HOP/NECC1, A Novel Regulator of Mouse Trophoblast Differentiation*

Kazuo Asanoma†‡, Hidenori Kato‡, Shinchiro Yamaguchi‡, Chong Hyun Shin*, Zhi-Ping Liu‡, Kiyoko Kato*, Takafumi Inoue**, Yoko Miyanari*, Koji Yoshikawa‡‡, Kenzo Sonoda**, Kotaro Fukushima**, and Norio Wake**

From the †Division of Molecular and Cell Therapeutics, Department of Molecular Genetics and the ‡Division of Pathology, Medical Institute of Bioregulation, Kyushu University and the **Department of Obstetrics and Gynecology, Kyushu University Faculty of Medicine, 3-1-1 Maidashi, Higashi-ku, Fukuoka City, Fukuoka 812-8582, Japan, the ‡‡Department of Gynecology, Hokkaido Cancer Center, 4-2-3-54 Kikusui, Shiraishi-ku, Sapporo City, Hokkaido, 003-0000, Japan, the §Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, and the ¶Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Texas 75390

Homeodomain-only protein/not expressed in choriocarcinoma clone 1 (HOP/NECC1) is a newly identified gene that modifies the expression of cardiac-specific genes and thereby regulates heart development. More recently, HOP/NECC1 was reported to be a suppressor of choriocarcinogenesis. Here, we examined the temporal expression profile of HOP/NECC1 in wild-type mouse placenta. We found that E8.5–E9.5 wild-type placentas expressed HOP/NECC1 in the giant cell and spongiotrophoblast layers. HOP/NECC1 (−/−) placentas exhibited marked propagation of giant cell layers and, in turn, reduction of spongiotrophoblast formation. We demonstrated SRF transcriptional activity increased in the differentiating trophoblasts and forced expression of SRF in a trophoblast stem (TS) cell line induces the differentiation into giant cells. Negative regulation of SRF (serum response factor) by the binding of HOP/NECC1 protein contributed at least in part to the generation of these placental defects. Gradual induction of HOP/NECC1 in response to differentiation stimuli may result in the decision to differentiate into a particular type of trophoblastic cell lineage and result in non-lethal defects shown by the HOP/NECC1 (−/−) placentas.

The development of the placenta starts with differentiation of the outer layer of the blastocyst, the trophoderm. After implantation, the molar trophodermal cells contribute to primary trophoblast giant (TG) cells and the polar trophoderm differentiates to extraembryonic ectoderm (ExE). ExE grows to form the ektoplacental cone (EPC), which contains precursor cells that give rise to spongiotrophoblasts (SpT) and secondary TG cells. Most TG cells originate from precursor cells located in the EPC, and partially from SpT (1). The TG cells are polyploid as a result of endoreduplication (repeated rounds of DNA synthesis without cell division) (2, 3).

In the course of exploring the molecular mechanism of gestational trophoblastic diseases, we have isolated a candidate choriocarcinoma suppressor gene (NECC1, not expressed in choriocarcinoma clone 1) (4). Normal placental villi express NECC1 but choriocarcinoma cell lines and tissue samples fail to express it. We transfected the NECC1 gene into choriocarcinoma cell lines to characterize its functions as a tumor suppressor gene and observed remarkable alterations in cell morphology (4).

This unusual type of homeodomain protein also functions during cardiac development. Genetic inactivation of the mouse orthologue HOP (homeodomain-only protein) results in a partially penetrant phenotype of embryonic heart failure and lethality (5, 6). HOP/NECC1-deficient adult mice display conduction defects (7). HOP/NECC1 contains a unique homeodomain that is incapable of sequence-specific DNA binding (5, 6). Instead, HOP/NECC1 functions by interacting with SRF to inhibit DNA binding by recruiting histone deacetylase (HDAC) activity (8). This interaction results in decreased transcription of SRF-dependent cardiomyocyte-specific genes (5, 6).

The fact that HOP/NECC1 is expressed in human placenta prompted us to investigate its biological role in placental development in the present study. First of all, we determined the temporal expression profile of HOP/NECC1 in wild-type mouse placentas; E8.5–E9.5 wild-type placentas expressed HOP/NECC1 in the TG cell and SpT layers. HOP/NECC1 (−/−) placentas exhibited marked propagation of the TG cell layers and, in turn, reduction of SpT formation. Forced expression of HOP/NECC1 in trophoblast stem cells (TS cells) and Rcho-1 cells inhibited the generation of TG cells and down-regulated the transcription of PI1, features, which were also observed in the case of dominant-negative SRF. Negative regulation of the activity of SRF as a transcription factor by the binding of HOP/NECC1 protein to SRF contributed at least in part to the generation of these placental defects. Induction of HOP/NECC1 expression in response to differentiation may negatively regulate the generation of TG cell lineage and result in non-lethal defects shown by the HOP/NECC1 (−/−) placentas.
A Novel Regulator in Murine Placentation

EXPERIMENTAL PROCEDURES

Mice—HOP/NECC1 (−/−) embryos were generated in the laboratory of Eric N. Olson (6). Genotyping was performed by PCR. Mutant phenotypes were analyzed in a mixed 129/Sv × C57Bl/6 background and isogenic 129/Sv × 129/Sv background.

