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Molecular Basis of Constitutive Production of Basement Membrane Components

GENE EXPRESSION PROFILES OF ENGELBRETH-HOLM-SWARM TUMOR AND F9 EMBRYONAL CARCINOMA CELLS*

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Engelbreth-Holm-Swarm (EHS) tumors produce large amounts of basement membrane (BM) components that are widely used as cell culture substrates mimicking BM functions. To delineate the tissue/organ origin of the tumor and the mechanisms operating in the BM overproduction, a genome-wide expression profile of EHS tumor was analyzed using RIKEN cDNA microarrays containing ~40,000 mouse cDNA clones. Expression profiles of F9 embryonal carcinoma cells that produce laminin-1 and other BM components upon differentiation into parietal endoderm-like cells (designated F9-PE) were also analyzed. Hierarchical clustering analysis showed that the gene expression profiles of EHS and F9-PE were the most similar among 49 mouse tissues/organs in the RIKEN Expression Array Database, suggesting that EHS tumor is parietal endoderm-derived. Quantitative PCR analysis confirmed that not only BM components but also the machineries required for efficient production of BM components, such as enzymes involved in post-translational modification and molecular chaperones, were highly expressed in both EHS and F9-PE. Pairs of similar transcription factor isoforms, such as Gata4/Gata6, Sox7/Sox17, and Cited1/Cited2, were also highly expressed in both EHS tumor and F9-PE. Time course analysis of F9 differentiation showed that up-regulation of the transcription factors was associated with those of BM components, suggesting their involvement in parietal endoderm specification and overproduction of the BM components.

Basement membranes (BMs)¹ are thin sheets of extracellular matrix (ECM) underlying the basal side of epithelial/paren-

chymal cells and consist of laminins, collagen IV, nidogens, and perlecan, all of which are specifically present in BMs but not in other types of ECMs. Through interactions with cell surface receptors such as integrins, BMs not only provide a structural basis for epithelial cells but also regulate their proliferation, migration, differentiation, and survival. Many growth factors (e.g. fibroblast growth factors, transforming growth factor- β , and hepatocyte growth factor) are also incorporated into the BMs through binding to various ECM molecules, further modifying the biological functions of BMs (1, 2). Despite their biological importance, biochemical and cell biological studies of BMs have been hampered by difficulties in preparing BM components on a large scale, since BMs are constantly maintained as very thin sheets in most tissues and organs. An exceptional source of BM components is the murine Engelbreth-Holm-Swarm (EHS) tumor, which produces extraordinary amounts of ECM that are readily extracted under nondenaturing conditions (3, 4). A crude extract prepared from EHS tumor contains nearly 1% (w/v) laminins and reconstitutes BM-like gels *in vitro*, which have been widely used as two- or three-dimensional cell culture substrates that support the differentiated functions of various cell types (5–8).

The molecular mechanisms operating in the overproduction of BM components in EHS tumor are poorly understood. Moreover, the tissue/organ origin of the tumor is obscure. EHS tumor spontaneously arose in an ST/Eh mouse strain and was initially designated as a chondrosarcoma based on its histological appearance (9). Later, biochemical analysis demonstrated that it did not produce cartilaginous proteins but secreted the major BM components (*i.e.* laminin-1, nidogen-1, collagen IV, and perlecan) (10). EHS tumor is still often called a “sarcoma,” and it is also referred to as a yolk sac tumor (3, 11–13).

The primary aims of the present study were to elucidate the origin of EHS tumor and the molecular basis of the BM overproduction. To this end, we utilized genome-wide gene expression profiling using RIKEN mouse 20K chip-1 and -2 containing ~40,000 cDNA clones corresponding to 24,000 nonredundant genes and expressed sequence tags (14, 15). To further elucidate the regulatory mechanisms of BM production, we also analyzed the gene expression profiles of murine F9 embryocarcinoma cells that differentiate into parietal endoderm-like cells (designated F9-PE) and produce large amounts of BM components upon treatment with all-*trans*-retinoic acid (RA) and dibutyryl cAMP (Bt₂cAMP) (16, 17). Utilizing these approaches, we characterized EHS tumor as a parietal yolk sac-

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¹ The abbreviations used are: BM, basement membrane; ECM, extracellular matrix; EHS tumor, Engelbreth-Holm-Swarm tumor; F9-PE, F9 differentiated into parietal endoderm-like cells; F9-S, undifferentiated F9 cells; RA, all-*trans*-retinoic acid; Bt₂cAMP, dibutyryl cAMP; CREB, cAMP-response element-binding protein; E17.5, whole mouse embryo at 17.5 days of gestation.

derived tumor. In both EHS tumor and F9-PE, not only secretory/ECM molecules, but also enzymes and chaperones involved in the post-translational modification of ECM molecules, were highly expressed, suggesting that parietal endoderm cells are an optimized “factory” producing the BM.

EXPERIMENTAL PROCEDURES

Engelbreth-Holm-Swarm Tumor—EHS tumor was maintained by intramuscular implantation in the hind limbs of C57BL/6J mice in the animal experiment facility of Aichi Medical University under approval of the Animal Care Committee of Aichi Medical University. Solid tumors that developed 3–4 weeks after transplantation were excised and used for RNA preparation.

Cell Culture—Murine F9 embryonic carcinoma cells were obtained from the Health Science Research Resource Bank (Osaka, Japan; available on the World Wide Web at www.jhsf.or.jp/English/index_e.html) and cultured on gelatin-coated culture dishes (Asahi Techno Glass Corp, Chiba, Japan) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under an atmosphere of 95% air, 5% CO₂, and 100% humidity. For induction of differentiation into parietal endoderm-like cells, F9 cells were treated with 1 μ M RA and 1 mM Bt₂cAMP for 96 h (16).

RNA Extraction—Total RNAs were extracted from EHS tumor and F9 cells using RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Poly(A)⁺ RNAs were purified using Oligotex dT-30 (TAKARA BIO Inc., Otsu, Shiga, Japan). RNAs from whole mouse embryos at 17.5 days of gestation (designated E17.5) were prepared as described (14).

Microarray Experiments—The RIKEN full-length enriched cDNA microarrays are composed of two chips, 20K-1 and 20K-2, with each chip containing 19,584 cDNA spots including 288 positive and 1,296 negative control spots. For positive controls, cDNA clones for glyceraldehyde-3-phosphate dehydrogenase and β -actin were spotted, whereas plant cDNAs, mouse Cot-1 DNA, salmon sperm DNA, and oligo(dA) were used as negative controls (14, 15).

Hybridization probes were prepared as described by Miki *et al.* (14) with modifications. Briefly, poly(A)⁺ RNAs were reverse-transcribed in the presence of amino-allyl dUTP (Sigma). After purification of the first strand cDNA, incorporated amino-allyl substrates were coupled with a Cy-3 or Cy-5 monofunctional reactive dye (Amersham Biosciences) in 0.1 M sodium bicarbonate buffer (pH 9.0) by incubating for 1 h at room temperature in the dark. Residual free dyes were removed using MicroSpin S-200 HR columns (Amersham Biosciences). Probes labeled with Cy-3 or Cy-5 were combined in 30 μ l of hybridization buffer per slide and competitively hybridized overnight. After hybridization, the slides were washed, dried, and subjected to fluorescence scanning. Hybridizations were performed in duplicate for each combination of samples and references. In duplicate experiments, probes were independently prepared from the same pool of poly(A)⁺ RNAs.

