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The Expression of Proprotein Convertase PACE4 Is Highly Regulated by Hash-2 in Placenta: Possible Role of Placenta-Specific Basic Helix-Loop-Helix Transcription Factor, Human Achaete-Scute Homologue-2

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PACE4 is a member of the mammalian subtilisin-like proprotein convertase (SPC) family, which contribute to the activation of transforming growth factor (TGF) β family proteins. We previously reported that PACE4 is highly expressed in syncytiotrophoblasts of human placenta [Tsuji *et al.* (2003) *Biochim. Biophys. Acta* 1645, 95–104]. In this study, the regulatory mechanism for PACE4 expression in placenta was analyzed using a human placental choriocarcinoma cell line, BeWo cells. Promoter analysis indicated that an E-box cluster (E4–E9) in the 5′-flanking region of the PACE4 gene acts as a negative regulatory element. The binding of human achaete-scute homologue 2 (Hash-2) to the E-box cluster was shown by gel mobility-shift assay. The overexpression of Hash-2 caused a marked decrease in PACE4 gene expression. When BeWo cells were grown under low oxygen (2%) conditions, the expression of Hash-2 decreased, while that of PACE4 increased. In both cases, other SPCs, such as furin, PC5/6, and PC7/8, were not affected. Further, PACE4 expression was found to be developmentally regulated in rat placenta. By *in situ* hybridization, Mash-2 (mammalian achaete-scute homologue 2) mRNA was found to be expressed in the spongiotrophoblast layer where PACE4 was not expressed. In contrast, the PACE4 mRNA was expressed mainly in the labyrinthine layer where Mash-2 was not detected. These results suggest that PACE4 expression is down-regulated by Hash-2/Mash-2 in both human and rat placenta and that many bioactive proteins might be regulated by PACE4 activity.

Key words: furin, Hash-2/Mash-2, PACE4, placenta, proprotein convertase.

Abbreviations: SPC, subtilisin-like proprotein convertase; bHLH, basic helix-loop-helix; Hash-2, human achaete-scute homologue 2; Mash-2, mammalian achaete-scute homologue 2; Hash-1, human achaete-scute homologue 1; Mash-1, mammalian achaete-scute homologue 1; BMP, bone morphogenetic protein; TGF, transforming growth factor; MMPs, matrix metalloproteinases; PLGF, placenta growth factor; RT-PCR, reverse-transcriptase-polymerase chain reaction.

A placenta-specific basic helix-loop-helix (bHLH) factor, mammalian achaete-scute homologue-2 (Mash-2), is crucially involved in the differentiation of trophoblast cells (*1*–*6*). The human Mash-2 homologue is called Hash-2 (*6*). Trophoblasts form the outermost cell layer of a preimplanting embryo, trigger implantation, and finally form the different, specialized epithelial structures of the placenta (*1*, *2*). Trophoblast cells are also important sources of pregnancy-associated hormones (*7*) and play a role in immune protection of the fetus (*8*, *9*). Many growth factors and cytokines are involved in regulating the differentiation, proliferation and invasion of trophoblast cells in pregnancy. Mash-2 mutant mouse conceptuses are arrested at E10.5 due to placental defects that include an absence of the spongiotrophoblast layer (derived from trophoblasts of the ectoplacental cone), excess trophoblast giant cells, and a poorly developed labyrinthine layer (*3*, *5*). Mash-2 is thus required to maintain spongiotrophoblasts at the expense of giant cell differentiation. However, it remains to be determined which gene is the target of Mash-2.

We previously reported that PACE4 is abundantly expressed within the syncytiotrophoblast layer of human placenta (*10*). It is apparent that the differentiation of cytotrophoblasts to syncytiotrophoblasts is associated with the generation of a cascade of regulatory signal; however, the molecular events that control this differentiation process are poorly understood. The placenta produces bioactive substances including many growth factors, adhesion factors, and peptide hormones that are synthesized as inactive precursors and then activated by limited proteolysis at an RXK/RR↓ site (*11*). Recent findings suggest that subtilisin-like proprotein convertases (SPCs) activate these factors (*12*–*15*). So far, seven members of the SPC family (furin, PC1/3, PC2, PACE4, PC4, PC5/6, and PC7/8) have been identified in mammals (*16*).

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All SPCs are Ca^{2+}-dependent serine proteases and share structural similarities, although they vary in tissue distribution and organelle localization. We have shown that PACE4 colocalizes with bone morphogenetic protein (BMP)-4 and that its expression is highly regulated during dentinogenesis (*17*, *18*) and embryogenesis (*19*).

