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A PDGF receptor mutation in the mouse (*Patch*) perturbs the development of a non-neuronal subset of neural crest-derived cells

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Summary

The *Patch* (*Ph*) mutation in mice is a deletion of the gene encoding the platelet-derived growth factor receptor alpha subunit (PDGFRα). *Patch* is a recessive lethal recognized in heterozygotes by its effect on the pattern of neural crest-derived pigment cells, and in homozygous mutant embryos by visible defects in craniofacial structures. Since both pigment cells and craniofacial structures are derived from the neural crest, we have examined the differentiation of other crest cell-derived structures in *Ph/Ph* mutants to assess which crest cell populations are adversely affected by this mutation. Defects were found in many structures populated by non-neuronal derivatives of cranial crest cells including the thymus, the outflow tract of the heart, cornea, and teeth. In contrast, crest-derived neurons in both the head and trunk appeared normal.

The expression pattern of PDGFRα mRNA was determined in normal embryos and was compared with the defects present in *Ph/Ph* embryos. PDGFRα mRNA was expressed at high levels in the non-neuronal derivatives of the cranial neural crest but was not detected in the crest cell neuronal derivatives. These results suggest that functional PDGFα is required for the normal development of many non-neuronal crest-derived structures but not for the development of crest-derived neuronal structures.

Abnormal development of the non-neuronal crest cells in *Ph/Ph* embryos was also correlated with an increase in the diameter of the proteoglycan-containing granules within the crest cell migratory spaces. This change in matrix structure was observed both before and after crest cells had entered these spaces. Taken together, these observations suggest that functional PDGFRα can affect crest development both directly, by acting as a cell growth and/or survival stimulus for populations of non-neurogenic crest cells, and indirectly, by affecting the structure of the matrix environment through which such cells move.

Key words: neural crest, PDGF receptor, PDGF, *Patch*, mouse embryo, mutant

Introduction

Neural crest cells of the vertebrate embryo are useful for analyzing the role of environmental factors on cell differentiation. Crest cells arise from the dorsal neural folds, migrate extensively to a variety of embryonic locations and ultimately produce a diversity of cell types, including pigment cells, glandular cells, connective tissue, cartilage and bone in the neck and face, and neurons and support cells of the peripheral nervous system. During their dispersal, neural crest cells encounter a variety of environmental factors (Weston et al., 1984; Newgreen and Erickson, 1986; Perris and Bronner-Fraser, 1989). Some of these environmental factors are known to influence the differentiation, survival, and motility of neural crest cells. For example, purified matrix macromolecules and complex matrices deposited by cells have been demonstrated to influence both crest cell migration and differentiation (for reviews, see Newgreen and Erickson, 1986; Perris and Bronner-Fraser, 1989). In addition, purified growth factors such as fibroblast growth factor and the neurotrophins have been demonstrated to affect the development of crest-derived cells (for review see, Weston, 1991).

One way to elucidate further the selective responses of crest-derived cells to different environmental cues is to analyze how these cells behave in embryos in which development has been mutationally perturbed. For example, examination of embryos homozygous for *Steel* and *Dominant Spotting* genes has helped to define one growth factor/receptor system required for the differentiation of crest-derived pigment cells (see Witte, 1990 and associated articles). Similarly, earlier work describ-

ing prominent defects in the head and neck structures of *Patch* (*Ph*) homozygotes (Grüneberg and Truslove, 1960) suggested that the development of cranial neural crest-derived cells is altered by the *Ph* mutation (Weston, 1980; Erickson and Weston, 1983; Morrison-Graham and Weston, 1989), and recent work has demonstrated that the platelet-derived growth factor receptor alpha subunit (PDGFRα) is deleted at the *Ph* locus (Stephenson et al., 1991). Clearly, a more thorough understanding of the defects present in *Ph/Ph* mutant embryos will help elucidate the role of PDGFRα during development.

In the accompanying paper, we correlate early defects in the mesodermally-derived mesenchyme of *Ph/Ph* embryos with sites of PDGFRα mRNA expression in normal embryos (Schatteman et al., 1992). In the present paper, we describe the defects observed in crest derivatives of *Ph/Ph* embryos and correlate expression of the PDGF receptor α-subunit mRNA within normal embryos with these defects. We demonstrate that non-neuronal cranial neural crest-derived cells express high levels of PDGFRα mRNA, and that many of the structures that rely on these cells are abnormal in *Ph/Ph* embryos in which the PDGFRα gene has been deleted. In contrast, the population of neuronal cells derived from the neural crest do not express PDGFRα mRNA and appear to develop normally within *Ph/Ph* embryos. These results suggest that PDGFRα is required for the normal development of many non-neuronal neural crest-derived cells and that the neuronal and non- neuronal subpopulations of crest cells express different growth factor receptors and have different growth factor requirements during development.

Materials and methods

Embryos

Homozygous *Ph/Ph* embryos were obtained by crossing *Patch* heterozygotes. The *Ph* mutation, carried on either a BALB/c or C57BL/6 background, produced a spotted coat color pattern in the heterozygotes. These mouse strains were originally obtained from the Jackson Laboratories (Bar Harbor, ME) and have been inbred by sibling matings for approximately 75 generations. E9 *Ph/Ph* embryos (E0 was the date of vaginal plug detection) were identified by the presence of subepidermal blisters and/or a distorted neural tube, while older *Ph/Ph* embryos ($\geq$ E12) were readily identified by a marked facial deformity. The presence of the *Ph* mutation in the older embryos was verified by Southern blot analysis (see, Schatteman et al., 1992). Control embryos were obtained by separate matings of inbred C57BL/6 mice. In some cases, when older mutant embryos were examined, normal looking littermates, which could be either heterozygotes or homozygous wild-type embryos, were used as controls.

Histology

Light microscopy

Pregnant animals were killed by cervical dislocation, and their embryos were placed into warmed buffered saline (Hanks' balanced salt solution, Gibco) and carefully dissected from the decidua to prevent damage of the fragile *Ph/Ph* embryos Embryos were fixed in 4% paraformaldehyde in PBS for 8 hours at room temperature or overnight at 4°C. Embryos for in situ hybridizations were fixed in 10% formalin, buffered at pH 7.0 with 0.1 M sodium phosphate buffer, for 1-7 days. Following fixation, embryos were dehydrated in a graded alcohol series, embedded in either Epon/Araldite or Paraplast and sectioned at 5 and 10 μm, respectively for histology and at 8 μm for in situ hybridization Epon/Araldite sections were stained with methylene blue-azure II/basic fuchsin (Humphrey and Pittman, 1974) and Paraplast sections were stained with 0.1% thionin buffered in acetic acid, pH 4.5.

For immunocytochemistry, embryos were first fixed for one hour at room temperature in PBS (pH 7.2) containing 4% paraformaldehyde followed by fixation overnight at 4°C in 0.1 M sodium borate buffer (pH 11) containing 4% paraformaldehyde (Berod et al., 1981). The embryos were washed, placed in 30% sucrose overnight and then frozen in OCT compound (Tissue Tek). Cryosections (14-16 μm) were air dried onto gelatin coated slides and stored at −20°C. To visualize neurons, sections were stained overnight at 4°C with either anti-neurofilament (mouse monoclonal RT97, Developmental Studies Hybridoma Bank) or anti-Hu-serum antibodies (Graus et al., 1986) using previously described procedures (Marusich and Weston, 1992) The anti-Hu antibodies are highly specific for central and peripheral neurons in adult (Budde-Steffen et al., 1988) and embryonic tissue (Marusich and Weston, 1992). Anti-neurofilament conditioned medium was used undiluted and the anti-Hu serum (sample no. 87/0054B, kindly provided by Dr. Jerome Posner) was used at 1:50 dilution. Antibody staining was visualized with rhodamine-conjugated anti-mouse or anti-human secondary antibodies (Jackson Immunologicals).