Data Analysis—Following the hybridization, fluorescence images were scanned and analyzed using the GenePix 4000B microarray scanner and GenePixPro 3.0 software (Axon Instruments, Union City, CA). Spots with abnormal appearance or with signal intensities lower than the local background were “flagged” and invalidated in the analysis that followed. Expression ratios were calculated by dividing the Cy-5 intensity by the Cy-3 intensity and normalized as the median of all validated ratios set to 1.0 by using GeneSpring 4.0 (SiliconGenetics, Redwood City, CA). Spots that showed more than a 2-fold discrepancy in the Cy-3/Cy-5 ratios in duplicate experiments were also eliminated. Normalized ratios from duplicate experiments were averaged and used for scatter plots and gene extraction based on the expression ratios.

For hierarchical clustering, the ratios were preprocessed by a filtering program, PRIM (18), and analyzed using the clustering software CLUSTER and TREEVIEW developed by Eisen *et al.* (19) (available on the World Wide Web at rana.lbl.gov/) as described (14, 15). Gene annotations of cDNA spots were according to the data base of functional annotation of RIKEN mouse cDNA clones (FANTOM DB) (20–22).

Northern Blotting, Quantitative PCR, and Criteria for Second Round Selection of Genes Highly Expressed in Parietal Endoderm Cells—For quantitatively stricter estimations of the gene expression levels, aliquots of the RNA were subjected to Northern blotting analyses using a previously described method (23). Information on the probes is available on request. Alternatively, gene expression levels were estimated by quantitative PCR. Total RNAs were reverse-transcribed by SuperScript-II (Invitrogen) with random primers. The reverse transcripts were used as templates for analysis of the gene expression levels using SmartCycler (Cepheid, Sunnyvale, CA) and a QuantiTect SYBR Green

PCR kit (Qiagen) according to the manufacturer's instructions. In general, PCR primers were designed to amplify 200–300 base pairs of the target sequences. Sequences of the primers are available on request. The kinetics of amplification were monitored by SYBR Green fluorescence intensity, and the numbers of cycles required for the fluorescence level to reach the defined threshold level in the logarithmic increase phase (threshold cycle: Ct) were calculated as exponents of the relative amounts of target cDNA in the templates. The differences in the expression levels between F9-PE and undifferentiated F9 (F9-S) were expressed as Δ Ct(F9). Similarly, the expression level in EHS relative to F9-S was expressed as Δ Ct(EHS). Typically, a Δ Ct of 4 corresponded to a 10-fold higher expression than F9-S. For second round extraction of genes highly expressed in both F9-PE and EHS tumor (Table I), a Δ Ct of 2 was used as the cut-off point.

For the time course analysis, F9 cells were treated with RA, Bt₂cAMP, or a combination of both reagents for 96 h in duplicates. Cells were harvested every 24 h, and total RNAs were extracted. The RNAs were also prepared from untreated cells. The expression levels of individual genes were quantified using standard curves drawn with serially diluted reverse transcripts obtained from F9-PE cells treated with RA/Bt₂cAMP for 96 h. Relative expression levels from duplicate experiments were averaged and expressed as mean \pm range.

Western Blotting—F9 cells were differentiated by stimulation with RA/Bt₂cAMP for up to 96 h. The medium was collected and replaced with fresh medium containing RA/Bt₂cAMP every 24 h during the differentiation. 24-h conditioned medium of F9-S was used as 0-h conditioned medium (*Undiff.*). 5 μ l of the conditioned medium were subjected to SDS-PAGE under reducing or nonreducing conditions. Following the transfer onto Immobilon-P membrane (Millipore, Bedford, MA), BM component proteins were detected using specific antibodies for mouse EHS-laminin (Sanbio BV, Uden, The Netherlands), mouse collagen IV (LSL, Tokyo, Japan), or mouse nidogen/entactin (Chemicon, Temecula, CA).

RESULTS

Global Gene Expression Profiles of EHS Tumor and Differentiated F9 Cells—To confirm that the overproduction of BM components by EHS tumor is regulated at the level of gene expression, total RNA from tumor cells was subjected to Northern blotting analysis using cDNAs encoding mouse laminin-1 subunits (α_1 , β_1 , and γ_1) as probes. Total RNAs from F9-S and F9-PE were also analyzed. Laminin α_1 subunit (Lama1) transcripts were almost absent in F9-S but were highly expressed in F9-PE and EHS tumor (Fig. 1A). Transcripts for β_1 (Lamb1) and γ_1 (Lamc1) subunits were also highly expressed in F9-PE and EHS tumor but were barely expressed in F9-S, demonstrating that the overproduction of laminin-1 in EHS tumor is due to the coordinated high expression of mRNAs encoding the three laminin subunits, as was the case with F9-PE.

To characterize the global gene expression profiles of EHS tumor and F9-PE, a Cy-3-labeled cDNA probe prepared from either EHS tumor or F9-PE cells was competitively hybridized to the 20K-1 or 20K-2 with a Cy-5-labeled reference probe prepared from E17.5. Gene expression ratios were calculated and normalized as described under “Experimental Procedures.” The variances of the normalized expression ratios of the duplicate experiments were within 2-fold in more than 90% of the 39,166 cDNA spots. The averaged normalized expression ratios of the duplicate experiments were logarithm-transformed in base 10 and compared between EHS/E17.5 and F9-PE/E17.5 (Fig. 1B). Consistent with the results of the Northern blotting analysis, the expression levels of Lama1, Lamb1, and Lamc1 in both EHS tumor and F9-PE (Fig. 1B, *red spots*) were more than 10-fold higher than those in E17.5. Spots including cDNAs for the other laminin subunits such as α_5 , β_2 , γ_2 , and γ_3 (*cyan spots* in Fig. 1B) showed much lower ratios than those of Lama1, Lamb1, and Lamc1 in both EHS tumor and F9-PE, indicating that laminin-1, composed of α_1 , β_1 , and γ_1 subunits, is the major laminin isoform expressed in EHS tumor and F9-PE.

To compare the global gene expression profiles of EHS tumor and F9-PE, a Pearson's correlation coefficient between the normalized expression ratios of EHS/E17.5 and F9-PE/E17.5 was