Previously, we identified the PACE4 gene as one of the targets of human and mammalian achaete-scute homologue 1 (Hash-1/Mash-1), which is especially important in the early stages of neural development (*23*). The 5′-flanking region of the PACE4 gene contains 12 E-box elements in the 1 kb upstream of the transcription initiation site (*20*). We showed that the E-box cluster (E4-E9) is a strong negative regulatory element: the promoter activity is greatly increased by the deletion or mutation of this cluster (*21*). Six tandem repeats (E4–E9) of a nanodecamer (GGCCTGGGGGTTCACCTGC) containing an E-box are located in this region and the CACCTG sequence is a specific binding site for Mash-1 (*42*). PACE4 gene expression is down-regulated by the specific binding of Hash-1/Mash-1 to an E-box (CACCTG) sequence in the PACE4 gene (*23*). In this study, we examined the effect of Hash-2 on PACE4 expression using the BeWo cell line to elucidate whether this bHLH transcription factor acts as a regulator of PACE4 gene expression. The DNA binding site of Hash-2 is 98% homologous to that of Hash-1, but the residual part differs completely. Hash-2 also binds to CACCTG E-box sequence as Hash-1 does. The BeWo cell line derived from human placental choriocarcinoma (*24*, *25*) has often been used as a model to study the mechanisms of the transplacental passage of nutrients and metabolites (*26*). The expression of SPC family proteases during rat placental development was examined by RT-PCR. Further, the cell-specific localization of PACE4 and Mash-2 in rat placenta was analyzed by *in situ* hybridization.

MATERIALS AND METHODS

Cell Culture and Transfection—The human choriocarcinoma cell line, BeWo, was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai). BeWo cells were maintained in Ham's F12 medium (Sigma Chemical, St. Louis, MO) containing 15% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 IU/ml penicillin. The cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 (20% O_2) or maintained in an atmosphere of 2% O_2/93% N_2/5% CO_2 (low oxygen condition: 2% O_2). Transfection was performed using Trans IT™-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's instructions (*21*).

Northern Blot Analysis—To analyze the mRNA expression of SPC family proteases, Northern blotting was performed using total RNAs isolated from BeWo cells and human placenta as described previously (*27*). Normal-term placentas from caesarean deliveries were obtained from patients in accordance with established guidelines of the ethical committee of the Tokushima University Hospital. Ten micrograms of total RNA was resolved by electrophoresis in a 1% agarose gel containing 6.7% formaldehyde, and transferred onto a nylon membrane, Hybond-N+ (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was prehybridized with Rapid-hyb buffer (Amersham Pharmacia Biotech) at 60°C for 3 h then hybridized with ^{32}P–labeled probe at 60°C for 16 h. The sites of the probes were as follows: human PACE4 (nt. 1687–2754), human furin (nt. 1561–2050), human PC5/6 (nt. 1801–2385), and human PC7/8 (nt. 1315–1992). The density of each band was quantified using NIH image software (*30*).

Construction of Hash-2 Expression Vector—The coding sequence of Hash-2 (*31*) was amplified from human placenta cDNA by PCR using a *Bam*HI-linked sense primer (5′-CGCGGATCCATGGACGGCGGCACACTG-3′, initial codon underlined) and *Eco*RI-linked antisense primer (5′-CCGGAATTCTCAGTAGCCCCCTAACCA-3′, terminal codon underlined). The PCR product was digested with *Bam*HI and *Eco*RI and cloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA).

Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA (5 μg) from BeWo cells was reverse-transcribed with SUPER SCRIPT™II (Gibco BRL, Gaithersburg, MD). PCR was performed with *Taq* DNA polymerase (Promega, Madison, WI) in accordance with the manufacturer's protocol. The sense and antisense primers for Hash-2 were 5′-ATGGACGGCGGCACACTG-3′ (nt. 545–562) and 5′-TCAGTAGCCCCCTAACCA-3′ (nt. 1108–1125), respectively. PCR was performed for 20 cycles of denaturing at 95°C for 1 min, annealing at 60°C for 1 min, and extension for 2 min at 72°C. The sequences of the sense and antisense primers for human placenta growth factor (PLGF) were 5′-ATGCCGGTCATGAGGCTG-3′ (nt. 331–349) and 5′-TTACCTCCGGGGAACAGC-3′ (nt. 753–771). The amplification of PACE4A and PLGF was carried out under the same conditions except that the cycle number was increased to 30. RT-PCR analysis of rat SPC family proteases (PACE4, furin, PC5/6, and PC7/8) was carried out as described previously (*18*).

Preparation of Anti-Hash-2 Antibody—Anti-Hash-2 antiserum was raised against a glutathione S-transferase (GST)-Hash-2 (aa 1–193) fusion protein in a rabbit. The fusion protein was expressed in *E. coil* (BL-20 strain) and purified to a homogeneous state by glutathione-Sepharose affinity chromatography according to the manufacturer's directions. The specificity of the antibody was confirmed by immunoprecipitation. The bHLH proteins, including Hash-2, Hash-1, Math-1, -2, E-47, Neurogenin-1, -2, -3, Hes-1, -5, and Id-3, were overexpressed in HEK293 cells. Cell lysates were prepared, immunoprecipitated with anti-Hash-2 antibody, and analyzed by SDS-PAGE. Hash-2 proteins (20 kDa) were detected, but neither other bHLH proteins nor the Hash-1 protein reacted with the anti Hash-2 antibody.