To examine bone and cartilage differentiation sequential staining with alcian blue 8GX (Sigma) and alizarin red S (Sigma) was used. Embryos (E16-17) were first anesthetized, eviscerated and placed in a solution of 80% ethanol and 20% acetic acid containing 0.015% alcian blue 8GX overnight, and then dehydrated in 100% ethanol for 4 days (changed daily). Following dehydration, embryos were placed in a solution of potassium hydroxide (0.5-0 7% depending on the size of the embryo) containing 0.01% alizarin red S until bone staining became visible (4-6 hours) Embryos were then cleared in a solution containing 1% potassium hydroxide and 20% glycerol. After the tissue became transparent (4-8 hours), embryos were transferred to 50% glycerol. All staining was done at room temperature. Embryos were stored in 100% glycerol.

Electron microscopy

E9 embryos were immersed in 2% glutaraldehyde, 2% formaldehyde, 3 mM $CaCl_2$, 1% CPC, and 0.1% ruthenium red (Polysciences, Inc.), buffered in 0.1 M sodium cacodylate at pH 7.0. After 3 hours specimens were washed in 0.1 M sodium cacodylate, and post-fixed for three hours in 0.1 M sodium cacodylate containing 2% OsO_4 and 0.1% ruthenium red. This fixation protocol has been shown to retain and precipitate proteoglycans into characteristic granules which can be identified using the electron microscope (Hay, 1978, Hascall, 1980). Ruthenium red was included in both primary and post-fixatives to increase the staining of the proteoglycans and glycosaminoglycans (Hayat, 1981) After washing in cacodylate buffer, the embryos were dehydrated in a series of graded ethanols and embedded in Epon/Araldite. Solutions were continuously stirred on a rotor at room temperature, and, in all cases, the fixatives were added to the ruthenium red immediately before use.

Some embryos were prepared by freeze substitution (Mjaatvedt et al., 1987). They were fixed briefly in formaldehyde, cryoprotected in 30% glycerol in 0.1 M sodium cacodylate, and rapidly frozen in liquid nitrogen cooled freon. Embryos were then subjected to freeze substitution for 1 week at −80°C in a solution of either 1% OsO_4 in absolute ethanol or 1% OsO_4 and 0.1% ruthenium red in absolute ethanol Samples were subsequently warmed and embedded in Epon/Araldite.

Sections for light microscopy were cut at 5 μm. Those sections chosen for thin sectioning were first photographed and then re-embedded by the method of Schabtach and Parkening (1974). Thin sections were stained for 2 hours at 45°C with 5% uranyl acetate and viewed using a Phillips CM-12 electron microscope.

Measurement of matrix

Regions of extracellular matrix were photographed at random in two embryonic locations, the truncus arteriosus of the developing heart and the ventral crest migratory pathway between the somite and neural tube. The photographs were coded, and the diameter of the ruthenium red-stained (proteoglycan-containing) granules within the extracellular matrix was measured in transects across each photograph (approximately 20 granules/photograph; final magnification, 30,000-51,000×). Approximately 10 photographs from each region of the embryos were measured.

Tissue culture

In vitro embryo culturing

E9-E9.5 embryos were carefully removed from the decidua leaving the visceral yolk sac intact. Embryos were transferred to 30 ml culture vials (sterile empties, MPL Solopac) containing 2 mls of prewarmed medium which had been equilibrated with a 5% CO_2/air (20% O_2) mixture (New et al., 1976). The medium contained 50% rat serum, 25% Hank's balanced salt solution and 25% oxypherol-ET, a fluorocarbon which serves as a synthetic oxygen carrier (Alpha Therapeutics; Sue O'Shea, personal communication). The rat serum was centrifuged immediately after withdrawal, heat inactivated (30 minutes, 56°C), aliquoted and frozen at −20°C until use (Steele and New, 1974; Sadler, 1979) After installing the embryos, the vials were re-equilibrated with the gas mixture, closed with a polyethylene stopper (Kimble), and rotated at 20 rev/minute in a 37°C incubator. Embryos were observed and re-equilibrated with the gas mixture at 12 hour intervals, and the culture medium was replaced after 24 hours.

In situ hybridization

The production of sense and antisense riboprobes and the in situ hybridization procedures used are detailed in the accompanying paper (Schatteman et al., 1992).

Results

Mutant embryos exhibit external defects in axial, neck and facial structures

Ph/Ph homozygotes were identified by visual inspection. Mutants were smaller compared with littermates (see Schatteman et al., 1992), and depending on embryonic age, they displayed various altered external features. External defects were first detected at E9-9.5 (14-25 somites) in >20% of the embryos. At this stage, embryos exhibited a distorted or wavy neural tube and/or one or more subepidermal blisters flanking the neural tube (see Erickson and Weston, 1983). The extent of the neural tube deformities and the number of blisters varied, but both of these features were most commonly observed between the otic vesicle and the first few somites. The somites were always present, but were often distorted or irregular in the region of the blisters.

Similar defects were only rarely observed in E10-11 embryos (see below), but were detected again in approximately 10% of the embryos older than E12. By this stage, the mutant embryos exhibited a characteristic facial cleft (see Grüneberg and Truslove, 1960; Weston, 1980). Although the maxillary process was of normal size, the frontonasal and mandibular processes were severely reduced in size and had failed to fuse at the midline. Most embryos also had a cleft palate, and consistently displayed a shortened neck and spina bifida beginning at the cervical level (see Fig. 4). In addition, large subepidermal blisters flanking the spinal cord, small blisters on one or more limbs, gastroschisis (gut not enclosed within the body wall), and abnormally shaped lenses, were observed with varying degrees of severity.

Early defects are transiently expressed in mutant homozygotes

As reported above, no obvious external defects are apparent at E10-11 in litters presumed to contain mutant homozygotes, and a smaller number of embryos, compared with E9-9.5, subsequently present visible defects after E12. These results can be explained in at least two ways. First, the subepidermal blisters characteristic of early embryos could be transiently expressed by *Ph/Ph* embryos. Alternatively, the defects observed in E9 embryos could result in immediate lethality of these embryos, while different defects could be expressed after E11 by a distinct portion of the surviving embryos.

To distinguish between these possibilities, we cultured E9.5 embryos that were either normal (from control matings) or *Ph/Ph* (identified by external phenotype) for 36-48 hours (see Methods). Four of the five cultured *Ph/Ph* embryos initially had blisters in the region of the first few somites. These blisters regressed and then disappeared during the first 24 hours in culture. At the end of the culture period, however, 3 of these embryos had developed a kink in the neural tube corresponding to the location of the blisters seen earlier. A fourth embryo developed a facial blister in the region where facial clefts normally appear. The fifth *Ph/Ph* embryo was extremely retarded developmentally at the beginning of the culture period. It remained retarded and still had blisters at the end of the culture period (36 hours). None of the seven control embryos developed neural tube kinks or other abnormalities. These results indicate that a least some of the early recognizable *Ph/Ph* homozygotes survive past E10, transiently cease to display subepidermal blisters, and subsequently develop defects characteristic of the older *Ph/Ph* embryos.

Many craniofacial defects in Patch *homozygotes reflect abnormal development of non-neuronal derivatives of the cranial neural crest*

The prominent visible defects observed in *Ph/Ph* embryos suggests that the cranial neural crest is altered by the *Patch* mutation. In avian embryos the cranial crest, in addition to supplying sensory, autonomic and enteric neurons, is involved in the development of many non-neuronal structures within the head and neck. These include the cornea, connective tissue elements, papillae of the teeth, septa in the outflow tract of the heart, and glandular tissue such as the thymus (LeDouarin, 1982; Kirby et al., 1983; Bockman and Kirby, 1984). To verify our inference that the *Patch* mutation affects cranial crest cell development, we compared the development of these structures in normal embryos and in embryos homozygous for the *Patch* mutation.

