Syncytin-A Mediates the Formation of Syncytiotrophoblast Involved in Mouse Placental Development

Rui Gong, Liqin Huang, Jian Shi, Kan Luo, Gang Qiu, Huixing Feng, Po Tien and Gengfu Xiao

State Key Laboratory of Virology and Modern Virology Research Centre, College of Life Sciences, Wuhan University, Wuhan

Key Words

Syncytin-A • Endogenous retrovirus • Syncytiotrophoblast cell • Fusion • Placental development

results present evidence in support of the direct role for syncytin-A in mouse TS cell fusion and differentiation involved in placental development.

Copyright © 2007 S. Karger AG, Basel

Abstract

Syncytin-A, a new mouse endogenous retroviral envelope protein expressed in placenta, can mediate cell fusion *in vitro*. But its physiological function was still unknown. We proposed a role for syncytin-A in syncytiotrophoblast (SynT) formation derived from the differentiation of trophoblast stem (TS) cells during placental development. To evaluate this hypothesis, we analyzed the involvement of syncytin-A in the differentiation of mouse TS cells. After withdrawing fibroblast growth factor 4 (FGF4), TS cells can fuse to form SynT cells. We found syncytin-A mRNA and protein expression are colinear with fusion index increase during TS cell differentiation. Expression of syncytin-A is localized in SynT cells through *in situ* immunofluorescent staining. By using specific antibody and antisense oligonucleotides, we demonstrated that inhibition of syncytin-A lead to obvious decrease of SynT cell formation. These

Introduction

Endogenous retroviruses (ERVs) may exist as “endogenized” variants of exogenous virus strains, which are present in the genome of all vertebrates [1, 2]. They are most probably the genomic traces of germ-line infections by exogenous retroviruses in ancient age. And during the longtime evolution, increase in their copy number has occurred, at least for some of them, via retrotransposition or germ-cell reinfection. This generated multigenic families containing a few to several hundred elements and now accounting for a substantial fraction of the genome (8% for the human [3] and 10% for the murine [4]).

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1015-8987/07/0205-0517\$23.50/0

Accessible online at:
www.karger.com/cpb

Gengfu Xiao, Ph.D.
State Key Lab of Virology, College of Life Sciences
Wuhan University, Wuhan 430072 (P.R.China)
Tel. + 86-27-68756272, Fax + 86-27-68752897
E-Mail gxiao@whu.edu.cn

ERV in the placenta of humans and other mammals has been known for many years. Recently, the biological significance of these retroviral elements attracts more and more attention. For example, syncytin-1, an envelope protein of human endogenous retrovirus family W (HERV-W), was possibly involved in the fusion of cytotrophoblast cells during human placental syncytiotrophoblast layer formation [5-7]. The envelope protein of a particular class of ERVs of sheep, endogenous Jaagsiekte sheep retroviruses (enJSRVs) regulates periimplantation placental growth and differentiation [8]. So, research on these retroviral elements in placenta becomes very important.

In mouse placental labyrinth, there are two layers of syncytiotrophoblast (SynT) cells that are in direct apposition to the endothelial cells of the fetal-derived blood vessels and an additional mononuclear cell type of unknown origin and function remains outside the syncytiotrophoblast layer [9]. SynT cells are multinucleated cells that form as a result of cell-cell fusion and function as the major transport surface for nutrient and gas exchange between the maternal and fetal circulation in the villous part of the placenta [10, 11]. Previous studies indicate that the formation of SynT cells is dependent on a distinct type of transcription factor called glial cells missing-1 (Gcm1), which is regarded as a marker of labyrinth [12-15].

Syncytin-A gene is a characterized mouse endogenous retroviral envelope gene, locates in the chromosome 5qG2 as a unique copy, which is specially expressed in mouse placental syncytiotrophoblast-containing labyrinthine zona and can mediate non-trophoblast derived cell such as 293T cell fusion *in vitro* [16]. Recently, we revealed the molecular fusion mechanism mediated by syncytin-A [17]. However, its physiological function was still obscure.

