UniGene cDNA array-based monitoring of transcriptome changes during mouse placental development

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The placenta is a highly specialized organ essential for embryonic growth and development. Here, we have applied cDNA subtraction between extraembryonic tissues of early- (day 7.5 of gestation) and late-stage embryos (day 17.5) to generate stage-specific cDNA pools that were used for screening of high-density mouse UniGene cDNA arrays containing 25,000 clones. A total of 638 clones were identified, 488 with the e7.5-specific probe and 150 with the e17.5-specific probe. Importantly, 363/638 (56.9%) of the hybridizing clones were not known to be expressed during placental development before. Differential regulation was confirmed by Northern blot and *in situ* hybridization for a total of 44/44 of positive clones. Thus, this combination of cDNA subtraction and array hybridization was highly successful for identification of genes expressed and regulated during placental development. These included growth factors and receptors, components of the transcriptional and translational machinery, cell cycle regulators, molecular chaperones, and cytoskeletal elements. The extensive *in situ* hybridization analysis revealed extraembryonic structures with a high density of differentially expressed genes, most strikingly the ectoplacental cone and the spongiotrophoblast. This large-scale identification of genes regulated during placentogenesis is extremely useful to further elucidate the molecular basis of extraembryonic development.

Genomic approaches by using DNA arrays are powerful tools ideal for mutation and polymorphism analyses and expression profile monitoring of known genes and uncharacterized expressed sequence tags (ESTs; ref. 1). Array technology is also well suited to replace the identification of expressed genes in a given tissue that was conventionally achieved by sequencing of individual clones of a cDNA library (2, 3).

Taking advantage of this technology, we have focused on gene expression and regulation in mouse placental development. The extraembryonic cell lineage is the first to differentiate in placental mammals after fertilization. In murine embryogenesis, the definitive placenta is formed at around day 10 of gestation (e10) and consists of four prominent cell layers, labyrinth, spongiotrophoblast, giant cell zone and maternally derived decidua (4, 5). In early- to mid-gestation conceptuses, trophoblast giant cells lie at the outside of the ectoplacental cone, a structure that mainly develops into the spongiotrophoblast. Two different cell lineages, the mesodermally derived allantois and the extraembryonic (chorionic) ectoderm, contribute to the formation of the labyrinth. This layer consists of a dense network of fetal blood vessels and maternal blood lacunae and is highly specialized for an efficient nutrient and gas exchange.

Placental development gained appropriate attention only recently despite the absolute requirement of a functional placenta for fetal development in all placental mammals. This importance is indicated by the high incidence of fetal death attributable to placental dysfunctions (4, 6, 7). Mostly in gene targeting approaches, several genes have now been identified that are essential for placental development and function. However, with few exceptions (2, 8), the placenta has not been in the focus of widespread gene expression analyses. Here, we report a large-scale expression profiling study comparing early- and late-stage mouse placentas by using a combination of cDNA subtraction and array hybridization techniques on high-density mouse UniGene cDNA filters. This approach identified both expression and regulation of a large number of genes in extraembryonic tissues and thus contributes largely to the understanding of the molecular basis of placentogenesis.

Materials and Methods

Mice and Tissue Preparations. For tissue collection and preparation, mouse strains C57BL/6, CD1, and ICR were used. The day of the vaginal plug was counted as day 0.5 (e0.5). Tissues used for cDNA subtraction have been described previously (9): (*i*) pooled e7.5 extraembryonic tissues including the ectoplacental cone, amnion, chorion, allantois, and trophoblast giant cells but excluding the embryo proper and decidual tissue; and (*ii*) complete e17.5 placentas including the maternally derived decidua and remnants of the yolk sac but excluding the uterine myometrium. For Northern analysis, e9.5 and e17.5 placentas free of myometrial tissue, but including the decidua, were used. For whole-mount *in situ* hybridizations, complete e7.5 conceptuses including the decidua were dissected and cut in halves along the longitudinal axis. E17.5 placentas were cut sagitally into slices of 2–3 mm thickness. Tissues for *in situ* hybridizations were fixed in 4% paraformaldehyde at 4°C overnight.