(A) Heart and thymus development

Sixteen mutant embryos (E15-18) were dissected and examined for heart and thymus defects. After gross examination, eight of the these embryos were examined histologically (see Methods). In control embryos (n=10) aorticopulmonary septation was completed by this developmental stage, and two separate vessels, the aortic and the pulmonary branches, were observed exiting the separate ventricles (Fig. 1A). In contrast, in 79% of the *Ph/Ph* embryos examined, aorticopulmonary septation failed to occur and only a single large vessel exited the heart (Fig. 1B).

Similarly, examination of some of the same mutant embryos (n=11) revealed that 91% of the *Ph/Ph* embryos also had thymus defects. The thymus defects ranged from a complete absence of the thymus (4/11), to the absence of one lobe (2/11), to a diminished size of one or both lobes (4/11) as compared with normal embryos. The three *Ph/Ph* embryos with apparently normal septation of the outflow vessel had exhibited abnormal (smaller or single lobed) thymus development. Histological sections of the thymus from *Ph/Ph* embryos revealed that although the glands had fewer and less well defined lobules, both thymocytes and epithelial cells were present (Fig. 2).

(B) Tooth development

Mesencephalic neural crest cells that contribute to the tooth-papillae participate in inductive interactions that lead to the formation of the enamel organ from the overlying dental epithelium (Kollar and Baird, 1970; Slavkin, 1974). In normal embryos (n=4) the enamel organ can be clearly distinguished by E16, whereas in two of the six *Ph/Ph* embryos, although the ingrowth of the dental epithelium occurred, the enamel organ did not form and the typical condensation of dental papillae was not observed (data not shown).

(C) Corneal development

Some of the mesencephalic crest cells surrounding the developing eye invade the matrix-containing space between the lens and corneal epithelium to form

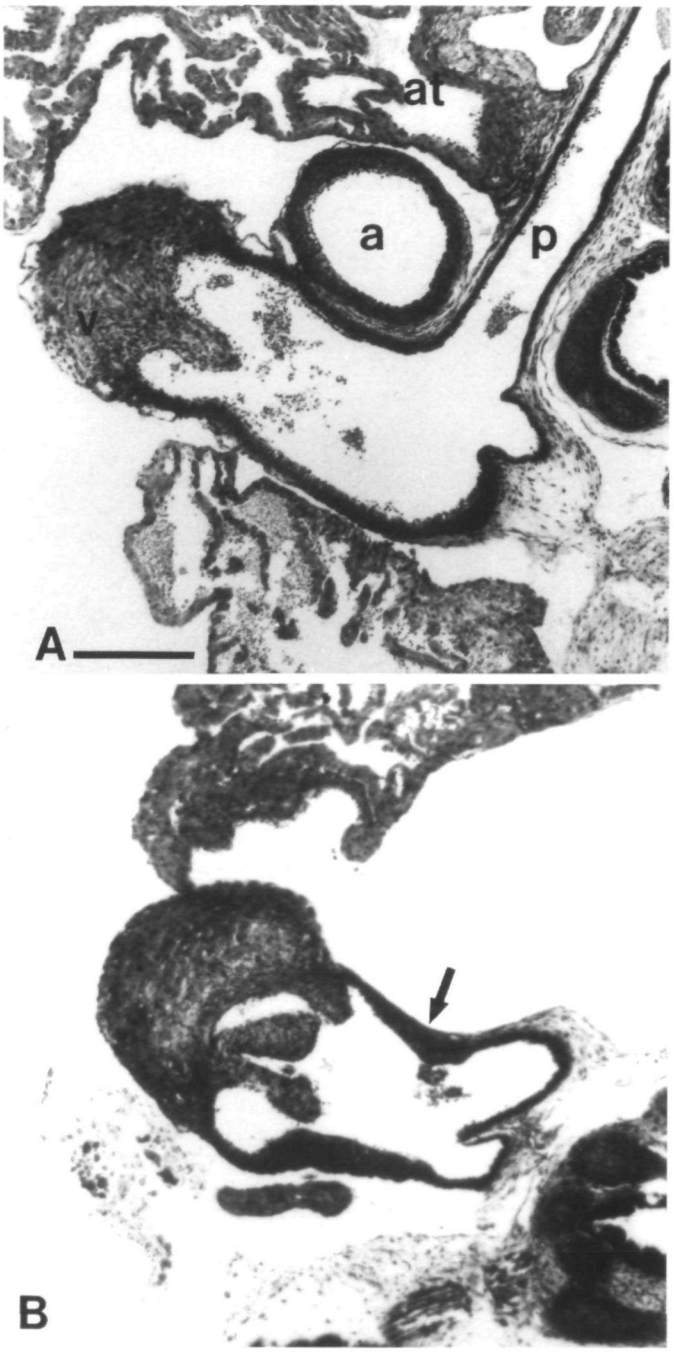


Fig. 1. Aorticopulmonary septation is abnormal in *Ph/Ph* embryos Light micrograph of the heart from an E17 normal embryo (A) demonstrates 2 clearly visible outflow vessels. The pulmonary vessel exits from the right ventricle, while a separate aortic vessel is present alongside. In contrast, a corresponding section from an E17 mutant embryo, reveals only a single outflow vessel (B, arrow). a, aortic vessel; p, pulmonary vessel; at, atrium; v, ventricle; Scale bar, 200 μm

corneal stroma (Hay, 1980). Whereas by E13 normal embryos had several layers of corneal fibroblasts, mutant embryos had at most 1-2 layers of corneal fibroblasts and a reduction in the amount of periorbital mesenchyme (Fig. 3). Similarly, the thickness of the