Trophoblast stem (TS) cells are induced to proliferate and maintain their stem cell phenotype in the existing of fibroblast growth factor 4 (FGF4) which activates signaling through the Fgfr2 receptor [18]. They have the potential to give rise to all differentiated trophoblast cell subtypes such as trophoblast giant (TG) cells and SynT cells. So, in order to investigate the relationship between syncytin-A and SynT cells, we carried out studies using these TS cells.

Here, we observed obvious syncytial formation during the differentiation of TS cells, which indicates the morphogenesis of SynT cells. Basing on this model, we performed a series of experiments to prove the role for

syncytin-A in SynT cell formation during mouse placental development.

Materials and Methods

Trophoblast stem cell culture and differentiation

A mouse TS cell line derived from E 3.5 blastocysts was used for all experiments (gift from Dr. Janet Rossant, The Hospital for Sick Children, Toronto) and was maintained as described previously [18, 19]. Briefly, the cells were grown in 30% TS medium (RPMI 1640 medium added with 20% fetal calf serum, glutamine, sodium pyruvate, β -mercaptoethanol, and penicillin/streptomycin) and 70% conditioned medium from mouse embryonic fibroblasts (CM), supplemented with 25 ng/mL fibroblast growth factor FGF4 (Invitrogen, Carlsbad, CA, USA) and 1 μ g/mL Heparin. TS cells were seeded in wells at ~70% initial density to evaluate their differentiation. Cells in different wells from 0-7d after FGF4/CM withdrawal were fixed by 2% paraformaldehyde, stained with 4', 6'-diamidino-2-phenylindole (DAPI). The fusion index, which represents the percentage of fusion events in a cell population is defined as $[(N - S)/T] \times 100$, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei counted. Only those syncytia contained at least three nuclei were counted. 293T cells which were transfected with syncytin-A expression plasmids (kindly provided by Dr. Thierry Heidmann, Institut Gustave Roussy, Villejuif) were used as markers in identification of SynT cells in differentiated TS cells. Experiments were repeated three times.

Syncytin-A mRNA isolation and analysis

Total RNA of cultured cells was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) with standard procedure. And its integrity was monitored by 1% agarose gel electrophoresis. RNAs used in following experiments were adjusted to same concentrations. After digesting these RNAs by RNA-free DNase, cDNAs were synthesized through oligo-dT primers. Using specific primers for syncytin-A, RT-PCR was achieved after 35 cycles of 94°C, 60s; 58°C, 45s; 72°C, 120s by using KOD plus DNA polymerase (Toyobo, Osaka-shi, Osaka-fu, Japan). The level of transcriptions was normalized using the β -actin gene, encoding mouse cytoplasmic β -actin protein as an endogenous RNA control. Primer sequences used for RT-PCR analysis of syncytin-A were 5'-ATG GTT CGT CCT TGG GTT TTC TGT CTC-3' and 5'-CTA GAC GGC ATC CTC CTC CGG GGG CTC-3'.

Anti-syncytin-A antiserum preparation

We used a recombinant pGEX-6P-1-syncytin-A-NHR plasmid describe in our previous work [17] to express this recombinant GST-NHR fusion protein. Then, polyclonal antibodies were produced by immunizing rabbits with this fusion protein. So, the anti-syncytin-A antiserum targeted the epitopes in syncytin-A transmembrane subunit.

Western blot analysis

Cell pellets in different wells were lysed in RIPA Lysis Buffer with PMSF and boiled in SDS loading buffer for 5 min. Lysates were run on SDS-PAGE and then transferred to nitrocellulose film. The anti-syncytin-A antiserum was added and detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sino-American Biotechnology Company, Luoyang, Henan Province, China). The level of total proteins was also normalized through examining β -actin protein expression by anti- β -actin antibody (Beyotime Institute of Biotechnology, Haimen, Jiangsu Province, China).