Luciferase Assay—Construction of reporter plasmids and luciferase assays were performed as described previously (*21*), and β-galactosidase activity was measured to normalize the luciferase activity. The results are presented as means of the luciferase activity ± SD of three independent experiments.

Preparation of Nuclear Extract and Gel Mobility-Shift Assay—The nuclear extract was prepared from BeWo cells as described elsewhere (*28*). The protein concentration was determined by the method of Bradford with bovine serum albumin as a standard (*29*). The sequences

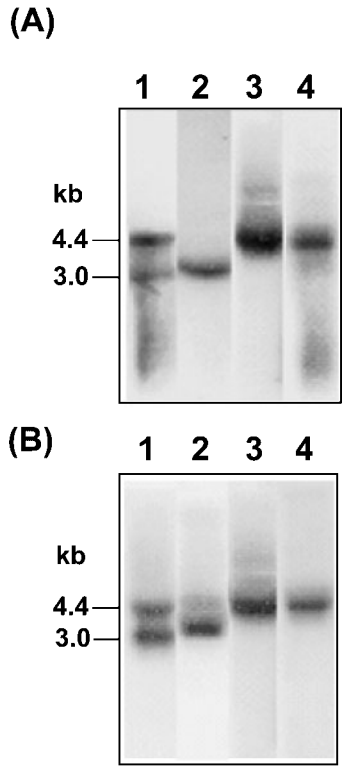

Fig. 1. **Northern blot analysis of SPC protease mRNAs in the human placenta and BeWo cells.** Total RNA (10 μg) isolated from human placenta (A) and BeWo cells (B) was analyzed as described under "MATERIALS AND METHODS." lane 1, PACE4; lane 2, PC5/6; lane 3, furin; lane 4, PC7/8.

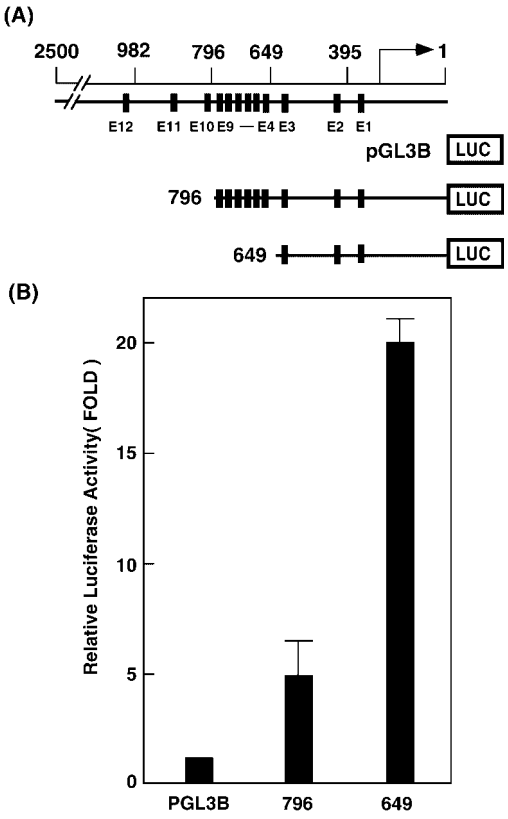

Fig. 2. **Effect of the deletion of the E-box cluster (E4-E9) on promoter activity in BeWo cells.** (A) Diagram of the 5′-flanking region of the PACE4 gene promoter. E1–E12 indicates the position of each E-box element. The 5′-end deletion mutants of the promoter fragment were ligated into luciferase plasmid (pGL3-Basic). (B) The luciferase activity of each construct was expressed relative to the baseline luciferase activity of a promoterless construct (pGL3-Basic vector), and normalized to the β-galactosidase activity. The results presented are means ± S.D.

of the top strands of oligonucleotides used as probes or competitors in the gel mobility-shift assay were described previously (*21*, *23*). The nuclear extract was incubated with anti-Hash-2 IgG antibody immobilized with protein A-Sepharose at 4°C for 2 h with occasional mixing. The complementary strands of each oligonucleotide were annealed and purified by PAGE: the double-stranded probe was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The DNA binding reaction was performed in 10 μl of a reaction mixture containing 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% (v/v) glycerol, 5 μg of pretreated nuclear extract and radiolabeled probe (5×10^4 cpm). The mixture was incubated at 4°C for 30 min and loaded onto a 4% (v/v) polyacrylamide gel. The gel was exposed to X-ray film for 6 h.