Generation of Complex cDNA Pools. Generation of the e7.5 extraembryonic tissue-specific cDNA pool has been described (9), and the same experimental strategy was used to generate the e17.5 placenta-specific probe. cDNAs from e7.5 extraembryonic portion and e17.5 placenta were used both as tester and driver in separate reactions to generate cDNA pools enriched for genes predominantly expressed in either early- or late-stage extraembryonic tissues. cDNA pools were digested with *Rsa*I and then subjected to two rounds of PCR amplification.

Complex Hybridization. High-density colony filters from RZPD library 952 (mouse UniGene) were obtained from the Resource Center of the German Human Genome Project (RZPD; www.rzpd.de). They contained a low-redundancy selection of

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Abbreviation: EST, expressed sequence tag.

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25,000 mouse I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) cDNA clones spotted in duplicate. Identical filters were used for hybridization with the two cDNA pools. After competition with 100 μg sonicated mouse DNA (10), hybridization was carried out with 500 ng of random-primed labeled subtracted cDNA material at 65°C over night. Complex hybridizations were performed in duplicate on a different filter set resulting in the same clone hybridization pattern for both probes.

Clone Analysis. Image files generated from hybridization experiments were analyzed by using the WINCLONE program (Kietzmann, unpublished; available as integral part of BIOCHIP EXPLORER software from GPC AG, Martinsried, Germany). All duplicate hybridization signals above background were counted as positive clones. Each cluster identified by hybridizing clones was individually checked by BLAST searches at NCBI (ncbi.nlm.nih.gov). Similarity scores >200 were designated as significant matches, and gene names and/or homologies of all identified clones are given in the supporting information (www.pnas.org). Few clusters (8) were represented by more than one clone pair, and repetitions were subsequently deleted from the clone lists. Bacterial stocks of hybridizing clones were obtained from the RZPD. All cDNA clones selected for further characterization were checked by sequencing on an Applied Biosystems sequencer.

Northern Hybridization. Fifteen to twenty micrograms of total RNA was electrophoresed and blotted onto GeneScreen Nylon membrane (NEN). Random-primed DNA labeling and Northern hybridizations were performed by using standard conditions (11).

Whole Mount *in Situ* Hybridizations. Digoxigenin-dUTP labeled riboprobes were generated according to the manufacturer's instructions (Roche). Whole-mount *in situ* hybridizations were conducted by using standard procedures. Signals were detected by using an anti-digoxigenin-alkaline phosphatase-conjugated antibody (Roche). Staining was carried out overnight by using NBT and BCIP (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt; GIBCO/BRL).

Results

Complex Hybridization. To identify genes regulated during extraembryonic development, the ectoplacental cone region of e7.5 mouse conceptuses and mature e17.5 placentas were comparatively analyzed. Two different cDNA pools were generated by subtractive hybridization, one of them enriched for genes expressed at e7.5 ("e7.5-specific probe"), the other one enriched for genes expressed in late-stage placentas ("e17.5-specific probe"). This step was carried out to quantitatively eliminate housekeeping genes and genes not regulated in their expression levels during development. The reduced-complexity cDNA probes were used to hybridize high-density filters containing a low-redundancy selection of 25,000 cDNA clones of the mouse I.M.A.G.E. cDNA collection. Because each clone was spotted in duplicate, hybridization signals could immediately be distinguished from unspecific background by the appearance of a twin hybridization pattern. Background was extremely low in these complex hybridizations.

A conspicuously different, nonoverlapping overall hybridization pattern was apparent between the two developmental stages (Fig. 1). None of the most strongly hybridizing clones showed equal signal intensities with the two different cDNA pools. A total number of 638 clones hybridized to the two probes; of these, 488 were identified with the e7.5 probe and 150 with the e17.5 probe (for complete clone lists, see Tables 3 and 4, which are published as supporting information on the PNAS web site, www.pnas.org). This hybridization pattern revealed a much more pronounced complexity of gene expression at early stages of placental development. Strikingly, for the majority of UniGene clusters identified (56.9%), expression in extraembryonic structures was a novel finding. Specifically, 54.1% (264/488) and 66% (99/150) of the genes/ESTs at e7.5 and e17.5, respectively, were not known to be expressed in extraembryonic tissues before as judged by gene identity and library derivation of ESTs. Where possible, the hybridizing genes were ordered into groups of functional similarity (Tables 5 and 6, which are published as supporting information on the PNAS web site, www.pnas.org). From this clustering, it became apparent that specific gene classes are differentially represented in early and late stages of trophoblast development (Table 1).