In situ immunofluorescent staining of syncytin-A

In situ immunofluorescent staining of syncytin-A was performed on 2% paraformaldehyde-fixed monolayers of differentiated cells derived from TS cell cultures. Then cells were rinsed three times with Tris-buffered saline contained 0.1% Triton X-100 (TBSTx), blocked in TBSTx contained 5% BSA. After 60 min, blocking buffer was removed and anti-syncytin-A antiserum diluted in TBSTx contained 5% BSA according 1:200 dilution was added and incubated for 60 min. After three times rinsing, Cy3-labeled goat anti-rabbit IgG (Beyotime Institute of Biotechnology, Haimen, Jiangsu Province, China) diluted in TBSTx contained 5% BSA according 1:1000 dilution was added and incubated for 60 min. After three times rinsing, samples were observed under fluorescence microscope.

Establishment of the TS cells and 293T cells fusion model

There were two methods used here. (I) 1:1 TS cells and 293T cells were plated overnight (about 12h), then pEGFP-N1 plasmids were transfected into the mixture cells and medium was changed to TS medium lacking FGF4/CM. After 24h, cells were fixed and stained with DAPI. (II) 293T cells were plated in a 24-well plate overnight (about 12h), and then pEGFP-N1 plasmids were transfected into them. After 6h, these cells were digested by trypsin and mixed with undifferentiated TS cells according to 1:1 proportion using TS medium lacking FGF4/CM. After 24h, cells were fixed and stained with DAPI.

Inhibition of SynT cell formation by anti-syncytin-A antiserum

Undifferentiated TS cells were plated in 24-well plate and medium was changed to 500 μ L TS medium lacking FGF4/CM. Then, the anti-syncytin-A antiserum was added into different well with different volume: 0, 1, 2, 4, 6, 8, 10 and 12 μ L respectively. Medium and anti-syncytin-A antiserum was refreshed everyday. After 7d, cells were fixed and stained by DAPI. Inhibition of formation of SynT cells was examined under fluorescence microscope. Anti-GST antiserum was used as control.

Inhibition of syncytial formation between the differentiated TS cells at 1d and 293T cells by anti-syncytin-A antiserum

According above procedures in establishment of the fusion model, 293T cells transfected pEGFP-N1 were mixed with undifferentiated TS cells in total 500 μ L TS medium lacking FGF4/CM. Then, the anti-syncytin-A antiserum was added into different well with different volume: 0, 1, 2, 4, 6, 8, 10 and 12 μ L

respectively. After 24h, cells were fixed and stained by DAPI. Inhibition of formation of syncytia was examined under fluorescence microscope. Anti-GST antiserum was also used as control.

Inhibition of syncytial formation between the differentiated TS cells at 1d and 293T cells by specific antisense oligonucleotides

Synthetic antisense oligonucleotides targeting the syncytin-A mRNA (GenBank accession no. AY849973) were designed and analyses of duplex and hairpin formations and of the absence of cross-reactivities with related sequences were performed using Prime Premier 5.0 software (PREMIER Biosoft International). The sequences of these oligonucleotides were: antisense 1-27 5' GAG ACA GAA AAC CCA AGG ACG AAC CAT 3' antisense 268-291 5' GAA ATT CTG GAG GCG CCT CTC AAG 3' antisense 1216-1240 5' GGC GAG AAG CGG GGA CAG AGG AGG 3' sense 1216-1240 5' CCC TCC TCT GTC CCC GCT TCT CGC C 3'

50 pmol of each was transfected to undifferentiated TS cells in the well of 24-well plate through Lipofectamine 2000 (Invitrogen). After 24h, cell lysates were examined by Western blot. The oligonucleotide which could most obviously down-regulate the expression of syncytin-A was determined by Western blot using anti-syncytin-A antiserum and was used in inhibition experiment. Control was cell transfected by Lipofectamine 2000 without any DNA.

Undifferentiated TS cells and 293T cells were transfected with oligonucleotide determined above and pEGFP-N1 separately. After 6h, they were digested, mixed with 1:1 proportion and re-plated in new wells. After 24h, cells were fixed and stained by DAPI. Syncytia formation was evaluated under fluorescence microscope.

