Relationships between homeoprotein EGAM1C and the expression of the placental prolactin gene family in mouse placentae and trophoblast stem cells

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Abstract

The mouse Crxos gene encodes three structurally related homeoproteins, EGAM1, EGAM1N, and EGAM1C, as transcription and splicing variants. Recently, we identified the functions of EGAM1 and EGAM1N in the regulation of differentiation in mouse embryonic stem cells. However, the function of EGAM1C remains unknown. To explore the additional roles of these proteins, the ontogenic expression of the respective mRNAs in post implantation mouse embryos and extraembryonic tissues, particularly from embryonic day (E) 10.5 to E18.5, was analyzed. The expression of Egam1n mRNA was specifically detected in embryos throughout this period, whereas that of Egam1 was undetectable in any of the tissues examined. However, in the placenta, Egam1c mRNA and its encoded protein were detected after E16.5, and these expression levels increased by E18.5 immediately before partum. Quantitative RT-PCR and in situ hybridization analyses in placentae revealed that the spatial and temporal expression patterns of the Egam1c mRNA were related to some extent with those of Prl3a1 and Prl5a1 and partially overlapped that of Prl3b1, which are members of the placental prolactin (PRL) gene family. When EGAM1C was overexpressed moderately in mouse trophoblast stem cells as a model for undifferentiated and differentiating placental cell types, the expression levels of endogenous Prl3b1 and Prl5a1 were enhanced under both undifferentiated and differentiating culture conditions. These results indicated that EGAM1C may play a role in the expression of members of the placental PRL gene family, such as Prl3b1 and Prl5a1.

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Introduction

In mammals, the prolactin (Prl) gene family is a large family of paralogous genes encoding hormones and cytokines (Soares et al. 2007). To date, 23 members in this family have been reported in mice (Simmons et al. 2008). PRL, PRL3D1 (also known as PL1), and PRL3B1 (PL2) are major hormones that bind specifically to the PRL receptor (Harigaya et al. 1988, MacLeod et al. 1989). These hormones stimulate the growth and differentiation of mammary epithelial cells responsible for lactation and help to maintain pregnancy via stimulation of the corpus luteum (Galosy & Talamantes 1995, Peters et al. 2000). Among the members of the PRL family, only PRL is secreted from the maternal pituitary gland, and the others are mainly from the placenta.

Until recently, some evidence has been accumulated regarding transcription factors for the Prl gene family. It is well established in primates and rodents that POU1F1 (PIT1), a pituitary-specific transcription factor with a homeodomain (Nelson et al. 1988), regulates the expression of Prl. In rodents, the expression of Prl3d1 in trophoblast giant cells is stimulated by HAND1 (Cross et al. 1995), a member of the basic helix-loop-helix transcription factors. GATA2 and GATA3, zinc finger containing transcription factors, are responsible for the placental gene expressions of Prl3d1 (Ng et al. 1994) and proliferin (Ma et al. 1997). TFAP2C (AP2γ) is also known as a critical factor for the expression of Prl3b1 (Ozturk et al. 2006). However, transcription factors that regulate the expression of the placental Prl gene family remain poorly understood.

The mouse Crxos gene (Alfano et al. 2005) was demonstrated to be expressed in the retina from the opposite strand of the mouse Crx gene, which plays an important role in eye development and function (Chen et al. 1997), and is located 15 kb away from the Crx gene. Recently, we identified three kinds of mRNAs that are transcribed from the Crxos gene as splicing or transcription variants. These transcripts encode structurally related homeoproteins, EGAM1 (also known as CRXOS1), EGAM1N (CRXOS1 sv2), and EGAM1C
(CRXOS1 tv3), and are expressed in preimplantation mouse embryos and pluripotent mouse embryonic stem (ES) cells (Saito et al. 2010). The function of these homeoproteins was estimated by enforced expression in mouse ES cells, which were established from the inner cell mass of blastocysts, as a model for pluripotent cells in preimplantation embryos (Saito et al. 2010). EGAM1 and EGAM1N may act as positive and negative regulators respectively in the differentiation of ES cells and seem to also regulate the differentiation of preimplantation embryos. However, the function of EGAM1C is still elusive.