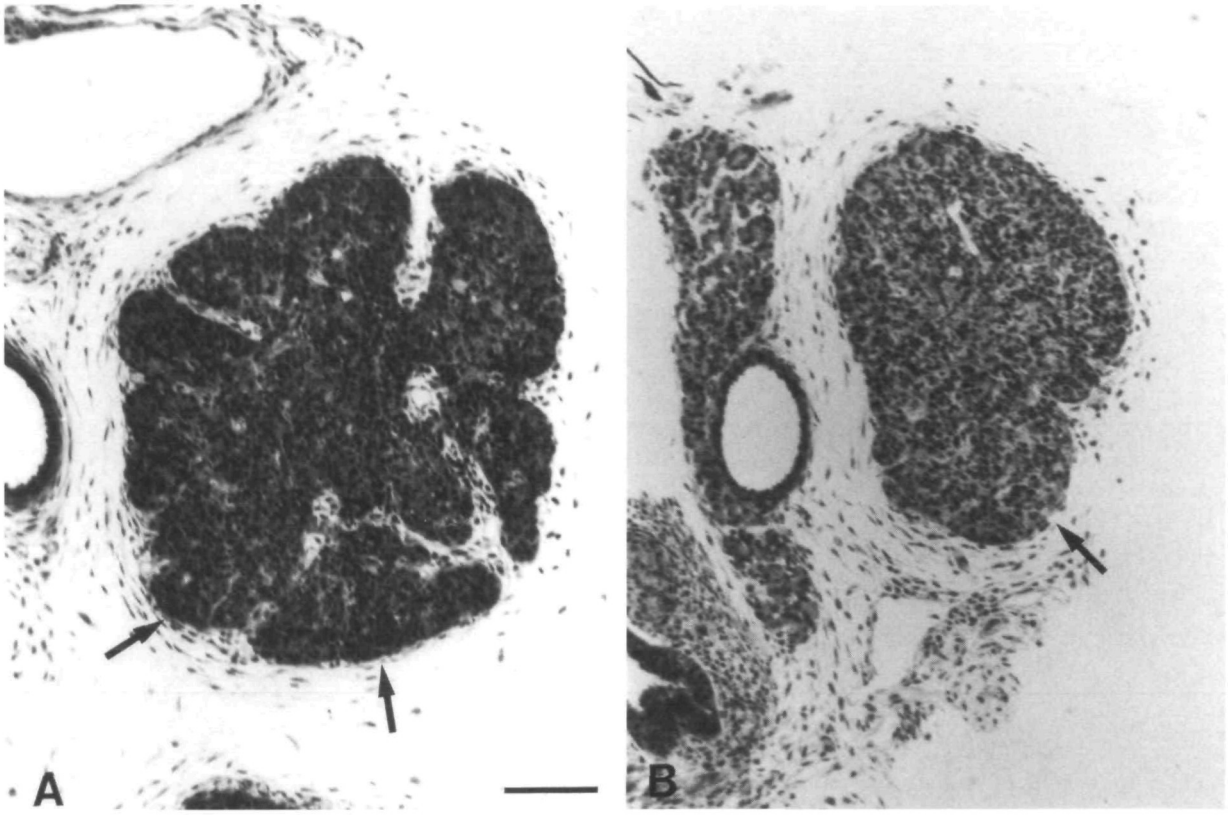


Fig. 2. Clear differences in thymus development are apparent in histological sections of normal (A) and *Ph/Ph* (B) E17 embryos. In transverse section, the thymus from *Ph/Ph* embryos is smaller in diameter and the lobes (arrows) are fewer in number and less well defined. Scale bar, 100 μm.

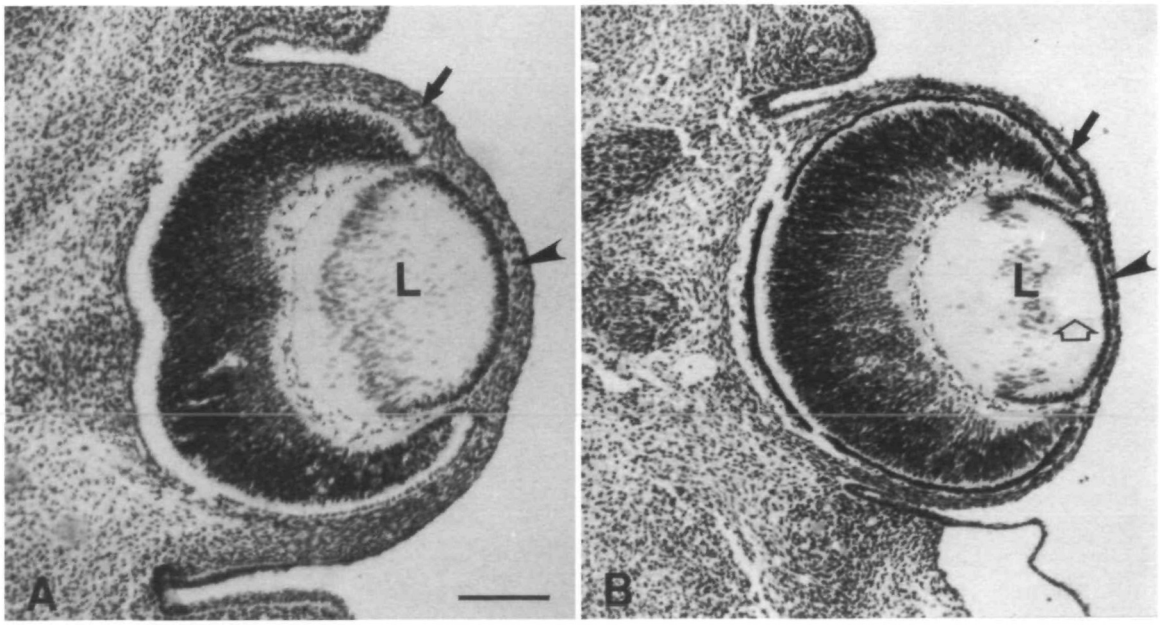


Fig. 3. Corneal fibroblasts are reduced in number in *Ph/Ph* embryos Thionin-stained sections through the developing cornea of E13 normal (A) and *Ph/Ph* (B) embryos show a significant decrease in both the number of neural crest-derived fibroblasts under the corneal epithelium (arrowhead) and neural crest-derived periocular mesenchyme (arrow). Note also the void in the lens matrix (open arrow) and the decreased number of fiber cells within the mutant lens (L). Scale bar, 100 μm

cornea in E16 *Ph/Ph* embryos was approximately half that of normal corneas.

(D) Bone development

Although most of the cranium originates from embryonic mesoderm, the bones of the face and jaw are derived from neural crest cells (Noden, 1982, 1984). Bone and cartilage development in mutant and normal embryos was compared at E16-17 using alcian blue and alizarin red to detect cartilage and bone respectively in whole-mount preparations (see Methods). Although bone ossification in the face and jaw appeared slightly delayed (by at most one day) in mutant embryos, the bones and cartilage in these regions appeared normal in shape and relative position. In contrast, the bones and cartilage of the cranium in mutant homozygotes were both smaller than normal and abnormal in position (Fig. 4A-B). Although the frontal bone which is derived from both mesoderm and neural crest (Noden, 1988) was present, it was found only in the region of the eye orbit and did not extend towards the front or the top of the skull. Also in most cases neither the parietal nor the interparietal bones met in the midline (Fig. 4A-B).

In contrast to the head, the stage of bone development in the trunk of mutant embryos was comparable to that of normal embryos, suggesting that the observed differences in formation of the cranium cannot be attributed to the embryos being developmentally delayed (Fig. 4C-D). However, the mutants were readily distinguished from normal embryos by the spina bifida caused by failure of the neural arch to form over the dorsal aspect of the spinal cord (see also Schatteman et al., 1992).

Neuronal derivatives of the neural crest are not affected in Patch *homozygotes*

To determine if neuronal derivatives of cranial neural crest cells are affected by the *Ph* mutation, crest-derived sensory, autonomic and enteric ganglia were compared in sections of mutant and normal embryos using thionin-staining as well as anti-neurofilament and anti-Hu immunofluorescence (see Methods). Both the size and the location of cranial sensory ganglia V, IX and X and the ciliary ganglion were grossly normal in the *Ph/Ph* embryos examined (Fig. 5). Similarly, the enteric ganglia were present in *Ph/Ph* embryos and were indistinguishable from the ganglia present in normal embryos (data not shown).

In the trunk of E12-E15 embryos, sensory and sympathetic ganglia were comparable in size and position. However, in some older embryos, even though the sensory ganglia appeared to have both neurons and support cells, the ganglia in the mutant embryos developed prominent intercellular spaces, whose etiology is presently unknown (data not shown).

The extracellular matrix within crest migration pathways is altered in Patch *homozygotes*

Erickson and Weston (1983) reported that the subepidermal blisters adjacent to the neural tube of E9.5 *Ph/Ph* embryos altered the distribution of extracellular matrix material and suggested that the migration of neural crest cells could be perturbed in these regions. To characterize the matrix differences in the regions of neural crest cell dispersal, we compared the appearance of the matrix in the interstitial spaces of normal and mutant E9-9.5 embryos. Transverse sections were examined by electron microscopy either in the region of dispersing vagal crest cells (at the level of the posterior hindbrain to somite 3) or in regions prior to crest cell dispersal (at the level of the last forming somite in the trunk).