Results

After FGF4/CM withdrawal, TS cells differentiate and obviously fuse to form SynT cells

Before studying the role of syncytin-A in TS cells differentiation, we first quantified the normal changes in TS cell populations during their differentiation with time *in vitro*. The differentiation of TS cells might show three primary cell morphologies: TS cell, TG cell and SynT cell. TS, TG and SynT cells have distinct cell morphologies that can be readily distinguished *in vitro*. TS cells are cuboidal, spindly and other shaped, which have a large nucleus-to-cytoplasmic ratio (Figures 1A and D). TG cells have a large expansive cytoplasm and large polyploid nuclei (Figures 1B and E). SynT cells have concentrative multiple nuclei (from several nuclei to some dozens of nuclei) (Figures 1C and F), just like syncytia in 293T cells

Fig. 1. Differentiation of TS Cells *in vitro*. (A and D) Undifferentiated TS cells observed directly and after DAPI staining. (B and E) TG cells derived from differentiated TS cells at 7d after FGF4/CM withdrawal observed directly and after DAPI staining. (C and F) SynT cells derived from differentiated TS cells at 7d after FGF4/CM withdrawal observed directly and after DAPI staining. (G) A syncytium contained six nuclei in the middle of the picture derived from differentiated TS cells at 4d after FGF4/CM withdrawal, which indicated the formation of SynT cells. (H) SynT cells derived from differentiated TS cells at 5d after FGF4/CM withdrawal. (I) Syncytial formation after 293T cells transfection with a mouse syncytin-A expression plasmid. These syncytial cells were similar as SynT cells in Fig 1C. (J) Relationship between differentiation time and formation of SynT cells. Fusion could not be observed until 4d after FGF4/CM withdrawal. Dramatic fusion occurred at 5d after FGF4/CM withdrawal. Values were presented as mean $\pm$ SEM.

induced by syncytin-A expression plasmids (Figure 1I). Formation of SynT cells was indicated by calculation of fusion index (see Materials and Methods). SynT cells began to appear at 4d after FGF4/CM withdrawal (Figure 1G). Dramatic increase of fusion index appeared at 5d after FGF4/CM withdrawal (Figure 1H). The fusion index arrived at maximum at 7d after FGF4/CM withdrawal (Figure 1C). The change of fusion index was illustrated in Figure 1J.

Syncytin-A mRNA and protein expression are colinear with fusion index increase during TS cell differentiation

Total RNA from TS cells was isolated at various differentiation days (0-7d) after withdrawal of FGF4/CM. RT-PCR was performed to examine the transcriptional level of syncytin-A mRNA (Figure 2A). To our slightly surprised, syncytin-A mRNA could be detected even in 0d, which was in an undifferentiation condition without withdraw of FGF4/CM. With the differentiation time

Fig. 2. Examination of mouse syncytin-A expression. (A) Examination of mouse syncytin-A in transcriptional level through RT-PCR. Total RNA was isolated from 0-7d differentiated TS cells. Syncytin-A mRNA (about 1.8kb) were clearly detected. Control was examination of mouse β -actin mRNA (about 1.1kb). (B) Examination of mouse syncytin-A in translational level through Western blot. Cell lysates from 0-7d differentiated TS cells were examined. A 24kD band was examined in every lane, which presented the transmembrane subunit in syncytin-A. Control was examination of mouse β -actin protein (43kD). (C and D) Localization of syncytin-A by anti-syncytin-A antiserum. Red fluorescence indicated the expression of syncytin-A. Two white arrows indicated SynT cell membrane. One black arrow indicated a TG cell membrane, which was not stained. One dashed arrow indicated undifferentiated TS cells, which was not stained too.

prolonging, the transcriptional level of syncytin-A mRNA improved and reached high level at 4-5d after FGF4/CM withdrawal. Then, it began to decrease but was still detectable at 7d after FGF4/CM withdrawal.

