

Developmental expression of the α receptor for platelet-derived growth factor, which is deleted in the embryonic lethal *Patch* mutation

AVI ORR-URTREGER¹, MARK T. BEDFORD¹, MYOUNG-SOOL DO², LEA EISENBACH² and PETER LONAI^{1,*}

Departments of ¹Chemical Immunology and ²Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

*Author for correspondence

Summary

The α receptor of PDGF (Pdgfra) is expressed in primitive endoderm and mesoderm derivatives throughout embryogenesis. In the early primitive streak stage the gene is transcribed in the visceral and parietal endoderm. Later it is expressed in the presomitic mesoderm, yolk sac and amnion. During somitogenesis its transcription localizes to the heart and the somites. Subsequently, it is transcribed in the dermatome, the sclerotome, the developing limb and in various mesenchymal tissues of visceral organs. Its wild-type expression pattern correlates well with the phenotype of homozygous mutant *Patch* (*Ph*) embryos, where the Pdgfra gene is deleted. The *Ph* phenotype is first detectable at the primitive streak stage with convoluted

and hypertrophic visceral yolk sac, deformed neural plate and disorganized or missing mesoderm. Most *Ph/Ph* embryos die before the 11th day of gestation. Those that survive till early organogenesis are very small, have hypertrophic yolk sacs, small and undifferentiated somites, convoluted neural tubes, large heart and pericardium, rudimentary limb buds and branchial arches. Our observations together suggest that the α PDGF receptor may be required for the normal development of visceral endoderm and mesoderm derivatives.

Key words: PDGF receptor, embryogenesis, *in situ* hybridization, *Ph* mutation, embryonic lethality

Introduction

PDGF (platelet-derived growth factor) is a major mitogen for connective tissue and glial cells *in vitro* (reviewed by Ross et al., 1986 and by Heldin and Westermark, 1989). It stimulates chemotaxis, it is possibly active in wound healing and it is thought to be involved in the development of atherosclerotic plaques (Rubin et al., 1988; Ferns et al., 1991). Abnormal regulation of PDGF, due to mutation or overexpression, can contribute to malignant growth and both the B and A chains of PDGF can cause malignant transformation (Beckmann et al., 1988). A derivative of PDGF-B, the *v-sis* oncogene, transforms cells that express the PDGF receptor (Downward et al., 1984; Heldin and Westermark, 1989). The role of PDGF in cell differentiation was demonstrated in the glial cells of the optic nerve, where PDGF is produced by type 1 astrocytes, acts on bipotential glial progenitor cells, and is required for the timing of their differentiation into oligodendrocytes (Noble et al., 1988; Raff et al., 1988).

PDGF is also expressed in embryonic carcinoma cells (Gudas et al., 1983), in the extraembryonic membranes and in the placenta (Goustin et al., 1985), suggesting

that it may have a role in embryogenesis. Numerous other polypeptide growth factors, such as those belonging to the FGF and the TGF β gene family, are active in mesoderm induction, as detected by the *Xenopus* animal cap model. PDGF however has no detectable effect in this system (Smith, 1989; Whitman and Melton, 1989), which may be one reason why we know comparatively little about its role in early development.

Biochemical analysis revealed that PDGF and its receptor (PDGF-R) each have two isoforms, designated A and B for the growth factor, α and β for the receptor. PDGF and PDGF-R isoforms function as dimers, and both homo- and heterodimers are formed. The α receptor (Pdgfra) recognizes the A and B chains, whereas the β receptor (Pdgfrb) is specific for PDGF-B. The PDGF AA homodimer is recognized exclusively by the $\alpha\alpha$ receptor homodimer (Seifert et al., 1989; Heldin et al., 1988).

Recent evidence drew attention to the involvement of Pdgfra in early development. Mercola et al. (1989) showed that Pdgfra and PDGF-A are expressed prior to Pdgfrb and PDGF-B in the 7.5- to 8.5-day-old mouse embryo, suggesting that Pdgfra and PDGF-A may have special roles at the primitive streak stage. RFLP

experiments by Stephenson et al. (1991) with interspecies crosses revealed that *Pdgfra* is deleted in *Patch* (*Ph*), an embryonic lethal mutation of the mouse. We report here similar results with DNA from homozygous *Ph* embryos. The genetic data are connected with an analysis of the developmental expression of *Pdgfra* and this is compared with the phenotype of homozygous *Ph* embryos. We demonstrate that *Pdgfra* is expressed in derivatives of the primitive endoderm and mesoderm of early mouse embryos and that its expression follows the differentiation of the two cell layers throughout embryogenesis. We also show that the mutant defects of the *Ph* homozygote correlates well with the wild-type expression pattern of *Pdgfra*. Nevertheless the mutant phenotype of our C57BL/6J congenic *Ph* stock manifested earlier, and the cranio-facial "cleft face" phenotype occurred much less frequently, than in Grüneberg and Truslove's (1960) original CBA stock, or in BALB/c-based stocks of others (Weston, personal communication). Requirement for *Pdgfra* receptors during early development and the effect of the genetic background on the *Ph* phenotype will be discussed.

Materials and methods

Embryos

Breeding nuclei of C57BL/6J-*Ph*/+ and congenic littermate C57BL/6J mice (4 registered males and 4 females from two litters) were purchased from the Jackson Laboratories (Bar Harbor, Maine). They were propagated by brother-sister mating, since 1989. For experiments they were kept under a 14 hour light, 10 hour dark (from 7.30 p.m. to 5.30 a.m.) regime. The time of pregnancy was established the morning following mating. This was regarded as day 0.5 post coitum (p.c.). Early embryos (6.5 to 8.5 days p.c.) were prepared for sectioning within the decidua. Later embryos were carefully dissected from their embryonic membranes. For in situ hybridization, the embryos were prefixed in 4% paraformaldehyde at 4°C. For histological investigations, Bouin fixative was used. The viability of the embryos after the age of 8.5 days (p.c.), was ascertained by their beating heart.

Probes

For the isolation of the mouse *Pdgfra* cDNA a *c-fms* probe was used to screen a mouse (BALB/c) brain λ gt10 cDNA library (a gift from Dr. Yoav Citri of the Weizmann Institute). Our full length murine *Pdgfra* cDNA is 6576 bp long (GenBank accession N° 84607). This is in good agreement with a single 6.5 kb band detected by RNA blotting in liver, metastatic murine sarcoma and embryo RNA (Do et al., unpublished data). The murine *Pdgfra* sequence displays high degree of homology to its human homologue (Claesson-Welsh et al., 1989; Matsui et al., 1989). Amino acid homology between the highly conserved tyrosine kinase domains, is 99%, whereas between the kinase insertion domains, which belong to the least conserved areas of receptor tyrosine kinases, it is 97%. The full length murine *Pdgfra* sequence of Do et al. (unpublished data) was also compared to the partial cDNA sequence of murine (from F9 tumor cells) *Pdgfra*, recently communicated by Wang and Stiles (GenBank, accession N° M57683). Nucleotide sequence homology was 99.2% over 3565 bp. The 0.8% difference caused either no change or conservative changes in the amino acid sequence. It

was concluded therefore that the cloned cDNA used in the present study is indeed a transcript of the murine *Pdgfra* locus.

Probes used for in situ hybridization: a 359 bp long cDNA fragment (corresponding to residue 2178 to residue 2536 of the human sequence of Claesson-Welsh et al., 1989) isolated from the interkinase region served as the *Pdgfra* probe for in situ hybridization. As positive control, *c-kit* (Orr-Urtreger et al. 1990) and *Hox-4.1* probes (from the area 3' to the homeobox; Lonai et al., 1987) cDNA fragments were used. All probes were cloned into BluescriptII KS+ T3 and T7 polymerase-catalyzed transcripts were synthesized in the presence of ³⁵S-UTP for in situ hybridization. Both sense and anti-sense transcripts were used. No significant signals were obtained with the sense (control) transcript.

Genomic hybridization

Genomic Southern blot hybridization was performed at high stringency (hybridization at 42°C in 50% formamide, post-hybridization wash at 67°C in 0.1×SSC) according to Maniatis et al. (1982). Probes were isolated from the full length murine cDNA (Do et al. unpublished data), they are identified here according to the respective residue number in the human cDNA sequence (Claesson-Welsh et al., 1989) and their position is indicated in Fig. 8A. Probe A: 558-1859; probe B: 1885-4036; probe C: 4037-5200, D: 5191-6576 (note, that the 3' noncoding regions of the two sequences are highly divergent).

In situ hybridization

Embedding, cryostat sectioning, post-fixation, hybridization and washing was as previously described (Orr-Urtreger et al., 1990).

Histological examination

Fixation in Bouin fixative and embedding in paraffin was as described by Kaufman (1990). Serial sections of 4 to 7 μ m thickness were stained with hematoxylin-eosin or with light green.

Results

Pdgfra expression at the egg-cylinder and primitive streak stages

In contrast to *c-kit* (Orr-Urtreger et al., 1990) and similarly to two FGF receptors, *bek* and *flg*, (Orr-Urtreger et al., 1991) the expression of *Pdgfra* was not detectable in ovarian oocytes (not shown). At the egg-cylinder stage, *Pdgfra* was expressed in the parietal and visceral endoderm, but not in the primitive (embryonic) ectoderm or trophectoderm (Fig. 1A-F). Fig. 1 shows a 6.5-day- (p.c.) old embryo before mesoderm induction and before the separation of the proamniotic cavity into the three cavities characteristic of the late primitive streak stage. It can be seen (Fig. 1B,F) that the extraembryonic visceral endoderm accumulated more *Pdgfra* transcripts than the embryonic part, which however is definitely positive, as it is apparent also in sections across the embryonic region (Fig. 1D). *Pdgfra* is expressed in the visceral endoderm-like derivatives of activated embryonic carcinoma and embryonic stem cell cultures (Schattelman et al., 1992 and our unpublished results). In addition to the visceral endoderm, *Pdgfra* was also expressed in the parietal endoderm

