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Identification of Transcripts Specific for Physiological Gene Activation by Platelet-Derived Growth Factor (PDGF)-B in Intact Brain Tissue

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Abstract Platelet-derived growth factor-B (PDGF-B) and its receptors play essential roles in the complex process of blood vessel maturation and they therefore constitute promising targets for therapeutic strategies against blood vessel-related diseases. Additionally, they are involved in the autocrine stimulation of tumor cells and have been suggested to regulate tumor stroma fibroblasts. Our study aimed to identify genes that are regulated directly by PDGF-B, or indirectly via the recruitment of perivascular cells, in the context of an intact tissue. We used a subtractive cloning technique to compare gene transcription in the brains of wild-type (WT) mice and syngenic mice deficient of PDGF-B leading to a defect in the recruitment of perivascular cells. The resulting 147 differentially expressed sequences contained early and late PDGF-B target genes, and genes implicated in blood vessel maturation-related pathways. Additionally, gene clusters for specific biological processes such as cell migration and intracellular transport were identified. Of eight randomly selected sequences, six were found expressed in cultured cells of mesenchymal origin, two of them inducible by exogenous PDGF-BB. The collection of cDNA presented here provides insights into the changes provoked by the removal of one growth factor of a complete tissue and might be the basis for the identification of novel players in the complex process of blood vessel maturation. J. Cell. Biochem. 95: 859–867, 2005.

Key words: subtractive cloning; tyrosine kinase receptor; growth factor; PDGF; blood vessel maturation; angiogenesis

The four platelet-derived growth factor (PDGF) polypeptide chains identified so far can dimerize to five PDGF isoforms: PDGF-AA, -AB, -BB, -CC, and -DD [Heldin et al., 2002]. The isoforms exert their cellular functions through tyrosine kinase α- and β-receptors. Aside from PDGF-DD, all isoforms induce PDGF α-receptor dimerization, whereas PDGF-BB and -DD activate PDGF β-receptor dimers. Furthermore, all isoforms with the exception of PDGF-AA activate both receptor types in cells where both receptor chains are expressed. Upon binding of the ligand, the receptor chains dimerize leading to receptor autophosphorylation. By recruiting SH2 domain-containing signaling molecules (e.g., phosphatidyl-inositol-3′-kinase, Grb2/Sos complex, c-Src, phospholipase C), different intracellular signaling pathways are activated causing various cellular reponses such as survival, migration, and proliferation. The PDGF ligand/receptor systems regulate important physiological functions during embryonic development [Betsholtz et al., 2001]. The binding of endothelial PDGF-B to its receptor β (PDGFR-β) is crucial for the recruitment of pericytes and vascular smooth muscle cells to the immature vasculature [Hellstrom et al., 1999]. PDGF-B, produced by endothelial cells, attracts PDGFR-β-positive mesenchymal cells. The close contact with the endothelium

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leads to their differentiation into pericytes and also to the production of extracellular matrix (ECM) components in which both the endothelial cells and the pericytes are tightly embedded [reviewed in Folkman and D'Amore, 1996]. Pericytes stabilize the endothelium and make it independent from survival factors [reviewed in Benjamin et al., 1998; Reinmuth et al., 2001]. Additionally, they regulate the proliferation of the endothelial cells [Darland and D'Amore, 1999; Hellstrom et al., 2001]. In PDGF-B deficient mice, PDGFR-β-positive mesenchymal cells are not recruited to the developing brain capillaries. The lack of covering pericytes leads to irregularly shaped unstable blood vessels, and ultimately to oedema formation [Lindahl et al., 1997]. Since it was shown recently that the multilateral crosstalk between the endothelium and surrounding cells and the ECM is crucial for the maturation of the vasculature, we studied PDGF-B-dependent gene transcription in physiologically normal brain tissue. Using a modification of the subtractive cloning method suppression subtractive hybridization (SSH) we compared gene expression in the brains of wild-type (WT) and syngenic mice deficient of PDGF-B at day 15.5 of embryonic development (E15.5). At this time point, pericytes have not yet developed around the capillaries of otherwise healthy PDGF-B-deficient embryos, while pericytes are covering the capillaries of WT mouse embryos of the same age [Lindahl et al., 1997]. We identified 147 sequences representing genes that are more expressed in WT mouse brains than in those of PDGF-B-deficient mice. The set of sequences consisted of 46 known genes, 77 cDNA sequences and chromosomal sequences of unknown function, and 24 unknown sequences. We show that a high proportion of clones represent rare mRNA species. Amongst the known genes established PDGF-B target genes as well as genes implicated in blood vessel maturation-related pathways could be identified.