Cell Culture, Transfection, Infection, and Analysis of Trophoblast Giant Cell Differentiation—The Rcho-1 cell line, a rat choriocarcinoma cell line, was kindly provided by Yoshiya Yamamoto, Osaka University, Japan and cultured as described previously (9). The TS cell line (kindly provided by Satoshi Tanaka, Tokyo University, Japan), derived from a 6.5-dpc conceptus of ICR mice, was cultured according to methods reported previously (10). Human wild-type (wt) SRF and dominant-negative-negative SRF (pm1) inserted into pCGN-HA were digested with XbaI-BamH1 and newly cloned into the pCDNA3 expression vector, which contains the CMV promoter. Rcho-1 cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA). Two micrograms of empty, wt-SRF or pm1 expression vector were used for transfection and several clones which stably expressed the genes were obtained from the transfected cells. An adenovirus expression vector of HOP/NECC1 was constructed by ligating a full-length human HOP/NECC1 cDNA into the pAxCACwt cosmid vector. 293a transfectants were derived and a high-titer adenovirus solution was obtained (Tokara, Japan). Rcho-1 cells and TS cells were infected at a multiplicity of infection (moi) of 10 or 20 1 day before the induction to differentiation at day 0. For trypsin resistance assays, after 2 days induction of Rcho-1 cells in 1% human horse serum (HS), stem cells were removed by trypsinization and several clones which stably expressed the genes were obtained from the transfected cells. An adenovirus expression vector of HOP/NECC1 was constructed by ligating a full-length human HOP/NECC1 cDNA into the pAxCACwt cosmid vector. 293a transfectants were derived and a high-titer adenovirus solution was obtained (Tokara, Japan). Rcho-1 cells and TS cells were infected at a multiplicity of infection (moi) of 10 or 20 1 day before the induction to differentiation at day 0. For trypsin resistance assays, after 2 days induction of Rcho-1 cells in 1% horse serum (HS), stem cells were removed by trypsinization (0.25% trypsin and 1 mM EDTA, incubation for 5 min and wash with phosphate-buffered saline twice), and then the adherent cells in 20 random high magnification fields for each clone were counted. Values were reported as the mean ± S.D., and the significance of differences between values were determined by Student’s t test. Nuclear DNA content was estimated by the area of 4’,6-diamidino-2-phenylindole (DAPI) staining using NIH Image software as described previously (2) with 200 cells from random fields.

Transient Transfection and Luciferase Assay—Promoter-reporter constructs were transiently transfected into Rcho-1 cells using Lipofectamine reagent according to the manufacturer’s instructions. A pSRSF construct containing five tandem SRF binding elements from c-fos promoter linked to bacterial luciferase (pSRSF-Luc) was purchased from Stratagene. A pm1 promoter/luciferase construct was generated from the previously characterized mouse pl1 promoter (11) using PCR. Briefly, genomic DNA from mouse trophoblast stem cells served as a template for PCR using an upstream primer with a Kpn1 restriction site (sense, −328 to −309) and a downstream primer with an Xho1 restriction site (antisense, +9 to +29). The amplified product was digested with Kpn1 and Xho1 and ligated into the pTA-Luc reporter plasmid (pmp1-Luc). The accuracy of the PCR-generated pl1 promoter/luciferase reporter construct was verified by DNA sequencing. Rcho-1 cells and TS cells (1 × 10⁶) were transfected with 3 µg of the pSRSF-Luc construct, pLuc-MCS basic vector (negative control) or pmp1-Luc. A CMV promoter-β-galactosidase construct (pCDNA3.1-His-LacZ, 0.5 µg) was cotransfected and used to evaluate transfection efficiency. 48 or 72 h after transfection, cells were collected, and lysates were prepared. Luciferase assays were performed using a luciferase assay kit (Promega, Madison, WI), and β-galactosidase activity was measured using a β-galactosidase assay kit (Invitrogen).