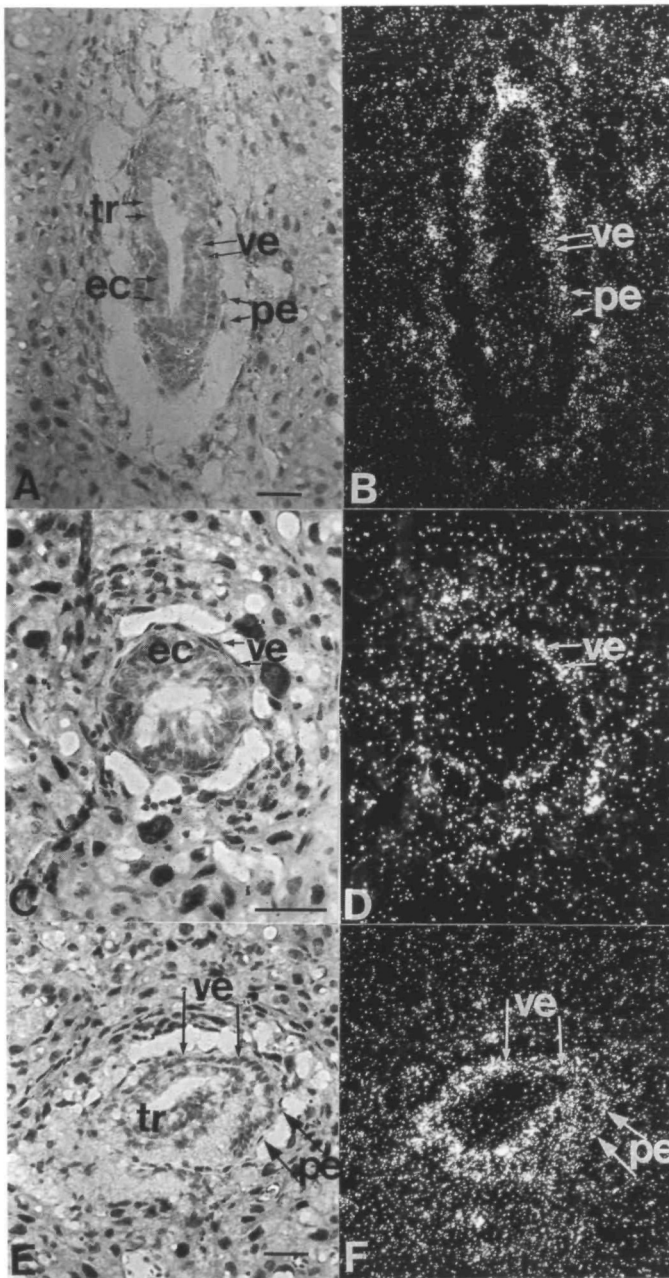


Fig. 1. Expression of *Pdgfra* in the primitive streak stage mouse embryo. (A,C and E) Bright-field, (B,D and F) dark-field illumination of the same sagittal (A,B) and transverse (C,D and E,F) sections of 6 5-days-old (p.c.) embryos. Abbreviations: ec, primitive ectoderm; pe, parietal endoderm; tr, trophoblast (extraembryonic ectoderm); ve, visceral endoderm. Bars: 50 μ m. Arrows point to the same cells in dark-field and bright-field photographs to help align the hybridization signals with their origin.

(Fig. 1B,D), a closely related derivative of the primitive endoderm (Gardner, 1983).

The mesoderm first appears in the posterior portion of the egg cylinder, it exfoliates through the primitive streak and invades both its embryonic and extraembryonic halves. Fig. 2A-D demonstrate that *Pdgfra* is

expressed in the embryonic and extraembryonic mesoderm and also in the visceral and parietal endoderm of the 7.5 day p.c. embryo. *Pdgfra* expression in the embryonic part of the visceral endoderm was greatly diminished by this stage and no hybridization was detectable in the neural plate-primitive ectoderm.

Expression of Pdgfra during somitogenesis and in differentiated somite derivatives

At around 8-8.5 days p.c., the neural folds start to close in the occipital region and a distinct head fold develops. At the same time along the longitudinal body axis paraxial mesoderm cells aggregate into pairs of cell blocks, the somites, and, ventrally from the head fold, the heart anlage forms. Parasagittal sections of 8.0-day-old embryos show that *Pdgfra* is strongly expressed both in the early, non-segmented and in the more mature, segmented paraxial mesoderm (Fig. 2E-H). No hybridization signals were detectable in the definitive endoderm of the foregut or in the neuroectoderm (Fig. 2F,H). *Pdgfra* at this stage was however transcribed in the extraembryonic mesoderm, in the blood islands in the head mesenchyme and in the heart anlage.

Starting with the formation of the first somites (Fig. 2E,F) and then throughout development, *Pdgfra* expression follows certain aspects of somite differentiation, as illustrated in Fig. 3. First the homogeneous somitic blocks separate into dermatomyotome and sclerotome. The myotome will form skeletal muscle, whereas vertebrae and other chondrogenic elements of the skeleton develop from the sclerotome. *Pdgfra* is expressed in all or most cells of the undifferentiated somitic block (Figs 2F and 3B). Later, however, *Pdgfra* transcription concentrates in the dermatome and sclerotome, and no hybridization is observable in the adjacent myotome (Fig. 3C,D).

During advanced organogenesis, the sclerotomes condense into prevertebrae and the chondrification of the vertebral body begins. Differentiation of these axial structures follows an anteroposterior direction. Thus in the tail of a 14.5-day-old embryo shown in Fig. 3E-J, the somites still appear as homogeneous blocks and they display strong accumulation of *Pdgfra* transcripts (Fig. 3I,J). At anterior positions, where axial development is more advanced vertebrae and intervertebral discs form at this stage. Fig. 3G,H show that the intervertebral discs accumulate high levels of *Pdgfra* transcripts, whereas in the body of the differentiating vertebrae, as in prospective ossification centers at other locations, their amount is greatly diminished.

Another differentiated somitic derivative that expresses *Pdgfra*, the dermatome, gives rise to the dermis, the mesodermal component of the skin. It can be seen in Fig. 3K that *Pdgfra* expression concentrates to the dermis and no transcripts are seen in the tail epidermis of a 14.5-day embryo.

Expression of Pdgfra during early organogenesis and limb development

At 9.5 days of development (Figs. 3A,B, 4A-D, 5A,B) *Pdgfra* transcripts accumulated in the lateral and

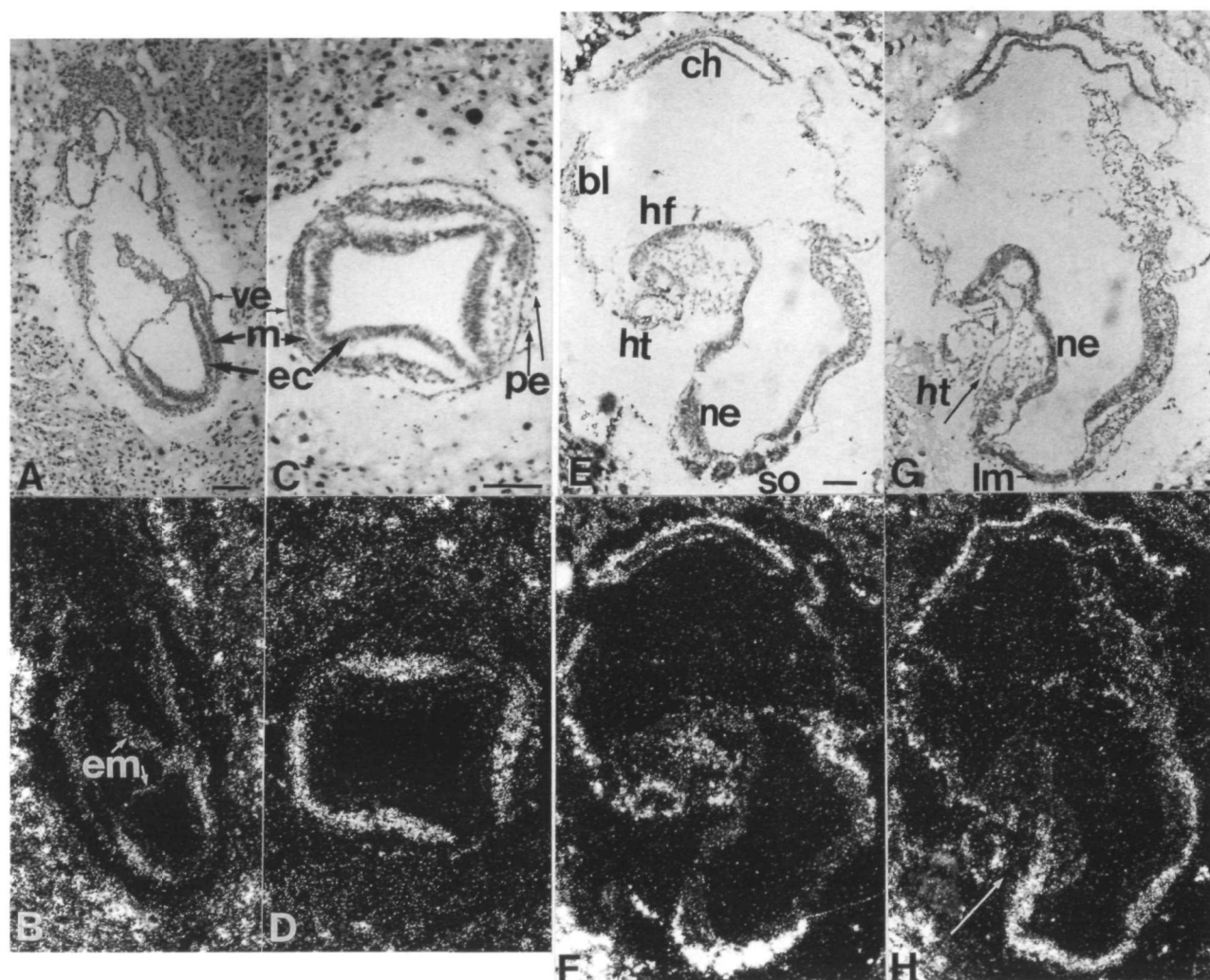


Fig. 2. *Pdgfra* expression at the late primitive streak - early somitic stage. Sections of 7.5- (A-D) and 8- (E-H) day-old embryos. (A,C,E and G) Bright-field, (B,D,F and H) dark-field illumination. (A) Sagittal section, (C) transverse section at the mid-embryo level, (E) sagittal section through the somites, (G) parasagittal section through the lateral plate mesoderm. Abbreviations: bl, blood island; ch, chorion; ec, primitive ectoderm; em, extraembryonic mesoderm (in the amnion and allantois), hf, head fold; ht, heart anlage; lm, lateral plate mesoderm; m, mesoderm; ne, neuroectoderm; pe, parietal endoderm; so, somites; ve, visceral endoderm. Bars: 100 μ m. Arrow in G points to the foregut.

splanchnic mesoderm. This signal was more prominent in the vicinity of the forelimb bud and in the anterior part of the lateral plate (Figs 4B,5B). *Pdgfra* transcripts were absent from the neuroectoderm. An exception at this stage was a weak hybridization signal in the dorsal region of the posterior neural tube (see small arrows in Figs 3B and 4B) and in the yet unclosed, caudal neural folds (Fig. 4C,D). This may suggest involvement of *Pdgfra* in early neural crest development and migration. Additional examples for this possibility are the *Pdgfra* transcripts observed along the ventral side of the neural tube (Figs 4B,H, 5B), that co-localize with the ventral pathway of neural crest migration (Rickmann, 1985). Sagittal and transverse sections of 10.5-day-old embryos (Fig. 4E-H) demonstrate the expression of *Pdgfra* in the mesenchyme of the face and branchial arches, a

destination for neural-crest-derived cells, which adds further support to the above suggestion.

