

# PDGF-A Is Required for Normal Murine

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Gina C. Schatteman,<sup>\*,1</sup> Carrie Loushin,<sup>\*</sup> Tong Li,<sup>\*</sup> and Charles E. Hart<sup>†</sup>

<sup>\*</sup>Department of Cardiovascular Research, St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135; and <sup>†</sup>Zymogenetics, Seattle, Washington

Several lines of evidence suggest that platelet-derived growth factor A chain (PDGF-A) is required for normal embryonic cardiovascular development. To test this directly, we introduced anti-PDGF-A neutralizing antibodies into mouse deciduas *in utero* at Embryonic Days (E) 8.5, 9.5, and 10.5. This resulted in the selective disruption of PDGF-A ligand–receptor interactions *in vivo* for a period of 18–24 hr and allowed us to assess both if PDGF-A is required for cardiovascular development and when it is required. Embryos collected 48 hr after antibody treatment displayed severe cardiovascular abnormalities. These included both atrial and ventricular myocardial hypertrophy, epicardial and endocardial abnormalities, and aortic dilation, among others. Although heart abnormalities were observed in embryos treated at all three ages, they were more common in embryos treated at E8.5. In contrast, only embryos treated at E10.5 exhibited significant aortic dilation. This work (1) demonstrates directly for the first time that PDGF-A is required for normal cardiovascular development, (2) identifies several processes that require PDGF-A, and (3) defines discreet developmental periods during which these PDGF-A-dependent processes require the factor. © 1996 Academic Press, Inc.

## INTRODUCTION

The platelet-derived growth factors (PDGF) are a group of three ligands composed of homo- and heterodimers of the PDGF-A chain (PDGF-A) and PDGF-B chain (PDGF-B) (Ross *et al.*, 1986). Both PDGF-A and B transcripts are detected in a variety of tissues during development (Sasahara *et al.*, 1991; Yeh *et al.*, 1991; Orr-Urtreger and Lonai, 1992). There are two known PDGF receptors,  $\alpha$ PDGF receptor ( $\alpha$ PDGFR) and  $\beta$ PDGF receptor ( $\beta$ PDGFR), both of which are expressed throughout most of postimplantation mouse development (Rappolee *et al.*, 1988; Mercola *et al.*, 1990; Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992; Schatteman *et al.*, 1992). These receptors dimerize to form both homo- and heterodimers which have different ligand binding phenotypes (Seifert *et al.*, 1989). PDGF-induced receptor dimerization appears to be required for signal transduction (Heldin *et al.*, 1989). Because PDGF-A chain interacts with  $\beta$ PDGFR only weakly, only six ligand receptor combinations result in high-affinity binding. High-affinity binding appears to be

necessary for signal transduction *in vivo*, although a low-affinity interaction of PDGF- $\beta\beta$  receptors with high concentrations of PDGF-AB may also result in PDGF signaling (Seifert *et al.*, 1989, 1993).

Homozygous deletion of either the PDGF-B chain (Leveen *et al.*, 1994) or  $\beta$ PDGFR (Soriano, 1994) gene results in prenatal or perinatal death. Similarly, mice homozygous for the naturally occurring *Patch* mutation, which includes a deletion of the  $\alpha$ PDGFR gene, die prenatally (Gruneberg and Truslove, 1960; Stephenson *et al.*, 1991). One-quarter of homozygous PDGF-B knockout mice die before birth, while the remaining three-quarters die immediately after birth. Both embryos and newborns are hemorrhagic and edematous (Leveen *et al.*, 1994). Embryonic Day 17.5 (E17.5) and older embryos and newborns have enlarged and deformed hearts and dilated thoracic arteries (Leveen *et al.*, 1994). Homozygous *Patch* (*Ph*) mutant mice exhibit even more severe cardiovascular abnormalities which include incomplete aorticopulmonary and heart septation, valve abnormalities, thinned and distorted myocardium, a reduced number of vascular smooth muscle cells, and aberrant patterning of large vessels (Morrison-Graham *et al.*, 1992; Schatteman *et al.*, 1995). Interestingly, while deletion of the  $\beta$ PDGFR gene results in hematological and other developmental abnormalities, the lack of  $\beta$ PDGFR does not result in severe cardiovascular abnormalities (Soriano, 1994).

<sup>1</sup>To whom correspondence should be addressed at Center for Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center, 15355 Lambda Drive, San Antonio, TX 78245-3207. Fax: (210) 567-7277.