In Situ Hybridization—Conceptuses were collected on day 12 of gestation from Wister rats. The tissues were immediately removed and immersed overnight in ice-cold Bouin's fixative lacking acetic acid. After dehydration, they were embedded in paraffin, serially cut into 7 μm-thick sections, mounted on slide glasses coated with 3-aminopropyltriethoxysilane (MATSUNAMI, Japan), and stored in the dark at –30°C until processed further. *In situ* hybridization was performed as described previously (*17*, *18*, *27*). A digoxigenin (DIG)-labeled antisense RNA probe was generated by *in vitro* transcription from pBluscript SK+ carrying rat PACE4 cDNA (nt. 3241–3820) as described previously (*17*) or Hash-2 cDNA (nt. 545–1124).

RESULTS

Expression of SPC Family Proteases in Human Placenta and BeWo Cells—The expression of SPC family proteases, including PACE4, furin, PC5/6 and PC7/8, in human placenta was first examined by Northern blot analysis. These SPCs function in a constitutive secretory pathway. Placenta contains the 4.4 kb (PACE4A) and 3.0 kb (PACE4C/CS) transcripts found in the megakaryoblastic cell line, Dami (lane 1) (*30*). These isofoms are produced by alternative splicing from a single PACE4 gene (*12*, *20*). The carboxyl terminal region varies in length and sequence between PACE4A and PACE4C/CS. PC5/6A (3.8 kb: lane 2), furin (4.4 kb: lane 3), and PC7/8 (4.4 kb: lane 4) mRNAs were also detected (Fig. 1A). BeWo cells exhibited similar expression profiles for PACE4, PC5/6A, furin, and PC7/8 mRNAs (Fig. 1B), although a higher level of PACE4C/CS expression was observed. Thus BeWo cells express PACE4 transcripts at a high level, and were used for promoter analysis.

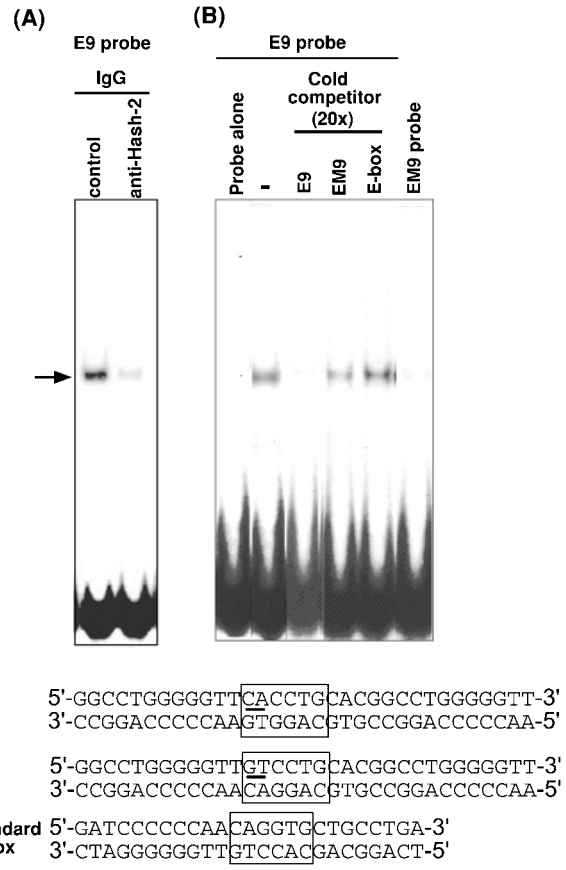

Fig. 3. **Gel mobility-shift assay of nuclear proteins interacting with the E9 E-box sequence.** (A) The ^{32}P-labeled double-strand E9 oligonucleotide (E9 probe) was incubated with nuclear proteins (5μg) from BeWo cells pretreated with control IgG or anti-Hash-2 IgG as described in "MATERIALS AND METHODS." (B) The E9 probe was incubated with the nuclear extract in the absence (–) and presence of cold excess E9, EM9 or standard E-box oligonucleotides as described under "MATERIAL AND METHODS." Competitors were added at a 20-fold excess relative to the radiolabeled DNA. Competitor oligonucleotides (E9, standard E-box, and EM9) are shown at the bottom of the figure.