MATERIALS AND METHODS

Cells and Animal Handling

The NIH 3T3 fibroblast cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM supplied with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin (all from Life Technologies, Inc., Grand Island, NY). WT and p53-deficient mouse embryonic fibroblasts (MEF) were generated at embryonic day 12.5. Animal handling and sacrifice were approved by the local ethics committee and governed by the corresponding national legislation.

Preparation of Original RNA Material for the Subtractive Cloning

We crossed PDGF-B heterozygote mice and checked conception by plug-control. At day 15.5 following conception, the embryos were removed by Cesarean section. To avoid any alteration in gene expression by a complicate and long tissue preparation, we removed the complete heads of the embryos by cutting directly under the skull thereby avoiding neck tissue. The heads were directly immersed in a phenol-containing solution (RNA-Gold, Peqlab) and subjected to the RNA-preparation (according to Peqlab's protocol), followed by an additional cleaning using the RNeasy system (Quiagen GmbH, Hilden, Germany).

Construction of Libraries and Subtracted Probe

Fifty nanograms total RNA from WT- and PDGF-B-deficient (KO) littermates was converted into cDNA and amplified using the SMART cDNA synthesis kit (CLONTECH Laboratories, Inc., Palo Alto, CA). The PCR-Select kit (CLONTECH Laboratories, Inc.) was then used for the suppression subtractive hybridization (SSH) procedure. In short, two pools of RsaI-digested cDNA from WT- and KO-littermates were used as tester material and ligated to two different oligonucleotide adapters. RsaI-digested cDNA from either KO- or WT-cDNA, respectively, were used as driver material without adapters. Two hybridizations were performed between the tester population and excess driver, ensuring the removal of cDNA-fragments present in both tester and driver material. In view of the design of the adapters, only the cDNA with different adapters at both ends were PCR amplified, thereby suppressing abundant cDNA fragments and enriching rare ones. The final result is a normalized pool of cDNA fragments that are more abundant in the WT- than in the KO- tissue. The subtraction efficiency was controlled by assessing the content of glyceraldehyde phosphate dehydrogenase (GAPDH) and PDGF-B cDNA by Southern blot hybridization: the consecutive decrease of GAPDH cDNA could be observed

while PDGF-B cDNA was gradually enriched (data not shown). Subtraction was maintained past the complete disappearance of the GAPDH signal. The subtracted and normalized cDNA (KO-cDNA subtracted from WT-cDNA) were cloned into the vector pCR2.1 (Promega Corp., Madison, WI) and transformed into Escherichia coli. By blue/white-selection, 2,304 colonies were picked and cultivated over night in 24, 96-well plates. Subsequently, the bacteria were lysed at 96°C for 10 min. The inserts were PCR-amplified from 1 μl of each lysate and separated on a high-density agarose gel electrophoresis. One hundred ninety-two amplified inserts were loaded on each gel. To control for equal loading and hybridization, a cDNA-fragment for GAPDH was loaded on each gel. All gels were blotted onto nylon membranes as described elsewhere [von Stein et al., 1997]. Southern blot hybridizations were performed with ^{32}P-labeled probes prepared from cDNA of WT and PDGF-B-deficient mice.