These findings collectively suggest that PDGF-A acting through  $\alpha$ PDGFR is the primary PDGF pathway required for normal embryonic cardiovascular development.

PDGF and PDGFR knockout mice demonstrate directly the requirement for the PDGF system in morphogenesis. However, they are of limited use in delineating the morphogenic mechanism through which the PDGF system exerts its embryonic effects. For example, although PDGF-A and  $\alpha$ PDGFR are expressed in the mouse central nervous system after E13 (Yeh et al., 1991; Schatteman et al., 1992; Yeh et al., 1993) few *Ph/Ph* mice survive past E14 (Gruneberg and Truslove, 1960). The few embryos that do survive past E14 exhibit central nervous system defects, but it is impossible to ascertain whether these abnormalities are due to the lack of  $\alpha$ PDGFR in the nervous system or are secondary to poor embryonic health. Thus, it is difficult to assess the role of  $\alpha$ PDGFR in central nervous system development. Even for structures that do form during the period of embryonic survival, information from mutants is limited. Because, for example, the *Patch* deletion critically affects the neural crest early in development, it is impossible to determine if the deletion also affects these cells at later stages of development (Gruneberg and Truslove, 1960; Morrison-Graham et al., 1992). In addition, the ventricular wall of both PDGF-B knockouts and *Ph/Ph* embryos is thin. Is this thinning a result of the requirement for PDGF-B or  $\alpha$ PDGFR at one critical development stage or the cumulative effect of the lack of PDGF signaling over an extended developmental period (Schatteman et al., 1992, 1995)?

To circumvent these limitations and to complement analyses of knockout mice, we adapted the method of Johnson et al. (1989) and Carroll et al. (1992) to determine the precise temporal requirements of the developing cardiovascular system for PDGF-A. Antibodies that block the function of PDGF-A were injected into the decidua of mouse embryos *in utero* at various times during development. Analysis of the embryos collected 48 hr after injection revealed that PDGF-A is required for normal embryonic cardiovascular development. Further, the specific abnormalities observed were associated with discreet embryonic periods of antibody administration.

## MATERIALS AND METHODS

### *Mating of Mice and in Utero Injection*

Males were mated with two females for 10 hr in the dark, and females were examined for the presence of a vaginal plug (indicating pregnancy) at the end of this period. Mid dark cycle was designated E0. To reduce variability among embryos, matings were done in the inbred strain C57Bl/6J. This is also the background in which *Patch* mice are normally maintained, and the strain has been found to be particularly susceptible to the mutation (Schatteman et al., 1992).

Pregnant mothers were anesthetized with sodium pentobarbital (90  $\mu$ g/gm), swabbed with alcohol, and a  $\frac{1}{2}$ -in longitudinal abdominal incision was made to expose the uterus. A glass injection pipette

(0.2–0.3 mm diameter) was passed through the uterus and inserted into the decidua. E8.5, E9.5, and E10.5 embryos were injected with 0.4–0.5, 0.6–0.7, or 0.7–0.8  $\mu$ l, respectively, of IgG in 0.9% saline or with 0.9% saline alone as a vehicle control. After injection the uterus was replaced and the mother was surgically closed with 5-0 silk and allowed to recover. Mothers were reanesthetized and killed by cervical dislocation at various times after injection and the embryos were removed. Embryos were either snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  or fixed in methyl Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) for 36 hr or more.

### *Concentration of Injected IgG in the Embryo*

To determine the time it takes for injected IgG to reach the embryo after decidual injection, and to determine how long the IgG remains in the embryo, 10 mg/ml rabbit or goat IgG stock solutions (Sigma, St. Louis, MO) in 0.9% saline or 0.9% saline alone were injected into the decidua of E8.5, E9.5, and E10.5 embryos. At 1, 3, 6, 12, 18, and 24 hr after injection embryos from two to four litters at each age were collected and snap frozen in liquid nitrogen. Lysates were prepared by dounce homogenization of frozen embryos (with extraembryonic tissues removed) in a minimum volume of ice-cold lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton, 0.9% NaCl, and 1 mM PMSF). The crude lysates were centrifuged (13g for 5 min) and the supernatants collected. For lysates from embryos injected at E8.5, all but 2 embryos in the litter were pooled. At E9.5 lysates were made from pools of 3–4 embryos, and at E10.5 lysates were made from 1–2 embryos.