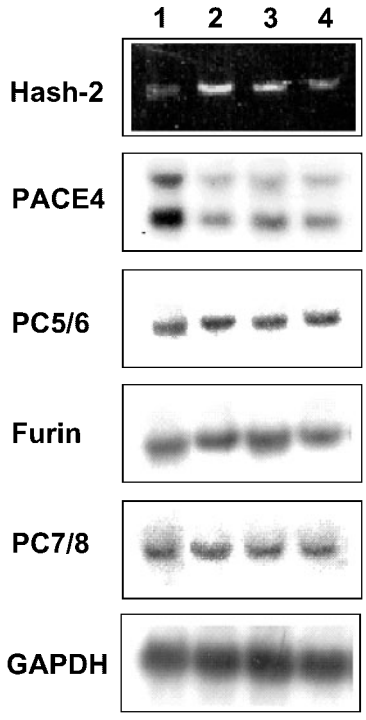

Fig. 4. **Effect of Hash-2 overexpression on the level of mRNA for SPC family proteases in BeWo cells.** The level of SPC mRNA after transfection with Hash-2 expression vector (lane 1, 0 h; lane 2, 4 h; lane 3, 8 h; lane 4, 12 h) was analyzed by Northern blotting as described in "MATERIALS AND METHODS." Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is also shown as a positive control.

The Promoter Activity of the Human PACE4 Gene in BeWo Cells—The 5′-flanking region of the PACE4 gene contains 12 E-boxes (E1-E12) within the region 1 kb upstream of the transcription initiation site. Previously, we showed that the E-box cluster (E4-E9) in the PACE4 gene acts as a major negative regulatory element in HepG2, GH4C1 (*21*), and neuroblastoma cells (*23*). As shown in Fig. 2A, deletion mutants lacking part of the 5′-flanking region of the human PACE4 gene were ligated to a promoterless luciferase reporter gene (pGL3B). The constructs were transfected into BeWo cells and the luciferase activities of the cell lysates were measured. Deletion of the region from –796 to –649, in which there were six repeats of the GGCCTGGGGGTTCACCTGC sequence containing the E-box (E4–E9), caused a large increase in luciferase activity (Fig. 2B). These results indicate that the E-box cluster (E4-E9) contains strong silencer activity in BeWo cells.

Hash-2 Binds to the E-Box Cluster in the PACE4 Gene—Hash-2 (human achaete-scute homologue-2) is one of the bHLH factors that regulate placental development, although the target genes of Hash-2 in trophoblast cells are unknown. To identify the proteins that interact with the E-box cluster, we performed a gel mobility-shift assay with a nuclear extract from BeWo cells and the E9 E-box oligonucleotide (GGCCTGGGGGTTCACCTGCACGGCCTGGGGGTT: –773 to –741 of the PACE4 gene). The expression of the Hash-2 transcript in BeWo cells was confirmed by PCR analysis (date not shown). The incubation of the E9 probe with the nuclear extract produced a single major band (Fig. 3B). The intensity of this band was markedly decreased when the nuclear extract was pretreated with anti-Hash-2 IgG, whereas control IgG had no effect (Fig. 3A). Most bHLH factors, such as MyoD, preferentially bind to the CACGTG or CAGCTG E-box element (*22*). Hash-1 and Mash-1, in contrast, are highly specific for the CACCTG sequence (*23*). The sequence specificity of E9-binding proteins was analyzed by performing competition experiments. The competition was complete with a 20-fold molar excess of unlabeled E9 probe (CA**CC**TG) but not with EM9 (**GT**CCTG) or standard E-box (CA**GG**TG) oligonucleotide (Fig. 3B). Furthermore, this band was not detected when the EM9 probe was incubated with the nuclear extract. These results clearly indicate that the Hash-2 protein is the major E9-binding protein in the BeWo cell nuclear extract. It is highly likely that heterodimers of Hash-2 and E-proteins (E2A/ALF1/E12) (*33*) bind to the CACCTG sequence in the E-box cluster of the PACE4 gene promoter.

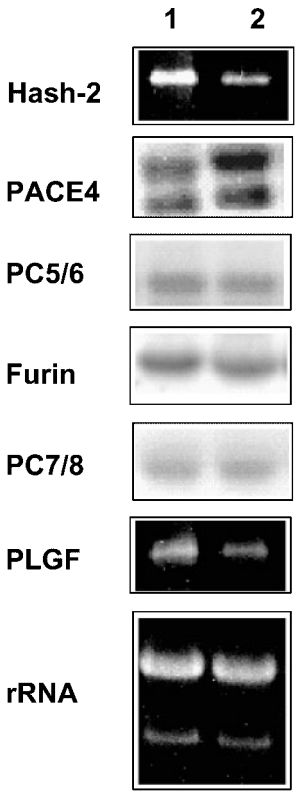

Fig. 5. **Effect of low oxygen tension on the level of SPC mRNAs and Hash-2 in BeWo cells.** BeWo cells were cultured under normal (lane 1; 20% O_2) and low (lane 2; 2% O_2) oxygen tension for one day. The levels of SPC mRNAs were analyzed by Northern blotting as described in "MATERIALS AND METHODS." The expressions of Hash-2 and placenta growth factor (PLGF) were examined by RT-RCR.