Comparison of Different Protocols for the Labeling of Probes to Screen the Clone Collection

A suitable probe should provide the detection of cDNA corresponding to abundant transcripts, as well as those resulting from rare mRNA species. On duplicate test membranes, we tested probes labeled by different protocols. First, we labeled 25 ng 1st strand cDNA radioactively by random priming. This method resulted in a few (11%) very strong signals, which can be detected easily after exposition to conventional film, and 20% weak spots (data not shown). The majority (69%) of the clones spotted on the membrane, however, cannot be detected even after exposition for 1 week. In a second approach, we labeled 1st strand cDNA in a 15 cycle-SMART PCR by using α-P^{32}-dCTP in the PCR reaction. As with the first approach, hybridization with these probes led to a few strongly labeled spots, while the majority of the clones could not be detected. These clones might represent rare transcripts and therefore, we used a third approach: 25 ng cDNA-material from WT and PDGF-B-deficient mice that had passed the normalization step of the subtractive cloning method, but had not undergone the subtraction step was labeled radioactively by random priming (described in detail in the PCR Select™ protocol). Hybridization of the duplicate membranes with these probes resulted in the detection of 75% of all clones (data not shown). For the following screening procedure, this latter labeling protocol was used.

Northern Blot and Semiquantitative RT-PCR Experiments

RNA from mouse brain tissue or cell cultures was extracted with peqGOLD RNA Pure™ (peqlab Biotechnologie GmbH, Erlangen, Germany). RT-PCR was performed as described previously [Renner et al., 2003], on the basis of one pair of WT/PDGF-B-deficient mRNA pools. For all amplifications shown here, 30–35 PCR cycles have been performed. For the Northern blot experiments, three pairs of WT/PDGF-B-deficient mRNA pools have been used. Each mRNA pool originates from one embryo (E15.5). Per lane, 0.6–1.6 μg mRNA were loaded. For the quantification, each signal was normalized to the respective GAPDH control signal.

RESULTS

Identification of 192 Clones Under-Represented in cDNA of PDGF-B Deficient Mice

Using the protocol described above, we labeled normalized, unsubstracted cDNA from WT and PDGF-B-deficient (KO) mouse brains. With these two probes we hybridized duplicates of membranes with the spotted 2,304 clones. The duplicate filters were washed and exposed identically to conventional film in the same exposition box. To facilitate the comparison of the duplicates, 50 ng GAPDH has been spotted onto each membrane. For the following comparison, the only duplicates that were considered were those with identical intensities of the GAPDH-signals. The duplicate films were compared by eye for clearly stronger signals on the film corresponding to the hybridization with the WT-probe on the film corresponding to the KO-probe. Comparison of the duplicates was carried out independently by two researchers. Of the 2,304 clones spotted on the membranes, 192 clearly differentially expressed clones were identified and analysed by DNA sequencing and BLASTN search (NCBI database): 27 sequences have been isolated more than once; the number of hits for each sequence is indicated in Tables I–IV. Of the 147 singular clone inserts, 46 were homologous to known genes, 23 were homologous to previously isolated cDNA clones, 54 represented chromosomal sequences without additional information, and 24 did not match any sequence in the database. As homologous

TABLE I. Genes Known to Be Transcriptionally Activated by Platelet-Derived Growth Factor-B (PDGF-B)

Gene	Accession no.	Type	Hits
Tenascin	D90343	Immediate early target	1
Glutamate receptor	AF483495	Late target (Glur3; G.r. subunit 3)	1
Fibroglycan (syndecan 2)	NM_008304	Cell surface proteoglycan	1

we assessed a sequence which matched one database sequence by over 80%, or which matched a sequence of the database with at least 80 nucleotides and a identity of over 90%, considering that preferably 5′- or 3′- fragments of cDNA are isolated by the chosen subtractive cloning method. The homologous sequences were comprised in categories: genes previously known to be transcriptionally activated by PDGF-B (Table I); genes implicated in PDGF-B pathways or blood vessel maturation (Table II); genes previously not associated with PDGF-B or blood vessel maturation (Table III); DNA sequences without described protein products (Table IV).