Western blot was also used to evaluate syncytin-A expression in protein level (Figure 2B). TS cell lysates in diverse differentiation days were tested through anti-syncytin-A antiserum. A 24kD band was examined, which indicated the transmembrane subunit in syncytin-A. Just like most retroviral envelope proteins, mature syncytin-A should be cleaved to two parts: surface subunit and transmembrane subunit (contained 200 amino acids). So,

the 24kD band identified by anti-syncytin-A antiserum presented the transmembrane subunit in syncytin-A. Obvious expression of syncytin-A could also be observed at 0d without FGF4/CM withdrawal. Peak value of protein level appeared at 4d after FGF4/CM withdrawal and became weak at 7d after FGF4/CM withdrawal. Changes of protein levels are accord with their mRNA counterparts.

Combining improvement of fusion index in TS cell differentiation with time, we observed that syncytin-A mRNA and protein expression are colinear with fusion index increase during TS cell differentiation.

Fig. 3. Fusion between differentiated TS cells at 1d after FGF4/CM withdrawal and 293T cells. (A-C) Mixture of TS cells/293T cells were transfected with pEGFP-N1 plasmids. A (bright), B (GFP fluorescence) and C (DAPI fluorescence staining) were in the same field of vision. (D-F) 293T cells were transfected with pEGFP-N1 plasmids and then mixed with TS cells. D (bright), E (GFP fluorescence) and F (DAPI fluorescence staining) were in the same field of vision. (G-I) 293T cells were transfected with pEGFP-N1. G (bright), H (GFP fluorescence) and I (DAPI fluorescence staining) were in the same field of vision. No syncytium formed.

Syncytin-A locates at SynT cells through in situ immunofluorescent staining

Through immunofluorescent staining on monolayers of differentiated TS cells at 7d. We found robust expression of syncytin-A distributed in SynT cells while expression of syncytin-A could hardly be detected in TS cells and TG cells (Figure 2C versus D), which implied that syncytin-A specially located at SynT cells and might be involved in cell fusion.

Differentiated TS cells at 1d after FGF4/CM withdraw can fusion with 293T cells

Because syncytin-A can mediate 293T cell fusion with high efficiency (Figure 1I), there might be abundant syncytin-A receptors in 293T cell membrane. And expression of syncytin-A can be detected in TS cells at 1d after FGF4/CM withdraw (Figures 2A and 2B). Therefore, we presumed that differentiated TS cells at 1d could fuse with 293T cells.

Fig. 4. Inhibition of syncytial formation by anti-syncytin-A antiserum and antisense oligonucleotides. (A) Inhibition of SynT cell formation by anti-syncytin-A antiserum. Fusion index decreased 80% when antiserum volume increased from 0 to 12μL. Fusion couldn't be inhibited completely even the antiserum volume exceeded 10μL. (B) Inhibition of fusion between differentiated TS cells and 293T cells by anti-syncytin-A antiserum. Fusion could be almost completely inhibited when the antiserum volume arrived at 10μL. (C) Inhibition of expression of syncytin-A in TS cells by specific antisense oligonucleotides. Four oligonucleotides described in Materials and Methods were transfected into undifferentiated TS cells. Through Western blot, the antisense oligonucleotides (1216-1240) could most obviously down-regulate expression of syncytin-A in four oligonucleotides. Control was transfection of TS cells without any DNA. (D) Inhibition of fusion between differentiated TS cells and 293T cells by antisense oligonucleotide (1216-1240). Sense oligonucleotide (1216-1240) was used as control. Fusion index declined from 10 to 4. Values were presented as mean ± SEM.

There were two methods used here. (I) mixtures of TS cells/293T cells were transfected with pEGFP-N1 plasmids. (II) 293T cells were transfected with pEGFP-N1 plasmids and then mixed with TS cells. In both cases, obvious syncytial formation could be seen (Figures 3A-F) and fusion index could arrive at 10. Control was 293T cells only transfected with pEGFP-N1 plasmids and no syncytium was observed (Figures 3G-I).