In Situ Hybridization, Immunohistochemistry, and Immunofluorescence—Freshly isolated placentas and embryos were fixed in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin, and sectioned (5 µm). RNA in situ hybridization was performed as described (12) using digoxigenin-UTP-labeled antisense cRNA probes for HOP/NECC1, PI1 (13), TbpB (14), Cea4 (15), Cdx2 (16), Mash2 (17), Pl2 (18), and proliferin (Pif) (19). Immunohistochemistry for SRF expression was performed using sections of placentas from wild-type mice. Standard immunohistochemical techniques were used, employing a polyclonal rabbit IgG antibody to SRF (Santa Cruz Biotechnology, Santa Cruz, CA) and a Dako LSAB kit (Dako, Denmark). Hematoxylin was used for counterstaining. For immunofluorescence, cells on coverslips were fixed in 4% PFA for 10 min and permeabilized in phosphate-buffered saline/0.1% Triton X-100 for 5 min. After three washes with phosphate-buffered saline for 3 min at room temperature, actin stress fiber staining was performed using rhodamine-labeled phalloidin (1:40, Molecular Probes, Eugene, OR). Slides were then washed, counterstained with DAPI, and mounted. For TS cells differentiation experiments, pCGN-HA-wt-SRF or pCGN-HA-pm1 transfected TS cells were cultured on stem cell condition for up to 48–72 h. The cells were fixed, permeabilized as described above, and blocked with rabbit serum (Nichirei Bioscience). The cells were incubated with FITC-conjugated anti-HA monoclonal antibody (1:100, Santa Cruz Biotechnology) for 1 h, counterstained with DAPI and mounted. Transfected cells were detected by their expression of HA tag. Two hundred cells were assessed for each transfection group, and experiments were repeated three times. The staining patterns of the cells were examined using a Carl Zeiss LSM 510 confocal microscope.

Northern Blot Analysis—Total RNA was isolated from Rcho-1, and TS cells using ISOGEN (Nippon Gene) following the manufacturer’s protocol. Northern blotting was performed as described previously (10).

Western Blot Analysis and Immunoprecipitation—To examine the expression of various proteins, subconfluent cells were lysed with ice-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 2% Nonidet P-40, 0.2% SDS) containing freshly added protease inhibitors (200 µM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A). After centrifugation at 13,000 × g for 10 min to remove debris, the lysate was subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Anti-HOP/NECC1 antibody was raised in a rabbit using the peptide RLAKWRRSEGLPSEC corresponding to the C-terminal portion of HOP/NECC1 and the specificity of the antibody was verified using Hela cells, which do not express HOP/NECC1, and HOP/NECC1-expressing adenovirus-infected Hela cells. Equal amounts of protein from each lysate were immunoprecipitated with anti-HOP/NECC1 or pmPI1-Luc. A CMV promoter-β-galactosidase construct (pCDNA3.1-His-LacZ, 0.5 µg) was cotransfected and used to evaluate transfection efficiency. 48 or 72 h after transfection, cells were collected, and lysates were prepared. Luciferase assays were performed using a luciferase assay kit (Promega, Madison, WI), and β-galactosidase activity was measured using a β-galactosidase assay kit (Invitrogen).
antibody bound to protein A/G-agarose (Calbiochem, San Diego, CA) and separated by SDS-PAGE. Separated proteins on the gel were transferred to polyvinylidene difluoride membranes, and the membranes were incubated with anti-HOP/NECC1 antibody after blocking. The membranes were washed with Tris-buffered saline-Tween-20 (TBS-T), and then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. The signal was visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ). The same amount of protein was loaded and blotted using anti-GAPDH antibody (Santa Cruz Biotechnology) for the loading control. Immunoprecipitations were carried out by incubating 500 μg of protein/500 μl of lysate with 10 μl of primary antibody and 20 μl of protein A/G-agarose at 4 °C. Immunoprecipitated proteins were analyzed by Western blotting using anti-SRF (Santa Cruz Biotechnology) antibody and anti-HOP/NECC1 antibody.

RESULTS

During mouse embryogenesis, HOP/NECC1 transcripts are first detected at E7.75 in trophoblast cells within extraembryonic membranes (6). To examine the temporal expression profile of HOP/NECC1 during wild-type mouse placental development, we performed in situ hybridization using E8.5–11.5 placentas. The results demonstrated that the highest levels of HOP/NECC1 transcript were present in E8.5 and E9.5 placentas, and then the level suddenly declined, and become almost undetectable in E10.5 and E11.5 placentas (Fig. 1, A–E). Abundant expression of HOP/NECC1 was detected in primary and secondary giant cells and a lower level of HOP/NECC1 expression was also observed in SpT layers (Fig. 1, B and C). The comparative presentation in Fig. 1C clearly shows that HOP/NECC1 was expressed in both Pl1-positive (TG) cells and Tpbp-positive (SpT) areas (Fig. 1C). The temporally and spatially restricted expression profiles of HOP/NECC1 during mouse placental development suggested its involvement in trophoblast cell differentiation.

HOP/NECC1 homozygous knock out (−/−) mice have been generated in 129/SV × C57 BL/6 mixed background and 129/SV × 129/SV isogenic background (6). First of all, we examined the expression of Pl1, which is an endocrine marker of TG cells in 10 (+/+), 15 (+/−) and 18 (−/−) placentas to explore the contribution of HOP/NECC1 to placental formation. No marked differences were found between (+/+) and (+/−) mice at any gestational age. TG cells identified histologically expressed Pl1 in E8.5 (+/+), (+/−) and (−/−) placentas (Fig. 2A). In contrast, there was a significant increase of the Pl1-expressing cell population in E9.5 (−/−) placentas, compared with E9.5 (+/+) or (+/−) placentas (Fig. 2B). Pl1-positive cells were markedly increased in E10.5 and E11.5 (−/−) placentas (Fig. 2, C and D).