Randomly Selected Clones Are Differently Expressed in WT- and KO- cDNA Pools

The subtraction of the KO-cDNA from the WT-cDNA pool was controlled by assessing the presence of GAPDH mRNA after each PCR-step. To prove that the identified sequences are differentially expressed in mRNA preparations from WT- and KO mouse embryonic brains, we performed Northern blot analyses for genes which we selected randomly from different categories of the aforementioned tables: the PDGF-regulated glutamate receptor (glutR1, Table I), the farnesylpyrophosphate synthase (FPP, Table II); from Table III the chaperone TCP-1, the intracellular transport protein P22, ARF4, and the signaling regulator NLRR-3; from Table IV the cDNA clone 12E5 (BC048158), and the unknown sequence of clone 16D6. The expression of the following genes was found to be elevated in the WT mRNA pool: TCP1 (1.5-fold $\pm$0.1); P22 (11.9-fold $\pm$3.6); ARF4 (2.7-fold $\pm$0.7); NLRR-3 (1.9-fold $\pm$0.5). A representative hybridization for each sequence is shown in Figure 1A; all shown hybridizations have been performed with the same pair of mRNA pools. The transcripts for glutR1, FPP, the cDNA clone sequence, and the unknown sequence could not be detected. We therefore established specific RT-PCR protocols for each of these sequences. As shown in Figure 1B, all four transcripts could be detected; the signals of the amplified cDNA were clearly stronger for the WT than for the PDGF-B-deficient mRNA pool.

Expression of Selected Clones in MEF

The clones glutR1, FFP, 12.E5, and 16.D6 represent a PDGF-B-dependent sequence, an independent sequence, a cDNA clone sequence, and an up to now unknown sequence, respectively. As shown in Figure 1B, they can be detected by RT-PCR in total mouse brain cDNA. Since the brains of normal E15.5 mouse embryos differ from PDGF-B-deficient brains in the presence of PDGF-B receptor-positive mesenchymal cells, we checked whether the selected representative RNA are expressed in MEF which express the PDGF-receptor chains. To this end, we performed RT-PCR with RNA preparations from primary MEF of C57/BL6 mice, as well as from immortalized MEF (p53-deficient) and from the MEF cell line NIH 3T3. Aside from the clones glutR1, FFP, 16.D6, and 12.E5, we also investigated the following clones: 9G12 (unknown sequence), 8F5 (D87901), AUF1, and 10A2 (unknown sequence).

TABLE II. Genes Implicated in PDGF-B Pathways or Blood Vessel Maturation

Gene	Accession no.	Remark	Hits
Hemoglobin alpha	AK019138	Anemia in PDGF-B –/– mice	3
Hemoglobin beta	AK011102 AK012551	Anemia in PDGF-B –/– mice	4
Farnesyl PP synthetase	BC048497	Stimulates proliferation of SMC	2
AUF1	MMU11274	Transcription of COX	1
Vdac1	NM_011694	Voltage-dependent anion channel	1
Ste20	BC054521	Phosphorylated by PDGF	1
Ubiquitin	X51703	PDGFR-beta degradation	1
Hmgb1	NM_010439	Attraction of SMC	1

TABLE III. Genes Previously Not Associated With PDGF-B or Blood Vessel Maturation

Gene	Accession no.	Type	Hits
RalGDS domain family	BC057402	Involved in cell migration or interacting with the actin cytosceleton	1
ARF4	D87901		1
ypt1	X15745		1
RAB13	BC050194		1
SCG10	BC026538		1
Actin	NM_007393		1
Sid2895p	AB025405	Intracellular transport	1
Importin beta 3	BC020012		1
RanBP2	AF279458		1
P22	AK045920		1
Gosr2	NM_019650		1
ERp72	J05186	Chaperone	1
Hsp 4	BC002056		1
HSP178	AF161526		1
TCP1	BC018459		1
BAG-5	XM_127149	Binding to HSP70	1
hnRNP D	BC049098	RNA-binding protein	2
RNA-bdg motif 9	NM_053104		2
RNA-binding motif	AK088039		1
Hu antigen D	BC048159		2
WDR1	BC049117	WD40 repeat protein	3
LAP	BC016536	Leucine aminopeptidase 3	2
NB-3	AB032602 NM_017383	Neural recognition	2
LEDGF	AF339082	Growth factor	1
NLRR-3	D49802 NM_010733	Regulation of EGF-RAS-MAPK signalling	2
Human SMP1 homolog	AK014282	Membrane protein	1
MGC-24/CD164	NM_016898	Cell-surface sialomucin	2
Cyclophilin F	BC004041	Peptidylprolyl isomerase	1
Dihydrolipoamide S-succinyltransferase	BC006702	E2 component of 2-oxo-glutarate complex	1
eIF4G2	BC057673	Translation initiation factor	1
Adprt12	NM_009632	ADP-ribosyltransferase-like 2	1
ATPase subunit 6	AF093677	Mitochondrial gene	1
Bleomycin hydrolase	BC027403	Protease	1
Zfp294	XM_128374	Zinc finger protein	2
Itm1	BC037612	Cell membrane protein	2