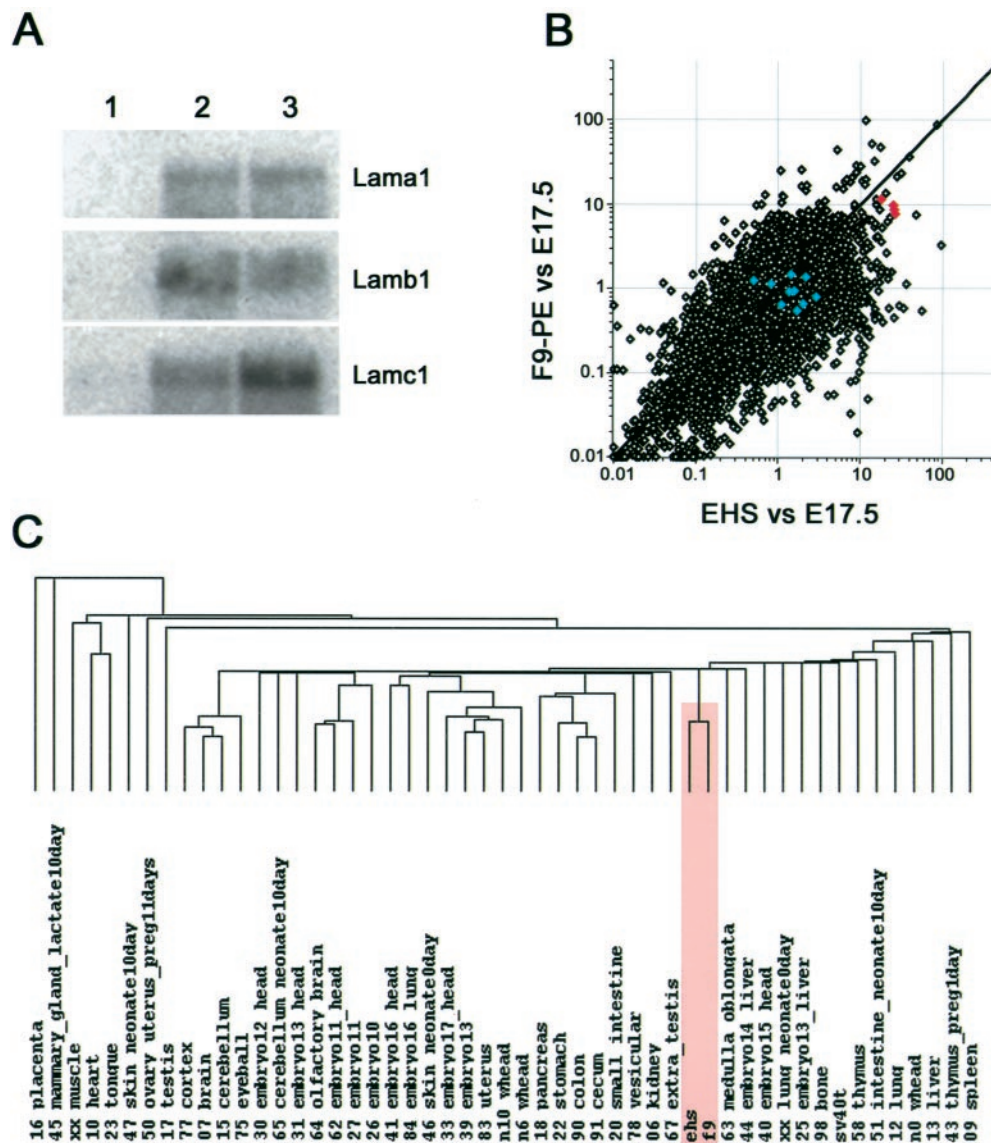


FIG. 1. Gene expression profiles in F9 cells and EHS tumor. A, expressions of laminin subunits. Expressions of laminin α_1 , β_1 , and γ_1 subunits (*Lama1*, *Lamb1*, and *Lamc1*, respectively) were analyzed by Northern blotting. 10 μ g of total RNA from undifferentiated F9 (lane 1), differentiated F9 (lane 2), and EHS tumor (lane 3) were fractionated, transferred to nylon membranes, and hybridized with individual probes. B, scatter plots of normalized expression ratios in EHS tumor and F9-PE cells relative to the E17.5 whole embryo reference. The normalized fluorescence intensity ratios of F9-PE versus E17.5 (y axis) and EHS versus E17.5 (x axis) were plotted in the logarithmic scale. Red spots represent the expression levels of laminin-1 subunits (i.e. α_1 , β_1 , and γ_1), whereas cyan spots represent those of other laminin subunits, including α_5 , β_2 , γ_2 , and γ_3 . C, hierarchical clustering of mouse tissues/cell types based on global gene expression profiles obtained with the RIKEN mouse 19K cDNA sets. The dendrogram covers 49 mouse tissues together with EHS and F9-PE (indicated by red shading) and provides a measure of the relatedness of their gene expression profiles.

calculated. The resulting coefficient was 0.72, indicating high similarity between EHS and F9-PE in terms of the gene expression profile. These expression profiles were further analyzed by hierarchical clustering together with the expression profiles of 49 mouse tissues in the READ (RIKEN Expression Array Database; available on the World Wide Web at read.gsc.riken.go.jp) (24), which were analyzed using the RIKEN cDNA microarray with E17.5 as a common reference (14). As shown in Fig. 1C, EHS and F9-PE were localized in one of the closest branches in the resulting dendrogram, indicating that the gene expression profiles of these cells are closer to each other than to any other tissues. Given the parietal endoderm phenotypes of F9-PE cells, it is likely that the EHS tumor is derived from parietal endoderm cells.

Identification of Genes Highly Expressed in Parietal Endoderm-like Cells—Since parietal endoderm differentiation of F9 cells is associated with a prominent up-regulation of the

expression of laminin-1 and other BM components (16, 25), we performed another round of gene expression profiling of F9-PE cells and EHS tumor, using F9-S cells as a reference to identify candidate genes involved in the excessive production of BM components. The expression ratios in EHS tumor and F9-PE against F9-S were calculated and plotted as shown in Fig. 2. The variances of ratios in EHS over F9-S were greater than those in F9-PE over F9-S, indicating that EHS tumor was more diverged from F9-S than F9-PE. Nevertheless, there was a group of spots showing high ratios both in F9-PE and EHS. These genes were expected to be up-regulated during F9 differentiation upon RA/Bt₂cAMP treatment and also highly expressed in EHS tumor and, hence, to be involved in the excessive production of BM components. To extract the genes highly expressed in these parietal endoderm-like cells, the following criteria were applied to the microarray results. First, the spots with ratios lower than 1.0 in any of the four conditions (i.e.

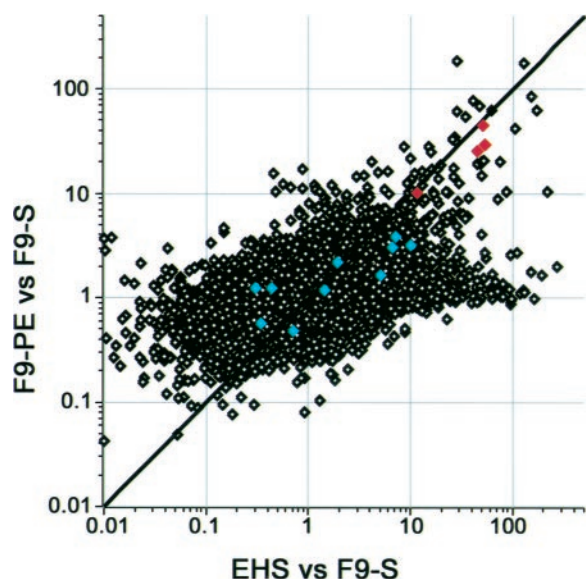


FIG. 2. Scatter plots of normalized expression ratios in EHS and F9-PE relative to F9-S. The normalized fluorescence intensity ratios of F9-PE versus F9-S (y axis) and EHS versus F9-S (x axis) were plotted in the logarithmic scale. Spots corresponding to the laminin-1 subunits (α_1 , β_1 , and γ_1) are shown in red. The cyan spots represent the expression levels of the other laminin subunits.

EHS/E17.5, F9-PE/E17.5, EHS/F9-S, and F9-PE/F9-S) were eliminated. Second, spots with ratios higher than 3.0 in three of the four conditions were selected. Through this strategy, 173 cDNA spots corresponding to 127 nonredundant sequences consisting of 78 known genes and 49 unknown or expressed sequence tag clones were selected. The relative expression levels of the selected genes in F9-PE, EHS, and F9-S were then individually analyzed by quantitative PCR. After the second round selection using the criteria described under "Experimental Procedures," we identified 54 known genes and 14 expressed sequence tags that were highly expressed in both F9-PE and EHS tumor. GenBank™ accession numbers, gene products, symbols, normalized ratios determined by microarray analysis, and relative expression levels determined by quantitative PCR (ΔC_t ; see "Experimental Procedures") of the 54 known genes are summarized in Table I.