In this study, in order to determine the roles of EGAM1, EGAM1N, and EGAM1C in subsequent developmental stages, we analyzed the ontogenic expression of their transcripts in mouse concepti. Subsequently, we investigated possible relations, especially for EGAM1C, in the expression of the placental Prl gene family.

Results

Expression of Egam1, Egam1n, and Egam1c mRNAs in embryos and extraembryonic tissues

In order to determine the expression profiles of Egam1, Egam1n, and Egam1c mRNAs, the expression of these transcripts in concepti from embryonic day (E) 10.5 to E18.5 was analyzed by RT-PCR (Fig. 1). The expression levels of mRNAs for Myog, a marker gene for skeletal muscle (Wright et al. 1989), and Tpbpa, a marker gene for the placenta (Lescisin et al. 1988), were restricted properly to embryo and extraembryonic tissues respectively, indicating that these tissues were dissected accurately throughout the experiment. Under these conditions, the expression of Egam1 mRNA was undetectable in both whole embryos and extraembryonic tissues (data not shown). The expression of Egam1n mRNA was detected in embryos, and the expression level decreased gradually with embryonic development. On the other hand, the Egam1c mRNA was detected in extraembryonic tissues after E14.5, and its expression level increased drastically until E18.5, immediately before partum. No expression of Egam members was detected in the maternal uterus adjacent to the conceptus.

Correlation between the expression levels of Egam1c and the placental Prl gene family

In order to examine the tissues expressing Egam1c, extraembryonic tissues were dissected into the placenta and amnion/yolk sac, and then the expression of Egam1c mRNA was analyzed by RT-PCR. As shown in Fig. 2A, proper expression profiles of Tpbpa and Afp, a marker gene for the yolk sac (Gitlin & Boesman 1967), were also detected in the placenta and amnion/yolk sac respectively throughout the experiment. In the placenta, tissue-specific expression of Egam1c transcript was clearly detected. The expression level of Egam1c increased ~30-fold from E16.5 to E18.5, as determined by quantitative RT-PCR (qRT-PCR; Fig. 2B). Western blot analysis revealed obvious expression of EGAM1C protein in placenta at E16.5 and E18.5 (Fig. 2C).

Drastic increases in the expression level of Egam1c and its encoded protein from E16.5 to E18.5 in placenta led us to hypothesize that Egam1c may play a role in the expression of genes involved in placental development or functions during this period. The expression patterns already reported of Plr3a1 (Plpi; Wiemers et al. 2003) and Plr5a1 (Plpi; Wiemers et al. 2003), members of the placental Prl gene family, seemed to associate well with Egam1c expression. Therefore, we examined the expression profiles of these genes, in addition to Plr3b1 (Pl2), a typical placental Prl family gene that is expressed in late pregnancy (Simmons et al. 2008), using qRT-PCR (Fig. 2D) and northern blot (Supplementary Figure 1, see section on supplementary data given at the end of this article) analyses in our hands. The expression of Plr3b1 was detected after E10.5, and its level increased gradually towards the end of pregnancy. The expression levels of Plr3a1 and Plr5a1 increased after E14.5, and their levels reached a maximum at E18.5, as reported previously (Wiemers et al. 2003). These results indicated...
that the expression pattern of \textit{Egam1c} correlated to some extent with those of \textit{Prl3a1} and \textit{Prl5a1} and partially overlapped with that of \textit{Prl3b1}.