Tpbp and Cea4 are specifically expressed in SpT (14, 15). As illustrated in Fig. 2A, no differences in the Tpbp-positive cell distribution were detectable in E8.5 (−/−) placentas, compared with (+/+) or (+/−) placentas. However, there was a significant reduction in the number of Tpbp-positive cells in E9.5 and E10.5 (−/−) placentas, suggesting the impairment of SpT development (Fig. 2, B and C). The expression profile of Cea4 was similar to that of Tpbp (data not shown). Interestingly, Tpbp-positive cells reappeared in SpT layers and Pl1-positive cells also expressed Tpbp in E11.5 (−/−) placentas, which is in strong contrast with the finding that the distributions of Pl1-positive and Tpbp-positive cells were markedly different in (+/+) placentas (Fig. 2D). Furthermore, the Pl1-positive cells in E11.5 (−/−) placentas expressed not only TG cell markers (Pl2 and Plf) but also another SpT markers, Cea4, and precursor marker, Mash2 (Fig. 2E). This result indicated that HOP/NECC1 knock-out in mice resulted in an abnormal increase of giant cells and a defect in SpT development. The abnormally increased of giant cells shared in part the biochemical characteristics with SpT in E11.5 (−/−) placentas.

Cdx2 and Mash2 is exclusively expressed in TS cells and precursor cells respectively located in the SpT and labyrinthine layers (16, 17). As illustrated in Fig. 2F, a marked reduction of the cell population expressing Cdx2 and Mash2 was detected in E10.5 (−/−) placentas compared with that in (+/+) placentas. The scarcity of Cdx2− and Mash2− positive cells was found in two types of knock-out mice derived from different back-
grounds. Additionally, hypoplastic labyrinthine layers were also detected in E9.5, E10.5, and E11.5 \( /H11002 /H11002 \) placentas, though the reason remains unknown.

The Rcho-1 cell line was derived from a rat choriocarcinoma and maintains in part the features of TS cells, since the cells have the ability to differentiate into giant cells in vitro \( (9) \). Therefore, we further addressed how HOP\( /\)NECC1 is involved in Rcho-1 cell differentiation in response to low horse serum. Northern blotting and RT-PCR demonstrated that the expression level of HOP\( /\)NECC1 remained low and no changes were observed following stimulation to differentiation by 1% horse serum (HS) \( (\text{data not shown}) \). To determine the function of HOP\( /\)NECC1 in the regulation of trophoblast cell differentiation, we infected Rcho-1 cells with HOP\( /\)NECC1-expressing adenovirus \( (\text{Ad-HOP}\( /\)NECC1) \). The infected cells were stimulated with 1% HS and observed for 4 days. The faintly detectable level of HOP\( /\)NECC1 expression at 12 h sharply increased up to a maximum at 72 h, followed by a gradual decrease. HOP\( /\)NECC1 protein was still detectable even at 168 h by Western blotting \( (\text{Fig. 3A}) \).

Rcho-1 cells spread and become more firmly attached to the substratum as assessed by resistance to release with trypsin in response to differentiation stimuli. Reorganization of actin filaments and endoreduplication of DNA that results in the appearance of cells with 4N-128N nuclei have also been shown in differentiated Rcho-1 cells \( (20) \). Infection with Ad-HOP\( /\)NECC1 resulted in marked suppression of Rcho-1 cell differentiation, as evidenced by the maintenance of sensitivity to trypsin release and the decrease of the appearance of cells with increased ploidy \( (\text{more than 16N}) \) and with actin filament reorganization \( (\text{Fig. 3, B–D}) \). These effects were dependent on the multiplicity of infection \( (\text{moi}) \) of adenovirus. In contrast to these results, control virus \( (\text{Ad-control}) \) had a minimal effect on the Rcho-1 differentiation, regardless of its moi \( (\text{Fig. 3, C and D}) \). This result was compatible with the block of Rcho-1 cell differentiation into giant cells in the presence of the continuous expression of HOP\( /\)NECC1, being supported by the evidence that PI1 expression was sharply suppressed by the Ad-HOP\( /\)NECC1 infection \( (\text{Fig. 3E}) \). In contrast, Ad-control infected Rcho-1 cells differentiated in vitro and expressed abundant levels of PI1. The expression of Tpbp in Rcho-1 cells stimulated by low horse serum did not show any significant alterations in the presence of HOP\( /\)NECC1 expression, com-