Expression of ECM Molecules and Endoplasmic Reticulum-resident Machineries—As expected, genes encoding BM components, such as laminin-1, collagen IV subunits, and nidogen-1, were prominently enriched in the 54 selected genes. Serglycin, a proteoglycan core protein abundantly present in Reichert's membrane (26), and structurally related glycoproteins, SPARC/osteonectin/BM40 (27) and SPOCK/testican (28), were also highly expressed in the parietal endoderm-like cells.

Interestingly, enzymes involved in post-translational modification and endoplasmic reticulum-resident proteins were also extracted. Prolyl 4-hydroxylase is essential for the assembly of collagen fibrils (29, 30). Heparan sulfate 3-O-sulfotransferase 1 is the rate-limiting factor in the biosynthesis of the heparan sulfate chains of proteoglycans (31). An endoplasmic reticulum-resident collagen-specific chaperone, HSP47 (32), a transcript encoding a murine homologue of endoplasmic reticulum lumen protein retention receptor 3 (Mm.29644), and other enzymes involved in glycosylation/post-translational modification were also highly expressed in the parietal endoderm-like cells. The network of these enzymes and proteins for protein biosynthesis and modification should support the efficient production of BM components in the parietal endoderm-like cells, indicating that the parietal endoderm-like cells are an optimized "factory" for excessive production of BM components.

Expression of Transcription Factors and Cofactors—Several genes encoding transcription factors known as parietal and/or primitive endoderm markers, such as Gata4, Gata6, Hnf1b, and Hnf3b (33–36), were present in the extracted genes. Although these genes have been reported to be induced during the F9 differentiation, this is the first demonstration of high expression of parietal endoderm marker transcription factor genes in EHS tumor. A pair of closely related transcription factors of the Sry-box family, Sox7 and Sox17 (37), were also extracted. The expressions of functionally redundant transcription factors such as Gata4/Gata6 and Sox7/Sox17 suggest their roles as fail-safe systems in parietal endoderm specification. In addition to these transcription factors, Atf3 (38), Epas1 (39), and Cited1 (a transcriptional cofactor that interacts with both CREB-binding protein/p300 and Smad proteins (40)) were identified as novel genes up-regulated in RA/Bt₂cAMP-induced differentiation of F9 cells. To further explore the regulatory roles of these transcription factors in parietal endoderm specification, the expression patterns of genes encoding ECM proteins (Fig. 3A) and the transcription factors (Fig. 3B) in various mouse tissues were analyzed using the data from the READ (read.gsc.riken.go.jp) (24). None of these transcription factors appeared to be uniquely expressed in EHS and F9-PE, but no tissues other than EHS and F9-PE highly expressed all of these factors (Fig. 3B), suggesting that parietal endoderm specification is conferred by the combinatorial expression of multiple transcription factors.

Other Genes Highly Expressed in Parietal Endoderm-like Cells—A group of genes encoding secreted molecules was also identified as highly expressed in EHS tumor and F9-PE. These were morphogens such as bone morphogenic protein 2 (Bmp2), fibroblast growth factor 3 (Fgf3), and wntless-related murine mammary tumor virus integration site 11 (Wnt11). Such secretory molecules may well be incorporated in the crude extract of EHS tumor, thereby modifying the biological functions of the extract. Tissue plasminogen activator (Plat), a classical parietal endoderm marker in early development, and midkine (Mdk), a growth factor originally identified as a RA-responsive gene in F9 cells (41), were also found to be expressed in EHS tumor at a high level. Several other secreted factors with ambiguous biological functions were also identified.

Intracellular signaling events evoked by binding of these soluble factors or ECM proteins to their receptors may also play an important role in specification and/or maintenance of the parietal endoderm. Disabled-2 (Dab2), an intracellular signaling molecule induced in F9 by RA stimulation (42), was also expressed at a high level in EHS tumor. None of the other extracted genes, such as a receptor tyrosine kinase (Tyro3) (43), small GTP-binding proteins (Rasd1, Rgnef) (44, 45), and a G-protein-coupled receptor (Edg7) (46), have been reported to be expressed in F9-PE or EHS tumor. These genes might be involved not only in specification of parietal endoderm by regulating the transcriptional network but also in alteration of cell morphology, motility, and cell-cell/cell-matrix interactions. The tissue distribution patterns of these genes are summarized in Fig. 3, C (secreted factors) and D (intracellular signaling molecules). As was the case with transcription factors, the expressions of these genes were not restricted to the parietal endoderm-like cells. Other genes identified as highly expressed in EHS tumor and F9-PE include those encoding channels/transporters, membrane proteins, and proteins with poorly defined functions including a number of expressed sequence tags.

Regulation of Genes Highly Expressed in Parietal Endoderm-like Cells during the Course of F9 Cell Differentiation—To gain further insights into the regulation of the selected genes, the

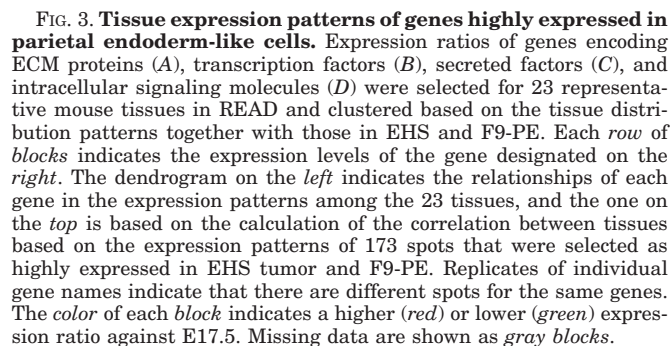
TABLE I
Transcripts expressed in F9-PE and EHS tumor