Anti-syncytin-A antiserum can inhibit SynT cell formation and fusion between differentiated TS cells and 293T cells significantly

Anti-syncytin-A antiserum can inhibit SynT cell formation (Figure 4A). Fusion index obviously declined from above 0.45 to 0.1 with increase of antiserum volumes from 0 to 12μL. Anti-syncytin-A antiserum was also used to inhibit syncytial formation from differentiated TS cell/

293T cell fusion. Fusion index dramatically declined from 10 to nearly 0 with increase of antiserum volumes from 0 to 12 μ L (Figure 4B), which suggested the role of syncytin-A in TS cell and 293T cell fusion. These results showed that syncytin-A might be a major agent responsible for SynT cell formation and fusion between differentiated TS cells and 293T cells.

Specific antisense oligonucleotides can down-regulate expression of syncytin-A, which leads to inhibit fusion between differentiated TS cells and 293T cells

Four oligonucleotides were first evaluated for their abilities in inhibiting expression of syncytin-A in differentiated TS cells. Western blot showed that the most efficient reduction of syncytin-A expression was obtained with the antisense oligonucleotide (1216-1240) (Figure 4C). A quantitative analysis of the influence of syncytin-A inhibition on fusion between differentiated TS cells at 1d and 293T cells was then performed. When the antisense oligonucleotide (1216-1240) was added, fusion index reduced from 10 to 4 (Figure 4D). Contrasting the case in sense oligonucleotide, fusion index decreased 60%. This result can confirm the role for syncytin-A in TS cells and 293T cell fusion, indicating syncytin-A mediate SynT cell formation.

Discussion

The rate of differentiation is influenced by several factors, including genotype and cell density. TS cell plated at a very low density differentiate very rapidly to giant cells and do not show an increased expression of intermediate markers of spongiotrophoblast and labyrinth cells [19]. It was also reported that TS cells plated at low initial density (1:50) differentiate into both giant cells and syncytiotrophoblast cells which contains only two nuclei after FGF4/CM withdrawal [15]. Comparing other previous differentiation experiment, we find TS cell can differentiate and fuse to form SynT cells contain multiple nuclei just like syncytia induced by many viral envelope proteins with high initial density. These findings imply that it is important to select an appropriate density for work.

The syncytiotrophoblast layer is important for fetus development because it is responsible for many functions performed by the placenta. *In vitro* studies have established that soluble factors such as epidermal growth factor [20-22], granulocyte-stimulating factor [23], glucocorticoids [24], estriol [25], and human chorionic

gonadotropin (hCG) [26, 27] activate different intracellular signaling pathways to stimulate the differentiation of villous cytotrophoblasts into the syncytiotrophoblast in placenta.

Although human syncytin-1 has been demonstrated to be involved in human placental syncytiotrophoblast formation [5-7], the cell-cell fusion process involved in other mammalian placental syncytiotrophoblast formation is still not very clear. In this study, we have shown for the first time that (I) syncytin-A, an envelope glycoprotein encoded by mouse retrovirus is specially expressed in mouse placental SynT cells and its expression is positive correlation with SynT cells formation; and (II) direct inhibition of this retroviral envelope protein inhibits trophoblast cell fusion remarkably. These results indicate that syncytin-A is directly involved in mouse TS cell differentiation and fusion during placental development. It is also noticed that anti-syncytin-A antiserum can not inhibit SynT cell formation completely (Figure 4A), which suggests that unidentified factors might also be involved in TS cell fusion.

Basing on previous findings and current results, it is very interesting that syncytin-1 and syncytin-A share similar structures and functions [5-7, 16, 17, 28]. Both of them, as the ERV ancestor active element entered their corresponding host lineage 25-40 million years ago [16, 29]. Therefore, whether these two proteins have common original and evolutionary courses is noteworthy problems. And, it could be also presumed that there should be similar endogenous retroviral envelope proteins involved in syncytiotrophoblast formations during the developments of other mammalian placentas which contain syncytiotrophoblast layers. So, detecting other endogenous retroviral envelope proteins involved in the formations of placental syncytiotrophoblast layers is significant to further confirm the hypothesis: these endogenous retroviral envelope proteins, which become different in primary sequences during long-term evolution, probably share common ancestor and still conserve similar structural and functional profilings.