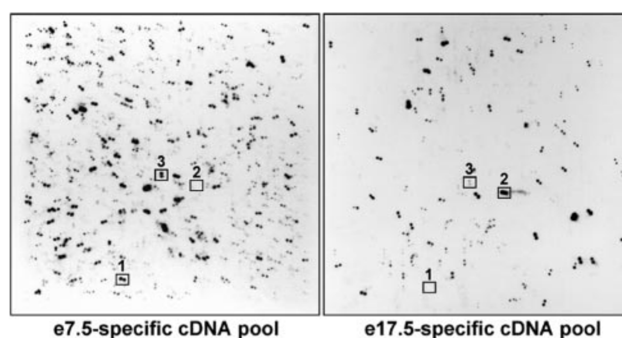

Fig. 1. Autoradiographs of high-density colony filters after hybridization with cDNA pools derived from e7.5 extraembryonic region and e17.5 placenta. The filters contain a low-redundancy selection of 25,000 mouse I.M.A.G.E. cDNA clones each spotted in duplicate. Three examples of hybridizing clones are highlighted on each filter showing the difference of hybridization intensities.

Differential Expression of Identified Unigene Clones. Several lines of evidence suggest the high efficiency of cDNA subtraction and the specificity of the complex hybridization: (*i*) The vast majority of housekeeping genes not differentially regulated between both stages were not identified in the filter hybridization. (*ii*) The pattern of strongly hybridizing clones was completely nonoverlapping between both filter sets. (*iii*) The complex hybridization identified several "marker" genes previously known to be differentially expressed, including *Pl1*, *Adm*, *Ctsl*, *Epcs26/Plac1*, *Ets2*, *Mmp9*, and *Pl2*, pregnancy-specific glycoproteins, *Afp* for e7.5 and e17.5, respectively.

Differential Expression of e7.5-Specific Clones. To further demonstrate expression and differential regulation for a selection of

Table 1. No. of genes in functional group

Functional group	No. of genes e7.5	No. of genes e17.5
Extracellular proteins	23	21
Receptors/transmembrane proteins	21	7
Transcription/chromatin	31	12
Cell signaling	23	3
Cell division cycle	11	1
Nucleolar proteins/nuclear import	9	0
Molecular chaperones	11	4
Cytoskeletal components	12	10
Carrier/transporter proteins	11	3
Protein trafficking	9	2
Energy/metabolism	62	4
Apoptosis-related proteins	4	2

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identified clones, Northern blot and *in situ* hybridizations were carried out with a total of 35 clones that were identified with the e7.5-specific cDNA probe (Table 2). Because of the limited amount of mRNA available in e7.5 ectoplacental cone samples, RNAs from e9.5 placentas and e17.5 placentas were used for Northern blot analyses. Of the 35 clones tested, 23 showed hybridization signals, and 19 of these were expressed at higher levels in the early-stage placental samples. The remaining 4 clones showed equal signal intensities at both developmental stages.

To investigate the tissue-specific expression pattern, whole mount *in situ* hybridizations were carried out with 33 e7.5-specific ESTs (Table 2). Expression of 30 of these genes was detectable, exhibiting a hybridization pattern consistent with a predominant expression in the e7.5 extraembryonic portion used for cDNA subtraction. Within this region, sites of expression were confined to chorionic ectoderm, allantois, ectoplacental cone, trophoblast giant cells, and yolk sac (Fig. 2). Expression in the embryo proper was also frequently observed, but this result was not surprising because the experimental strategy was not aimed to eliminate embryo-expressed genes. Notably, the 30 clones detectable by *in situ* hybridization included all of the genes that exhibited equal signal intensities, and 10 of the undetectable genes in the Northern blot approach. Thus, the high number of unconfirmed clones in the Northern blot analysis was clearly caused by the use of later-stage placental tissue samples (e9.5 vs. e7.5). Trophoblast-specific expression of only 2 genes, *Ptch* and *Rasa3*, could not be confirmed by either of our approaches.