In the early forelimb bud (9.5 days p.c.), *Pdgfra* transcription was unevenly localized (Fig. 5A,B). Concentration of the transcripts increased proximally, towards the body wall. Similar observations were made also in the limb bud of 10.5-day-old embryos. Later, at 11.5 days p.c. (Fig. 5C,D), *Pdgfra* expression significantly decreased at the site of mesenchymal condensation, which precedes chondrification in the center of the limb. Later the anlagen of the limb bone undergo chondrogenesis leading to osteogenesis. As can be seen in Figs 5G-I and 6E,F, *Pdgfra* is not expressed in these chondrification (carpals and digits) and ossification centers (humerus, ulna and radius). Whereas the two FGF receptors, *bek* and *flg*, occupy characteristic

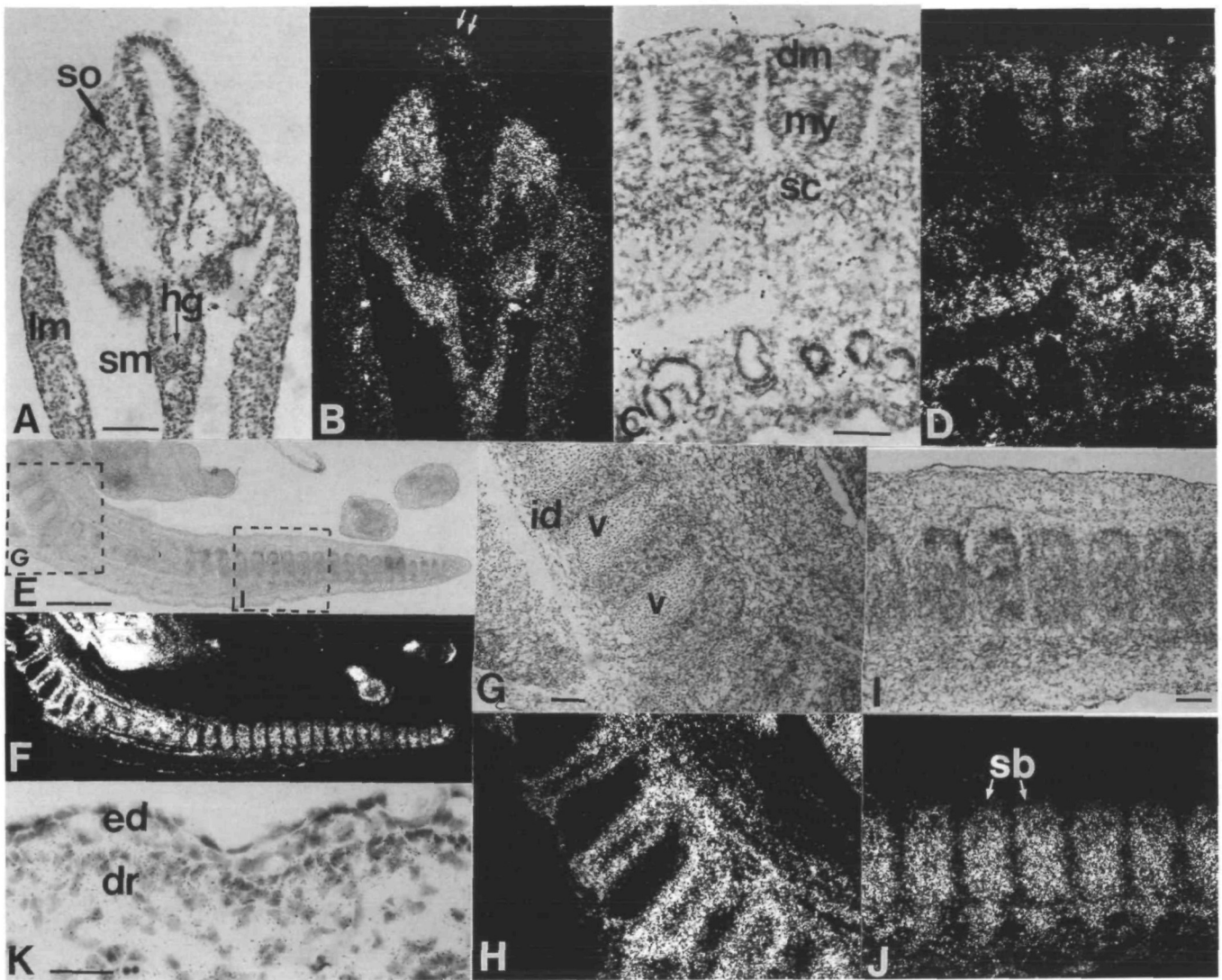


Fig. 3. Expression of *Pdgfra* during somite and vertebral column development. (A,C,E,G and I) Bright-field photographs, (B,D,F,H and J) dark-field photographs of the same sections, respectively. (A,B) Transverse section through a posterior somite of 9.5 day p.c. embryo. (C,D) Parasagittal section through a 10.5 day p.c. embryo. (E,F) Sagittal section through the tail of 14.5 day p.c. embryo. G and I are magnifications of the frames in E. (K) High-power magnification of the tail integument of 14.5 days p.c. embryo. Abbreviations: dm, dermatome; dr, dermis; ed, epidermis; hg, hindgut; id, intervertebral disc; lm, lateral plate mesoderm; my, myotome; sb, somitic blocks; sc, sclerotome; sm, splanchnic mesoderm; so, somite, v, vertebrae. Bars: A,C,G and I: 100 μ m; E: 500 μ m; K: 30 μ m

positions at the same sites during this stage of development (Orr-Urtreger et al., 1991). *Pdgfra* in contrast is detected in the mesenchymal tissue around the bone anlage and also in the joints between the prospective bones (Fig. 5H). Absence of *Pdgfra* expression in the centers of bone formation was also detected in the vertebral column and sternum (Figs 3H, 6D,G). This suggests that the PDGF system is less involved in bone development than the FGF system. It rather seems to contribute to the development of structures, which connect and surround prospective bones, like muscles and joints. *Pdgfra* transcripts were detected also in the apical ectodermal ridge (AER) of the forelimb (Fig. 5C-F) as early as 9.5 days p.c. and one day later in the hind limb (not shown), showing further involvement of *Pdgfra* in limb development.

Pdgfra expression during later stages of organogenesis
Sagittal and transverse sections of 12.5- (Fig. 6A,B) and 14.5- (Fig. 6C-F) day-old embryos demonstrate the expression of *Pdgfra* in the mesenchymal components of numerous organs. Transcripts can be detected in the facial mesenchyme with a clear border of expression in the epithelium of the nose and mouth (Fig. 6), and in the mesenchyme of the lung, bladder, kidney and genital ridge (Fig. 6B,D). Cross-sections of the bronchi and midgut loops (Fig. 7A-D) show transcripts accumulating between the epithelium and the outer layer, in the supporting mesenchyme of these tubular structures. This pattern is consistent with *Pdgfra* expression in the early precursors of these structures. Figs 3B and 4B show that the mesenchyme of the hindgut copiously transcribes this receptor. Another example of *Pdgfra*