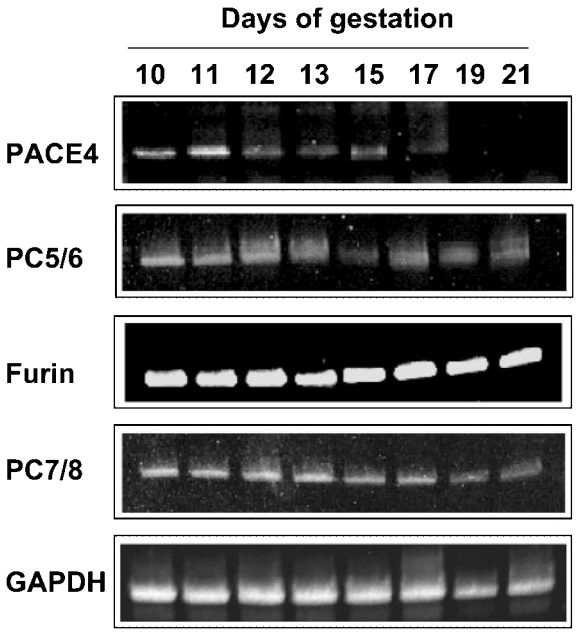

Fig. 6. **Expression of SPC family proteases in the conceptus during rat gestation.** Total RNA (5 μg) from rat conceptus at various developmental stages was isolated and analyzed by PT-PCR as described in "MATERIALS AND METHODS."

Effect of Hash-2 on the mRNA Level of PACE4 and Other SPC Family Proteases in BeWo Cells—To see whether Hash-2 directly affects the expression of PACE4, the effect of ectopic Hash-2 expression on the level of the PACE4 mRNA was analyzed by Northern blotting. The Hash-2 transcript (582 bp) was expressed at a high level 4 h after transfection. Although this level of expression gradually decreased, it remained high for 12 h as determined by RT-PCR. In contrast, the expression of both PACE4 mRNAs (PACE4A and PACE4C/CS) decreased rapidly after transfetion with the Hash-2 expression vector (Fig. 4). Hash-2 expression has no effect on the mRNA expression of other SPCs such as PC5/6, furin, and PC7/8. To confirm the effect of Hash-2, the expression levels of PACE4 mRNA in BeWo cells cultured under different concentrations of oxygen were compared. As shown in Fig. 5, Hash-2 mRNA expression decreased to 50% of the control level when BeWo cells were cultured under 2% oxygen. This effect of oxygen tension was confirmed by a decrease in the expression of PLGF (Placenta growth factor) (*34*, *35*). PLGF, a member of the vascular endothelial growth factor family, is known to be down-regulated in placental trophoblasts at low concentrations of oxygen. The amount of PLGF transcript gradually decreased. In contrast, the expression of PACE4 mRNA was up-regulated 2-fold by the decrease in oxygen concentration, whereas other SPCs were not affected. These results indicate that the expression of the PACE4 gene is efficiently suppressed by the induction of Hash-2, and this event is specific for PACE4 among SPCs.

Control of PACE4 Expression during Rat Placental Development—To clarify the developmental regulation of SPC expression in placenta, total RNA was isolated from rat placenta at various stages of development and SPC transcripts were analyzed by RT-PCR (Fig. 6). The sense and antisense primers for rat PACE4A were 5′-CCCTCTGGAACCAAGTCTCAACTT-3′ (nt. 1658–1681) and 5′-TGAAGCCAGCTTTACATCTGCTGC-3′ (nt. 2676–2699), respectively (*18*). The PACE4A transcript (1,042 bp) was expressed. Although PC5/6, furin, and PC7/8 showed nearly almost constant expression profiles at all stages examined, PACE4A mRNA expression varied greatly. The expression of PACE4A was highest on day 11, but the transcript was hardly detectable on day 19 or 21. Rat placenta consists of three kinds of the trophoblast cells, labyrinthine trophoblast, spongiotrophoblast and giant cells, and each is complex as a labyrinth (Fig. 7 A). The cell-specific expression profiles of Mash-2 (rat homologue of Hash-2) and PACE4 in rat placenta were analyzed by *in situ* hybridization. The results showed that a distinction exists in trophoblast cells. Mash-2 was found to be expressed in spongiotrophoblast cells (Fig. 7, G and H), while little was detected in the labyrinthine layer (Fig. 7, G and I). This is in good agreement with the localization of mouse Mash-2 mRNA (*3*, *5*, *36*). In contrast, PACE4 showed specific expression in the labyrinthine layer of the rat placenta, and was not expressed in the spongiotrophoblast layer (Fig. 7, D, E, and F). Incubation with sense RNA probes showed no specific hybridization to adjacent sections of day 12 placenta (Fig. 7, B and C).