Differential Expression of e17.5-Specific Clones. A total of 14 e17.5-specific genes were analyzed for expression and differential regulation during development (Table 2). Northern blot hybridizations using RNAs from e9.5 and e17.5 placentas confirmed higher expression levels of 7 genes at e17.5. A reversed hybridization pattern with stronger signals at the early stage was observed for 3 clones (putative glycine-*N*-acyltransferase, putative diamine acetyltransferase, and *Vezf*). However, *in situ* hybridization revealed high expression of these genes in the decidua (Fig. 2). The decidua was included in the e9.5 placentas used for Northern blot analysis, but not in the extraembryonic region used for cDNA probe generation. Higher decidual expression of these genes at mid-gestation therefore explains the results obtained in the Northern blot analysis. *In situ* hybridizations confirmed differential expression of 10 of the 14 e17.5-specific genes (Fig. 2). No specific hybridization signals were observed for *Cpr2, Dnajc7, Kcnc1*, and *Matn4*. Differential expression of *Cpr2* was, however, detected by Northern blot hybridization.

Taken together, of the 49 genes assessed by Northern blot and/or *in situ* analyses, 44 (exceptions: *Dnajc7, Kcnc1*, *Matn4*, *Ptch,* and *Rasa3*) showed hybridization signals. All of these 44 clones exhibited differential expression as expected from the direction of cDNA subtraction. For the 5 ESTs not detectable in our approaches, database searches revealed the presence of ESTs derived from extraembryonic libraries in the cDNA clusters for *Dnajc7* and *Rasa3*. This finding shows that these genes are indeed expressed in tissues of extraembryonic origin. Although a lack of expression cannot be fully ruled out for *Kcnc1, Matn4*, and *Ptch*, it is more likely that they represent low-abundant transcripts not detectable by Northern blot and *in situ* hybridization.

Expression Analysis To Identify Genes Contributing to Tissue Specification and Function. Apart from confirmation of stage-specific expression, the extensive *in situ* analysis also allowed us to assess tissue specificity of these cDNAs (Table 2). At e7.5, only 2 genes, muscle-specific serine kinase *Mssk1* and leukemia gene *Ell*, showed ubiquitous expression in all embryonic and extraembryonic structures. The tissue exhibiting expression of the largest number of e7.5-specific genes was the ectoplacental cone. Genes identified to be expressed in this region included several cell cycle regulators (*Cdc71l*, *Cdc2a*, and *Cdc23*), transcription factors (*Sc1*, zinc finger protein), growth factor-related proteins (restricted expressed proliferation associated protein, *Fgfbp1*), and heat shock/stress-induced proteins (*Hsc70*, *Hsp60*, and *Stip1*). The chorion harbors the trophoblast stem cell population that continues to proliferate during early placental development. Although none of the genes investigated was exclusively detected in chorion, expression of several cDNAs in this tissue reflected its proliferative activity, e.g., epidermal growth factor receptor kinase substrate 8 (*Eps8*), oncoprotein-induced transcript 1 (*Oit1*), antioxidant protein 2 (*Prdx5*), Ras-related protein (*Rab3a*), and restricted expressed proliferation-associated protein. Prominent giant cell-specific expression was observed for genes encoding the tumor rejection antigen P1A (*Trap1a*), heat-shock proteins Hsc70 and Hsp60, Csf1-receptor (*Csf1r*, *c-fms*; ref. 12), and vasodilator stimulated phosphoprotein (*Vasp*). It is also noteworthy that expression of the stem cell growth factor *Scgf* was most pronounced in the distal tip of the allantois, indicating a potential role of this gene product in allantoic outgrowth and/or chorioallantoic fusion.

At e17.5, strongest hybridization signals were observed for four spongiotrophoblast-expressed genes, a carcinoembryonic antigen-related gene, cathepsin J (*Ctsj*), diamine-*N*-acetyltransferase (*SSAT*)-homologue, and transferrin (*Trf*). For *Ctsj*, equally strong expression was also observed throughout the labyrinth layer. Furthermore, several genes were expressed in the decidua (*Vezf*, glycine-*N*-acyltransferase(*HP33*)-homologue, and *Tgfb1i4*), whereas transcripts of genes expressed in the labyrinth and/or the chorionic plate were generally less abundant.