Human and mouse placentas have many similarities, especially both of them contain syncytiotrophoblast layers. Abnormal syncytin-1 expression and protein localization in human placenta may cause some diseases such as preeclampsia and HELLP (hemolysis, elevated liver enzymes and low platelets) during pregnancy [30-33]. Unfortunately, we can not research on these diseases directly in human body. So, revealing the role of syncytin-A in mouse placental development can give us a great chance to develop mouse model *in vivo*, which will highlight the way to overcome the diseases.

Acknowledgements

We are very grateful to Dr. Janet Rossant for kindly providing the TS cells. This work was supported by grant from the China National Major Scientific Research Plan (2006CB933104) and Program for New Century Excellent Talents in University.

References

- Boeke JD, Stoye JP: Retrotransposons, endogenous retroviruses, and the evolution of retroelements; in Coffin JM, Hughes SH, Varmus HE (eds): *Retroviruses*. New York, Cold Spring Harbor Laboratory Press, 1997, pp 343–435.
- Bannert N, Kurth R: Retroelements and the human genome: new perspectives on an old relation. *Proc Natl Acad Sci U S A* 2004;5:14572-14579.
- Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, et al.: Initial sequencing and analysis of the human genome. *Nature* 2001;409:860-921.
- Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, et al.: Initial sequencing and comparative analysis of the mouse genome. *Nature* 2002;420:520-562.
- Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC Jr, McCoy JM: Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 2000;403:785-789.
- Frendo JL, Olivier D, Cheynet V, Blond JL, Bouton O, Vidaud M, Rabreau M, Evain-Brion D, Mallet F: Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol* 2003;23:3566-3574.
- Mallet F, Bouton O, Prudhomme S, Cheynet V, Oriol G, Bonnaud B, Lucotte G, Duret L, and Mandrand B: The endogenous retroviral locus ERVWE1 is a bona fide gene involved in hominoid placental physiology. *Proc Natl Acad Sci U S A* 2004;101:1731-1736.
- Dunlap KA, Palmarini M, Varela M, Burghardt RC, Hayashi K, Farmer JL, Spencer TE: Endogenous retroviruses regulate periimplantation placental growth and differentiation. *Proc Natl Acad Sci U S A* 2006;103:14390-14395.
- Rossant J, Cross JC: Placental development: lessons from mouse mutants. *Nat Rev Genet* 2001;2:538-548.
- Cross JC: Genetic insights into trophoblast differentiation and placental morphogenesis. *Semin Cell Dev Biol* 2000;11:105-113.
- Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG, Yamamoto H, Kingdom JC: Genes, development and evolution of the placenta. *Placenta* 2003;24:123-130.
- Basyuk E, Cross JC, Corbin J, Nakayama H, Hunter P, Nait-Oumesmar B, Lazzarini RA: Murine Gcm1 gene is expressed in a subset of placental trophoblast cells. *Dev Dyn* 1999;214:303-311.
- Anson-Cartwright L, Dawson K, Holmyard D, Fisher SJ, Lazzarini RA, Cross JC: The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nat Genet* 2001;25:311-314.
- Stecca B, Nait-Oumesmar B, Kelley KA, Voss AK, Thomas T, Lazzarini RA: Gcm1 expression defines three stages of chorioallantoic interaction during placental development. *Mech Dev* 2002;115:27-34.
- Hughes M, Dobric N, Scott IC, Su L, Starovic M, St-Pierre B, Egan SE, Kingdom JC, Cross JC: The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promote terminal differentiation of trophoblast stem cells. *Dev Biol* 2004;271:26-37.
- Dupressoir A, Marceau G, Vernochet C, Benit L, Kanellopoulos C, Sapin V, Heidmann T: Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae. *Proc Natl Acad Sci U S A* 2005;102:725-730.
- Peng X, Pan J, Gong R, Liu Y, Kang S, Feng H, Qiu G, Guo D, Tien P, Xiao G: Functional characterization of syncytin-A, a newly murine endogenous virus envelope protein. Implication for its fusion mechanism. *J Biol Chem* 2007;282:381-389.
- Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J: Promotion of trophoblast stem cell proliferation by FGF4. *Science* 1998;282:2072-2075.
- Kunath T, Rossant J: Mouse trophoblast stem cells; in Soares MJ, Hunt JS (eds): *Placenta and Trophoblast*. Totowa, Humana Press, 2005, pp 123-146.
- Alsat E, Haziza J, Evain-Brion D: Increase in epidermal growth factor receptor and its messenger ribonucleic acid levels with differentiation of human trophoblast cells in culture. *J Cell Physiol* 1993;154:122-128.
- Alsat E, Haziza J, Scippo ML, Frankenne F, Evain-Brion D: Increase in epidermal growth factor receptor and its mRNA levels by parathyroid hormone (1-34) and parathyroid hormone-related protein (1-34) during differentiation of human trophoblast cells in culture. *J Cell Biochem* 1993;53: 32-42.
- Morrish DW, Linetsky E, Bhardwaj D, Li H, Dakour J, Marsh RG, Paterson MC, Godbout R: Identification by subtractive hybridization of a spectrum of novel and unexpected genes associated with in vitro differentiation of human cytotrophoblast cells. *Placenta* 1996;17:431-441.