Based on the presence of known crest cell derivatives at distal locations in the mutant embryos (see above), and the appearance of mesenchymal cells at the appropriate time in the interstitial spaces normally

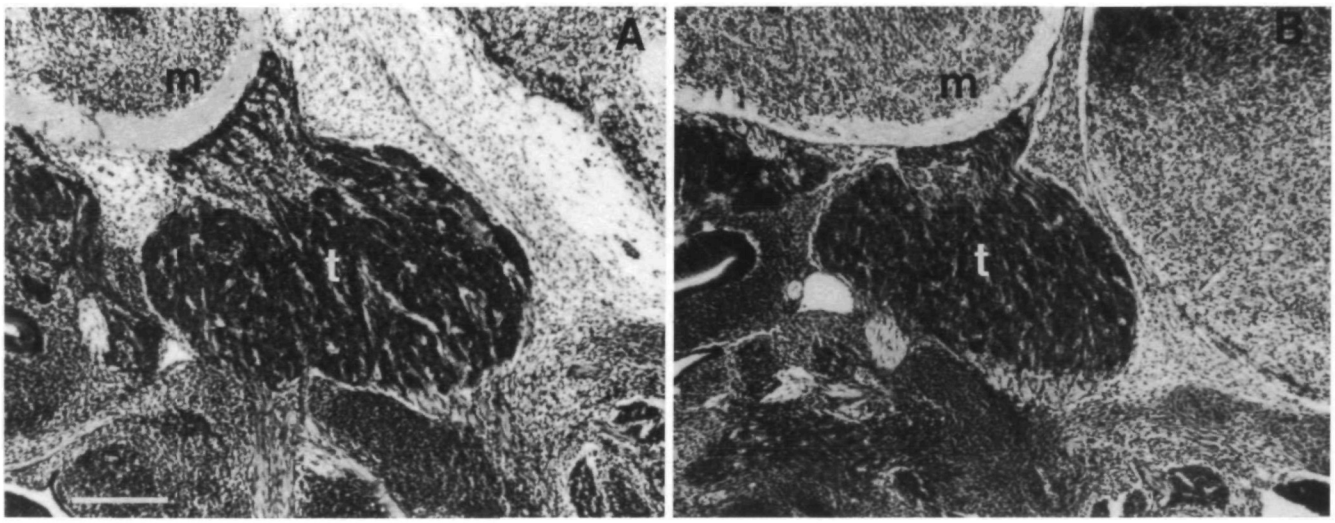


Fig. 5. The trigeminal ganglia present in *Ph/Ph* embryos are comparable to those observed in normal embryos. Transverse sections through the myelencephalon (m) of E14 normal (A) and *Ph/Ph* (B) embryos reveal trigeminal ganglia (t) of comparable size and location within the developing cranium. Scale bar, 200 μm.

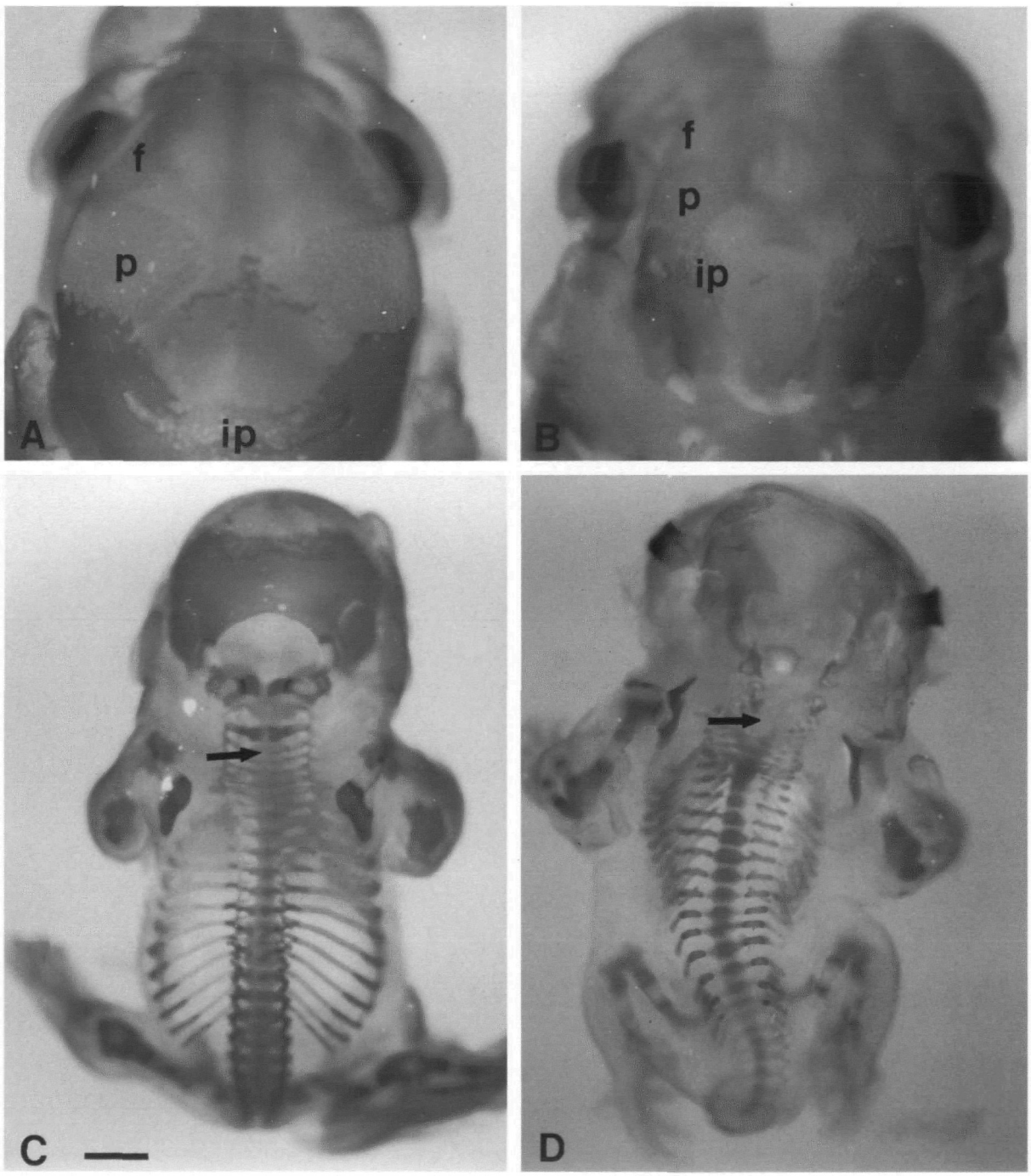
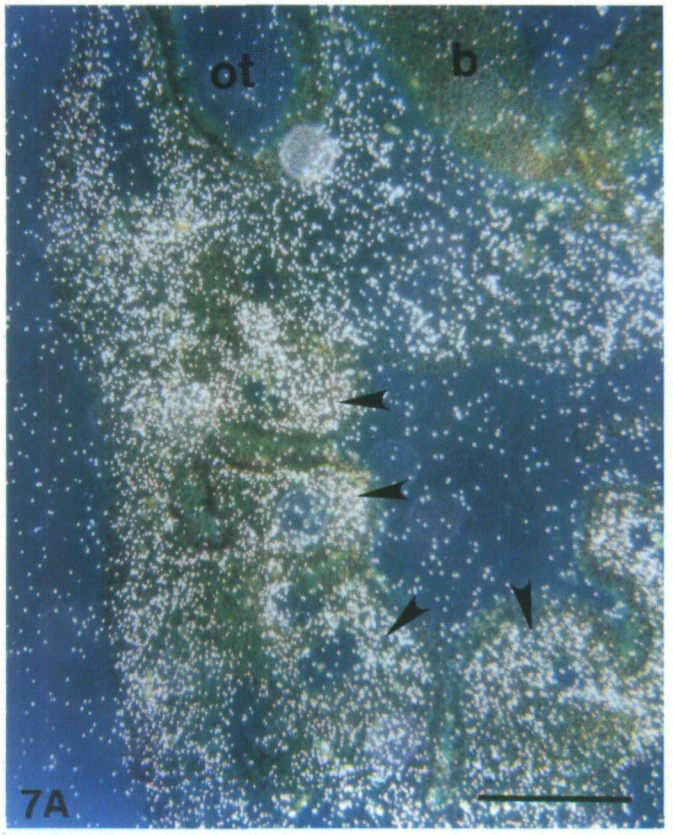
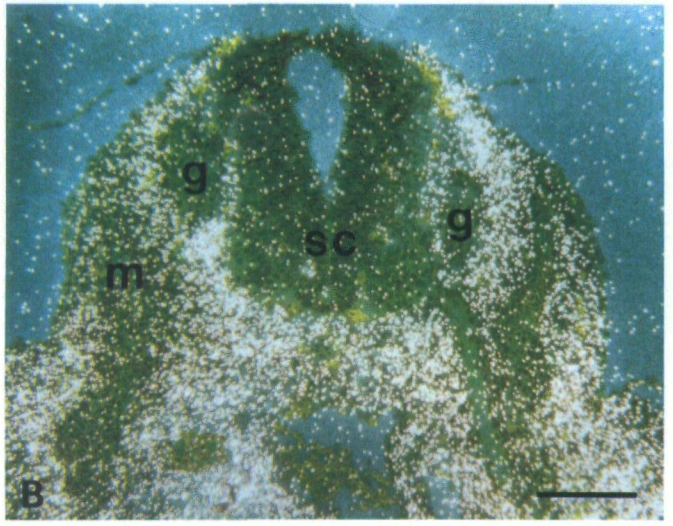
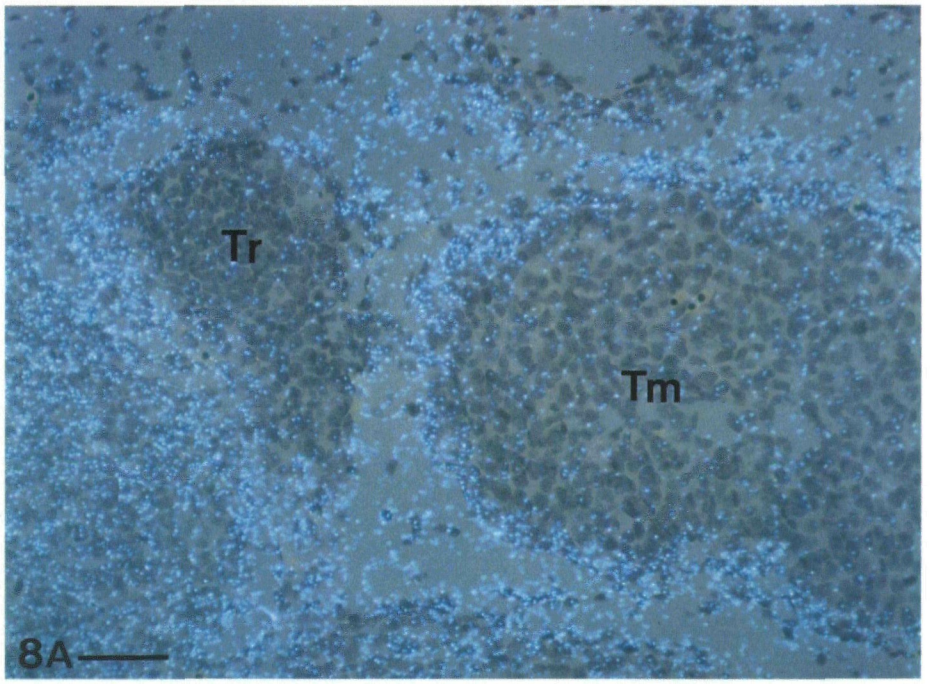