**FIGURE 2. Expression of trophoblast-specific markers in the mutant placentas.** In situ hybridization of trophoblast-specific markers in E8.5–E11.5 wild-type \( (+/+) \) or hetero-mutant \( (+/-) \) and HOP\( /\)NECC1-deficient \( (-/-) \) placental serial sections. PI1 and Tpbp expression in E8.5 \( (A, \text{scale bar: } 200 \mu\text{m}) \), E9.5 \( (B, \text{scale bar: } 300 \mu\text{m}) \), E10.5 \( (C, \text{scale bar: } 500 \mu\text{m}) \), and E11.5 placenta \( (D, \text{scale bar: } 500 \mu\text{m}) \). The right panels in \( D \) are amplified pictures of the boxed areas in the middle panels. White arrows indicate the Tpbp-positive spongiosotrophoblast layer. E, in situ hybridization of the indicated markers in E11.5 wild-type \( (+/+) \) and mutant \( (-/-) \) placenta. These panels show the same areas as the boxed areas in \( D \). F, in situ hybridization of stem cell marker, Cdx2, and precursor cell marker, Mash2, in E10.5 placenta. The middle panels are amplified pictures of the boxed area in the left panels.
ened compared with Ad-control infection (data not shown). These findings suggest that transient induction followed by down-regulation of HOP/NECC1 is required for the proper differentiation from TS cells to TG cells.

HOP/NECC1 regulates cardiomyocyte development by inhibiting SRF transcriptional activity, which controls the expression of genes involved in cell proliferation and cardiomyogenesis (5, 6, 21). However, whether SRF-mediated signaling involved in trophoblast differentiation are unknown. First of all, we examined the expression profile of SRF during Rcho-1 cell differentiation in response to serum starvation. SRF protein was detected as two bands with different mobilities, and its expression level remained similar regardless of differentiation stimulation (Fig. 4A). As phosphorylation of SRF alters its migration in SDS-PAGE (22), we treated Rcho-1 cell extracts with lambda-protein phosphatase but did not detect any alterations in band intensity, suggesting that these two bands were alternative splicing products of SRF (data not shown and Ref. 23).

To examine SRF activity in differentiating Rcho-1 cells, Rcho-1 cells transfected with pSRF-Luc were cultured in medium with 20% fetal calf serum (FCS) (stem cell condition) or with 1% HS for up to 72 h. Because target genes of SRF involved in trophoblast differentiation are unknown, we instead used pSRF-Luc vector which contained five tandem CArG box elements from c-fos gene promoter. The relative luciferase activity increased after cultivation with 1% HS for 60 h and increased furthermore to 6.2 times higher than that with 20% FCS at 72 h (Fig. 4B). Co-transfection of wild-type (wt) SRF induced SRF activity under cultivation with 20% FCS. In contrast to the effect of wt-SRF, ectopic expression of HOP/NECC1 and dominant-negative (DN) mutant SRF (pm1) suppressed the luciferase activity induced by differentiation in dose-dependent manners (Fig. 4C). There were minimal changes observed using empty luciferase vector, pTA-Luc (data not shown). This data indicated that SRF activity as a transcriptional factor on a SRF binding element increased in the differentiating Rcho-1 cells and was inhibited by HOP/NECC1 and pm1.

Next, we established a series of Rcho-1 cell clones that stably expressed wt-SRF or pm1 to examine the involvement of SRF in trophoblast differentiation. Pm1 inhibits DNA binding of endogenous SRF and blocks transcriptional activation of its target genes (24). These clones expressed abundant levels (5–10-fold) of transfected wt-SRF or pm1, seen as increased signal intensity of the upper band (Fig. 4D). The Rcho-1 cells expressing wt-SRF exhibited a similar pattern of differentiated cell properties as shown in the vector-alone-transfected control, including resistance to trypsin release and increased DNA ploidy in response to serum starvation.
In turn, pm1 expression resulted in the suppression of Rcho-1 cell differentiation, as evidenced by the maintenance of sensitivity to trypsin release and the impairment of increased ploidy and actin filament reorganization (Fig. 4, E and F and data not shown). The features shown by pm1-expressing Rcho-1 cells were identical with those of HOP/NECC1-expressing clones. Additionally, pm1 expression resulted in the absence of Pl1 up-regulation at 4 days after serum starvation (Fig. 4, G).

We next addressed whether SRF and HOP/NECC1 physically interact in Rcho-1 cells. Immunoprecipitation followed by immunoblotting using specific antibodies showed that HOP/NECC1 was co-precipitated with SRF and in turn, SRF was also co-precipitated with HOP/NECC1 (Fig. 5, A). The positive signal intensity shown in lanes 2 and 6 reflected the abundance of intrinsic SRF protein in Rcho-1 cells. To further characterize the physical interaction of HOP/NECC1 with SRF, we examined the localization of SRF protein immunohistochemically and compared the expression pattern of HOP/NECC1 in wild-type mouse placenta. As mentioned above, HOP/NECC1 was localized in the TG cell and SpT layers of E8.5 to E10.5 mouse placenta. SRF was observed to be localized in the nuclei of TG cells and SpT in addition to decidual cells (Fig. 5, B–E). In labyrinthine trophoblasts, the localization of SRF is restricted to the cytoplasm. Although the role of SRF in labyrinthine trophoblasts remains unclear, several studies showed that the subcellular localization of SRF prescribes its function (25, 26). Our results showed that SRF and HOP/NECC1 were colocalized in TG cells and SpT, and negative regulation of SRF by HOP/NECC1 was important for the regulation of trophoblast differentiation.