GenBank™ accession no.	Gene Product	Symbol	Normalized ratios by microarray (average ± range of duplicate experiment)				Difference of PCR cycles to F9-S by quantitative PCR	
			EHS/E17.5	F9PE/E17.5	EHS/F9S	F9PE/F9S	ΔCt(EHS)	ΔCt(F9)
Extracellular matrix								
NM_008480	Laminin, α ₁	Lama1	18.1 ± 1.7	11.4 ± 1.5	51 ± 2.2	45.2 ± 1.2	3.37	10.22
XM_126863	Laminin, β ₁	Lamb1	25.7 ± 2.3	8.6 ± 0.2	45.7 ± 3.8	25.3 ± 0.8	7.86	8.20
			26.3 ± 0.7	7.7 ± 0.7	54 ± 5.4	29.5 ± 4.4		
J02930	Laminin, γ ₁	Lamc1	25.3 ± 1.8	9.7 ± 0.6	12.0 ± 3.1	10.2 ± 0.4	6.42	5.70
XM_134042	Procollagen, type IV, α ₁	Col4a1	4.1 ± 0.2	1.7 ± 0.3	8.8 ± 0.6	8.5 ± 0.5	11.99	11.51
			15.2 ± 2.4	5.9 ± 0.7	36.2 ± 11.1	54.2 ± 4.8		
			7.6 ± 0.1	3.1 ± 0.4	178.7 ± 43.0	62.6 ± 3.8		
XM_134014	Procollagen, type IV, α ₂	Col4a2	5.6 ± 1.2	4.0 ± 0.4	104.7 ± 5.5	42.3 ± 1.6	11.55	11.36
			9.4 ± 0.0	3.8 ± 0.2	129.0 ± 0.6	177.0 ± 18.1		
			8.0 ± 0.0	4.6 ± 0.1	157.5 ± 42.5	86.2 ± 10.5		
			6.3 ± 0.7	3.3 ± 0.1	47.8 ± 2.9	68.1 ± 3.7		
NM_010917	Nidogen 1	Nid1	17.1 ± 0.1	1.2 ± 0.0	86.8 ± 9.8	10.4 ± 0.1	6.64	4.47
NM_011157	Serglycin	Prg	18.0 ± 0.3	1.6 ± 0.0	72.5 ± 11.9	8.8 ± 0.1	7.18	12.17
			10.4 ± 1.1	19.1 ± 3.1	28.2 ± 1.4	190.9 ± 45.1		
			8.9 ± 0.5	19.4 ± 2.7	20.6 ± 7.9	65.2 ± 14.3		
NM_009242	Secreted acidic cysteine-rich glycoprotein	Sparc	8.9 ± 1.5	29.1 ± 4.6	40.9 ± 4.0	80.9 ± 22.6	6.67	5.53
			8.4 ± 0.1	14.0 ± 1.9	7.8 ± 4.0	91.0 ± 9.7		
			4.4 ± 0.5	1.1 ± 0.2	27.4 ± 5.7	34.9 ± 0.1		
NM_009262	Sparc/osteonectin, cwcy, and kazal-like domains proteoglycan 1	Spock 1	3.9 ± 0.2	1.2 ± 0.2	29.0 ± 4.6	61.5 ± 4.7	11.06	10.26
			9.3 ± 2.2	2.5 ± 0.0	25.2 ± 10.2	6.5 ± 0.3		
			6.4 ± 0.4	2.2 ± 0.1	7.2 ± 3.1	3.2 ± 0.1		
Post-translational modification, protein processing								
NM_011031	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide	P4ha2	10.7 ± 1.2	7.2 ± 1.6	16.9 ± 4.1	17.2 ± 2.5	5.21	5.48
			10.1 ± 2.7	5.5 ± 0.5	13.8 ± 3.4	13.8 ± 1.3		
			10.8 ± 0.5	4.3 ± 0.2	26.9 ± 4.0	17.3 ± 0.1		
			5.3 ± 0.7	3.4 ± 0.2	6.5 ± 0.8	4.9 ± 0.2		
NM_011961	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	Plod2	10.4 ± 1.6	5.1 ± 0.5	14.2 ± 2.5	8.8 ± 0.8	2.77	4.86
NM_011962	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 3	Plod3	5.5 ± 0.8	2.9 ± 0.2	3.4 ± 0.3	5.4 ^a	2.06	2.19
NM_009825	Serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1	HSP47	3.1 ± 0.5	1.6 ± 0.3	7.7 ± 2.2	5.3 ± 1.7	2.52	3.71
NM_134090	Similar to KDEL (LYS-ASP-GLU-LEU) endoplasmic reticulum protein retention receptor 3	(KDEL) Mm.29644	5.7 ± 0.7	1.4 ± 0.2	15.8 ± 0.9	25.5 ± 3.9	6.36	7.86
NM_010474	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Hs3st1	12.2 ± 0.2	3.5 ± 0.3	9.8 ± 2.1	4.2 ^a	11.12	8.75
			51.4 ± 3.0	10.6 ± 1.6	102.6 ± 14.5	38.1 ± 13.3		
			42.9 ± 0.7	8.3 ± 1.1	36.1 ± 21.0	19.3 ± 0.3		
NM_029935	B cell RAG-associated protein	Galnac4s-6st	3.7 ± 1.4	1.1 ± 0.4	5.2 ± 0.1	5.7 ^a	7.53	4.58
NM_013792	α-N-acetylglucosaminidase (Sanfilippo disease IIIB)	Naglu	5.0 ± 0.2	2.6 ± 0.3	8.3 ± 0.2	4.8 ^a	3.85	2.97
NM_010893	Neuraminidase 1	Neu1	5.8 ± 0.8	4.6 ± 0.5	3.0 ± 0.5	3.9 ± 0.3	3.28	3.22
			5.8 ± 0.2	3.2 ± 0.2	5.6 ± 0.3	5.2 ± 0.2		
			9.8 ± 1.7	5.9 ± 1.6	3.8 ± 0.4	5.4 ^a		
NM_011992	Reticulocalbin 2	Rcn2	4.2 ± 0.3	2.1 ± 0.4	4.4 ± 0.9	4.2 ± 0.7	3.49	3.23
			5.3 ± 1.2	2.0 ± 0.4	5.4 ± 1.2	3.7 ^a		
Transcription factors								
NM_009330	Transcription factor 2	Hnflb/Tcf2	7.3 ± 0.5	6.6 ± 0.8	22.4 ± 7.4	6.0 ± 0.9	9.60	10.51
NM_010446	Forkhead box A2	Hnf3b/Foxa2	21.4 ± 0.1	6.3 ± 1.6	28.7 ± 1.0	4.9 ± 0.0	4.92	4.84
XM_128828	Transcription factor GATA-6	Gata6	15.7 ± 3.4	11.3 ± 1.3	35.7 ± 19.3	27.3 ± 0.8	11.96	13.23
			11.5 ± 0.0	2.8 ± 0.1	13.5 ± 3.0	16.1 ± 0.3		
			3.8 ± 0.0	1.6 ± 0.0	3.9 ± 1.6	3.5 ± 0.4		
NM_011446	SRY-box containing gene 7	Sox7	4.1 ± 0.7	4.3 ± 0.8	8.2 ± 2.5	6.1 ± 0.6	4.07	7.14
			2.7 ± 0.3	5.1 ± 0.8	9.6 ± 1.5	10.4 ± 2.7		
NM_011441	SRY-box containing gene 17	Sox17	15 ± 0.2	2.2 ± 0.1	16.8 ± 6.3	6.9 ± 0.6	12.93	10.04