Fig. 4. Development of the cranium is perturbed in *Ph/Ph* embryos. Dorsal view of normal E16 (A,C) embryos and mutant E16 (*D*) and E17 (B) embryos stained with alcian blue and alizarin red to show cartilage and bone development, respectively. Ossification of the ribs is comparable between normal (C) and *Ph/Ph* (D) embryos. However, comparison of normal and *Ph/Ph* embryos shows the absence of the vertebral arch formation over the dorsal aspect of the spinal cord along its length (i.e. see arrows). The views of the cranium show that the interparietal (ip) bones are smaller in the mutant and do not fuse in the midline (A and B). The higher magnification of the cranium shows that the parietal (p) and that the frontal (f) bones are also smaller in the mutant. Also note that the frontal bone does not extend towards the top of the skull in the front. These differences are apparent even though the *Ph/Ph* embryo is older than the normal embryo. Note also the reduced amount of cartilage in the region. Scale bar A and B, 2 mm; C and D, 1.6 mm.







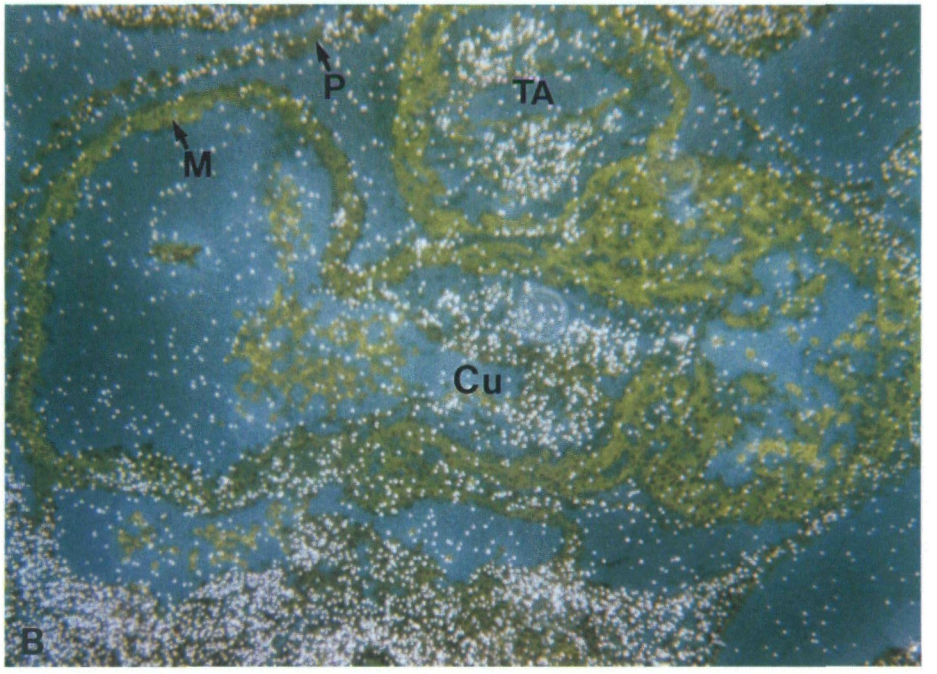


Fig. 7. PDGFRα transcripts are detected in non-neuronal crest cell derivatives but not in neuronal derivatives. Dark-field images of sections from E11.5 normal embryos processed with in situ hybridization using PDGFRα riboprobe. (A) The branchial arches (arrowheads) are intensely labeled while the epithelia of the pharyngeal pouches which separate the arches, the otic vesicle (ot) and the developing brain (b) are negative. (B) A section through the trunk shows that PDGFRα transcripts are also not expressed in the spinal cord (sc), sensory ganglia (g) or myotome (m). Scale bars, 100 μm.