With the exception of 9G12 and 8F5 clones, we could detect all respective mRNA in the three different MEF (Fig. 2). For AUF1 and clone 12E5, we noted an upregulation of expression in response to added PDGF-BB protein.

DISCUSSION

The aim of the work presented here is to contribute to the investigation of the complex process of blood vessel maturation, the step following the initial sprouting of endothelial cells. We constructed a cDNA library representing genes involved in this process. Blood vessel maturation involves the paracrine/juxtacrine signaling between the endothelium and the perivascular cells, and the contact between the cells and the ECM [Folkman and D'Amore, 1996; Hellstrom et al., 2001]. Here we studied brain tissue from WT- and PDGF-B-deficient mice at day 15.5 of embryonic development. At this time point, blood vessel maturation has proceeded to the recruitment of mesenchymal cells to the endothelial tubes of the brain capillaries and their subsequent differentiation to pericytes in the normal mouse. In contrast, the brain capillaries of PDGF-B-deficient embryos are not covered by pericytes. While E15.5 embryos look phenotypically normal, the missing pericyte coverage leads to the dilation of the brain capillaries and hemorrhagies in the brains of E17.5 PDGF-B-deficient embryos. Comparing the whole brain mRNA of WT and PDGF-B-deficient mice, we identified direct and indirect PDGF-B target genes involved in blood vessel maturation. For the comparison of the two pools of mRNA, we used a recently described subtractive cloning method, SSH, which combines a high subtraction efficiency with an equalized representation of differentially expressed sequences [Diatchenko et al., 1996]. By combining this method with a high density gel-based screening, the possibility of isolating false positive clones can virtually be excluded [von Stein et al., 1997]. The resulting 192 clones were analyzed through sequencing