TABLE I—continued

GenBank™ accession no.	Gene Product	Symbol	Normalized ratios by microarray (average \pm range of duplicate experiment)				Difference of PCR cycles to F9-S by quantitative PCR	
			EHS/E17.5	F9PE/E17.5	EHS/F9S	F9PE/F9S	Δ Ct(EHS)	Δ Ct(F9)
NM_007709	CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	Cited1	14.6 \pm 0.3 17.0 \pm 0.7	5.6 \pm 1.8 8.7 \pm 1.4	15.0 \pm 1.6 51.9 \pm 15.4	29.1 \pm 6.7 14.7 \pm 0.2	5.15	8.12
NM_010137	Endothelial PAS domain protein 1	Epas1	6.9 \pm 0.2	2.1 \pm 0.1	7.9 \pm 0.8	3.4 \pm 0.2	6.23	3.33
NM_007498	Activating transcription factor 3	Atf3	3.7 \pm 0.3	4.1 \pm 0.5	4.1 \pm 1.1	4.5 \pm 0.0	5.07	9.61
Intracellular signaling factors, receptors								
NM_023118	Disabled homolog 2	Dab2	3.0 \pm 0.0 3.6 \pm 0.4	1.8 \pm 0.3 1.9 \pm 0.2	8.4 \pm 1.7 91.9 \pm 41.9	4.0 \pm 0.5 40.3 \pm 2.8	8.55	9.03
NM_011309	S100 calcium binding protein A1	S100a1	1.8 \pm 0.1 2.0 \pm 0.2 2.1 \pm 0.5	3.5 \pm 0.1 3.6 \pm 0.1 4.0 \pm 0.7	5.8 \pm 1.9 7.8 \pm 0.6 4.2 \pm 0.3	11.2 \pm 2.1 7.8 \pm 1.6 20.4 \pm 2.7	3.75	5.92
NM_019392	TYRO3 protein tyrosine kinase 3	Tyro3	16.5 \pm 2.1 18.0 \pm 0.7	4.2 \pm 0.5 3.6 \pm 0.2	10.8 \pm 2.9 19.5 \pm 2.3	5.0 \pm 0.2 4.7 \pm 1.5	5.90	4.26
NM_009026	RAS, dexamethasone-induced 1	Rasd1	9.4 \pm 1.2	1.8 \pm 0.4	8.1 \pm 0.7	7.2 \pm 1.5	7.61	6.19
NM_012026	Rho-guanine nucleotide exchange factor	Rgnef	4.1 \pm 0.6 3.8 \pm 0.2	3.4 \pm 0.6 3.7 \pm 0.1	3.6 \pm 0.6 4.1 \pm 0.3	2.4 \pm 0.2 4.1 \pm 0.3	4.00	5.24
NM_010884	N-Myc downstream regulated 1	Ndr1	5.7 \pm 0.3 6.6 \pm 1.1	2.4 \pm 0.1 2.9 \pm 0.5	7.8 \pm 0.4 6.2 \pm 1.7	5.6 \pm 0.4 4.4 \pm 0.0	3.38	4.33
NM_022983	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 7	Edg7	5.8 \pm 1.9	4.5 \pm 1.1	9.2 \pm 2.9	5.6 \pm 0.3	8.66	7.87
XM_141384	Fibronectin leucine-rich transmembrane protein 3	Flrt3	22.8 ^a 13.5 \pm 0.6	6.9 \pm 0.4 4.8 \pm 0.6	75.7 ^a 60.5 \pm 5.4	20.0 \pm 1.0 20.8 \pm 7.2	7.40	7.05
NM_010171	Tissue factor precursor (coagulation factor III)	F3	10.9 \pm 0.6	6.8 \pm 0.8	9.2 \pm 0.3	12.5 \pm 0.5	6.00	6.68
NM_033603	Amnionless precursor protein	Amn	4.5 \pm 0.6	1.3 \pm 0.1	6.7 \pm 2.3	4.4 \pm 0.2	4.58	3.25
Secreted factors								
NM_008872	Plasminogen activator, tissue	Plat	12.1 \pm 1.4	5.4 \pm 1.6	29.1 \pm 10.1	24.8 \pm 2.1	9.69	10.38
NM_009519	Wingless-related MMTV integration site 11	Wnt11	5.3 \pm 0.3	1.1 \pm 0.2	13.2 \pm 0.0	3.2 \pm 0.3	3.96	4.64
NM_007553	Bone morphogenetic protein 2	Bmp2	4.7 \pm 0.3 3.6 \pm 0.3	1.4 \pm 0.2 1.2 \pm 0.1	5.8 \pm 0.0 6.2 \pm 0.2	4.8 \pm 1.4 3.7 \pm 0.5	12.64	9.84
NM_010784	Midkine	Mdk	4.8 \pm 0.3	3.9 \pm 0.3	5.5 \pm 1.0	7.2 \pm 0.2	2.31	4.31
NM_008007	Fibroblast growth factor 3	Fgf3	5.1 \pm 3.1	6.3 \pm 0.2	4.9 \pm 0.6	7.1 \pm 0.9	3.83	5.96
S79463	Semaphorin 4C (semaphorin 1)	Semal	3.4 \pm 0.3	1.1 \pm 0.1	9.5 \pm 4.3	5.5 \pm 0.3	6.31	5.29
NM_023476	Lipocalin 7	Lcn7	21.1 \pm 3.8	2.6 \pm 0.2	37.6 \pm 3.2	9.1 \pm 0.5	8.76	6.54
NM_023395	Prostate stromal protein PS20/WAP four-disulfide core domain 1	PS20/Wfdc1	12.7 \pm 0.1 3.7 \pm 0.0 10.7 \pm 0.5 14.1 \pm 1.4	3.2 \pm 0.7 1.8 \pm 0.1 2.8 \pm 0.5 2.4 \pm 0.0	31.3 \pm 7.9 4.2 \pm 1.2 25.8 \pm 3.6 23.7 \pm 10.6	2.9 \pm 0.5 4.7 \pm 0.1 6.6 \pm 1.4 3.7 ^a	14.52	5.54
Miscellaneous								
XM_125842	B-cell translocation gene 1, anti-proliferative	Btg1	4.6 \pm 0.0 4.6 \pm 0.1	1.2 \pm 0.1 1.4 \pm 0.1	6.8 \pm 0.6 7.1 \pm 0.9	3.4 \pm 0.3 3.1 \pm 0.2	3.51	2.43
NM_010174	Fatty acid binding protein 3, muscle and heart	Fabp3	3.4 \pm 0.2 4.6 \pm 0.2 4.0 \pm 0.0	4 \pm 0.1 7 \pm 0.2 6.5 \pm 0.1	1.5 \pm 0.2 1.5 \pm 0.3 1.8 \pm 0.4	3.5 \pm 0.1 3.8 \pm 0.3 3.7 \pm 0.2	2.56	2.24
XM_131373	SCP-like extracellular protein containing protein		3.3 \pm 0.6	4.5 \pm 0.8	1.4 \pm 0.1	3.2 ^a	3.03	3.41
NM_026097	FRING	Fring	5.4 \pm 0.2 7.8 \pm 0.3	2.4 \pm 0.3 3.0 \pm 0.0	4.6 \pm 0.3 6.6 \pm 0.4	3.1 \pm 0.2 3.3 \pm 0.0	4.72	4.41
NM_020052	Similar to CEGP1 PROTEIN	Cegf1	15.6 \pm 0.5	4.1 \pm 0.2	36.9 \pm 7.9	6.0 \pm 0.6	7.81	7.93
NM_009292	Stimulated by retinoic acid gene 8	Stra8	4.0 \pm 0.3	5.6 \pm 1.2	2.7 \pm 0.1	3.7 \pm 1.3	4.41	8.29
NM_007474	Aquaporin 8	Aqp8	9.4 \pm 0.3	5.3 \pm 0.2	62.6 \pm 12.4	65.7 \pm 19.6	12.68	12.11
NM_033314	Solute carrier family 21 (prostaglandin transporter), member 2	Slc21a2	7.6 \pm 0.2	1.1 \pm 0.1	50.1 \pm 8.3	4.0 \pm 0.1	9.72	5.31
NM_031251	Cystinosis, nephropathic	Ctns	5.3 \pm 0.1	7.4 \pm 1.0	3.9 \pm 1.1	4.8 \pm 0.4	3.55	3.86

^a No replicates.



The time course analyses of the expressions of other secreted molecules and intracellular signaling molecules are depicted in Fig. 4, *D* and *E*, respectively. Among these genes, *Mdk* and *SemaI* were up-regulated in almost identical patterns by RA