A sandwich ELISA was used to determine the concentration of IgG in the clarified embryo lysate (Harlow and Lane, 1988). Microtiter plate wells were coated overnight (O/N) at  $4^{\circ}\text{C}$  with 50  $\mu$ l 20  $\mu$ g/ml swine anti-rabbit or rabbit anti-goat IgG (DAKO) in Dulbecco's phosphate-buffered saline (PBS), blocked with 150  $\mu$ l 3% bovine serum albumin (BSA) and 0.02% sodium azide in PBS for 2 hr at room temperature (RT), and incubated O/N at  $4^{\circ}\text{C}$  with 50  $\mu$ l embryonic extracts. Plates were washed with 500  $\mu$ l PBS per well  $4 \times 5$  min, incubated with 0.1  $\mu$ g/ml biotinylated horse anti-goat IgG (or goat anti-rabbit) (Vector) for 2 hr, washed again, and incubated with 0.3  $\mu$ g/ml alkaline phosphatase-conjugated streptavidin (Zymed) for 2 hr. Plates were washed  $3 \times 5$  min with PBS, then rinsed twice with 0.5 mM  $\text{MgCl}_2$  in 10 mM diethanolamine, and reacted with 50  $\mu$ l 0.5 mg/ml disodium *p*-nitrophenyl phosphate in the rinse buffer. The absorbance at 405 $\lambda$  was read and the concentration of IgG determined by comparison to absorbance of IgG standards.

Protein content of the clarified extracts was determined by a modified Lowry assay (1951) using Bio-Rad (Hercules, CA) protein reagent according to manufacturer's instructions and compared to bovine serum albumin standards. IgG concentrations were expressed as ng/mg of total soluble protein.

### *Distribution of Injected IgG in the Embryo*

Two embryos from each of the litters examined for IgG concentration by ELISA were set aside and fixed in methyl Carnoy's. These embryos were dehydrated, paraffin embedded, sectioned at 8  $\mu$ m, and examined by immunocytochemistry (ICC) to determine the distribution of injected IgG. After deparaffinization, sections were incubated 30 min in 0.6%  $\text{H}_2\text{O}_2$  in PBS, washed  $2 \times 10$  min in PBS, then incubated 1 hr in PBS with 1% BSA. Slides were then incu-

bated 1 hr at room temperature in 0.5  $\mu\text{g}/\text{ml}$  biotinylated goat anti-rabbit IgG or horse anti-goat IgG (Vector), washed  $3 \times 20$  min in PBS, incubated 1 hr at room temperature with 1  $\mu\text{g}/\text{ml}$  horseradish peroxidase-conjugated streptavidin (Vector), and washed  $3 \times 20$  min in PBS. Slides were reacted 5–20 min with 760 ng/ml 3,3'-diaminobenzidine (DAB) in 0.3%  $\text{H}_2\text{O}_2$  in 0.1 M Tris, pH 7.6.

### Characterization of Anti-PDGF-A Neutralizing Antibody

An anti-PDGF-A rabbit polyclonal neutralizing antibody was generated by immunizing rabbits with purified human recombinant PDGF-AA homodimer that had been produced in yeast *Saccharomyces cerevisiae* (Hart *et al.*, 1990). The antibody containing serum and preimmune serum were purified by protein A-Sepharose chromatography and then dialyzed against PBS. The anti-PDGF-A antibody has less than 1% cross-reaction to PDGF-B chain as detected by immunoprecipitation of  $^{125}\text{I}$ -PDGF-BB and neutralization of PDGF-BB mitogenic activity (Hart *et al.*, 1990).

To verify that the antibody recognizes mouse PDGF-A, whole E14 mouse embryos lysates were prepared as for the ELISA assays above. Clarified lysates were mixed with an equal volume of  $2 \times$  sample buffer containing 7.7 mg/ml dithiothreitol (SB-DTT). As a control, 500  $\mu\text{l}$  of whole blood from heart puncture of anesthetized (90  $\mu\text{g}/\text{gm}$  pentobarbital) adult female mice was drawn into a plastic syringe containing 50  $\mu\text{l}$  of 100 mM sodium citrate. The blood was centrifuged (20 min at 250g) in a plastic tube to pellet red blood cells. After removing the supernatant, the pellet was washed  $2 \times$  by resuspending the cells in PBS and centrifuging (20 min at 250g). The pellet was resuspended in 20  $\mu\text{l}$  of  $2 \times$  SB-DTT. Both samples were vortexed vigorously 30 sec, boiled 5 min, and then centrifuged 5 min at 8200g. The supernatants were loaded on a 12% SDS-PAGE gel as described (Harlow and Lane, 1989).