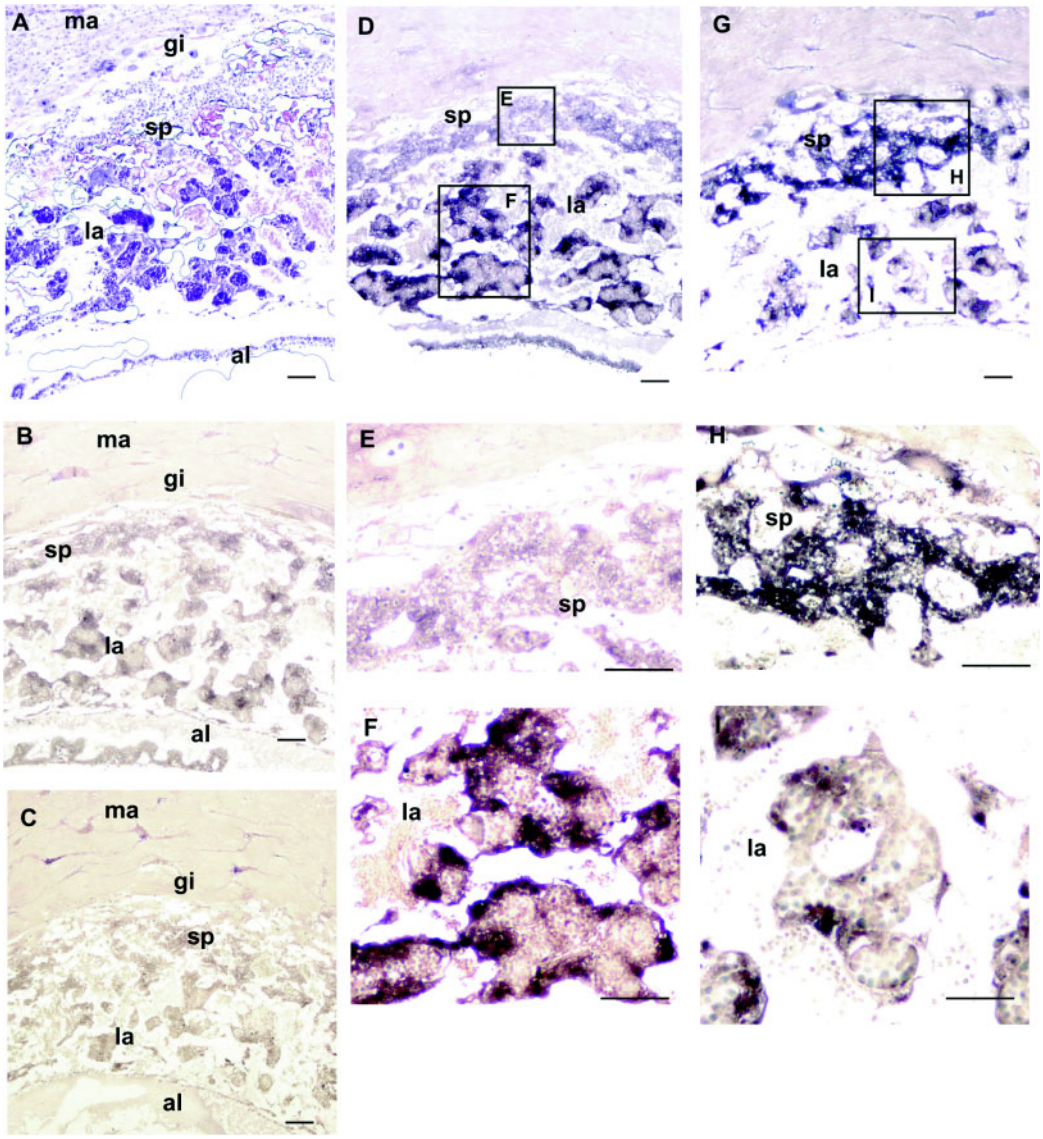

Fig. 7. **Cellular distributions of PACE4 and Mash-2 mRNAs in the rat placenta.** Adjacent sections from a rat placenta at day 12 of gestation were stained with hematoxylin and eosin (A) or hybridized to PACE4 antisense RNA probes (D, E, and F) or Mash-2 antisense RNA probes (G, H, and I). Mash-2 mRNA was detected in the spongiotrophoblast layer (H), while PACE4 mRNA was localized to the labyrinthine layer (F). Negative control with PACE4 sense RNA probe (B) and Mash-2 sense RNA probe (C). ma, maternal deciduas; gi, giant cell layer; sp, spongiotrophoblast; la, labyrinthine trophoblast; al, allantois. Scale bars: A-I, 100 μm.

These results suggest that PACE4 expression is down-regulated by Hash-2 in placenta similar to the relationship between Hash-1 and PACE4 in neuroblastoma cells.

