and *Tpbpb*. Except for *PL-1*, which showed the maximum expression levels at E9.5 and E10.5, all other eight genes exhibited the highest level at E16.5.

Consistent with the literature, we showed that after the decrease of *PL-1* expression at E11.5, expression of the analog *PL-2* increased in pTGCs (Harigaya et al., 1988; Nieder and Jennes, 1990). Simmons et al. (2007) identified 4 subtypes of TGCs where expression of *PL-1*, *PL-2*, and *Plf* in several cells is equal to our findings. The observation of different genes within TGCs suggests different ways of differentiation. Partially TGCs arise from the ectoplacental cone. One marker of these cells is the protein *Tpbpa*, which is mainly expressed in STs and GlyTs (Hu and Cross, 2011). *Tpbpa* level of expression was 1,604,372.8 mol/ng cDNA the highest of all analyzed nine genes. TGCs, GlyTs and other placental cells are derived from *Tpbpa*-expressing cells, suggesting an essential role of *Tpbpa* for placental cell and maternal vasculature development (Hu and Cross, 2011). We further showed the expression profile and localization of *Tpbpb*, which was also expressed in STs and GlyTs. The level of expression during gestation was similar to *Tpbpa*, although lower (supplemental Table 3). Since both *Tpbpa* and *Tpbpb* throughout development show a similar pattern of expression by ISH and absolute qPCR we predict that both gene family members most likely function similarly.

In order to analyze the process of cell–cell fusion in more detail, we established a primary mouse trophoblast cell culture system comparable to Zuckermann and Head (1986). A comparison showed that our fractionation and the fractionation of Zuckermann and Head (1986) resulted in ~90% cytokeratin positive (epithelial) cells and ~10% “contaminating” cells, like CD45-positive leukocytes (6.7%), or mesenchymal macrophages. In addition, analyzing the fractionated trophoblasts, we detected 0.42% CD34 positive cells, which we interpreted as minimal amounts of placental endothelial cells or hematopoietic stem cells. This supports that both fractionation methods isolated a mix of trophoblasts, and possibly precursor cells, which could develop into TGC and multinucleated syncytia after 24–48 h (Zuckermann and Head, 1986). To identify the different trophoblast subtypes more precisely, the gene expression of the nine marker genes was quantified via qPCR. The results showed expression of all markers directly after isolation indicating that many subtypes were present. While *Peg10* and *PL-1* remained highly expressed past 24 h, other marker genes decreased after cell seeding (*Syncytin-B*, *Gcm1*) or decreased in a stepwise manner following 24 h of culturing (*Syncytin-A*, *PL-2*, *Plf*, *Tpbpa*, *Tpbpb*). Although the marker of giant cells, like *PL-1*, *Plf* and *Peg10* were highly expressed in the first 24–48 h of culturing, the number of cells containing giant nuclei decreased after 6 h and then was maintained at approximately 5%. A comparison of *PL-1* expression in cell culture to embryonic stages E8.5–E18.5 showed contrasting results (Figs. 4B and 5A). This *in vitro* discrepancy could reflect a lack of for example, specific placental cell–cell interactions, changes in oxygen content or even feedback signaling from the fetus occurring during placental development.

In addition to the formation of giant cells the fractionated primary trophoblasts were especially suited to form multinucleated syncytia (up to 28.8% after 72 h). We detected a 4.1-fold induction of *Syncytin-A* expression after 24 h compared to initial seeded cells. Previously, Dupressoir et al. (2009, 2011) demonstrated that *Syncytin-A* and *Syncytin-B* were essential for cell–cell fusion and the forming of multinucleated SCT-I and SCT-II, respectively, during murine placenta development. Evidence supports that SCT-I and SCT-II are derived independently during mouse placentogenesis, where at E8.5 *Syncytin-A* expression starts from the apical side of the chorion and *Syncytin-B* and *Gcm1* expression starts at the basal side of the chorion at the interface with the allantois (Simmons et al., 2008a). Furthermore, *Syncytin-B* was important for placental architecture, especially involving the vascular blood system where knockout mice

demonstrated a significant 5-fold enlargement of the blood filled spaces of maternal lacunae (Dupressoir et al., 2011). Knockdown of *Syncytin-A* with specific siRNA in fractionated trophoblasts showed in agreement a significant reduction of the fusion index by 1.5-fold, supporting a functional role of *Syncytin-A* in cell fusion *in vitro*. Surprisingly, *Syncytin-B* and *Gcm1* showed a continuous decrease of expression following each culture set-up till 72 h, although *Syncytin-B* increased in placental tissues till E16.5. The discrepancy to maintain *Syncytin-B* and *Gcm1* levels during cell culture could be linked with the absence of fractionated placental CD34+ endothelial and/or hematopoietic cells, which may be important for SCT-II differentiation along with placental vascular architecture. Considering the comparable high *Syncytin-A* expression in the fractionated trophoblasts (70-fold higher *Syncytin-A* than *Syncytin-B* at 24 h) and the high FI of 23.15% at 24 h, this finding supports that multinucleated SCT-I-like syncytia (*Syncytin-A* positive) formed, but only minimal SCT-II like syncytia (*Syncytin-B* positive) *in vitro*. These results support that SCT-I-like syncytia can differentiate independently of *Syncytin-B* and SCT-II-like syncytia. Importantly, this is similar to *Syncytin-A* knockout mice with normal SCT-II and *Syncytin-B* knockout mice with normal SCT-I (Dupressoir et al., 2009, 2011). Comparing the cell culture of murine fractionated trophoblasts with human fractionated trophoblasts, similar cell fusion processes were observed *in vitro* (Langbein et al., 2008; Ruebner et al., 2010). It should be noted, however, that regarding human placenta and fractionated human trophoblasts, a different expression and localization of *Syncytin-1* and *Syncytin-2* (not orthologous to *Syncytin-A* and *Syncytin-B*) was noted compared to the mouse. *Syncytin-1* was more generally expressed in different human trophoblasts, whereas *Syncytin-2* was restricted to the villous cytotrophoblasts at the membrane, specifically towards the SCT (Malassine et al., 2005, 2007). siRNA mediated knockdown of *Syncytin-1* resulted in a reduction of cell–cell fusion, whereas the knockdown of *Syncytin-2* resulted in an almost complete inhibition of cell fusion *in vitro* (Frendo et al., 2003; Vargas et al., 2009). These results revealed that *Syncytin-2* was responsible for a more fundamental role of cell–cell fusion.