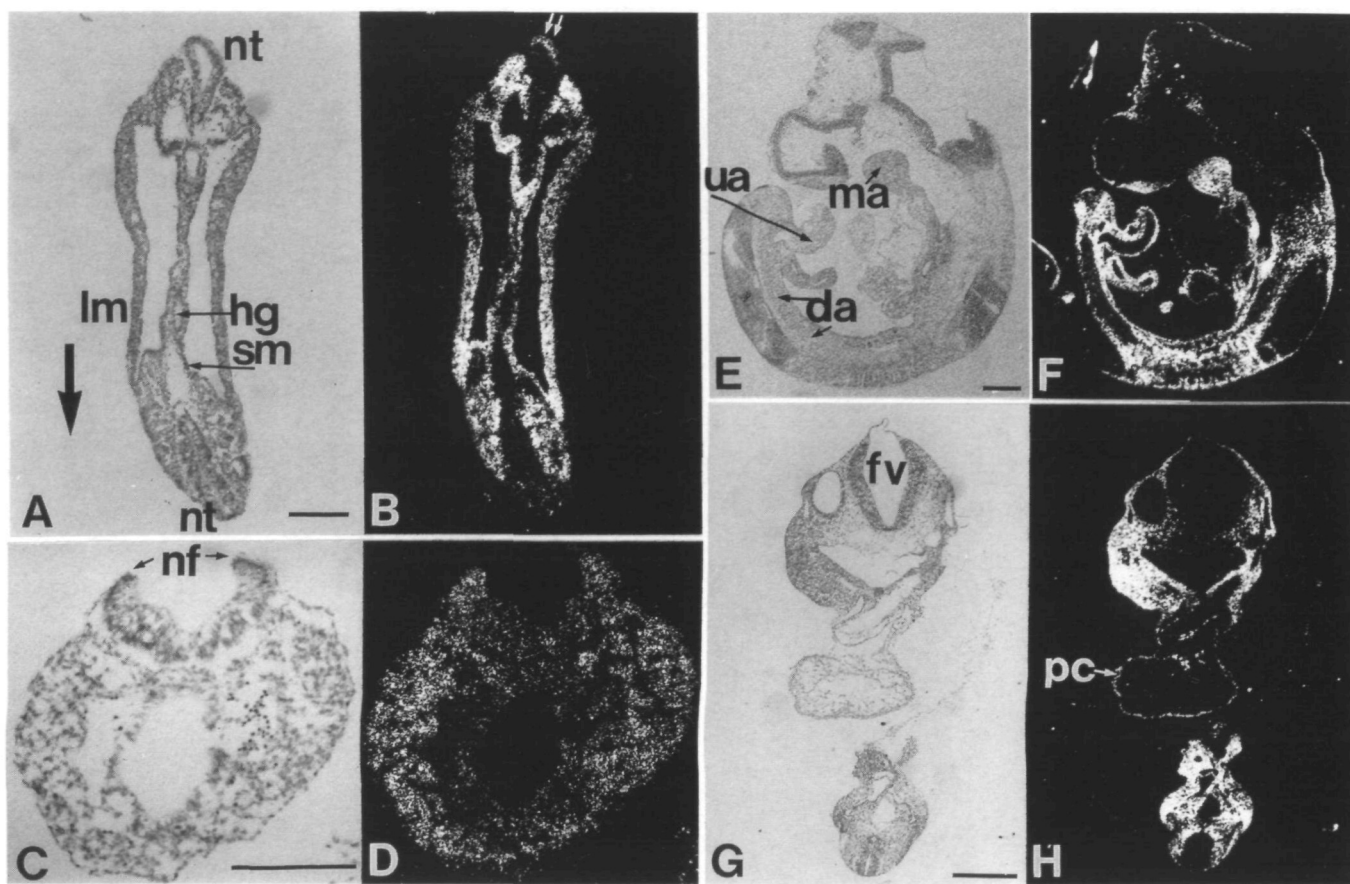


Fig. 4. Expression of *Pdgfra* during early organogenesis (A-C) Transverse sections through the body (A,B) and tail (C,D) at 9.5 days p.c. embryos. (E,F) Sagittal and (G,H) transverse sections of 10.5-day-old embryos. (A,C,E and G) Bright-field photographs, (B,D,F and H) dark-field photographs of the same sections. Abbreviations: da, dorsal aorta; fv, fourth ventricle of the brain; hg, hindgut; lm, lateral plate mesoderm; má, mandibular arch; nf, neural folds; nt, neural tube; pc, pericardium; sm, splanchnic mesoderm, ua, umbilical artery. Bars: A-D: 200 μ m; E-H: 500 μ m. Bold arrow in A points to anterior part of the embryo.

activity in mesenchymal components is its expression in the supporting capsules of the kidney, adrenal gland and thymus and in the central part of the diaphragm (Fig. 6B,D).

In the heart, *Pdgfra* expression was detected from the earliest stages of its development. At 8 days, *Pdgfra* hybridization was detectable in mesenchymal cells around, but not in, the heart tube epithelium (Fig. 2F,H). At later stages (10.5 days p.c.: Fig. 4F,H; 14.5 days p.c.: Figs 6D,F, 7F) transcripts were detected mostly in the pericardium. They were consistently detectable in all four heart valves of 12.5- and 14.5-day-old embryos (Figs 6E,F, 7E,F). This specific expression in the epithelial elements of the heart indicates that *Pdgfra* may have a role in cardiac valve development. Similarly it was expressed in the wall of the major blood vessels (dorsal aorta, umbilical and branchial arch arteries) of 9.5- and 10.5-day-old embryos (Fig. 4). The PDGF system was implicated in the pathogenesis of atherosclerosis (Ross et al., 1986). Experimental evidence demonstrates selective induction of PDGF-A synthesis during the regeneration of arterial injury (Rubin et al., 1988; Ferns et al., 1991). Transcription of

the PDGF α receptor in the inner wall of developing major arteries and in the heart may have bearing on these observations.

As has been mentioned earlier, very little or no *Pdgfra* expression was detectable in the neuroectoderm before 12.5 days p.c. An exception to this was the expression of *Pdgfra* in the neural fold. By 12.5 and 14.5 days, *Pdgfra* expression did become detectable in the central nervous system (CNS). Sporadic signals could be seen both in the vertebral column and in the fore-, mid- and hindbrain (Fig. 6). At higher magnification, the signals were found in the cell body surrounding some, but not all nuclei of the developing central nervous system (Fig. 6G-I). Strong hybridization was observed in the differentiating choroid plexus (Fig. 6H,I), which develops from the roof of the fourth ventricle and expressed *Pdgfra* already at day 10.5 p.c. (Fig. 4H).

Correlation of Pdgfra expression in wild-type and Ph mutant embryos

Homozygous *Ph* mutants were obtained from C57BL/6J-*Ph*/+ females crossed to C57BL/6J-*Ph*/+

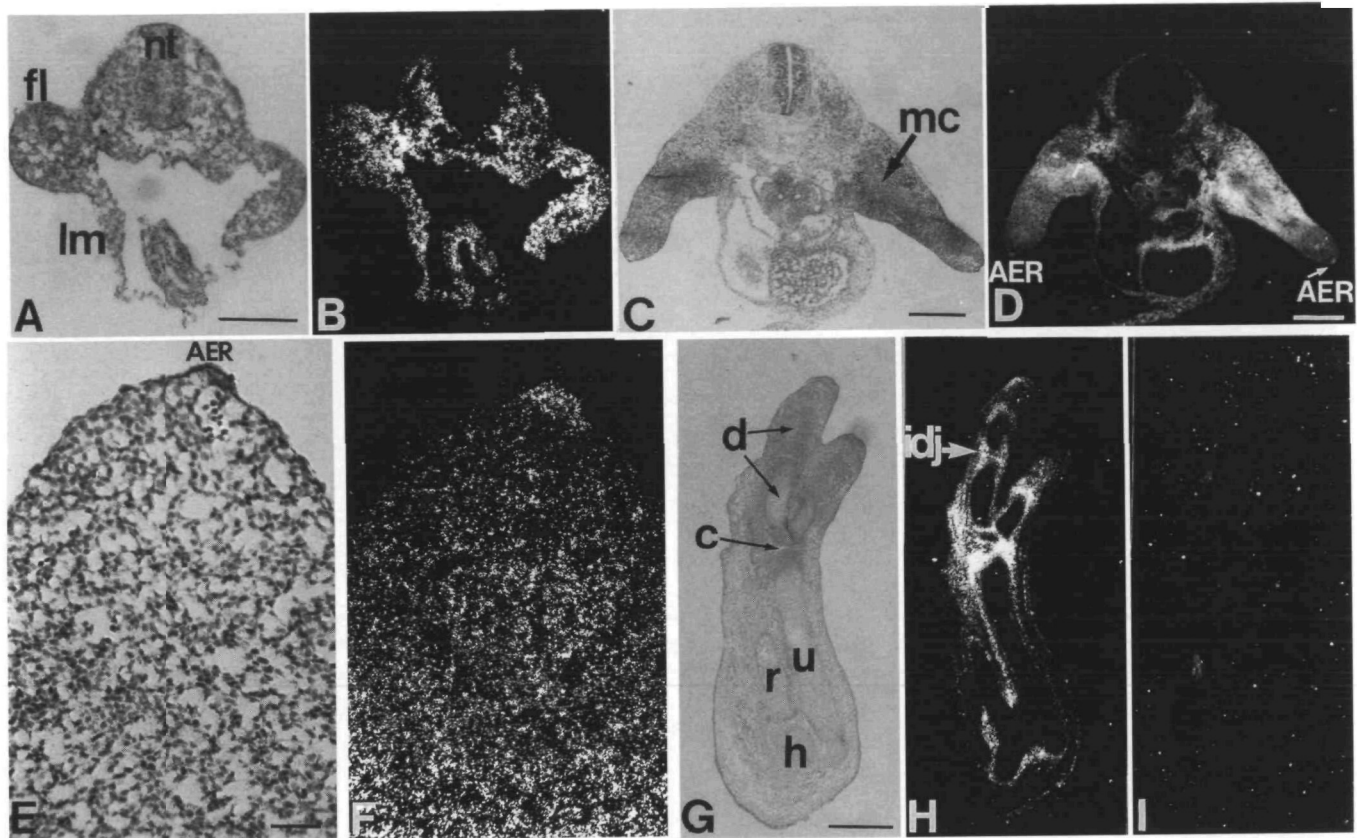


Fig. 5. *Pdgfra* expression during limb development. (A,B and C,D) Transverse sections through the forelimb of 9.5- and 11.5-day-old embryos. (E,F) High magnification of the tip of the forelimb (see arrow in D). (G-I) Sections through the longitudinal axis of 14.5-day-old forelimb. (A,C,E and G) Bright-field photograph; (B,D,F and H) dark-field photographs of the same sections. (I) Dark-field photograph of the section consecutive to H hybridized with the sense (control) probe. Abbreviations: AER, apical ectodermal ridge; c, carpal bones; d, digits; fl, forelimb; h, humerus; idj, interdigital joint; lm, lateral plate mesoderm; mc, mesenchymal condensation; nt, neural tube; r, radius; u, ulna. Bars: A: 200 μ m; C and G: 500 μ m; E: 50 μ m.

males. More than 1000 embryos were investigated between days 6.5 and 12.5 of gestation (Table 1). Most died by 11.5 days p.c. and hemorrhagic 'deciduomata' were found in their place (Table 1, lanes 1 and 2). Earlier, up to 10.5 days p.c., approximately 1/4 to 1/3 of the embryos were distinguishable by their small size, enlarged, fluid-filled pericardium, subepidermal blisters and thick and convoluted yolk sac (Fig. 10). Grüneberg and Truslove (1960) in their original report also described a later phenotype, which was distinguished by craniofacial and neural tube defects. Among 647 embryos investigated between days 11.5 and 12.5 p.c., only four survived over 11.5 days p.c. These four were the only ones that corresponded to Grüneberg and Truslove's (1960) description of the "cleft face" embryo (Table 1, line 2). The frequency of cleft face embryos in

our population was 0.6%, which was more than fivefold less than the normal frequency of fetal defects in the wild type at the same gestational age (see line 2 in Table 1).