Discussion

In the present study, we have investigated gene expression and regulation during mouse placental development by using a combination of cDNA subtraction and array hybridization technology. This proved to be a powerful approach (*i*) to identify a large number of genes previously unknown to be expressed in this cell lineage and (*ii*) to determine their differential regulation between early and late stages of placentogenesis.

DNA array technology is an attractive tool, ideal to investigate expression profiles in a large-scale fashion, commonly achieved by the use of glass slides as carrier for spotted oligonucleotides or clones and hybridization with fluorescent dye-labeled probes. This technique, however, was described to produce inconsistent results with conventional transcript quantitation methods and to vary dependent on dye combination (13), whereas the use of nylon membranes and radioactively labeled probes seems to be more reliable (2, 14, 15). Here, we followed the latter strategy and applied a combination of cDNA subtraction and high-density array hybridization resulting in an extremely high reproducibility of hybridization patterns and intensities. In fact, the combination of techniques was superior to both cDNA subtraction and array hybridization methods individually where commonly a certain background of false-positive clones is observed. In our approach, only one single clone was observed with the e7.5-specific probe that inconsistently hybridized on two separate filters. Of all clones whose expression was detected by Northern blot and *in situ* hybridization, 100% (44/44) were differentially regulated in the expected pattern. These results demonstrate convincingly the specificity of the combined cDNA subtraction and complex hybridization approach.

One of the major achievements of this study was the identification of a large number of genes that are expressed in extraembryonic tissues, irrespective of their regulation during placental development. A total of 56.9% of all hybridizing clones were not known to be expressed in the trophoblast cell lineage before. This result reflects the fact that extraembryonic tissues have been less comprehensively analyzed in cDNA sequencing projects than most other cell types. Even in the recent annotation

Table 2. List of genes analyzed for differential expression during extraembryonic development

EST	Gene/homology	Symbol	Northern (e9.5 ↔ e17.5)	ISH (e7.5 ↔ e17.5)	Am	All	Ch	EPC	TGC	YS
e7.5-specific genes										
AA596853	Amyloid beta (A4) precursor protein-binding family B member 1	*Apbb1*	n.s.	+		(+)				
AA035888	Cell division cycle 2 homolog A	*Cdc2a*	+	+		(+)	(+)	(+)		(+)
AA111385	Cell division cycle 23	*Cdc23*	+	+				(+)		(+)
AA588985	Cdc71 homolog-like 1	*Cdc71l*	n.s.	+	(++)			(+)		
AA553029	Cadherin 5, VE-cadherin	*Cdh5*	+	+	(+)			(++)	(+)	(+)
AA473813	Colony stimulating factor 1 receptor = c-fms	*Csf1r*	n.d.	+					(++)	
AA259654	Eleven-nineteen lysine-rich leukemia gene	*Ell*	n.d.	+	(+)	(+)	(+)	(+)	(+)	(+)
AA512777	Epidermal growth factor receptor kinase substrate 8	*Eps8*	+	+		(+)	(+)			
AA509414	Fibroblast growth factor-binding protein 1	*Fgfbp1*	+	+				(++)		
AA419967	LIM protein FHL2	*Fhl2*	n.s.	+	(+)					(+)
AA616844	ESTs similar to *Hairless*		n.s.	+	(+)	(+)				
AI119357	Heat shock protein cognate 70	*Hsc70*	+	+				(++)	(++)	
AI255269	Heat shock protein, 60 kD	*Hsp60*	+	+				(++)	(++)	
AA575200	Interferon-rel. dev. regulator 2	*Ifrd2*	+	+			(+)	(+)		(+)
AA681067	Migration inhibitory factor	*Mif*	+	+	(+)			(++)		
AA500064	Muscle-specific serine kinase 1	*Mssk1*	n.d.	+	(+)	(+)	(+)	(+)	(+)	(+)
AA545507	Neuronal cell death-related gene in neuron-7, DN-7		+	not det.						
AA451058	Oncoprotein induced protein 1	*Oit1*	n.s.	+	(+)		(+)			
AA869039	Pre-B-cell colony enhancing factor	*Pbef*	+	not det.						
AA269461	Phosphatase inhibitor 2	*Ppi2*	+	+		(+)		(+)		(+)
AA611470	Protein phosphatase 4 reg. sub. 1	*Ppp4r1*	+	+					(+)	(+)
AI196467	Peroxiredoxin 5; antioxidant protein 2	*Prdx5*	+	+			(++)	(++)		
AA517095	Patched homolog	*Ptch*	n.s.	n.s.						
AA509606	Ras-related protein Rab-3A	*Rab3a*	n.s.	+		(+)	(+)	(+)		(+)
AA756830	Ras p21 protein activator 3	*Rasa3*	n.s.	n.s.						
AA607867	Sim. to restricted expressed proliferation associated protein		+	+			(+)	(+)		
AI194861	Ribosomal protein S6	*Rps6*	+	+		(+)	(+)	(+)		
AA544542	Ral guanine nucleotide dissociation stimulator, -like 2	*Rgl2*	+	+			(+)			(+)
AA619328	SC1 protein		n.s.	+				(++)		
AA544018	Stem cell growth factor	*Scgf*	n.s.	+	(+)	(+)				(+)
AI173974	Stress-induced phosphoprotein 1	*Stip1*	n.s.	+		(+)		(+)		(+)
AA538219	Tumor rejection antigen P1A	*Trap1a*	n.s.	+				(++)	(+)	(+)
AA466417	Vasodilator-stimulated phosphoprotein	*Vasp*	n.d.	+				(++)	(++)	
AA607860	Zinc finger protein		+	+			(+)	(++)		
BB310355	EST		+	n.s.						