TABLE IV. DNA Sequences Without Described Protein Products

Gene	Accession no.	Type	Hits
RIKEN cDNA clone	AK030162	Unknown	1
RIKEN cDNA clone	AK041555	Unknown	2
RIKEN cDNA clone	BC048158	Unknown	1
RIKEN cDNA clone	NM_026617	Unknown	1
RIKEN cDNA clone	AF161526	Unknown	1
RIKEN cDNA clone	AK045152	Unknown	1
RIKEN cDNA clone	BC004726	Unknown	1
RIKEN cDNA clone	BC049072	Unknown	1
RIKEN cDNA clone	AK084007	Unknown	1
RIKEN cDNA clone	AK047625	Unknown	2
RIKEN cDNA clone	BC052371	Unknown	3
RIKEN cDNA clone	AK013011	Unknown	1
RIKEN cDNA clone	BC043711	Unknown	1
RIKEN cDNA clone	BC027158	Unknown	1
cDNA clone	BC025546	Unknown	1
cDNA clone	BC057919	Unknown	1
cDNA clone	NM_172596	Unknown	1
cDNA clone	BC061209	Unknown	1
cDNA clone	BC062125	Unknown	1
cDNA clone	BC057919	Unknown	2
cDNA clone	BC059872	Unknown	1
cDNA clone	BC055684	Unknown	1
cDNA clone	AB093210	Unknown	1
Chromosome 1 clone	AL645669	Unknown	1
Chromosome 2 clone	AL772292	Unknown	1
Chromosome 2 clone	AL929138	Unknown	1
Chromosome 2 clone	AL591882	Unknown	1
Chromosome 2 clone	AL954315	Unknown	1
Chromosome 2 clone	AL928545	Unknown	1
Chromosome 2 clone	AL928959	Unknown	1
Chromosome 2 clone	AL844561	Unknown	1
Chromosome 2 clone	AL928793	Unknown	2
Chromosome 3 clone	AC092855	Unknown	2
Chromosome 3 clone	AL606750	Unknown	1
Chromosome 3 clone	AL663099	Unknown	1
Chromosome 4 clone	AL691440	Unknown	2
Chromosome 4 clone	AL837505	Unknown	2
Chromosome 4 clone	AL772210	Unknown	2
Chromosome 4 clone	AC099413	Unknown	1
Chromosome 6 clone	AC122410	Unknown	1
Chromosome 7 clone	AC126274	Unknown	1
Chromosome 7 clone	AC128661	Unknown	1
Chromosome 7 clone	AC139572	Unknown	1
Chromosome 8 clone	AC087183	Unknown	1
Chromosome 8 clone	AC121999	Unknown	1
Chromosome 9 clone	AL592222	Unknown	1
Chromosome 9 clone	AC130551	Unknown	2
Chromosome 9 clone	AC132474	Unknown	1
Chromosome 10 clone	AC101677	Unknown	1
Chromosome 10 clone	AC123812	Unknown	1
Chromosome 10 clone	AC093316	Unknown	1
Chromosome 11 clone	AL596181	Unknown	3
Chromosome 11 clone	AL645947	Unknown	1
Chromosome 11 clone	AL663108	Unknown	1
Chromosome 11 clone	AL669902	Unknown	2
Chromosome 11 clone	AL731728	Unknown	1
Chromosome 12 clone	AC124530	Unknown	1
Chromosome 12 clone	AC122023	Unknown	2
Chromosome 14 clone	AC108430	Unknown	2
Chromosome 16 clone	AC079044	Unknown	1
Chromosome 16 clone	AC129320	Unknown	1
Chromosome 18 clone	AC144672	Unknown	1
Chromosome 19 clone	AC121940	Unknown	1
Chromosome 19 clone	AC121967	Unknown	1
Chromosome X clone	AL672308	Unknown	4
Chromosome X clone	AL663073	Unknown	1
Chromosome X clone	AL671908	Unknown	1
Chromosome X clone	AL840642	Unknown	1
Chromosome X clone	BX005468	Unknown	1
Chromosome X clone	AL671853	Unknown	1
Clone	AL731820	Unknown	1
Clone	AL731820	Unknwon	1
Clone	BX322546	Unknown	1
Clone	AL670236	Unknown	1
Clone	AC123065	Unknown	1
Clone	AC124194	Unknown	1
Clone	AL691491	Unknown	1

No hit was found for 24 clones (default settings of BLAST search, NCBI).

and comparing with the NCBI database (BLASTN, January 2004). Twenty-four clones did not show any homology to the NCBI sequence collection; 68 were homologous to 54 different chromosomal DNA sequences of unknown function. Twenty-seven of the clones corresponded to 23 described cDNA sequences of unknown function. Sixty-three clones were homologous to 46 known genes. Of these 46 genes, 3 are known as targets of signal pathways induced by PDGF-B (Table I). In Table II, genes are summarized which are either linked to PDGF function or to pathways influenced by PDGF, or which are implicated in blood vessel maturation. Hmbg1, for example, has been described recently to stimulate the migration of smooth muscle cells [Degryse et al., 2001]. It was recently shown that the leucine aminopeptidase is expressed in murine endothelial cells and plays an important role in angiogenesis [Yokoyama et al., 1995; Miyashita et al., 2002]. Amongst the genes for which no direct connection to blood vessel maturation has been described (Table III), functional clusters were defined. Six genes involved in cell migration and/or connected to the cytoskeleton have been identified. These genes may be linked to the recruitment of mesenchymal cells to the endothelial tubes, the prominent feature of the experimental system used in this study. Other clusters consist of genes implied in intracellular transport mechanisms, of RNA-binding proteins, and of chaperones. Bag-5 was identified, a Hsp70-binding protein; another member of the BAG-family, Bag-1, has been described to enhance the PDGF-mediated protection from apoptosis, and to associate with the PDGF receptor [Bardelli et al., 1996]. Furthermore, it has been reported to colocalize with actin filaments and to accelerate cell motility in human gastric cancer cells [Naishiro et al., 1999]. Amongst the genes involved in intracellular transport, two components of the transport into the nucleus were identified,