\textbf{Identification of cells expressing mRNAs for \textit{Egam1c} and the \textit{Prl} gene family in placentae by in situ hybridization}

Expression of mRNAs for \textit{Egam1c}, members of \textit{Prl} family genes, and \textit{Tpbpa}, a typical marker for the junctional zone (JZ; Lescisin \textit{et al.} 1988), in placental tissues at E18.5 was analyzed by \textit{in situ} hybridization. As indicated in Fig. 3, the expression patterns of \textit{Tpbpa} and \textit{Prl3a1} (\textit{Plpi}) were restricted to the JZ. \textit{Prl3b1} (\textit{Pl2}) mRNA was detected in both the JZ and labyrinth zone (LZ). These expression patterns were identical to observations reported previously (Simmons \textit{et al.} 2008). Interestingly, the expression of \textit{Egam1c} mRNA was detected in almost all placental tissues, including the JZ, LZ, and maternal decidual zone (DZ).

Cell types expressing \textit{Egam1c} mRNA were identified (Fig. 4 and see enlargements in Supplementary Figure 2, see section on supplementary data given at the end of this article). In general, the expression of \textit{Tpbpa} was restricted to spongiotrophoblast (SpT) cells and glycogen trophoblast (GlyT) cells in the JZ (Fig. 4E; Adamson \textit{et al.} 2002, Simmons \textit{et al.} 2008), and the expression of \textit{Prl3b1} was detectable in SpT cells (Fig. 4E) and sinusoidal trophoblast giant (S-TG) cells (Fig. 4H; Simmons \textit{et al.} 2008). In addition to SpT or GlyT cells, the expression of \textit{Tpbpa} and \textit{Prl3b1} was detected in some but not all of the parietal trophoblast giant (P-TG) cells (Fig. 4G). P-TG cells were distinguishable as populations carrying differential hematoxylin- and eosin-stained cytosol, and herein referred to as ‘purpuric (indicated by black arrow)’ and ‘pinky (white arrow)’ P-TG cells. One more remarkable feature of ‘pinky’ P-TG cells is their configuration; they lay adjacent to SpT cells. Co-expression of \textit{Tpbpa} and \textit{Prl3b1} in the same cells was observed specifically in ‘pinky’ P-TG cells. In the study, localization of \textit{Prl3a1} mRNA in specific cell types failed to detect such weak signals (data not shown).

In the JZ, \textit{Egam1c} mRNA was detectable in both SpT (Fig. 4E) and GlyT cells (Fig. 4F). It was also detected in S-TG cells in the LZ (white arrowhead, Fig. 4H). However, no signals of \textit{Egam1c} mRNA were detected in syncytiotrophoblast (SynT) cells comprising the LZ (black arrowhead, Fig. 4H). ‘Pinky’ P-TG cells were also found to express \textit{Egam1c} transcripts (white arrow, Fig. 4G). In the DZ, \textit{Egam1c} mRNA was detected in invasive trophoblast (InvT) cells (white arrowhead, Fig. 4I), spiral artery-associated trophoblast giant
Forced expression of EGAM1C enhances the expression of endogenous Prl3b1 and Prl3a1 in undifferentiated and differentiating mouse trophoblast stem cells

To investigate the expression patterns of EGAM1C protein and mRNAs for the placental Prl family genes, the expression level of EGAM1C was analyzed during the course of trophoblast stem (TS) cell differentiation (Fig. 5) as a model for undifferentiated and differentiating placental cell types. An unexpected finding was that the expression of EGAM1C protein decreased after the induction of differentiation (Fig. 5A). On the other hand, the expression of Cdx2, a marker for undifferentiated TS cells (Tanaka et al. 1998), decreased (Fig. 5B). The expressions of Prl3b1 (Pl2) and Prl5a1 (PlPl) transcripts increased after the induction of differentiation. However, the expression of Prl3a1 mRNA was below the threshold level (data not shown).