Southern blot hybridization of DNA from 9.5- and 10.5-days-old homozygous *Ph* embryos showed the presence of a genomic deletion, which included most or all of the *Pdgfra* locus (Fig. 8). Four cDNA fragments were used as probes. Fig. 8B and C demonstrate hybridization with the 5' coding region probe, A, and with the 3' non-coding region probe C. It can be seen that both were deleted in *Ph/Ph* DNA. However, the same DNA digests gave normal RFLP with other gene-probes, like *c-kit* and *Hox-4.1*. Approximately 10 kb of genomic DNA upstream from the initiator AUG was also used for hybridization. Probes from this source did

Table 1. Frequency of homozygous *Ph* mutants

Homozygous mating	Litters	Age (days, p.c.)	Normal	Deciduoma	<i>Ph</i>	Cleft face	Total	% Mutant	% Cleft face
C57BL/6J- <i>Ph</i>	24	9.5 - 10.5	138	—	63	—	201	31.3	—
	76	1.5 - 12.5	459	180	4	4	647	29.1	0.6
C57BL/6J	17	11.5	150	6	—	—	156	3.8	—

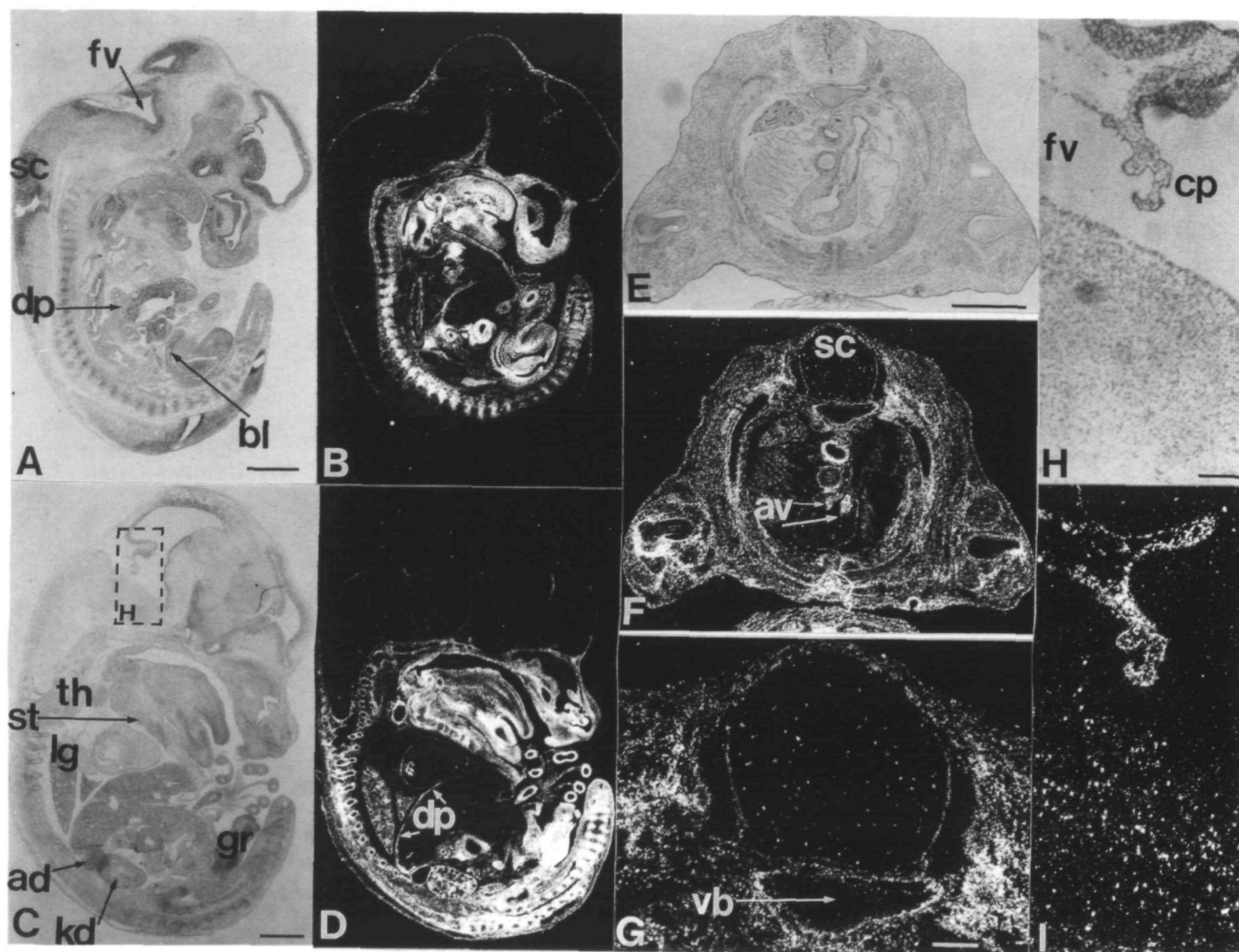


Fig. 6. Expression of *Pdgfra* during late organogenesis. (A,B) Midsagittal section of 12.5-days-old embryo; (C,D) sagittal section and (E,F) transverse section at the level of the heart of 14.5-day-old embryos. (G) Higher magnification of the spinal cord and vertebral body from section F. (H,I) Higher magnification of the frame in C. (A,C,E and H) Bright-field photographs, (B,D,F and I) dark-field photographs of the same sections, respectively. Abbreviations: ad, adrenal gland; av, aortic valves; bl, bladder; cp, choroid plexus; dp, diaphragm; fv, fourth ventricle of the brain; gr, genital ridge; kd, kidney; lg, lung; sc, spinal cord; st, sternum; th, thymus; vb, vertebral body. Bars: A-F: 1 mm; G-I: 200 μ m.

not reveal rearrangement in heterozygous *Ph*/+ DNA, indicating that the upstream deletion breakpoint may be farther from *Pdgfra* than 10 kb. Additional evidence for the deletion came from PCR assays of individual 9.5- to 10.5-day-old *Ph/Ph* embryos. Embryos distinguished by their small size, enlarged pericardium, wavy neural tube and subepidermal blebs consistently failed to contain *Pdgfra* sequences amplifiable with primers from the interkinase region, in contrast to their normal littermates (data not shown).

In agreement with a genomic deletion encompassing most of the *Pdgfra* locus, homozygous *Ph* mutants failed to transcribe this receptor. Figs 9H-J and 10F-H demonstrate examples of the *in situ* hybridization analysis of 7.75- and 10.5-day-old *Ph/Ph* embryos, respectively. It can be seen that these embryos displayed no hybridization with the *Pdgfra* probe. In contrast, the adjacent maternal decidua (Fig. 9I) transcribed *Pdgfra* and the *Ph* mutant embryo ex-

pressed the *bek* receptor (Fig. 9J; Orr-Urtreger et al., 1991). Taken together, our data lend support to those of Stephenson et al. (1991) and demonstrate that the early *Ph* embryo is associated with a null mutation of *Pdgfra*.

Experiments with homozygous mutant embryos sectioned in the decidua, also allowed us to distinguish zygotic and maternal *Pdgfra* transcription. Hybridization in the wild type (Fig. 9G) demonstrated strong, aggregated signals in the area of mural trophoblastic giant cells. Much less signal could however be seen in the same area of the homozygous mutant (Fig. 9I), suggesting that most of the mural giant cells are of embryonic origin. In addition the same figures show increased maternal *Pdgfra* transcription in the decidua distal to the mutant embryo, as compared to wild type. This could be a compensatory reaction connected to interactions between trophectoderm and maternal decidua (Goustin et al., 1985).

To analyze the *Ph* phenotype, serial sections were

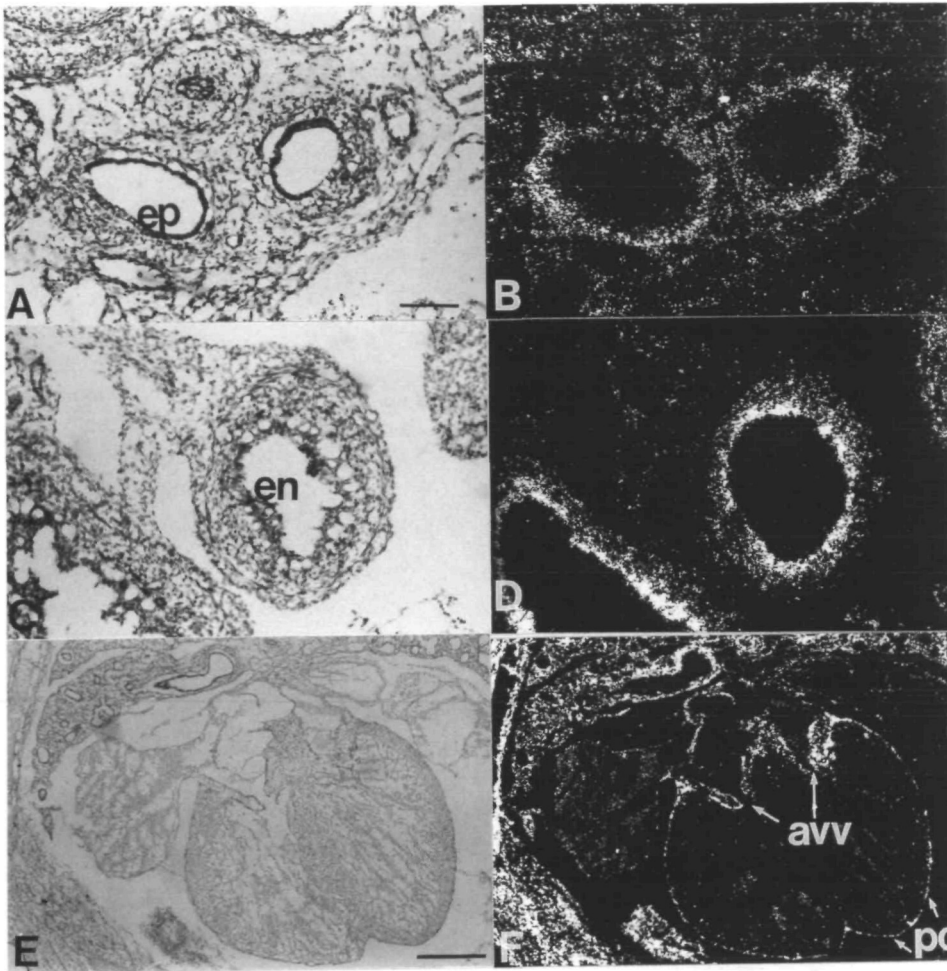


Fig. 7. Expression of *Pdgfra* in the bronchi, midgut and heart of 14.5 days p.c. embryos. (A,C and E) Bright-field photographs; (B,D and F) dark-field photographs of the same sections, respectively. (A,B) bronchi, (C,D) midgut, (E,F) heart. Abbreviations: avv, atrio-ventricular valves; en, endothelium of the gut; ep, epithelium of the bronchus; pc, pericardium. Bars: A-D: 100 µm; E,F: 400 µm.

prepared from 165 *Ph*/+ x *Ph*/+ embryos collected between 6.5 and 11.5 days p.c. Table 2 demonstrates that the mutant phenotype, distinguished by convoluted visceral endoderm, was first visible at 6.5 days, when its frequency was significantly less than 1/4. By 7.5 days p.c., a quarter of the embryos had this phenotype (see also Fig. 9B-D) and between days 8.5 and 10.5 several disorganized partially autolytic embryos were seen. This indicates that, after 7.5 days p.c., the *Ph* homozygote in our crosses start to lose viability and only a minority survives beyond 10.5 days p.c.