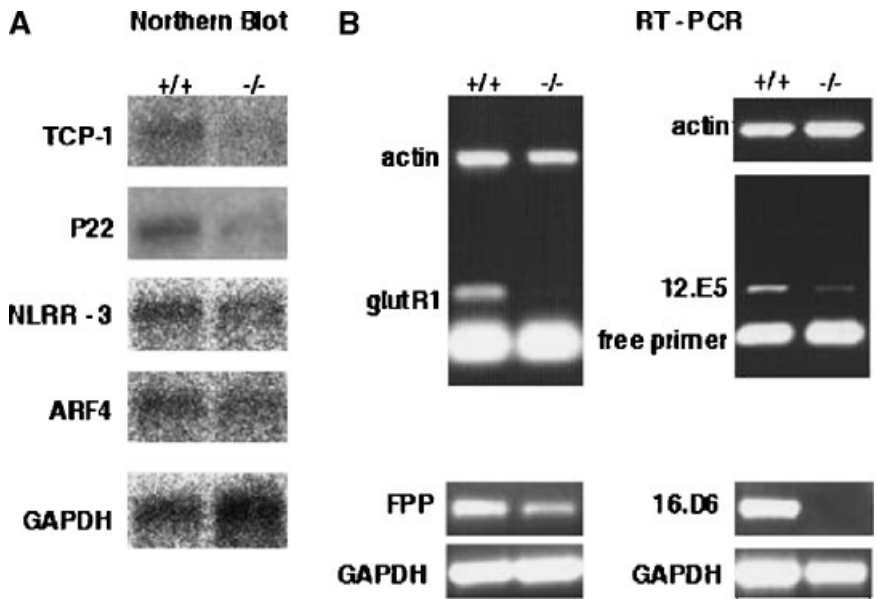

Fig. 1. Validation of selected clones by Northern-blot (**A**) and RT-PCR (**B**) analyses. A: All Northern-blot analyses shown have been performed on the same membrane. As a loading control, a glyceraldehyde phosphate dehydrogenase (GAPDH)-hybridization has been performed. B: For the genes farnesylpyrophosphate synthase (FPP) and the unknown sequence 16.D6, a GAPDH RT-PCR reaction has been performed to check for the comparability of the wild-type (WT)-template (+/+) and the template generated from the platelet-derived growth factor-B (PDGF-B)-deficient mouse (−/−). For the gene of the glutamate receptor 1 (glutR1) and the EST-sequence 12.E5, the actin gene has been amplified as an internal control in the same reaction.