DISCUSSION

PACE4 exhibits cell-specific expression unlike furin, and, especially, its expression is strictly regulated during embryogenesis (*19*). Recently we found that PACE4 is highly expressed in syncytiotrophoblast cells of human placenta, but not in cytotrophoblast cells (*10*). Trophoblast cells are a specialized cell type peculiar to eutherian mammals that give rise only to the placenta. As cytotrophoblasts mature, they stop dividing and fuse to form the terminally differentiated syncytiotrophoblast layer (*37*, *38*). Throughout pregnancy, the syncytiotrophoblast layer is the major site of many placental functions required for the maintenance of pregnancy and for fetal growth and development, including nutrient and gas exchange, and the biosynthesis of steroid and polypeptide hormones necessary for initiating maternal recognition of pregnancy (*39*). Later in the development of the mouse embryo, the placenta contains three trophoblast layers, namely, the labyrinthine trophoblasts, the spongiotrophoblasts, and the giant cells, each of which is morphologically distinct (*40*). As a first step in elucidating the role of PACE4 in placental development, we characterized the regulatory mechanism of PACE4 expression in the placenta. The most interesting feature of the 5′-flanking region of the PACE4 gene is the presence of 12 E-boxes (CANNTG, E1–E12), a target sequence for bHLH transcription factors. E-boxes are developmental regula-

tors of transcription in a variety of tissues including neuronal tissues and muscle (*41*, *42*). The Mash-2 gene has been shown to encode a trophoblast-specific bHLH transcription factor required for the development of a subset of trophoblast cells (*3*, *33*), although little is known about the target gene of Mash-2. Targeted mutagenesis of Mash-2 has revealed that loss of function results in embryonic lethality at midgestation due to placental failure associated with a lack of spongiotrophoblasts and a reduced labyrinthine trophoblast layer (*5*). Previously, we reported that PACE4 gene expression is suppressed by Hash-1/Mash-1 transcription factors in neuroblastoma cells (*23*). Mash-1 is a bHLH transcription factor that is important in neural development (*41*). In the present study, the effect of Hash-2, a human homologue of Mash-2 (*6*), on the transcription of PACE4 in the human trophoblastic cell line BeWo was analyzed. This cell line is widely used as a trophoblast model system (*43*). BeWo cells express not only PACE4 but also furin, PC5/6 and PC7/8. The ectopic expression of Hash-2 selectively suppressed the transcription of PACE4. The expression of other proprotein convertases was not influenced by Hash-2. Reporter gene assays indicate that the E-box cluster (E4–E9, –796 to –649) is a negative regulatory element for the promoter activity of the PACE4 gene (*21*). The specific binding of nuclear protein to the CACCTG sequence of the E-box (E4-E9) was demonstrated by gel mobility assay, and this binding was abolished by pretreating the nuclear extract from BeWo cells with anti-Hash-2 antibody. Furthermore, in BeWo cells, the level of endogenous Hash-2 mRNA was decreased whereas the PACE4 mRNA level was increased. These results show that Hash-2 acts as a negative regulator of PACE4 expression similar to Hash-1 in neuroblastoma cells. During gestation in the rat, we found that the expression of PACE4 is inversely proportional to that of Mash-2. The expression of mouse Mash-2 has been reported to be still apparent in the spongiotrophoblast layer and patchy in the labyrinthine layer at 12.5 days (*5*, *36*). In particular, the PACE4 transcript was found at high levels in the labyrinthine layer but was not detectable in the spongiotrophoblast layer. In contrast, Mash-2 was barely detectable in the labyrinthine layer, whereas it was expressed at a high level in the spongiotrophoblast layer. We previously showed that the structure of the 5′-regulatory regions of PACE4 from human and rat are very homologous (*21*). Thus, it is highly likely that Hash-2 plays a role in the regulation of PACE4 in human placenta.

TGFβ-related growth factors and matrix metalloproteinases (MMPs) are candidate substrate proteins for PACE4 (*11*, *44*, *48*). These proteins have a recognition site for PACE4 between the propeptide and the mature protein. Recent studies strongly suggest a significant role for PACE4 in the proteolytic activation of TGFβ-related growth factors during embryogenesis (*45–47*). In addition to TGFβ-related factors, a precursor of MMP-11 (stromelysin-3) has also been shown to be activated by PACE4. Placentation is mediated by fetal trophoblastic cells, which penetrate the decidualized uterine endometrium. Human extravillus trophoblasts invading the maternal decidua produce MMP-11 (*44*). In floating villi, MMP-11 expression is restricted to the syncytiotrophoblasts. Taken together, our results and recent findings suggest that Hash-2 plays a critical role in the regulation of PACE4 mediated activation of proproteins such as MMP-11 and TGFβ-related growth factors in placenta.

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