During placental development, the other retrotransposon-derived gene *Peg10* showed a comparable high expression to other genes increasing from E8.5 to E18.5. *Peg 10* along with *Plf* was the highest expressed gene in early developmental stages (E8.5–E10.5). Similar to Ono et al. (2006) we also found expression of *Peg10* at stages E8.5 and E9.5 in TGCs and precursor cells in the EPC and chorion and at E10.5 in all extraembryonic tissues. In the following developmental stages *Peg10* expression increased peaking at E16.5 as noted by gene expression (24.3-fold total increase). Furthermore, compared to *Syncytin-A*, *Syncytin-B* and *Gcm1*, *Peg10* had the highest expression levels in the labyrinth layer.

Although *Peg10* knockout mice demonstrated normal implantation, morphology and expression at E7.5 and E8.5, an essential role for *Peg10* was found at E9.5–10.5 where an absent ST and labyrinth was discovered (Ono et al., 2006). This result points to a possible role for *Peg10* in precursor differentiation, esp. ST and labyrinth. Furthermore, our ISH findings confirm and bring forth new information demonstrating that *Peg10* was expressed in all extraembryonic tissues, specifically in the sinusoidal TGCs of the labyrinth layer and the parietal TGCs of the junctional zone. Compared to the TGCs only a low expression in the STs and GlyTs could be shown after E10.5. Both TGC types are involved in hormone production and implantation of the placenta where the sinusoidal TGCs are in direct contact to the maternal blood and with SCT-I (Hernandez-Verdun, 1974). Therefore, due to the expression of *Peg10* in the sinusoidal TGCs we propose that *Peg10* may have an essential functional role in the fetal–maternal blood system. In line with early placental development of the

vascular system, the findings support that the lack of mediating nutrients and gas through the sinusoidal TGCs in *Peg10*<sup>+/-</sup> knockout mice halts development of the labyrinth beyond E8.5.

In addition to the above, we also found that knockdown of *Peg10* with siRNA in primary fractionated trophoblasts showed no difference in the FI. These results indicate that the absent labyrinth layer in *Peg10*<sup>+/-</sup> knockout mice is not linked with SCT fusion, but probably important for the formation or function of the sinusoidal TGC. However, we observed that knockdown of *Peg10* had no significant effect in the formation of giant nuclei *per se*, which states that genetic redundancy is existent for TGC development. In accordance with these results, placenta from *Peg10*<sup>+/-</sup> mice showed normal giant cells at E8.5–E10.5 (Ono et al., 2006).

The dominant (–1)-frameshift of *Peg10* during murine placentogenesis resulted in a protein of 150 kDa following E9.5 (Clark et al., 2007) and was also noted in our analyses using Immunoblotting with trophoblasts fractionated from placenta of E14.5. To date the functional role of murine or human *PEG10* in placentogenesis is still uncertain; however several cellular and protein interactions of human *PEG10* have been demonstrated. For example, *PEG10* was detected in human cell lines, where it bound to the TGF- $\beta$  receptor *activin receptor-like kinase 1 (ALK1)*, inhibited TGF- $\beta$  signaling and increased cell adhesion and migration (Lux et al., 2005). Furthermore co-expression of *ALK1* and *PEG10* affected cell morphology by development of lamellipodia and filipodia (Lux et al., 2005).

In summary, nine important murine genes including three retroviral/retrotransposon genes, *Syncytin-A*, *Syncytin-B* and *Peg10*, demonstrated a temporal pattern of expression and specific cellular localization during murine placental development. All three retroviral/retrotransposon genes localized to different cells in the labyrinth layer, which have cellular contacts: *Peg10* to the sinusoidal TGCs and *Syncytin-A* and *Syncytin-B* to the SCTs. The established cell culture system with fractionated primary trophoblasts demonstrated the expression of marker genes important for murine placental development. In addition, trophoblast culturing showed significant SCT development occurring within 24 h and confirmed *Syncytin-A* as a key fusogenic protein, however *Peg10* showed no functional effect in cell–cell fusion.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at: <http://dx.doi.org/10.1016/j.diff.2013.02.002>.

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