Besides the Rcho-1 cell line, cultured TS cells are also available cells to analyze the process of differentiation of trophoblast cells (10). Northern blotting demonstrated that the expression of HOP/NECC1 was faintly detectable 5 days after the start of differentiation and increased thereafter (Fig. 6, A). The expression pattern of HOP/NECC1 was almost parallel to that of Pl1 and Tpbb (Fig. 6, A).

To determine the function of HOP/NECC1 in the regulation of trophoblast cell differentiation, we infected TS cells with Ad-HOP/NECC1. HOP/NECC1 protein started to be detectable at 12 h and was still detectable even at 144 h by Western blotting (data not shown). Control adenovirus-infected TS cells cultured under differentiation conditions for 6 days showed an increased population of giant cells and abundant expression of Pl1. Ad-HOP/NECC1-infected TS cells showed resistance to differentiation and fewer cells
showed the giant cell phenotype (Fig. 6B). This result was compatible with the inhibition of the differentiation of TS cells into TG cells in the presence of the continuous expression of HOP/NECC1, being supported by the evidence that Pl1 expression was suppressed by the Ad-HOP/NECC1 infection (Fig. 6C). TS cells are known to express Pl1 progressively even when cultured in the stem cell condition (10). As expected, HOP/NECC1 also inhibited Pl1 induction under stem cell conditions (Fig. 6D). Mash2 expression in the presence of HOP/NECC1 expression persisted to some extent at day 6 compared with Ad-control. Tpbp expression was not remarkably changed (Fig. 6C).

The Rcho-1 cells stably overexpressing wt-SRF exhibited no remarkable phenotypic alterations compared with control vector transfectants (Fig. 4, E–G). To define SRF function on TS cells by transient transfection, TS cells transiently transfected with HA-tagged wt-SRF or pm1 expression constructs were cultured under stem cell conditions. The successfully transfected cells were detected with FITC-conjugated anti-HA antibody. Notably, about 50% of wt-SRF-transfected cells had become post-mitotic TG cells by 48 h (Fig. 7, A and B). In contrast to the effect of wt-SRF, pm1-transfected cells suppress the spontaneous differentiation observed in untransfected cells (Fig. 7, A and B).

To confirm the suppressive effect of the HOP/NECC1-SRF pathway on Pl1 expression, we performed Pl1 reporter assays using luciferase vector, which contains the critical promoter region for Pl1 (11). After 72 h of spontaneous differentiation, as expected, Pl1 luciferase activity increased to 3.9 times higher than that under stem cell conditions (Fig. 7C). Co-transfection of wt-SRF further increased the luciferase activity in a dose-dependent manner. Wt-SRF also induced Pl1 transcription under stem cell conditions. In contrast to the effect of wt-SRF, ectopic expression of HOP/NECC1 and pm1 suppressed the luciferase activity in dose-dependent manners. To examine the inhibitory effect of HOP/NECC1 on Pl1 reporter activity induced by wt-SRF, we analyzed the Pl1 luciferase activity under conditions of co-transfection of wt-SRF and HOP/NECC1. As expected, pCDNA3-HOP/NECC1 also decreased the Pl1 luciferase activation by wt-SRF in dose-dependent manners (Fig. 7E). These results suggest that regulated expression of HOP/NECC1 in the differentiating trophoblast is required for the proper development of the placenta.

**DISCUSSION**

The present study provided evidence demonstrating important roles of HOP/NECC1 in early placentation. HOP/NECC1 (-/-) placenta exhibiting the disorganization, including enhanced formation of TG cell layers in parallel with reduced SpT and labyrinthine populations (Fig. 2, B–D). Although the appearance of TG cells expressing both Pl1 and Tpbp markers in E11.5 placenta may compensate in part for this placental defect, the mechanism behind the unexpected phenomenon remains to be clarified (Fig. 2, D and E). Our present data demonstrated that the expression of HOP/NECC1, which increased gradually during TS differentiation, suppressed the expression of Pl1 and giant cell phenotypes in both Rcho-1 cells and TS cells (Figs. 3 and 6). These results demonstrated that the HOP/NECC1 pathway functions as a negative feedback regulator for the differenti-
A Novel Regulator in Murine Placentation

FIGURE 6. HOP/NECC1 inhibits trophoblast differentiation to giant cells in vitro. A, Northern blot analysis of various markers in differentiating TS cells. HOP/NECC1 expression was gradually induced in differentiating TS cells, being parallel to the expression of PI1 and Tpbp. B, the relative proportion of giant cell type at 4 days after induction was assessed by scoring cells at ×200 magnification. About fifty fields were counted for each virus infectant. *, p < 0.05 Student’s t test. C and D, PI1, Tpbp, and Mash2 expression levels were examined in HOP/NECC1-virus-infected cells in response to differentiation (C) and in stem cell conditions (D) by Northern blotting. β-Actin (Actb) expression is shown as an internal control. The figures below the upper panels are relative signal densities compared with day 0 of control-virus infectant.