To explore the potential functions of EGAM1C as a transcriptional activator for Prl3b1 or Prl5a1 expression, EGAM1C protein was overexpressed in TS cells (Fig. 6). Western blot analysis revealed that the expression level of EGAM1C in Egam1c transfectants (Fig. 6A) was evaluated as approximately sixfold higher than that in placentae at E18.5 (Fig. 2C). In Egam1c transfectants, no visible morphological changes were found (Supplementary Figure 3, see section on supplementary data given at the end of this article, C Fibroblast growth factor 4 (FGF4)); however, the expression levels of endogenous Prl3b1 (Pl2) and Prl5a1 (PlPl) increased significantly by 4.1- and 3.3-fold respectively, relative to control TS cells (Fig. 6B). After the induction of differentiation, morphological differences between transfectants were also not found (Supplementary Figure 3, K FGF4). However, moderate, but significant increases in the expression levels of Prl3b1 and Prl5a1 were detected in TS cells overexpressing EGAM1C (Fig. 6C and D). In contrast, the expression level of Cdx2 was comparable to that of the control.

No expression of Egam1c mRNA in the maternal pituitary

In order to clarify the contribution of Egam1c to the induction of Prl expression, the correlation of expression profiles for Egam1c and Prl in the maternal pituitary was investigated. As indicated in Fig. 7, co-expression of Pou1f1 (Pit1) and Prl was found, while the expression of Egam1c mRNA was barely detected.

Discussion

Three kinds of transcripts, Egam1, Egam1n, and Egam1c, encoding structurally related homeoproteins have been detected in mouse ES cells (Saito et al. 2010). These transcripts were expressed differentially during in vitro ES cell differentiation, and the encoded proteins probably act as transcriptional regulators. In this study, we further analyzed the ontogenic expression of these transcripts in mouse concepti to address the functions of these proteins in the subsequent developmental stages. Tissue-specific expression of Egam1c was found in the placenta; therefore, the relationship between Egam1c and expression of the placental Prl gene family was
further investigated. Major results obtained in the present study can be summarized as follows: 1) the expression pattern of *Egam1c* mRNA and its encoded protein correlated to some extent with those of *Prl3a1* (*Plpi*) and *Prl5a1* (*Plph*) and partially overlapped with that of *Prl3b1* (*Pl2*) in the mouse placenta from E16.5 to E18.5, 2) the spatial expression profile of *Egam1c* mRNA and those of *Prl3a1* and *Prl3b1* overlapped in placentae at E18.5, and 3) the forced expression of EGAM1C protein in TS cells resulted in stimulating both *Prl3b1* and *Prl5a1* expression levels, although *Prl3a1* expression was not detected during TS cell differentiation. It is noteworthy that the spatial expression profile of *Prl5a1* in the mouse placenta as reported

Figure 4 Cell-specific localization of *Egam1c* mRNA in the mouse placenta at E18.5. (A) E18.5 placenta stained by hematoxylin and eosin (HE). (B–D) *In situ* hybridization of mRNAs for *Egam1c*, *Tpbpa*, and *Prl3b1* (*Pl2*). Boxes labeled with the corresponding letters represent the enlarged areas shown in E through K. (E–K) Magnified images of spongiotrophoblast (SpT; E) cells, glycogen trophoblast (GlyT; F) cells, parietal trophoblast giant (P-TG; G) cells, sinusoidal trophoblast giant (S-TG; H) cells, invasive trophoblast (InvT; I) cells, spiral artery-associated trophoblast giant (SpA-TG; J) cells, and decidual (Dec; K) cells. White arrowheads with broken circles show representative cell clusters (InvT; I) or cells (others). In panel F, a broken rectangle with white arrowhead indicates that there are no specific signals for *Prl3b1*; therefore, the cell shapes are hardly detectable. This area contains a considerable number of GlyT cells that are not expressing *Prl3b1*. Black arrowheads in panel H indicate syncytiotrophoblast (SynT) cells, in which no *Egam1c* mRNA was detected. Black arrows in panel G are ‘purpuric’ P-TG cells that are HE-stained differently from ‘pinky’ P-TG cells, as indicated by white arrows. A series of panels derived from the same photographs are shown in Fig. 3. *, Specific signals for respective mRNAs were detected in almost all of cells in the panel; **, no specific signals were detected in the panel; and ***, decidual (Dec) cells could not be identified; Sp, spiral artery. Scale bar represents 1.0 mm and 40 μm in panels A–D and E–K respectively.
expressed as mean ± S.E.M. (n = 4).