Fig. 8. PDGFRα transcripts are expressed in regions populated by crest-derived mesenchyme. In situ hybridization of antisense PDGFRα probe to sections through the thymus and thyroid (A) and the heart (B) of an E11.5 normal embryo. In A the silver grains are visualized by reflected light and the cells by low levels of transmitted light. The mesenchyme of the thymus (Tm) and the thyroid (Tr) are intensely labeled while the epithelial components of the developing organs are negative. In B silver grains, which appear as bright spots, are visualized by dark-field microscopy and are detected in the cushion tissue (Cu) of the atrioventricular canal and the truncus arteriosus (TA) and within the pericardium (P), while expression is undetectable in most regions of the myocardium (M). Scale bar, A, 50 μm; B, 100 μm.

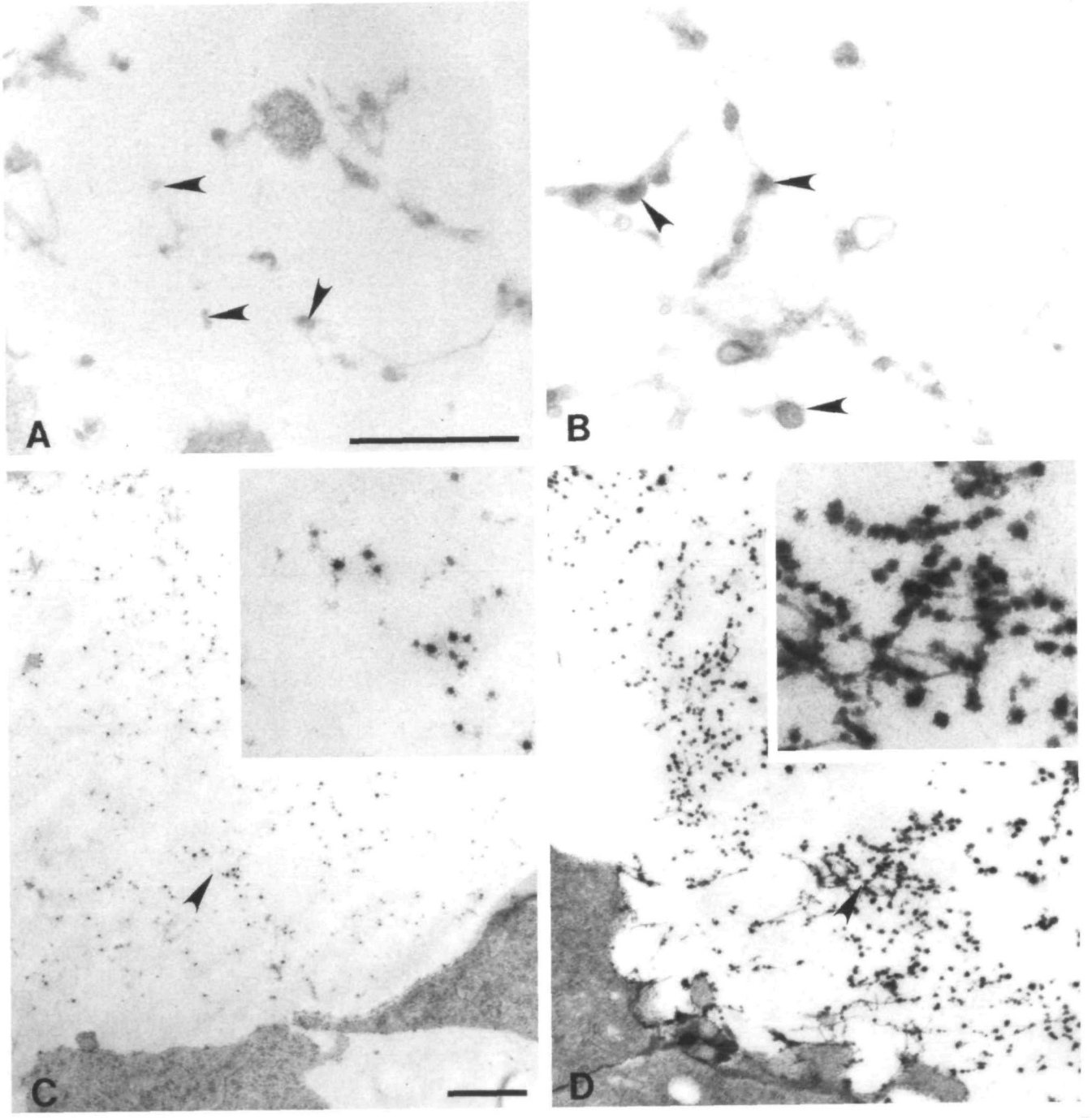


Fig. 6. The matrix granules observed in the interstitial spaces of E 9 5 *Ph/Ph* embryos (right panel) are larger than those present in E9.5 normal embryos (left panel) both prior to (A,B) and during (C,D) crest cell migration. The electron micrographs in A and B show that the granular matrix component (arrowheads) present in the cell free space bounded by the neural tube, somite and surface ectoderm, at the level of the last somite (prior to crest cell dispersal), of a 14 somite embryo is characteristically larger in *Ph/Ph* embryos. Similarly, the micrographs taken through the region of the developing truncus (C,D) at the time of crest cell dispersal reveal clear differences in the diameter of the matrix granules. Higher magnification inserts of regions denoted by the arrowheads in C and D demonstrate that in both normal and mutant embryos, the granules are often clustered on a fibrillar component of the matrix Scale bars, A,B, 1.0 μm; C,D 1 μm, inserts, 0.3 μm.

occupied by dispersing crest cells, it seems clear that crest cells do disperse in the mutants. However, consistent differences in the appearance of the proteoglycan granules were observed in normal and *Ph/Ph* embryos. These differences were apparent in crest cell pathways and perinotochordal interstitial spaces before (Fig. 6A-B), during (Fig. 6C-D), and after (Table 1) crest cells were present on these migration pathways.

Table 1. *Average diameter of the granular matrix component in* Ph *and normal E9 embryos*

	Truncus	Neural crest pathway
Patch	140.6±29.2 nm* (8)†	130.1±17 9 nm (10)
Normal	73 3±7.1 nm (6)	74 8±12.4 nm (7)

*Approximately 200 granules were measured from approximately 10 photographs taken in each region and an average size was determined for each embryo The average size/embryo was then averaged with the values obtained from the other embryos.

†Number of embryos

'Blindfold' measurements of the granules present in two separate regions (the ventral crest cell pathway following migration and within the truncus during migration; see Methods) revealed that the diameter of the glycosaminoglycan-containing matrix granules was consistently larger in mutant embryos (Table 1). Differences in granule size between normal and mutant matrix were also present in embryos prepared by rapid freezing followed by freeze substitution (see Methods; data not shown). Although the absolute size of the granules varied with the different fixation paradigms, the granule size was always greater in *Ph/Ph* embryos. These results indicate that the abnormal matrix is present prior to crest cell dispersal and could be a cause rather than a consequence of the abnormal development of crest-derived structures (see Discussion).

PDGFRα mRNA is expressed by non-neuronal crest derivatives that develop abnormally in Patch *homozygotes*

To understand the differential effect of the *Ph* mutation on the development of neural and non-neuronal crest cell populations, we examined the expression of the PDGF receptor α-subunit (PDGFRα) mRNA in normal embryos by in situ hybridization. We then compared the normal expression pattern of this gene in regions populated by neural crest cells with the sites of defects present in mutant embryos. The expression pattern within mesodermally-derived mesenchyme is reported in the accompanying paper (Schatteman et al., 1992).