...the SpT population in the (−/−) placenta suggest that appropriate formation of the SpT layer also needs HOP/NECC1 (Fig. 2, B–D). However, forced HOP/NECC1 expression in TS cells down-regulated PI1 and somewhat up-regulated Mash2 but did not cause remarkable effects on Tpbp expression (Fig. 6C). The data suggested that hypoplasia of the SpT layer in HOP/NECC1-deficient mice may be a secondary change following expansion of the giant cell layer and may be caused by depletion of the TS cells or precursor cells. The reduction of the cell population expressing Cdx2 and Mash2 in the (−/−) placentas also supports this assumption (Fig. 2F).

Forced expression of wt-SRF by stable transfection failed to cause any phenotypic alterations in Rcho-1 cells compared with the control cells (Fig. 4, E–G). In contrast to that, transient overexpression of wt-SRF in TS cells promoted differentiation into TG cells and transactivates PI1 expression (Fig. 7). These differences in the response to forced SRF expression might result from different transfection methods. The activation of SRF was reported to depend on the phosphorylation status of serine or threonine residues (22, 30, 31, 32). The decreased level of the active SRF form following long-term culture might be involved in the case of stable transfection.

These data indicate the importance of HOP/NECC1-mediated negative control of SRF in the process of giant cell formation. Of course, other binding partners may participate in the formation of SRF complexes. Nkx2.5 is known to be a component of the myogenic SRF complex and to up-regulate the HOP/NECC1 transcription in myocytes (5, 6). Our data suggest that transient expression of exogenous Nkx2.5 is able to upregulate the HOP/NECC1 promoter activity in Rcho-1 cells (data not shown). However, Nkx2.5 expression is absent in mouse and human placental trophoblasts, indicating the possibility that other Nkx family members are components of the SRF complex in trophoblasts and promote HOP/NECC1 transcription. As for other components of the SRF transcriptional complex, GATA family, GATA-2 and GATA-3 are reported to be expressed in placenta and regulate transcription of trophoblast-specific genes, PI1, proliferin and syncitin (33, 34). These reports suggested that the interaction between SRF and GATA-2 and/or GATA-3 might regulate the trophoblast differentiation.

The dramatic differences in structure between the rodent and human placenta would be expected to be associated with...
different expression patterns of HOP/NECC1 between rodent and human trophoblast subtypes. HOP/NECC1 is expressed both in TG cells and SpT subtypes in mice, in contrast to its exclusive expression in the syncytiotrophoblast in humans. However, extravillous trophoblasts, which are human orthologues of TG cells, do not express HOP/NECC1 (data not shown). Comparative dissection of HOP/NECC1 functions in each rodent and human trophoblast subtype will clarify its detailed role in trophoblast differentiation.

Mice homozygous for a HOP/NECC1-null allele segregate into two phenotypic classes characterized by an excess or deficiency of cardiac myocytes (6). Prior to E11.5, HOP/NECC1 is involved in expansion of the ventricular myocardium, and later in fetal development, it restricts cardiomyocyte proliferation. Although the dual functions of SRF as a regulator of muscle cell proliferation and differentiation have to be considered in heart development, early embryonic cardiac defects are often secondary to placental dysfunction (35, 36). Further investigation of the role of HOP/NECC1 in placental development may provide new insights into the opposing phenomena of proliferation and differentiation in cardiomyocytes, and also in trophoblasts.

Acknowledgments—We thank Prof. Eric N. Olson for inviting us to his laboratory for analysis of the HOP/NECC1 knockout mice and valuable advice. Dr. Satoshi Tanaka at Tokyo University for valuable advice and trophoblastic stem cells, Drs. Takashi Takeda and Toshiya Yamamoto at Osaka University for Rho-1 cells.