One of the major morphological characteristics of these embryos was their relatively hypertrophic and convoluted visceral endoderm. The exocoelom and the ectoplacental cavity were separated into numerous

compartments, and the amniotic cavity, surrounded by the primitive ectoderm and mesoderm, was compressed (Fig. 9B-D). A change in cell morphology could also be observed in the visceral endoderm. The columnar vesiculated cells of the extraembryonic visceral endoderm descended more towards the embryonic region of the egg-cylinder in the mutant embryo, where they in part replaced the flattened squamous cells of the wild-type embryonic visceral endoderm (Dziadek, 1978). This distinction could be also seen in transverse sections (Fig. 9E,F). They also revealed abnormalities in the mesoderm; in some *Ph* embryos the mesoderm was irregularly shaped, whereas in others, like in Fig. 9F, it failed to develop altogether.

Next 9.5- and 10.5-day-old homozygous *Ph* embryos were studied. Most striking was their very small size (Fig. 10A) and that they were found within hypertrophic, cauliflower-like yolk sacs (Fig. 10E). They also had a greatly extended fluid-filled pericardium, characteristic subepidermal blisters, retarded limb buds and branchial arches, open abdominal walls with enlarged umbilical cord and wavy neural tube (Fig. 10A-D). These mutant embryos, like the earlier phenotype failed to transcribe *Pdgfra* (Fig. 10G), whereas the unrelated *Hox-4.1* gene was transcribed normally (Fig. 10H). Histological sections revealed small, irregular somites in the homozygous mutant, which did not

Table 2. *Homozygous Ph mutants, summary of histological analysis*

Embryonic age (days p.c.)	Live mutants	Dead	Total	% mutant
6.5	4	—	38	10.5
7.5	7	—	30	23.3
8.5	2	4	20	30.0
10.5	2	6	29	27.5
11.5	—	13	48	27.1
			165	

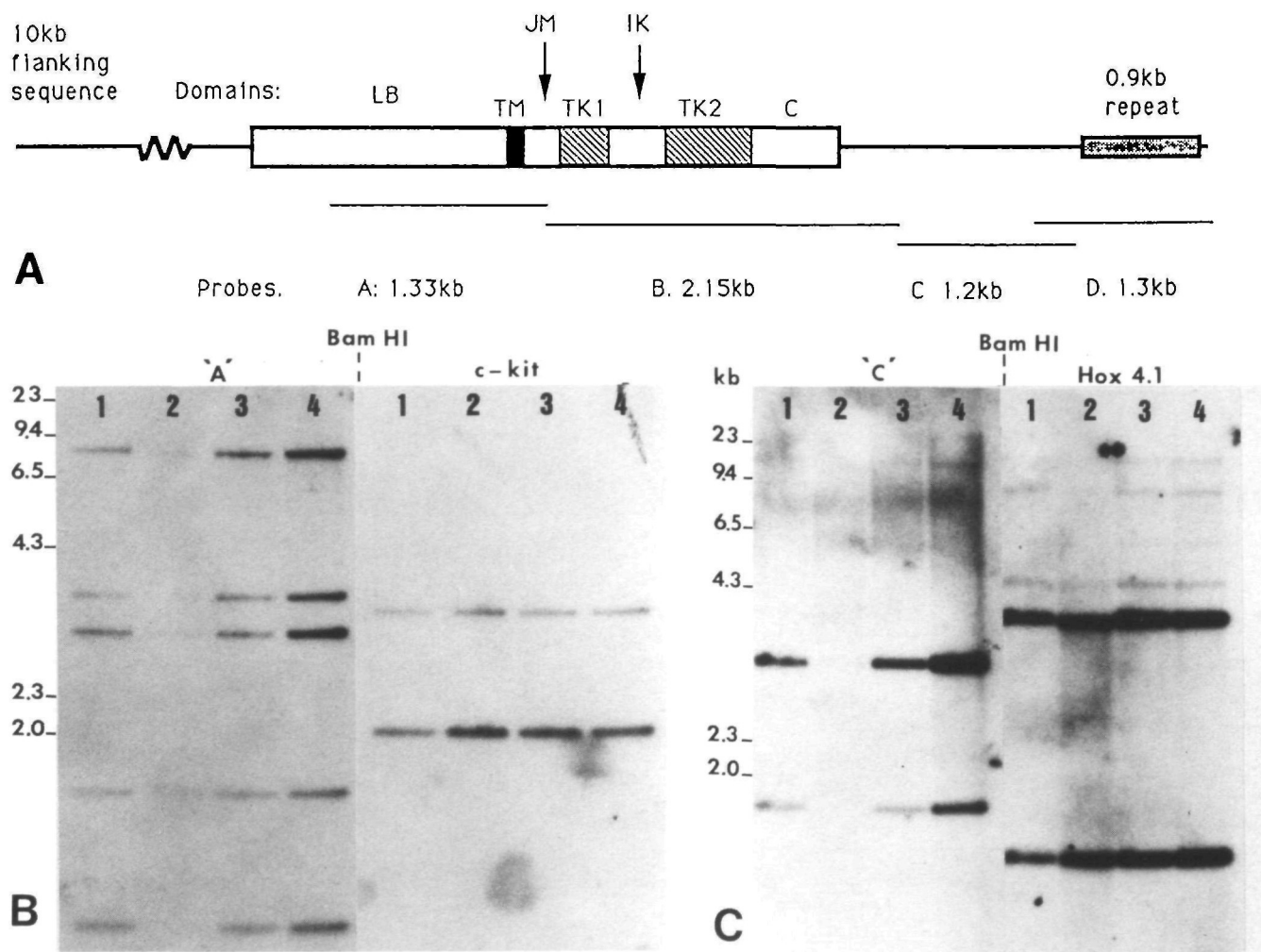


Fig. 8. The *Pdgfra* gene is deleted in homozygous *Patch* embryos. Genomic Southern blot hybridization. (A) Schematic representation of the murine *Pdgfra* cDNA. Abbreviations. LB, ligand binding domain; TM, transmembrane domain, JM, juxta-membrane domain; TK1 and TK2, tyrosine kinase domains; IK, interkinase domain. A,B,C and D, DNA fragments used as probes (B) Hybridization with the A probe of *Pdgfra* and with the *c-kit* probe (5' end of the coding region) to *Bam*HI-digested DNA isolated from: normal 10.5 days p.c. embryos of a *Ph*/+ \times *Ph*/+ cross (lane 1); from early (9.5 and 10.5 days p.c.) *Ph* mutants (lane 2), which were littermates of the above; from adult *Ph*/+ liver (lane 3); and from adult wild-type liver tissue (lane 4). (C) Hybridization of the *Pdgfra* C probe and the homeodomain probe, *Hox 4.1* with the same DNA samples.

differentiate into myotome, dermatome and sclerotome (Fig. 10D). The interstitial spaces contained few and comparatively sparsely spaced mesenchymal cells and the waviness of the neural tube extended to its entire length (Fig. 10C).

Discussion

The developmental localization of *Pdgfra* has general characteristics common to other receptor tyrosine kinases, like *c-kit* (Orr-Urtreger et al., 1990) and the two FGF receptors, *bek* and *flg* (Orr-Urtreger et al., 1991). All four are expressed in more than one major cell lineage soon after implantation and they stay active throughout development (and sometimes also during

adult life) in some, but not all, differentiated derivatives of the lineages where they were first detected.

The *Pdgfra* receptor is first expressed in derivatives of the primitive endoderm, and its transcripts are detectable in the parietal and visceral yolk sac also during later embryogenesis. *Pdgfra* is not expressed in the primitive ectoderm or in the definitive endoderm. However, it is copiously expressed in the nascent mesoderm, and later in the paraxial and lateral mesoderm, though apparently not in the notochord. During mesoderm differentiation this receptor is expressed in all cells of the early mesenchymal somitic block, but later only in the sclerotome and in the dermatome of the ensuing dermomyotome. With the formation of vertebrae, *Pdgfra* concentrates to the intervertebral disc and it is not detectable in the