In E9.5-11.5 embryos, abundant PDGFRα mRNA expression was detected in regions populated by cranial crest-derived mesenchyme. PDGFRα mRNA was expressed by the mesenchyme within the branchial arches of E9.5-11.5 embryos (Fig. 7A) and by the majority, if not all, of the cells within the migratory pathways to the branchial arches (data not shown; see also Schatteman et al., 1992). In addition both the mesenchyme surrounding the epithelial cells of the developing thymus, and thyroid glands (Fig. 8A), and the mesenchyme within the cushion tissue of the truncus, which includes cells involved in aorticopulmonary septation (Fig. 8B), were intensely labeled. The associated epithelial cells in each case, whether derived from mesoderm (endothelial cells) and or endoderm, did not express detectable PDGFRα mRNA. Similarly, PDGFRα mRNA was expressed within the facial mesenchyme of older embryos, including the crest-derived mesenchyme underlying the tooth epithelium and the crest-derived cells of the developing cornea (not shown). However, PDGFRα mRNA was not expressed within crest-derived cartilage of the jaw and face.

In contrast to the intense labeling of most of the non-neuronal derivatives of the neural crest, PDGFRα expression was not detectable in the neuronal derivatives of the crest during the developmental period examined in this study (<E16). Labeling was absent from the sensory, autonomic and enteric ganglia derived from both the cranial and trunk neural crest (Fig. 7B). In addition, labeling was not observed in the developing central nervous system prior to E13.

Discussion

Deletion of the gene for the PDGFRα perturbs the development of structures that normally express PDGFα mRNA

Cranial neural crest cells that first populate the branchial arches and the frontonasal process of avian embryos normally participate in the formation of the skeletal components and connective tissue of the face and neck (LeDouarin, 1982; Noden, 1984). Cranial crest-derived cells also contribute to the aorticopulmonary septum within the coronary outflow tract (Kirby et al., 1983), the corneal stroma (Hay, 1980), and the mesenchyme involved in inductive interactions with epithelia that lead to morphogenesis of the thymus (Bockman and Kirby, 1984), the dental rudiments (Kollar and Baird, 1970), and possibly the lung (Weston, 1984). Finally, the cranial crest contributes populations of cells that produce support cells of some cranial sensory ganglia, and both neurons and glia of other cranial sensory, autonomic and enteric ganglia (see, LeDouarin, 1982; D'Amico-Mattel and Noden, 1983).

The *Ph* mutation is a deletion in the gene encoding the alpha subunit of the PDGF receptor (Stephenson et al., 1991), and embryos we identified as mutant homozygotes can be shown by Southern blot analysis to lack this gene (see Schatteman et al., 1992). It is of particular interest, therefore, that the embryonic structures adversely affected by the mutation express abundant PDGFRα mRNA in normal embryos. As a consequence of the mutation, such cells presumably cannot respond to PDGF A-chain. It should be emphasized that the cells could still respond to the PDGF B-chain if they express the PDGFβ receptor subunit. Cells that do not express detectable levels of PDGFRα mRNA in normal embryos (i.e., neurons), and thus would normally not respond to PDGF A-chain, appear to be unaffected by the deletion. This suggests that the ability to respond to PDGF A-chain is required for the normal early development of many

non-neuronal crest derivatives but not for the neuronal derivatives. However, since the occurrence and severity of the tissue defects in *Ph/Ph* embryos varied, it is important to emphasize that other factors must also play a role in the normal early development of non-neuronal crest-derivatives. Moreover, although our results demonstrate a lack of dependence upon the PDGF A-chain during the early development of neuronal populations, dependence upon PDGF A-chain may arise later in the development of the nervous system. In normal embryos, expression of PDGFRα can be detected within the central nervous system after E13.5 (Schatteman et al., 1992) and within trunk sensory ganglia after E16 (Schatteman, unpublished observations). In addition, it has been reported that PDGF A-chain transcripts are present in neurons of both the central and peripheral nervous systems during later stages of development (Yeh et al., 1991), and that PDGF plays an important role in normal gliogenesis in the central nervous system (Noble et al., 1988; Richardson et al., 1988). For these reasons, it seems likely that PDGFRα plays a role in later development of the nervous system. However, since *Patch* homozygotes rarely survive much beyond E16, this inference cannot yet be verified in this system.

The absence of the PDGFα receptor could affect the development of the non-neuronal derivatives of the crest directly or indirectly

Our studies of PDGF receptor expression coupled with our analysis of the defects in *Ph/Ph* embryos suggest that the normal growth and differentiation of most non-neuronal mesenchymal derivatives of the cranial neural crest depend, at least in part, on PDGF A-chain. The role of PDGF A-chain in the regulation of development might be exerted in a number of ways. For example, the deficiencies in cranial crest development could arise because the ectomesenchymal crest cells require the direct action of PDGF for their survival or differentiation. The expression of PDGFRα mRNA in the majority, if not all, of the cells within the branchial arches and within the crest cell pathways to the arches supports this suggestion.

Alternatively, the absence of the PDGFRα in mutants could affect the development of the non-neuronal derivatives of the crest by causing changes in the composition or structure of the extracellular matrix through which these cells migrate. Thus, the deficiencies in development of the cephalic crest-derived structures could arise because abnormal matrix in the crest cell migration spaces causes either a delay in the migration of the crest cells or a reduction in the number of such cells.

Components of the extracellular matrix are known to affect both the migration (see, Newgreen and Erickson, 1986; Perris and Bronner-Fraser, 1989) and differentiation of specific subpopulations of crest-derived cells (i.e. see, Derby, 1982; Loring et al., 1982; Perris and Löfberg, 1986; Tucker and Erickson, 1986; Maxwell and Forbes, 1987; Perris et al., 1988; Morrison-Graham et al., 1990a; Rogers et al., 1990) of neural crest cells. As previously reported for the *Steel* mutant (Morrison-Graham et al., 1990b), the *Patch* mutation also results in a marked alteration of the structure of the extracellular matrix. In *Steel* embryos, there is an apparent change in the stability of interaction between hyaluronic acid and other matrix components (Morrison-Graham et al., 1990b), although how this difference relates to the changes in the availability of Steel factor is presently unknown (see Flanagan et al., 1991). In *Ph* homozygotes, the difference is manifest in the appearance of proteoglycan granules in the matrix spaces. At present no information is available concerning the molecular differences that might exist between mutant and normal matrices.

It should be emphasized, however, that both neural crest cells and mesenchymal cells are known to produce extracellular matrix material (Pintar, 1978; Weston et al., 1978; Hay, 1980). Since the altered matrix structure in *Ph/Ph* embryos is manifest whether or not crest cells are present within the interstitial spaces, it seems unlikely that the altered matrix structure is due to abnormal matrix production by the crest cells. It seems more likely that the observed matrix changes result from the loss of the PDGFRα within the somitic mesenchymal cells associated with the crest cell migratory spaces. In normal embryos these cells express abundant PDGFRα mRNA (Schatteman et al., 1992), and interaction of PDGF A-chain with its receptor may be required to elicit their contribution to the interstitial matrix in the adjacent crest migration spaces. Whatever the cause of the altered matrix, its effect must have to be selective, since the migration, localization and early differentiation of crest-derived neuronal populations appear unaffected by the mutation. Although *Patch* homozygotes cannot be identified early enough to examine the initial migration of cranial crest cells, preliminary evidence suggests that trunk crest cell migration may indeed be delayed in *Ph* embryos (Morrison-Graham and Bork, unpublished observations). If a similar delay were manifest in the cranial region, the altered timing of crest cell migration could affect the development of epithelial structures with which the crest-derived mesenchymal cells normally interact.

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