REFERENCES

1. Rossant, J. (1995) Semin. Dev. Biol. 6, 237–247
2. MacAuley, A., Cross, J. C., and Werb, Z. (1998) Mol. Biol. Cell 9, 795–807
3. Ohgane, J., Aikawa, J., Ogura, A., Hattori, N., Ogawa, T., and Shiotani, K. (1998) Dev. Genet. 22, 132–140
4. Asanoma, K., Matsuda, T., Kondo, H., Kato, K., Kishino, T., Niikawa, N., Wake, N., and Kato, H. (2003) Genomics 81, 15–25
5. Chen, F., Kook, H., Milewski, R., Gitler, A. D., Lu, M. M., Li, J., Nazarian, R., Schnepp, R., Jen, K., Biben, C., Runke, G., Mackay, J. P., Novotny, J. L., Schwartz, R. J., Harvey, R. P., Mullins, M. C., and Epstein, J. A. (2002) Cell 110, 713–723
6. Shin, C. H., Liu, Z. P., Passier, R., Zhang, C. L., Wang, D. Z., Harris, T. M., Yamagishi, H., Richardson, J. A., Childs, G., and Olson, E. N. (2002) Cell 110, 725–735
7. Ismat, F. A., Zhang, M., Kook, H., Huang, B., Zhou, R., Ferrari, V. A., Epstein, J. A., and Patel, V. V. (2005) Circ. Res. 96, 898–903
8. Kook, H., Lepore, J. J., Gitler, A. D., Lu, M. M., Wing-Man Yung, W., Mackay, J., Zhou, R., Ferrari, V., Gruber, P., and Epstein, J. A. (2003) J. Clin. Investig. 112, 863–871
9. Faria, T. N., and Soares, M. J. (1991) Endocrinology 129, 2895–2906
10. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., and Rossant, J. (1998) Science 282, 2072–2075
11. Shida, M. M., Ng, Y. K., Soares, M. J., and Linzer, D. I. (1993) Mol. Endocrinol. 7, 181–188
12. Wilkinson, D. G., and Nieto, M. A. (1993) Methods Enzymol. 225, 361–373
13. Colosi, A., Talamantes, F., and Linzer, D. I. (1987) Mol. Endocrinol. 1, 767–776
14. Lescisin, K. R., Varmuza, S., and Rossant, J. (1988) Genes Dev. 2, 1639–1646
15. Rudert, F., Saunders, A. M., Rebstock, S., Thompson, J. A., and Zimmermann, W. (1992) Mammm. Genome 3, 262–273
16. Beck, F., Erler, T., Russell, A., and James, R. (1995) Dev. Dyn. 204, 219–227
17. Guillenot, F., Caspy, T., Tilghman, S. M., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Anderson, D. J., Joyner, A. L., Rossant, J., and Nagy, A. (1995) Nat. Genet. 9, 235–242
18. Faria, T. N., Ogren, L., Talamantes, F., Linzer, D. I., and Soares, M. J. (1991) Biol. Reprod. 44, 327–331
19. Lee, S. J., Talamantes, F., Wilder, E., Linzer, D. I., and Nathans, D. (1988) Endocrinology 122, 1761–1768
20. Takahashi, Y., Carpino, N., Cross, J. C., Torres, M., Parganas, E., and Ihle, J. N. (2003) EMBO J. 22, 372–384
21. Rescy, J. M., Belaguli, N. S., and Schwartz, R. J. (1998) Heart Development, pp. 273–290 Academic Press, New York
22. Fluck, M., Booth, F. W., and Waxham, M. N. (2000) Biochem. Biophys. Res. Commun. 270, 488–494
23. Belaguli, N. S., Zhou, W., Trinh, T. H., Majesky, M. W., and Schwartz, R. J. (1999) Mol. Cell. Biol. 19, 4582–4591
24. Croissant, J. D., Kim, J. H., Eichele, G., Goering, L., Lough, J., Prywes, R., and Schwartz, R. J. (1996) Dev. Biol. 177, 250–264
25. Ding, W., Gao, S., and Scott, R. E. (2001) J. Cell Sci. 114, 1011–1018
26. Camoretti-Mercado, B., Liu, H. W., Halayko, A. J., Forsythe, S. M., Kyle, J. W., Li, B., Fu, Y., McConville, J., Kogut, P., Vieira, J. E., Patel, N. M., Hershenson, M. B., Fuchs, E., Sinha, S., Miano, J. M., Parmacek, M. S., Burkhardt, J. K., and Solway, J. (2000) J. Biol. Chem. 275, 30387–30393
27. Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998) EMBO J. 17, 6289–6299
28. Philippar, U., Schratt, G., Dieterich, C., Muller, J. M., Galgoczy, P., Engel,
A Novel Regulator in Murine Placentation

F. B., Keating, M. T., Gertler, F., Schule, R., Vingron, M., and Nordheim, A. (2004) Mol. Cell 22, 867–880
29. Hughes, M., Dobric, N., Scott, I. C., Su, L., Starovic, M., St-Pierre, B., Egan, S. E., Kingdom, J. C., and Cross, J. C. (2004) Dev. Biol. 271, 26–37
30. Rivera, V. M., Miranti, C. K., Misra, R. P., Ginty, D. D., Chen, R. H., Blenis, J., and Greenberg, M. E. (1993) Mol. Cell. Biol. 13, 6260–6273
31. Wheaton, K., and Riabowol, K. (2004) Mol. Cell. Biol. 24, 7298–7311
32. Iyer, D., Chang, D., Marx, J., Wei, L., Olson, E. N., Parmacek, M. S., Balasubramanyam, A., and Schwartz, R. J. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4516–4521
33. Ma, G. T., Roth, M. E., Groskopf, J. C., Tsai, F. Y., Orkin, S. H., Grosveld, F., Engel, J. D., and Linzer, D. I. (1997) Development 124, 907–914
34. Cheng, Y. H., and Handwerger, S. (2005) Biol. Reprod. 73, 500–509
35. Adams, R. H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R., and Nebreda, A. R. (2000) Mol. Cell 6, 109–116
36. Hemberger, M., and Cross, J. C. (2001) Trends Endocrinol. Metab. 12, 162–168