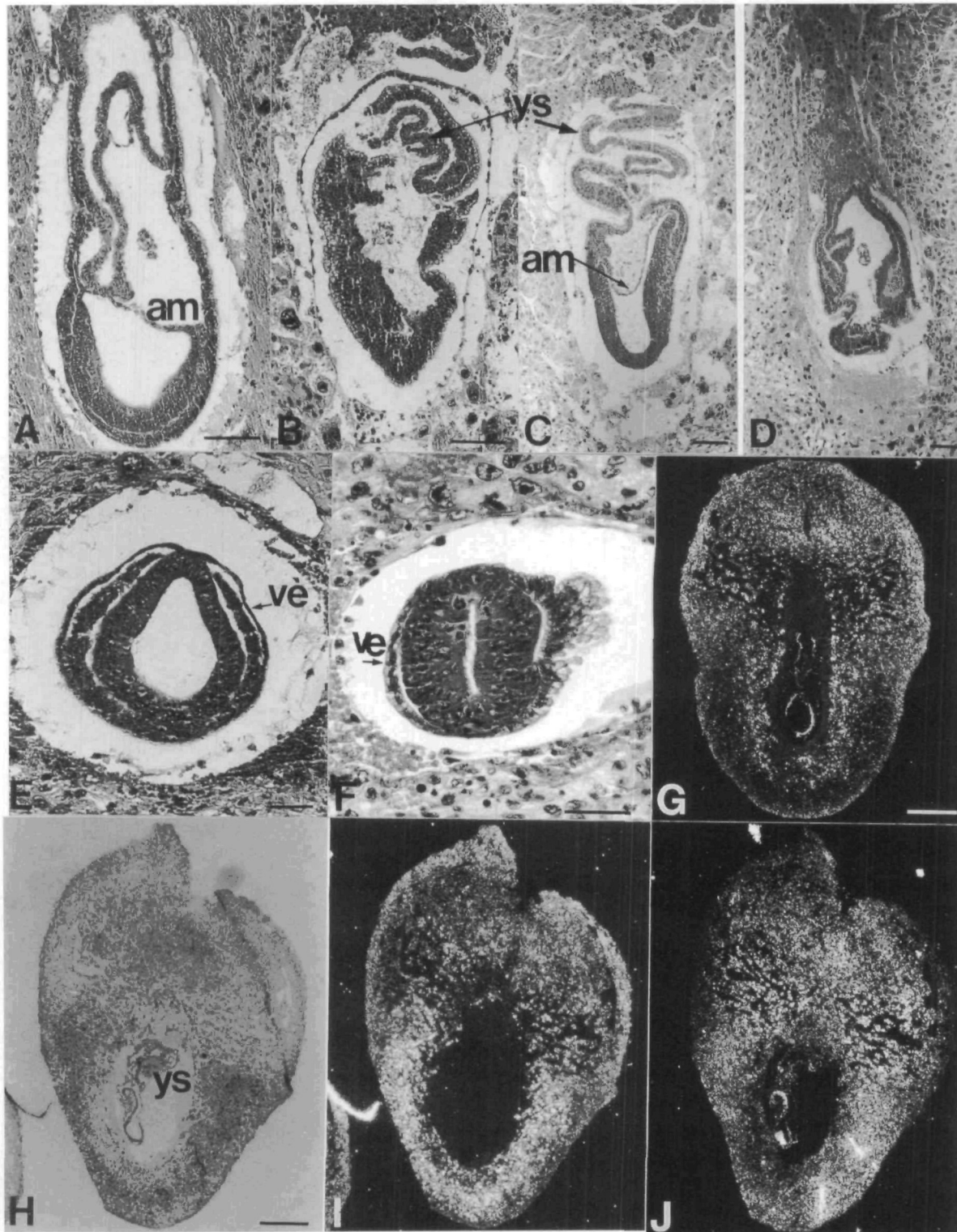


Fig. 9. The *Patch* (*Ph*) mutant phenotype at late gastrulation. (A) Phenotypically normal 7.5-day-old embryo derived from a *Ph/+* × *Ph/+* cross. (B,C and D) Phenotype of three different mutant embryos from the same cross. (E and F) Transverse sections (7 mm) at about the same level of normal (E, section N° 15 from the antimesometeric end of the embryo) and mutant (F, section 14) 7.5-days-old embryos. Note the difference between the visceral endoderm cell types in the two embryos. (G) Dark-field photograph of a normal 7.75-day-old embryo within the decidua, hybridized with the *Pdgfra* probe. (H and I) Bright-field and dark-field photographs of the same section of homozygous *Ph* mutant hybridized with the *Pdgfra* probe (hybridization signals are absent from the embryo and from the mural trophoblastic giant cells). (J) hybridization of a section adjacent to the one, shown in I with the *bek* probe. Note that the homozygous *Ph* mutant does express *bek* transcripts. Abbreviations: am, amnion; ve, visceral endoderm; ys, yolk sac. Bars: A-D: 100 μm; E and F: 50 μm; G-J: 500 μm.

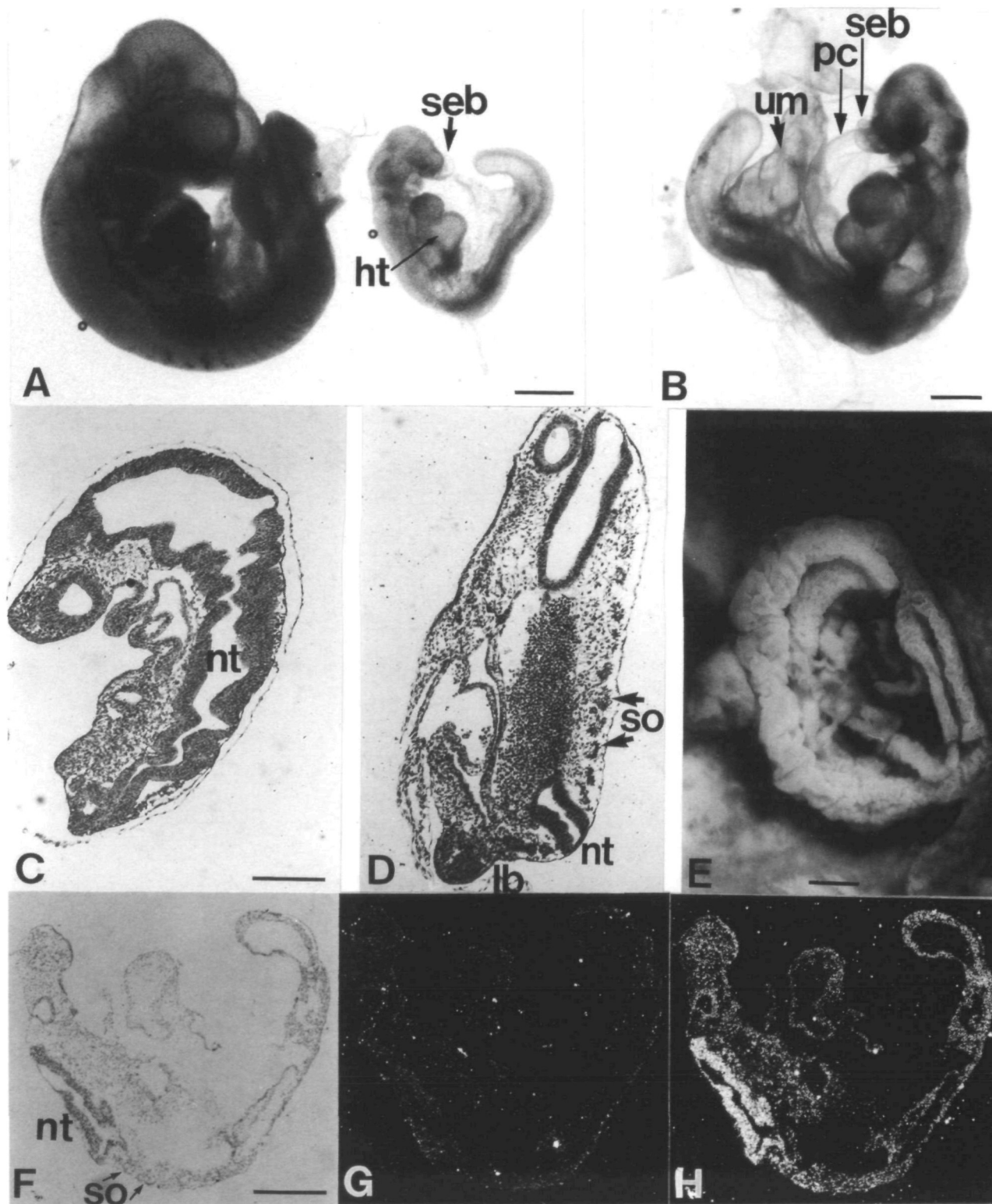


Fig. 10. The homozygous *Ph* mutant phenotype at 9.5 and 10.5 days of gestation. (A) Normal and mutant 10.5 days p.c. embryos from the same litter. (B) 10.5-day-old homozygous *Ph* embryo with fluid filled pericardium. (C) Sagittal section through a 9.5-day-old *Ph* embryo to demonstrate the abnormal neural tube. (D) Transverse section through 10.5-day-old mutant embryo, to demonstrate the abnormal somites. (E) Hypertrophic yolk sac of a 10.5-day-old mutant embryo. (F and G) Bright-field and dark-field photographs of the same section of the mutant embryo in A, which was hybridized with *Pdgfra*. (H) Hybridization of the consecutive section with a *Hox-4.1* probe. Abbreviations. ht, heart; lb, limb-bud; nt, neural tube; pc, pericardium; seb, subepidermal blister; so, somites, um, umbilicus. Bars: A: 1 mm, B, E and F-H: 500 μm; C and D: 200 μm

chondrification centers of the developing vertebral body. An additional characteristic is its localization in the parietal, and visceral endoderm of the primitive streak embryo. The functional importance of this localization gains support from the abnormal development of the yolk sac in homozygous mutants surviving into midgestation.

During the first half of gestation no *Pdgfra* expression could be detected in the central nervous system. A probable exception was its expression in the dorsal part of the neural tube and in the caudal neural folds, which may point to involvement with early neural crest development. This is consistent with *Pdgfra*'s later expression in the branchial arches and in facial structures of neural crest origin. Because *Pdgfra* was not expressed in the peripheral nervous system (dorsal ganglia), it is possible that during early stages of neural crest differentiation, this receptor could be preferentially expressed in non-neuronal derivatives of the neural crest, as was suggested before by Weston and his colleagues (Weston, 1991; Morrison-Graham et al. 1992).

We have observed sporadic *Pdgfra* expression in cells of the spinal cord and brain at the later stages investigated in this study (12.5, 14.5 days p.c.). A decisive part of mammalian CNS development does not start until midgestation and the definitive brain structure is built largely during the prenatal and early postnatal period. Our findings may indicate the involvement of *Pdgfra* in the beginning of this definitive period of CNS maturation. Recent reports describe the involvement of both PDGF isotypes (A and B) in multiple neuron and glial cells of the developing and mature CNS (Yeh et al., 1991; Sasahara et al., 1991). Additional studies are required for a comprehensive evaluation of PDGF and PDGF-R expression in the development of the brain and spinal cord.

The expression pattern of *Pdgfra* is distinctly different from that of the related FGF receptors. This could be seen already at the egg-cylinder stage. *bek* and *flg* were first expressed in the primitive ectoderm and they retained more or less extensive ectodermal and neuroectodermal expression throughout development (Orr-Urtreger et al., 1991). In contrast, *Pdgfra* was expressed in the visceral and parietal endoderm and later in the nascent mesoderm. Mesodermal expression of *bek* and *flg* was initiated only later, just prior to mesoderm segmentation. If in addition we take into account that FGFs are involved in mesoderm induction, whereas no such indications were found for PDGF (Whitman and Melton, 1989), we may conclude that the respective contribution of the FGF and PDGF systems to early development is essentially different. The expression pattern of *Pdgfra* and the two FGF receptors was also distinguishable during limb development and osteogenesis. *Pdgfra* was expressed in the mesenchyme and in the AER of the early limb bud, but not in the chondrification centers, where transcripts of the two FGF receptors could be found (Orr-Urtreger et al., 1991).

The developmental mechanism served by the PDGF

system is not known. A few clues however may have emerged from our data. Visceral endoderm differentiation was specifically altered in the early *Ph* mutant. Its extraembryonic cellular phenotype prevailed in areas where the embryonic phenotype is normally found (Fig. 9). Previous evidence suggests that the alternative extraembryonic or embryonic cell fate of the visceral endoderm depends on its interaction with the trophoctoderm and the primitive ectoderm, the cell layers with which it comes into contact (Dziadek, 1978). Another example for the effect of *Pdgfra* on cell fate may be its expression in the AER and its absence from the chondrification centers of the developing limb. This localization may indicate the contribution of *Pdgfra* in the AER's inhibitory effect on cartilage differentiation (Solursh and Reiter, 1988). Both examples suggest that *Pdgfra* may be involved in interactions by which one cell layer influences the differentiation of another (Saunders, 1972). Support for this interpretation was found when the localized expression of *Pdgfra* and its ligand, PDGF-A, were compared. We found that the two are localized in separate but adjacent cell layers during primitive streak stage development, axial skeleton development and organogenesis. Hence their localization suggested that this hormone-receptor pair may serve interactions across cell layers (unpublished data).