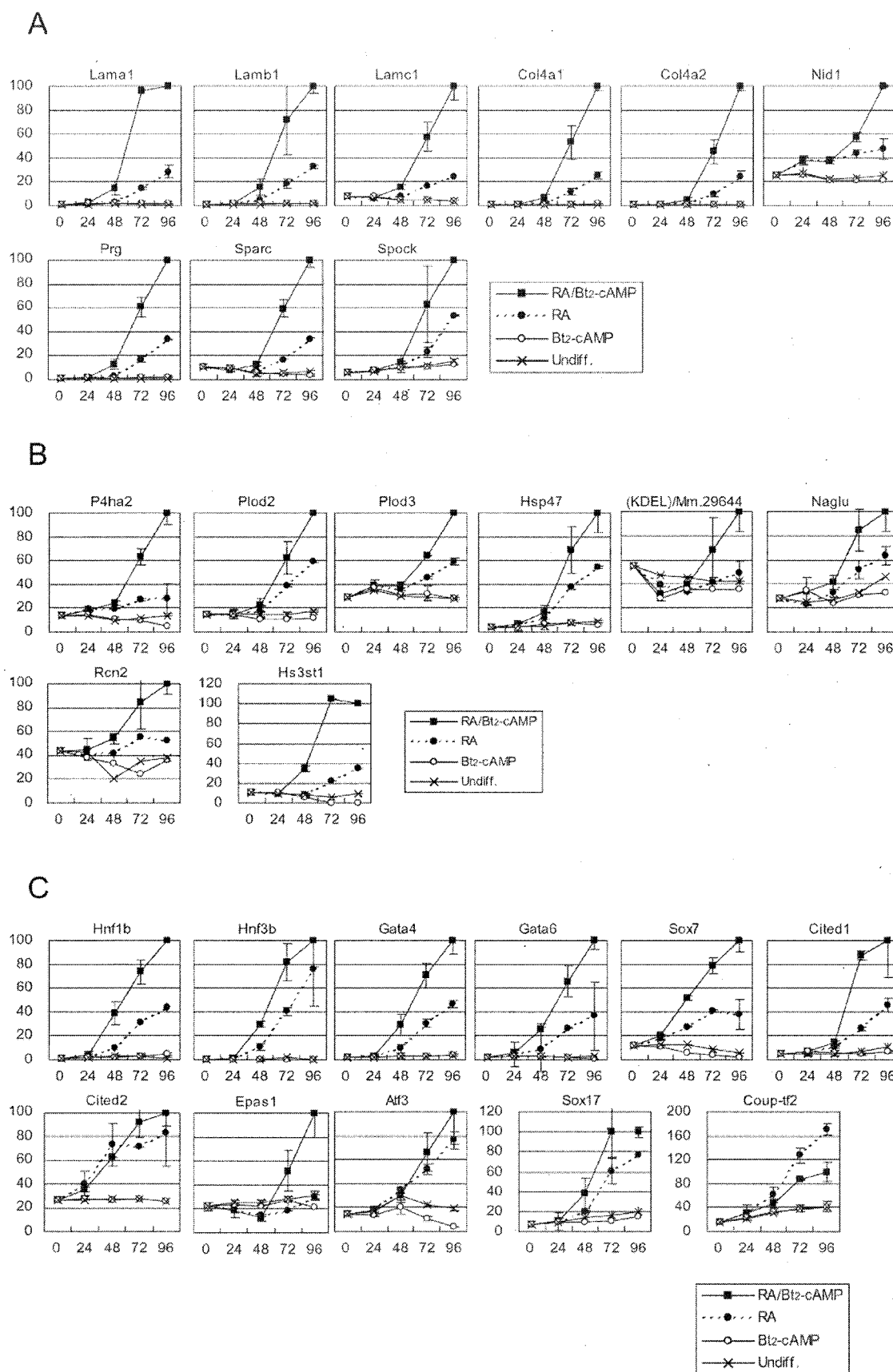


FIG. 4. Time course analysis of the expression levels of genes highly expressed in parietal endoderm-like cells. The expression levels of the genes encoding ECM proteins (A), post-translational modification enzymes (B), transcription factors (C), secreted molecules (D), and intracellular signaling molecules (E) were examined by real time reverse transcriptase-PCR during the course of F9 cell differentiation. F9 cells were stimulated by RA and Bt₂cAMP (RA/Bt₂cAMP), by either RA or Bt₂cAMP alone (RA and Bt₂cAMP, respectively), or by neither (Undiff.). Total RNA samples were collected from duplicate experiments. The expression levels are shown as the percentages relative to those of RA/Bt₂cAMP sample at 96 h after stimulation and expressed as mean \pm range of the duplicates.

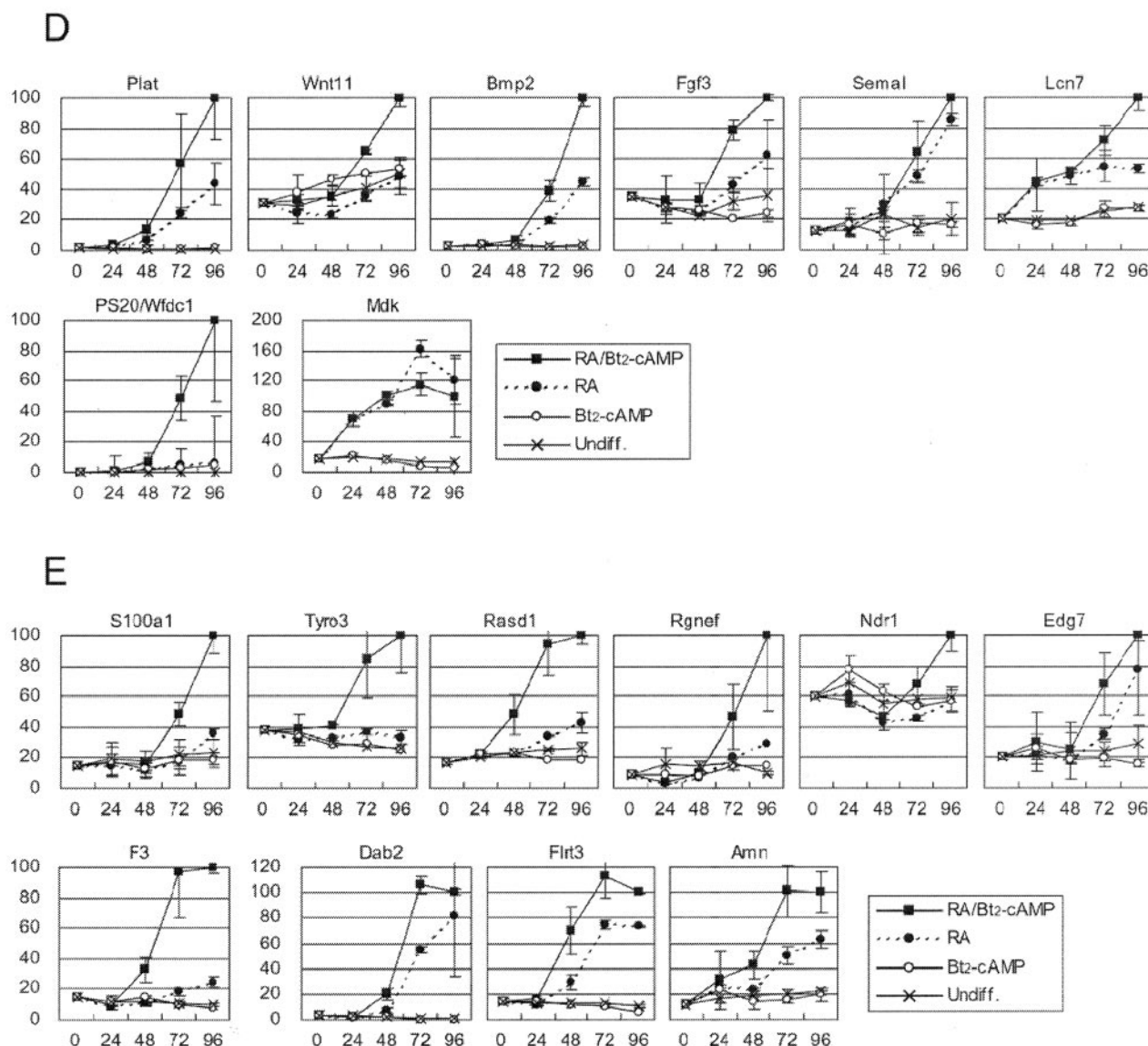


FIG. 4—continued

alone and by RA/Bt₂cAMP, indicating that these genes are directly regulated by RA. On the other hand, the expressions of the other genes were increased at 48 h or later by RA/Bt₂cAMP. A marked difference between induction patterns by RA/Bt₂cAMP treatment and those by RA alone was noted in *Wnt11*, *PS20/wfdc1*, *S100a1*, *Tyro3*, *RasD1*, *Rgnef*, *Ndr1*, and *F3*, indicating that high expression of these genes is specifically associated with the parietal endoderm-like phenotype.