Previously (Watson & Cross 2005, Simmons et al. 2008), and were confirmed in this study. Nevertheless, to our knowledge, the transcription factors that stimulate these genes are as yet unidentified.

In the present study, Egam1c expression was not found in the maternal pituitary (Fig. 7), indicating that Egam1c is likely to be unrelated to Prl expression. On the other hand, the temporal expression profiles of Prl3b1, Prl3a1, and Prl5a1 overlapped, at least in part, with that of Egam1c especially during late pregnancy (Fig. 2). Tissue-specific expression of the Egam1c mRNA was detectable in the placenta (Fig. 4); in placenta at E18.5, almost all cell types in the JZ, except for 'purpuric' P-TG cells, the LZ except for SynT cells, and the DZ expressed Egam1c. In 'pinky' P-TG and SpT cells of the JZ and S-TG cells of the LZ, we identified the co-localization of Egam1c and Prl3b1 mRNAs. Egam1c expression was also detectable in SpT cells expressing both Prl3a1 mRNA, as indicated in this study, and Prl5a1 mRNA, as reported previously (Wiemers et al. 2003). These reciprocal expression domains of Prl3a1, Prl3b1, Prl5a1, and Egam1c led us to hypothesize that EGAM1C could play a role in the expression of these placental Prl family genes in the respective cells. Furthermore, in placenta at E18.5, Egam1c mRNA was also detectable in GlyT, InvT, and SpA-TG cells in spite of the absence of Prl3a1 and Prl3b1 expression, suggesting that Egam1c may conceivably exhibit additional functions in these cells. In addition, Egam1c was expressed not only in the trophoblast cell lineage but also in the maternal decidual cells. The results suggested that the expression of Egam1c is inducible in the uterine endometrium. Factors that induce the Egam1c expression are still unknown, but hormones, including placental PRL family and steroid hormones, or cell communications between the trophoblasts and Dec cells are likely to affect the induction of Egam1c expression.

The successful derivation of mouse TS cells offers a model system to dissect the functions of genes that are crucial to the development and function of the mammalian trophoblast lineage (Tanaka et al. 1998). In this system, FGF4 promotes TS cells derived from polar trophectoderm to proliferate as an undifferentiated state. Removal of FGF4 results in a rapid decline in cell proliferation and an acceleration of differentiation towards cell types seen in developing placentae, including P-TG, SpT, and InvT cells (Tanaka et al. 1998, Hemberger et al. 2004, Hughes et al. 2004) expressing Egam1c mRNA as indicated by *in situ* hybridization. During the course of TS cell differentiation, the expression of EGAM1C decreased to a low level, while the expression levels of Prl3b1 (Pl2) and Prl5a1 (Plpi) increased substantially after the induction of differentiation (Fig. 5). This indicates that the up-regulation of EGAM1C in differentiating TS cells is dispensable for the expression of these Prl family genes. Unexpectedly, the expression level of endogenous EGAM1C protein decreased in differentiating TS cells,
and thus we tried to overexpress the protein in TS cells. The enforced expression of EGAM1C was capable of stimulating Prl3b1 and Prl5a1 expression both in undifferentiated and in differentiating TS cells without changes in the expression of Cdx2 or cell morphology. Considering that the placenta also contains cell types other than the trophoblast lineage, including connective tissues, blood vessels, and stromal cells, the forced expression level of EGAM1C in TS cell transfectants could be predicted to be below sixfold of that in placenta. Therefore, we considered that the effects of EGAM1C observed in TS cells were not experimental artifacts because it is possible to assume that the forced expression level of EGAM1C was a moderate degree. Homeoproteins often act as transcriptional regulators by forming heterodimers, such as a heterodimer OCT4/SOX2 (Niwa 2007). EGAM1C might function via heterodimerization as a co-activator for essential, but unidentified transcription factors expressed at a basal level in undifferentiated TS cells with members of the placental Prl family genes. Therefore, it likely that this resulted in the moderate, but significant, induction of Prl3b1 and Prl5a1 expression in undifferentiated TS cells. During the progression of differentiation, it is also likely that the forced expression of EGAM1C led to the significant elevation in endogenous Prl3b1 and Prl5a1 expression levels. We would like to speculate that, in differentiating TS cells transfected with Egam1c expression vector, sufficient amounts of EGAM1C might further activate the essential transcription factors for Prl3b1 or Prl5a1 that are expressed at physiological levels without promoting cellular differentiation. Another possible mechanism of EGAM1C action is acting as a biphase transcriptional regulator depending on its expression levels: EGAM1C at a high expression level, such as in forced EGAM1C-expressing TS cells, might act as a transcriptional co-activator, and conversely, at a relatively low expression level, such as in undifferentiated TS cells, it might act as a transcriptional repressor for the expression of members of the placental Prl gene family. Direct analysis including a knockdown experiment will be required to elucidate the transcriptional repressive activity of EGAM1C in undifferentiated TS cells. In differentiating TS cells, the expression of EGAM1C might be inducible, as observed in uterine endometrium, by hormones or other stimuli.