- 23 Garcia-Lloret MI, Morrish DW, Wegmann TG, Honore L, Turner AR, Guilbert LJ: Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion. *Exp Cell Res* 1994; 214:46-54.
- 24 Robinson BG, Emanuel RL, Frim DM, Majzoub JA: Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta. *Proc Natl Acad Sci U S A* 1988;85:5244-5248.
- 25 Cronier L, Guibourdenche J, Niger C, Malassine A: Oestradiol stimulates morphological and functional differentiation of human villous cytotrophoblast. *Placenta* 1999;20:669-676.
- 26 Shi QJ, Lei ZM, Rao CV, Lin J: Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts. *Endocrinology* 1993;132:1387-1395.
- 27 Cronier L, Bastide B, Herve JC, Deleze J, Malassine A: Gap junctional communication during human trophoblast differentiation: influence of human chorionic gonadotropin. *Endocrinology* 1994;135:402-408.
- 28 Gong R, Peng X, Kang S, Feng H, Huang J, Zhang W, Lin D, Tien P, Xiao G: Structural characterization of the fusion core in syncytin, envelope protein of human endogenous retrovirus family W. *Biochem Biophys Res Commun* 2005;331:1193-1200.
- 29 Voisset C, Blancher A, Perron H, Mandrand B, Mallet F, Paranhos-Baccala G: Phylogeny of a novel family of human endogenous retrovirus sequences, HERV-W, in humans and other primates. *AIDS Res Hum Retroviruses* 1999;15:1529-1533.
- 30 Lee X, Keith JC Jr, Stumm N, Moutsatsos I, McCoy JM, Crum CP, Genest D, Chin D, Ehrenfels C, Pijnenborg R, van Assche FA, Mi S: Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. *Placenta* 2001;22:808-812.
- 31 Knerr I, Beinder E, Rascher W: Syncytin, a novel human endogenous retroviral gene in human placenta: evidence for its dysregulation in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2002;186:210-213.
- 32 Kudo Y, Boyd CA, Sargent IL, Redman CW: Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BeWo cells: implications for impaired trophoblast syncytialisation in pre-eclampsia. *Biochim Biophys Acta* 2003;1638:63-71.
- 33 Knerr I, Weigel C, Linnemann K, Dotsch J, Meissner U, Fusch C, Rascher W: Transcriptional effects of hypoxia on fusogenic syncytin and its receptor ASCT2 in human cytotrophoblast BeWo cells and in ex vivo perfused placental cotyledons. *Am J Obstet Gynecol* 2003;189:583-588.