DISCUSSION

The present study was primarily aimed at elucidating the origin of EHS tumor, an extract of which has been widely used as a practical source of BM in many cell biological/tissue-engineering studies, and the molecular basis of its overproduction of BM. Analyses of the gene expression profiles of EHS tumor and F9-PE cells, combined with the expression profiles of various mouse tissues compiled in the READ (24), indicated the origin of EHS tumor to be the parietal yolk sac. Whereas the EHS tumor is still often referred to as a sarcoma, it was also regarded as a yolk sac tumor based simply on its overproduction of BM components (3, 11–13). The present study is the first report providing strong evidence for the nature of EHS tumor being a parietal endoderm tumor based on detailed gene ex-

pression profiling. In early mouse embryogenesis, parietal endoderm arises from the inner cell mass in the blastocyst as a result of the first differentiation events and produces a large amount of ECM to form Reichert's membrane, an extraordinarily thick BM-like structure that separates the yolk cavity from the maternal tissue (17, 49). The constitutively high production of BM components in EHS tumor should reflect the properties of the parietal endoderm *in vivo* as well as that in F9-PE cells.

To understand the molecular basis underlying the overproduction of BM components, we extracted genes highly expressed in EHS tumor and differentiated F9 cells, but not in undifferentiated F9 cells, from expression data compiled by the analyses using the RIKEN cDNA microarray. Through this approach, not only the genes encoding BM components, but also genes encoding machineries for post-translational modification of ECM molecules, were extracted, indicating that EHS tumor as well as F9-PE cells are a specialized "factory" optimized for overproduction of BM components. Although not extracted by the present criteria, other chaperone molecules, such as GRP78/BiP (50) and protein-disulfide isomerases (51), were moderately up-regulated during the differentiation of F9 cells and were highly expressed in EHS tumor (data not

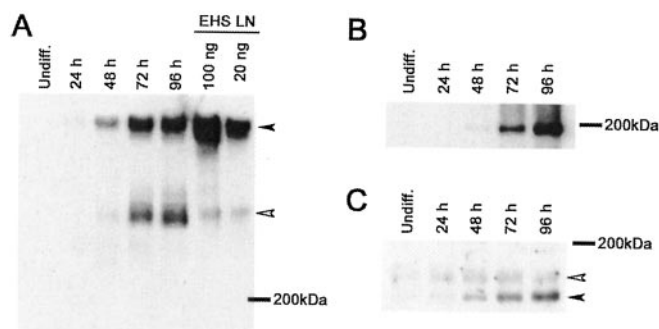


FIG. 5. Western blotting of major BM proteins secreted during the course of parietal endoderm differentiation of F9 cells. F9-S cells were cultured in differentiation medium containing RA/Bt₂cAMP. The conditioned medium was removed every 24 h and replaced with fresh differentiation medium. 5 μ l aliquots of conditioned medium were subjected to SDS-PAGE, followed by Western blotting with specific antibodies. The time the medium was collected after the start of stimulation is indicated at the top of each blot. 24-h conditioned mediums of undifferentiated F9 cells were also analyzed (Undiff.). A, expression of laminin-1. Aliquots of medium were separated under nonreducing conditions. In the right two lanes, 100 and 20 ng of purified laminin-1 from EHS tumor (indicated as EHS LN) were applied as standards for quantification. The polyclonal antibody recognizes $\alpha_1\beta_1\gamma_1$ heterotrimer (solid arrowhead) as well as $\beta_1\gamma_1$ heterodimer and α_1 monomer (open arrowhead). B, expression of collagen IV. Medium separated under reducing conditions. C, expression of nidogen-1. Medium separated under reducing conditions. The solid arrowhead indicates nidogen-1, and the open arrowhead indicates a nonspecific signal.

shown), lending support to our conclusion that these cells are well equipped for excessive production of large ECM molecules.

The present approach also produced a catalogue of secreted molecules possibly incorporated in the crude extract from EHS tumor or in Reichert's membrane *in vivo*. Among these genes, *Bmp2*, *Fgf3*, and *Mdk* have been reported to be induced during the course of F9 cell differentiation (41, 52–54), although expression of Wnt11, another secreted molecule that plays important roles in the morphogenesis of early vertebrate development (55, 56), has not previously been identified in either F9 cells or EHS tumor. Indian hedgehog (Ihh), which has been reported to be up-regulated in F9 cell differentiation (57) and to play a role in yolk sac angiogenesis (58), was also highly expressed in EHS tumor but was not extracted by the present criteria due to the moderate up-regulation in F9-PE (data not shown). These factors might be secreted by parietal endoderm cells *in vivo* and could affect the environment of the yolk cavity. Moreover, these growth factors/morphogens might be present in trace amounts in the crude extract from EHS tumor, since these factors are known to bind proteoglycans or to be incorporated into BMs (59).

The time course analyses of the gene expression profiles during F9 differentiation indicated that the up-regulation of several transcription factors is closely associated with, or rather preceded, those of BM components, suggesting that such transcription factors play important roles in the specification of parietal endoderm cells. Interestingly, three pairs of transcription factors and cofactors closely related to each other, Gata4/Gata6, Sox7/Sox17, and Cited1/Cited2, were identified as up-regulated during the course of F9 differentiation and were highly expressed in EHS tumor. Although the expressions of these genes are not limited to parietal endoderm cells, no other tissues express all of these transcription factors simultaneously at levels comparable with the parietal endoderm lineage. It seems likely, therefore, that the combinatorial expression of these transcription factors is instrumental in ensuring parietal endoderm specification. It can be easily presumed that deficiency of the genes involved in parietal endoderm specification or in Reichert's membrane formation would result in

embryonic lethality at the early postimplantation stage unless other genes can compensate for the resulting defects. Indeed, loss of Gata6 function leads to a lethality at 5.5 days of gestation, due to a defect in an extraembryonic tissue (34), and overexpression of Gata6 or Gata4 in ES cells is sufficient to induce differentiation to extraembryonic endoderm (60). On the other hand, homozygous mutant mice of Sox17 (61) and Cited2 (62) survive longer, suggesting that ablation of one of these genes does not have a catastrophic effect on parietal endoderm development. Although the redundant expression of closely related factors could well be regarded as multiple safeguards securing the progression of early development, it remains to be clarified whether Sox7/Sox17 or Cited1/Cited2 are critical for parietal endoderm specification. Double-knockout mice for these functionally redundant transcription factors have yet to be produced.

In the present study, we characterized EHS tumor as a parietal endoderm-derived tumor through gene expression profiling. The efficient production of BM components by parietal endoderm cells appears to be based on high expressions of post-translational modification enzymes and molecular chaperones. Multiple transcriptional factors and intracellular signal transducers that control the coordinated expressions of ECMs and the machineries for posttranslational modifications were efficiently identified through the present approach. Further studies with overexpression and ablation of genes encoding transcription factors and/or signal transducers should lead to findings useful for the development of efficient production systems of BM components/ECMs that would serve as invaluable materials for tissue regeneration/engineering.

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**Molecular Basis of Constitutive Production of Basement Membrane Components:
GENE EXPRESSION PROFILES OF ENGELBRETH-HOLM-SWARM TUMOR
AND F9 EMBRYONAL CARCINOMA CELLS**

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