Taken together, the present study indicated a clear relationship between EGAM1C and the expression of members of the placental Prl family genes, such as Prl3b1 (Pl2) and Prl5a1 (Plp1). To the best of our knowledge, this is the first delineation of the capability of homeoproteins for involving the expression of placental Prl family genes. Further work will be required to address the regulatory mechanisms for the transactivation of Egam1c and by which EGAM1C regulates the expression of Prl3b1 and Prl5a1.
Figure 7 Temporal expression profiles of mRNAs for Egam1c, Prl, and Pou1f1 in the maternal pituitary. Expression of respective mRNAs in the maternal pituitary (E6.5 to E18.5) in each sample prepared from several mice was analyzed by RT-PCR. The cDNA sample prepared from placenta (E18.5) was used as a positive control for Egam1c expression. NP, cDNA sample prepared from the pituitaries of non-pregnant mice. Results indicated in the figure were derived from a representative of duplicate samples.

Materials and Methods

Animals

CD-1 and BDF1 mice (Charles River Japan, Yokohama, Japan) were housed in an environmentally controlled facility with a 12 h light:12 h darkness cycle and allowed free access to food and water. Female mice were mated with males, and the presence of a copulatory plug was designated as E0.5 after fertilization. All animal procedures conformed to the Guidelines for the Care and Use of Laboratory Animals of Akita Prefectural University.

Construction of an Egam1c expression vector

An expression vector for Egam1c cDNA was constructed using a dicistronic mRNA-expressing pMN1Pur vector (Saito et al. 2010). This vector contains a powerful and versatile chicken Actb (β-actin) promoter with the cytomegalovirus immediate early enhancer and rabbit Hbb (β-globin) intron II acceptor site (CAG promoter (Niwa et al. 1991)). The vector expresses a single mRNA concomitantly encoding a gene of interest, an internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus, and a puromycin resistance gene. In cells transfected with this vector, the high degree of secondary structure of the IRES in the transcript is recognized by ribosomes, and the puromycin resistance gene placed downstream of the IRES is translated by IRES dependently. Such a dicistronic vector is advantageous to ensure the co-expression of the desired gene in cells expressing a selectable marker gene. The cDNA corresponding to the coding sequence for Egam1c was inserted into the cloning site (Xbal) of pMN1Pur in the sense orientation. The resultant pMN1Pur vector expressing Egam1c was designated as pMN1Pur/Egam1c.