EST	Gene/homology	Symbol	Northern (e9.5 ↔ e17.5)	ISH (e7.5 ↔ e17.5)	CP	Lab	Sp	TGC	Dec
e17.5-specific genes									
AA017867	Carcinoembryonic antigen (*Cea*)-related protein		+	+			(++)		
W87077	Sim. to cell cycle progression 2	*Cpr2*	+	n.s.					
AA013726	Cathepsin J	*Ctsj*	+	+		(++)	(++)		
AA475449	DnaJ (Hsp40)-hom.	*Dnajc7*	n.s.	n.s.					
AA097230	Growth arrest specific 1	*Gas1*	not det.	+	(+)	(+)	(+)	(+)	(+)
AA276166	Put. glycine-N-acyltransferase, HP33-like		−	+					(++)
AA097374	Potassium voltage gated channel	*Kcnc1*	n.s.	n.s.					
AA289820	Matrilin 4	*Matn4*	n.s.	n.s.					
AA000322	Put. diamine-N-acetyltransferase (SSAT)		−	+	(+)		(++)		(+)
AA794777	Stanniocalcin	*Stc2*	+	+	(+)				
AI114976	TGF beta 1 induced transcript 4	*Tgfb1i4*	+	+			(+)		(+)
AI266895	Transferrin	*Trf*	+	+			(++)		
AA097970	Notch4-like protein/vascular endothelial zinc finger 1	*Vezf*	−	+	(+)	(+)			(+)
W20703	Zinc finger protein 100	*Zfp100*	+	+	(+)	(+)			

+, expected differential expression; n.s., no hybridization signal; n.d., no difference in hybridization intensities; −, expression level difference opposite to subtractive hybridization; not det., not determined. Sites of expression as determined by *in situ* hybridization (ISH) are given on the right. (+) and (++) indicate the presence of transcripts; (++) indicates high levels of expression.

All, allantois; Am, amnion; Ch, chorion; CP, chorionic plate; Dec, decidua; EPC, ectoplacental cone; Lab, labyrinth; Sp, spongiotrophoblast; TGC, trophoblast giant cells; YS, yolk sac.