Our studies on the genomic structure of the *Ph* allele support and extend those of Stephenson et al. (1991). They studied interspecies crosses. Our results were obtained with DNA from homozygous *Ph* embryos. They suggest that most or all of the *Pdgfra* gene is deleted in this mutation. Using the full-length *Pdgfra* cDNA as probe, Stephenson et al. (1991) observed laboratory mouse specific RFLP-s in *Ph/Mus spretus* heterozygote DNA, which was interpreted to suggest that the deletion, or at least one breakpoint of it, is within the *Pdgfra* locus. We could not confirm this observation, because no upstream breakpoint was found within 10 kb 5' of the *Pdgfra* initiator methionine and we have not been able to use the full-length cDNA for RFLP analysis, because of a 900 bp long reiterated B1 type repeat in its 3' end. Hence at present the extent of the *Ph* deletion is not known, therefore, in a strict sense, we cannot rule out the possibility that the deletion may involve other genes besides *Pdgfra*.

The homozygous *Ph* phenotype was investigated here in considerable detail. The genotype of the embryos was ascertained by in situ hybridization and by PCR applied to individual embryos. Diagnostic for the early mutant phenotype was the abnormality of the visceral endoderm and mesoderm at the egg-cylinder stage. Embryos with abnormal visceral endoderm could be detected in our *Ph/+* × *Ph/+* material as early as 6.5 days p.c. and the full complement of the *Ph* phenotype became apparent by 7.5 days p.c.; however, by 10-11 days of gestation, most homozygous *Ph* embryos died. Those that survived suffered from defects in the yolk sac and had functionally connected defects in somite and neural tube development (Watterson et al., 1955; Van Straaten et al., 1989), all of which correlate with

Pdgfra expression during early development. Thus, there was a good correlation, both in time and space, between the deletion phenotype and the developmental expression of Pdgfra. Hence we conclude that the normal *Ph* allele is required for visceral endoderm and mesoderm differentiation.

One of our observations is at variance with Grüneberg and Truslove's (1960) original description of the *Ph* mutation. In our hands, the 'cleft face' phenotype was considerably less frequent. We think that this was due to differences in the genetic backgrounds used to maintain the *Ph* allele by the various research groups. We used the C57BL/6J-*Ph*/+ strain from the Jackson Laboratories (Bar Harbor, Maine). Although the Grüneberg and Truslove (1960) stock originated in C57BL, most of their original studies of the *Ph* mutation were performed on the CBA background. In another *Ph* stock, which was crossed to BALB/c mice over a long time and many generations, cleft face embryos with Pdgfra deletion were obtained regularly (Weston and Bowen-Pope, personal communication). It appears, therefore, that the *Ph* phenotype may be sensitive to the effect of unlinked alleles residing in various inbred backgrounds.

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References

- Beckmann, P. M., Betsholtz, C., Heldin, C.-E., Westermark, B., Di Marco, E., Di Fiore, P. P., Robbins, K. C. and Aaronson, S. A. (1988) Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. *Science* **241**, 1346-1349.
- Claesson-Welsh, L., Erikson, A., Westermark, B. and Heldin, C.-H. (1989). cDNA cloning and expression of the human A-type platelet-derived growth factor receptor establishes structural similarity to the B-type PDGF receptor. *Proc Natl Acad Sci U S A* **86**, 4917-4921.
- Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. D. (1984) Close similarity of epidermal growth factor receptor and *v-erb* B oncogene protein sequences. *Nature* **307**, 521-527.
- Dziadek, M. (1978) Modulation of alpha-fetoprotein synthesis in the early post-implantation mouse embryo. *J Embryol Exp Morph.* **46**, 135-146.
- Ferns, G. A., Raines, E. W., Sprugel, K. H., Motani, A. S., Reidy, M. A. and Ross, R. (1991) Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* **253**, 1129-1132.
- Gardner, R. L. (1983) Origin and differentiation of extraembryonic tissues in the mouse. *Int Rev Expt Path* **24**, 64-131.
- Goustin, A. S., Betsholtz, C., Pfeifer-Ohlson, S., Persson, H., Rydnert, J., Bywater, M., Holmgren, G., Heldin, C.-E., Westermark, B. and Ohlson, R. (1985) Coexpression of the *sis* and *myc* protooncogenes in developing human placenta suggests autocrine control of trophoblast growth. *Cell* **41**, 301-312.
- Grüneberg, H. and Truslove, G. M. (1960) Two closely linked genes of the mouse. *Genet Res* **1**, 69-90.
- Gudas, L. J., Singh, J. P. and Stiles, C. D. (1983). Secretion of growth regulatory molecules by teratocarcinoma stem cells. In *Teratocarcinoma Stem Cells*, Cold Spring Harbor Conferences of Cell Proliferation (eds. L. M. Silver, G. R. Martin and S. Strickland) Vol. 10, pp. 229-236, New York: Cold Spring Harbor Laboratory.
- Heldin, C.-E., Backstrom, G., Ostman, A., Hammacher, A., Ronnstrand, L., Rubin, K., Nister, M. and Westermark, B. (1988) Binding of different dimeric forms of PDGF to human fibroblasts. evidence for two separate receptor types. *EMBO J* **7**, 1387-1393.
- Heldin, C.-E. and Westermark, B. (1989) Platelet derived growth factor. three isoforms and two receptor types. *TIG* **5**, 108-111.
- Kaufman, M. H. (1990) Morphological stages of post-implantation embryonic development. In *Postimplantation Mammalian Embryos* (eds. A. J. Copp and D. L. Cockcroft) pp. 81-91, Oxford. IRL Press.
- Lonai, P., Arman, E., Czosnek, H., Ruddle, F. H. and Blatt, C. (1987) New murine homeoboxes, structure and chromosomal assignment. *DNA* **6**, 409-418.
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (eds) (1982). *Molecular Cloning, a Laboratory Manual*. New York: Cold Spring Harbor.
- Matsui, T., Helderan, M., Miki, T., Popescu, N., LaRochelle, W., Kraus, M., Pierce, J. and Aaronson, S. (1989) Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. *Science* **243**, 800-804.
- Mercola, M., Wang, C., Kelly, J., Brownlee, C., Jackson-Grusby, L., Stiles, C. and Bowen-Pope, D. (1990) Selective expression of PDGF A and its receptor during early mouse embryogenesis. *Dev Biol* **138**, 114-122.
- Morrison-Graham, K., Schattman, G. C., Bork, T., Bowen-Pope, D. F. and Weston, J. A. (1992) A PDGF receptor mutation in the mouse (*Patch*) perturbs the development of a non-neuronal subset of neural crest-derived cells. *Development* **115**, 133-142.
- Noble, M., Murray, K., Strooban, E. P., Waterfield, M. D. and Riddle, P. (1988) Platelet derived growth factor promoter division and motility and inhibits premature differentiation of the oligodendrocyte/tyre 2 astrocyte progenitor cell. *Nature* **333**, 560-562.
- Orr-Urtreger, A., Avivi, A., Zimmer, I., Givol, D., Yarden, Y. and Lonai, P. (1990) Developmental expression of *c-kit*, a proto-oncogene encoded by the *W* locus. *Development* **109**, 911-923.
- Orr-Urtreger, A., Givol, D., Yayon, A., Yarden, Y. and Lonai, P. (1991) Developmental expression of two murine fibroblast growth factor receptors, *flg* and *bek*. *Development* **113**, 1419-1434.
- Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F. and Noble, M. D. (1988) Platelet derived growth factors from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* **333**, 562-565.
- Rickman, M., Fawcett, J. W. and Keynes, R. J. (1985) The migration of neural crest cells and the growth of motor neurons through the rostral half of the chick somite. *J Embryol Exp Morph* **90**, 437-455.
- Ross, R., Raines, E. W. and Bowen-Pope, D. F. (1986) The biology of platelet derived growth factor. *Cell* **46**, 155-169.
- Rubin, K., Tingström, A., Hansson, G. K., Larsson, E., Ronnstrand, L., Klareskog, L., Claesson-Welsh, L., Heldin, G.-H., Fellström, B. and Terraclo, L. (1988) Induction of B-type receptors for platelet-derived growth factor in vascular inflammation: possible implications for development of vascular proliferative lesions. *Lancet* **i**, 1353-1355.
- Sasahara, M., Fries, J. W. U., Raines, E. W., Goner, A. M., Westrum, L. E., Frosch, M. P., Bonthron, D. T., Ross, R. and Collins, T. (1991) PDGF-B-chain in neurons of the central nervous system, posterior pituitary, are in a transgenic mode. *Cell* **64**, 217-227.
- Saunders, J. W., Jr (1972) Developmental control of three dimensional polarity in the avian limb. *Ann N Y Acad Sci* **193**, 29-42.
- Schattman, G. C., Morrison-Graham, K., van Koppen, A., Weston, J. A. and Bowen-Pope, D. F. (1992) Regulation and role of PDGF receptor α subunit expression during embryogenesis. *Development* **115**, 123-131.
- Selfert, R. A., Hart, C. A., Phillips, P. E., Forstrom, J. W., Ross, R., Murray, M. J. and Bowen-Pope, D. (1989) Two different subunits

- associate to make isoform-specific platelet derived growth factor receptors. *J Biol Chem* **264**, 8771-8778
- Smith, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development *Development* **105**, 667-677
- Solursh, M. and Reiter, R. S. (1988) Inhibition and stimulatory effects of limb ectoderm on in vitro chondrogenesis *J Exp Zool* **248**, 147-154
- Stephenson, D. A., Mercola, M., Anderson, E., Wang, C., Stiles, C. D., Bowen-Pope, D. F. and Chapman, V. M. (1991) Platelet derived growth factor α -subunit gene (*Pdgfra*) is deleted in the mouse *Patch (Ph)* mutation *Proc Natl Acad Sci U S A* **88**, 6-10
- Van Straaten, H. W. M., Hekking, J. W. M., Beurgens, J. P. W. M., Terwindt-Rouwenhorst, E. and Drukker, J. (1989). Effect of the notochord on proliferation and differentiation in the neural tube of the chick embryo. *Development* **107**, 793-803
- Watterson, R. L., Goodheart, C. R. and Lindberg, G. (1955) The influence of adjacent structures upon the shape of the neural tube and neural plate of chick embryos *Anat Record* **122**, 539-559
- Weston, J. A. (1991). Sequential segregation and fate of developmentally restricted intermediate cell populations in the neural crest lineage *Curr Topics Dev Biol* **25**, 133-153.
- Whitman, M. and Melton, D. A. (1989) Growth factors in early embryogenesis *Ann Rev Cell Biol* **5**, 93-117
- Yeh, H.-J., Ruit, K. G., Wang, Y.-X., Parks, W. E., Snider, W. D. and Deuel, T. F. (1991) PDGF-A chain gene is expressed by mammalian neurons during development and in maturity. *Cell* **64**, 209-216.

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