Cell culture and transfection

Isolation and culture of mouse TS cells were performed in accordance with the method reported previously (Tanaka et al. 1998). TS cells established from a CD-1 blastocyst (Hosoi et al. 2010) were trypsinized and resuspended in Ca2+- and Mg2+-free PBS at a concentration of 1 × 105 cells/0.8 ml, and then electroporated with 50 μg of linearized pMN1Pur/Egam1c at 200 V and 500 μF in a 0.4 cm cuvette using a Gene Pulser (Bio-Rad). Transfected cells were plated onto gelatinized plates (10 cm in diameter). After a 48 h period, stable transfectants were selected with 3.0 μg/ml puromycin for 11 days in undifferentiated state-maintaining culture (+FGF4) conditions supplemented with FGF4 and the medium conditioned by mouse embryonic fibroblasts (MEFCM). Differentiation of TS cells was induced by the withdrawal of FGF4 and MEFCM from the medium (−FGF4).

RNA extraction, RT-PCR, and qRT-PCR analyses

Tissues isolated from concepti from pregnant females at E10.5, 12.5, 14.5, 16.5, and 18.5 were frozen in liquid nitrogen. Total RNA was extracted from the frozen tissues or transfected TS cells using an acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987). Contaminating DNA was degraded with RNase-free DNase I (Takara-Bio, Kyoto, Japan). DNase I-treated total RNA was purified again by acid guanidinium thiocyanate–phenol–chloroform extraction. First-strand cDNA was synthesized using reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) and an oligo-dT20 primer in accordance with the manufacturer’s instructions.

PCR analysis was performed using an Ex-Taq DNA polymerase (Takara-Bio) with a set of gene-specific primers (Supplementary Table 1, see section on supplementary data given at the end of this article). Hydroxymethylbilane synthase (Hmbs), a housekeeping gene, was used as a positive control to monitor the quality and quantity of cDNA. Aliquots of the PCR products were electrophoresed on 1.5 or 3.0% agarose gels and visualized after ethidium bromide staining. QPCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer’s protocols. A standard curve was obtained using serial dilutions of each amplicon cloned in a pGEM-T Easy vector (Promega).

In situ hybridization analysis of mRNAs in the placenta and identification of trophoblast cell types

cDNAs were amplified using gene-specific primers (Supplementary Table 2, see section on supplementary data given at the end of this article) from E18.5 placental cDNA. Digoxigenin (DIG)-labeled sense and antisense riboprobes were synthesized using a DIG RNA labeling kit (SP6/T7, Boehringer Mannheim) using the respective cDNAs cloned in the pGEM-T Easy vector as templates in accordance with the manufacturer’s instructions. The labeled probes were shortened to about 150 bases by limited alkaline hydrolysis.

Placentae (E18.5) were fixed in Ca2+- and Mg2+-free PBS containing 4% paraformaldehyde at 4°C for 22 h.
Fixed placenta were dehydrated with a graded series of ethanol (50, 70, 80, 90, and 99.5%), cleared with xylene, infiltrated with paraffin (50% xylene/50% paraffin), and embedded in paraffin. Sections (10 μm thick) were air-dried on 3-aminopropyltriethoxysilane-coated glass slides. Deparaffinized and rehydrated sections were further treated with 5 μg/ml proteinase K in water at 37°C for 10 min. Thereafter, the sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, and followed by washing with 4× SSC. Following incubation with a hybridization buffer (50% deionized formamide, 2× SSC, 1 μg/ml tRNA, 1 μg/ml salmon sperm DNA, 1 μg/ml BSA, and 10% dextran sulfate) at 42°C for 30 min, the sections were incubated with hybridization buffer containing a DIG-labeled probe (1.5 μg/ml) in a humidified chamber at 42°C for 16 h. After hybridization, the sections were sequentially washed with 50% formamide/2× SSC at 42°C (20 min, three times), and NTE buffer (0.5 M NaCl, 10 mM Tris–HCl, and 1 mM EDTA, pH 8.0) at 37°C (5 min). Then, the sections were further treated with 20 μg/ml RNase A in NTE buffer at 37°C for 30 min, followed by washing with NTE buffer at 37°C (5 min) and with 0.1× SSC at 42°C (20 min, three times). After washing with a maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl, pH 7.5) at 22°C for 1 min, the sections were incubated with blocking solution (25% normal rabbit serum, 0.5% Tween 20, and 74.5% maleic acid buffer) at 22°C for 30 min. Then, the sections were incubated in a humidified chamber for 16 h at 4°C with sheep anti-DIG Fab fragments conjugated with alkaline phosphatase (1:500, Roche Diagnostics). Finally, immunoreactions were visualized by incubating the sections with a solution containing nitroblue tetrazolium and bromo-4-chloro-3-indolyl-phosphate. The sections were mounted in a mounting reagent (Aqua Poly Mount, Polysciences, Warrington, PA, USA).