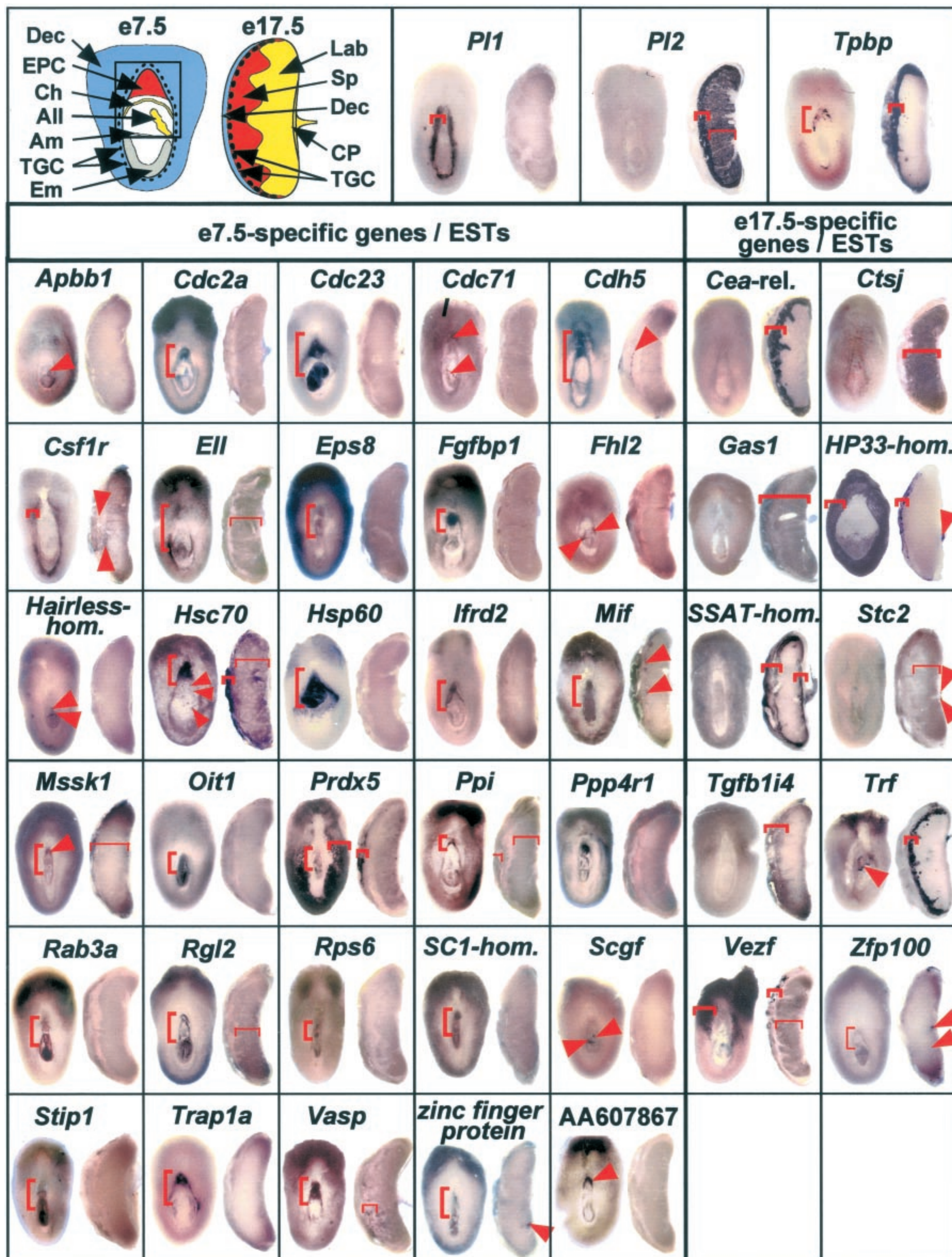

Fig. 2. Whole mount *in situ* analysis of genes and ESTs identified in the filter hybridization. For all samples, the e7.5 conceptus is shown on the left side, the e17.5 placenta on the right. The schematic diagram shows the tissues used for *in situ* hybridization. Colors indicate placental layers and their early-stage derivatives. The boxed area at e7.5, excluding the maternal decidua, was used for cDNA subtraction. Red arrowheads and brackets mark sites of expression. *Pl1*, *Pl2*, and *Tpbp* (*4311*) were used as controls. All genes exhibited differential expression as expected from the filter hybridization. All, allantois; Am, amnion; Ch, chorion; CP, chorionic plate; Dec, decidua; Em, embryo; EPC, ectoplacental cone; Lab, labyrinth; Sp, spongiotrophoblast; TGC, trophoblast giant cells.

of a full-length mouse cDNA collection (16), term placenta was the only extraembryonic structure included in the analysis. Description of overall gene expression during placental development is therefore still far from complete. This result demonstrates the impact of the present array hybridization in enhancing knowledge on overall gene expression in extraembryonic tissues.