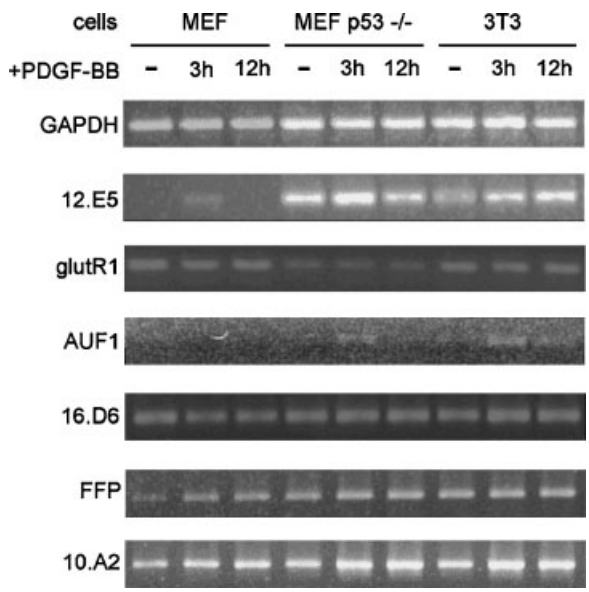

Fig. 2. Expression of selected genes in fibroblasts. Primary mouse embryonic fibroblasts (MEF), immortalized MEF (p53−/−), and the mouse fibroblast cell line NIH 3T3 have been deprived of serum for 24 h and treated with PDGF-BB for 0, 3, and 12 h. RT-PCR has been performed for the unknown sequences 10.A2 and 16.D6, the cDNA clone sequence 12.E5, and the genes glutR1, AUF1, and FFP. As a loading control, a RT-PCR reaction for GAPDH has been performed.

importin-β and RanBP2 [reviewed in Yokoyama et al., 1995; Gorlich, 1998].

To experimentally confirm that the identified sequences are indeed differentially expressed, we performed Northern blot and RT-PCR analyses. The expression of eight genes, which were randomly selected of all four categories (Tables I–IV), was assessed. All tested genes were higher expressed in the WT cDNA pools than in the ones from PDGF-B-deficient embryos.

The construction of the cDNA library described here is based on complex interactions of different cell types present in the mouse brain. It has been shown, by example, that astrocytic endfeet form part of the brain capillaries and influence vascular permeability [reviewed in Abbott, 2002]. It can be assumed that a part of the differential transcription described here results from such interactions. Despite this, we demonstrated the expression of six of eight tested genes in single cell cultures of mesenchymal origin; and in addition that the expression of two of these genes could still be enhanced by the addition of PDGF-BB protein. The fact that six of the eight genes tested are not detectable in cultured mesenchymal cells or do not respond to exogenous PDGF-BB could be

due to the differences in gene expression which are frequently observed between intact tissue and cell culture. It is also likely, that the transcription of some of the identified genes is regulated indirectly by PDGF-B via the recruitment of pericytes, the prominent feature of the experimental system used in this study. By comparing PDGF-B-deficient embryos with corresponding WT embryos, RGS5 has been identified as a marker for pericytes and vascular smooth muscle cells [Bondjers et al., 2003]. Although, the absence of RGS5 expression in PDGF-B-deficient embryos correlated with pericyte loss in these mice, residual RGS5 expression in rare pericytes suggested that RGS5 is expressed independently of PDGF-B/PDGFR-β signaling. This conclusion is consistent with in vitro data, showing that RGS5 expression is only marginally influenced by exposure of cultured vascular smooth muscle cells to PDGF [Cho et al., 2003]. It remains to be established, whether genes identified by us are regulated by pericyte coverage, independently of PDGF-B/PDGFR-β signaling.

With regard to the early effects of PDGF-B activation in cell culture systems, several studies have been published [Rollins and Stiles, 1989; Herschman, 1991; Fambrough et al., 1999; Chen et al., 2004]. With a high number of genes that do not match the list of reported PDGF-B targets, our set of genes complements these studies and will help to elucidate the PDGF-regulated cellular functions, for example cell movement and intracellular transport.

Since the discovery that the transforming retroviral v-sis oncogene is derived from the *PDGF-B* gene, PDGF has been considered a promising target for cancer therapy. As recently reviewed by Pietras et al. [2003], targeting PDGF pathways may not only serve as a anti-angiogenic strategy, but could also inhibit the paracrine stimulation of stromal fibroblasts and the autocrine stimulation of cancer cells. The recent development of clinically useful PDGF receptor antagonists has led to the evaluation of these approaches in clinical trials. The outcome of clinical studies will be dependent on the selection of the optimal patient subset. As identified in a natural system for long-term effects of PDGF-B activation, the clone collection described in our study might be a valuable tool for the categorization of tumor samples and the evaluation of patient groups in clinical trials.

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