Trophoblast cell types were identified in accordance with the classification described by Simmons et al. (2008). In brief, the fully developed placenta has three distinct layers comprising several trophoblast cell types: the JZ consisting of P-TG cells, SpT cells expressing Tpbpa and Pl3b1 (P12), and GlyT cells expressing Tpbpa; the LZ, in which TG cells that line maternal blood canals enter the labyrinth leading into small sinusoïds lined with S-TG cells and SynT cells; and the DZ which contains maternal Dec cells, Spa-TG cells, and InvT cells that invade into the DZ and form cell clusters. These cell types were classified by the configuration in placenta and the size of the cell or nucleus. Expression of non-specifically expressed genes was also considered. In general, TG cells have a larger nucleus than the other cells, such as SpT, GlyT, SynT, InvT, or Dec cells.

Protein extraction and western blotting

Transfected TS cells were lysed by sonication in Ca²⁺- and Mg²⁺-free PBS. Concentrations of protein in cell lysates were determined using a BCA Protein Assay reagent (Pierce, Rockford, IL, USA) using BSA as a standard. Placentae (E12.5, 14.5, 16.5, and 18.5) were lysed by sonication in SDS-sample buffer. Placental lysates were boiled for 5 min and centrifuged at 20 000 g or 5 min at 4°C, and the supernatant was used for western blotting.

Lysates prepared from placentae (450 μg wet weight/lane) or TS cells (40 μg of protein/lane) were separated on SDS–14% polyacrylamide gels and subsequently electrophoretically transferred onto a PVDF membrane (Immobilon, Millipore, Bedford, MA, USA). Thereafter, the membranes were blocked with a PVDF Blocking Reagent (Toyobo) and incubated with rabbit anti-EGAM1C antiserum (1:20 000) or preimmune rabbit serum (1:20 000) in immunoreaction enhancer solution 1 (Can Get Signal kit, Toyobo). The avidity of anti-EGAM1C antiserum raised against bacterially expressed, glutathione-S-transferase (GST)-fused EGAM1C protein separated by polyacrylamide gels used in our previous study (Saito et al. 2010) was extremely low; therefore, new anti-EGAM1C was prepared by immunizing rabbits with bacterially expressed, soluble GST-fused protein. The resultant new anti-EGAM1C antiserum recognized cellular EGAM1C with high sensitivity in western blotting. The membrane was incubated with HRP-conjugated goat anti-rabbit IgG antibodies (1:1 000 000) as secondary antibodies in immunoreaction enhancer solution 2 from the kit. The enzyme reaction was detected using an ECL plus Western Blotting Reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and a LAS-4000 image analyzer (Fuji Film, Tokyo, Japan).

Statistical analysis

The statistical significance of the difference between sample means was determined by Student’s t-test. A statistically significant difference was considered to be present at P<0.05.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0355.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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