The expression profiling approach revealed the abundance of several functionally related gene groups for both stages. The

early developmental stage was characterized by the high representation of growth factors and receptors, components of the transcriptional and translational machinery, metabolic components, cell cycle regulators and signal transduction molecules. This finding in part reflects the high proliferation rate of the chorionic ectoderm and ectoplacental cone (17). Furthermore, the importance of a functional signal transduction cascade for early placental development has been suggested in knockout experiments of several receptor kinases, adapter molecules, and mitogen-activated protein (MAP) kinases (7). Also, many transcription factors have been shown to be indispensable for extraembryonic development (7). Our study has identified a considerable number of other signaling molecules and transcriptional regulators to be expressed in early- to mid-gestation extraembryonic structures. It is very likely that some of them are of similar importance for placenta formation.

Other groups highly represented at e7.5 included genes involved in nuclear import, molecular chaperones, and cytoskeletal elements. Whereas the former is a completely novel finding, a few heat-shock proteins were known to be essential for early placental development, notably Hsp90β (18) and Mrj (19). The massive cytoskeletal re-organizations that occur during the process of giant cell differentiation (20) were reflected by the large number of cytoskeletal components, some of which were already known to be essential for trophoblast function (21, 22). Giant cell formation is also characterized by a transition from a mitotic to an endoreduplicative cell cycle, resulting in large, polytene nuclei (23). Both, mitotic activity in chorionic stem cells and onset of endoreduplication in differentiating trophoblast giant cells might account for the abundance of cell cycle regulators at e7.5.

In contrast, genes up-regulated in late-stage placenta reflected the transition to a specialized organ with less proliferative activity, acquirement of endocrine functions, and adapted for metabolic exchange. This transition is mirrored by gene groups specific for growth arrest, hormones and glycoproteins, vascular differentiation, and a dramatic decrease in genes involved in general cell metabolism.

The majority of all e7.5-specific genes analyzed (64%) were expressed in the ectoplacental cone. This finding might reflect the various commitments of cells in this region that still proliferate, but also start to differentiate into spongiotrophoblast precursors and trophoblast giant cells (17). The diversity of these cells might therefore require a high degree of transcriptional complexity to account for all of these different processes. The same reason is also a likely explanation for the overall enhanced complexity of gene expression at e7.5, as compared with e17.5, that was evident from the filter hybridization patterns (Fig. 1). Another remarkable observation was that, except for the ubiquitously expressed *Mssk1* and *Ell* genes, expression in proliferating chorionic ectoderm and the most differentiated cell type, trophoblast giant cells, was mutually exclusive for all of the e7.5-specific genes.

Strikingly, only one of the e17.5-specific genes analyzed (cathepsin J) exhibited high expression levels in the labyrinth, a layer that constitutes the largest fraction of a mature mouse placenta and that doesn't even form until after e8.5. This finding might indicate that the majority of genes expressed in mature labyrinth are already expressed in early extraembryonic tissues and are therefore eliminated from the cDNA pool during subtractive hybridization. This speculation is supported by expression patterns of several labyrinth-specific genes that are already detected in early extraembryonic tissues, as e.g., *Dlx3* (24), *Esx1* (25), *Gcm1* (26), *Tcfeb* (27), *Tead1*, *Tead4*, and *Tead5* (28). An opposite regulation is present in the spongiotrophoblast that seems to acquire at least some of its specialized functions as a result of gene expression only later in development. This different regulation is indicated by strongest expression of four genes (Cea-rel. protein, *Ctsj*, SSAT-homolog, *Trf*) in this cell layer.

In summary, we have applied a combination of cDNA subtraction and array hybridization to identify a large number of genes expressed and differentially regulated during murine placental development. Extensive expression analyses confirmed convincingly the specificity of this approach. Therefore, this comprehensive analysis of gene expression and regulation during placentogenesis provides important insights into the molecular basis of this process. Because homologous cell types have been identified in human and mouse extraembryonic tissues (7), this analysis is also very useful to further elucidate the underlying aspects of human placental dysfunctions that are often accompanied by fetal and maternal lethality (6).

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