After SDS-PAGE, samples were transferred to nitrocellulose (Gibco-BRL, Gaithersburg, MD) for Western analysis as described (Harlow and Lane, 1989). Briefly, after blocking for 2 hr in TTBS (0.1 M Tris, 0.9% NaCl, 0.1% Tween 20) containing 5% nonfat dry milk, membranes were incubated for 3 hr in TTBS with 2% nonfat dry milk (Blotto) containing anti-PDGF-A IgG at 5.3  $\mu\text{g}/\text{ml}$ . Membranes were then washed  $3 \times 10$  min in Blotto, incubated 1 hr in 0.5  $\mu\text{g}/\text{ml}$  horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Arlington Heights, IL) in Blotto, and washed  $3 \times 10$  min in TTBS. Blots were developed in chemiluminescence reagent (Dupont, Boston, MA) diluted 1:1 with  $\text{H}_2\text{O}$ .

### In Utero Injection of Embryos with Anti-PDGF-A IgG

To verify that injection of anti-PDGF IgG results in antibody being distributed in a manner similar to control IgG, two litters of six E10.5 embryos were injected with 0.7–0.8  $\mu\text{l}$  of a 5.28 mg/ml solution of anti-PDGF-A IgG. The embryos were collected 6 hr after injection and analyzed by ICC for the presence of injected IgG as above.

To determine if PDGF-A is required for normal cardiovascular development, embryos were treated with anti-PDGF-A IgG. Seven E8.5, 4 E9.5, and 7 E10.5 litters were injected with the appropriate volume of a 5.28 mg/ml solution of anti PDGF-A IgG as described above. Three E8.5, 3 E9.5, and 5 E10.5 litters were injected with nonimmune rabbit IgG, 5.28 mg/ml stock solution (Sigma) to serve as controls. Embryos were collected 48 hr after injection. Addition-

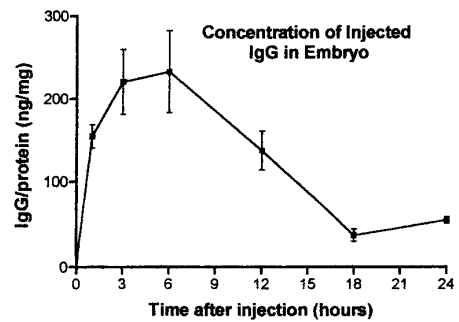


FIG. 1. Concentration of injected IgG in embryos. The concentration of injected IgG in the embryo was measured at various times after injection by ELISA and expressed as nanograms of injected IgG per milligram of total protein in the embryo extracts. Bars, SEM.

ally, four E8.5 litters injected with anti-PDGF IgG and three E8.5 litters injected with control IgG were collected 60 hr after injection. Finally, one litter of uninjected embryos was also collected for each time point.

### Analysis of Anti-PDGF-A IgG-Injected and Control Embryos

At the time of removal from the mother, embryos were examined under a dissecting microscope and photographed. Embryos were then fixed in methyl Carnoy's for 36 hr or more, paraffin embedded, sectioned at 8  $\mu\text{m}$ , and, after deparaffinization, stained with hematoxylin and eosin. Developmental ages were determined according to Theiler (1989) from photographs and histological analysis. Of embryos injected at E8.5 and collected 48 hr later, 34 anti-PDGF-A IgG-treated, 14 control-injected, and 5 uninjected control embryos were examined histologically. Of embryos injected at E9.5, 18 anti-PDGF-A IgG-injected, 12 control-injected, and 3 uninjected control embryos were examined. Of embryos injected at E10.5, 32 anti-PDGF-A IgG-injected, 29 control-injected, and 6 uninjected control embryos were examined. Not all features were examined in all embryos due to loss of sections or damage during collection. For all statistical analyses,  $\chi^2$  analysis was used, except that a *t* test was used to compare aortic circumference of E10.5 injected anti-PDGF-A-treated embryos with that of controls.

## RESULTS

### Embryonic Distribution and Titer of Injected IgG

Decidua were injected *in utero* at E8.5, E9.5, and E10.5 with IgG and collected at various times after injection to establish how long injected IgG takes to reach an embryo, and what its titer is in the embryo. Treated embryos were homogenized for analysis of IgG content by ELISA. Figure 1 summarizes the ELISA data for E9.5 and E10.5 injected embryos. Data from embryos injected at E8.5 is not included because although IgG could be detected in the embryos, the embryos were too small to allow protein concentrations to

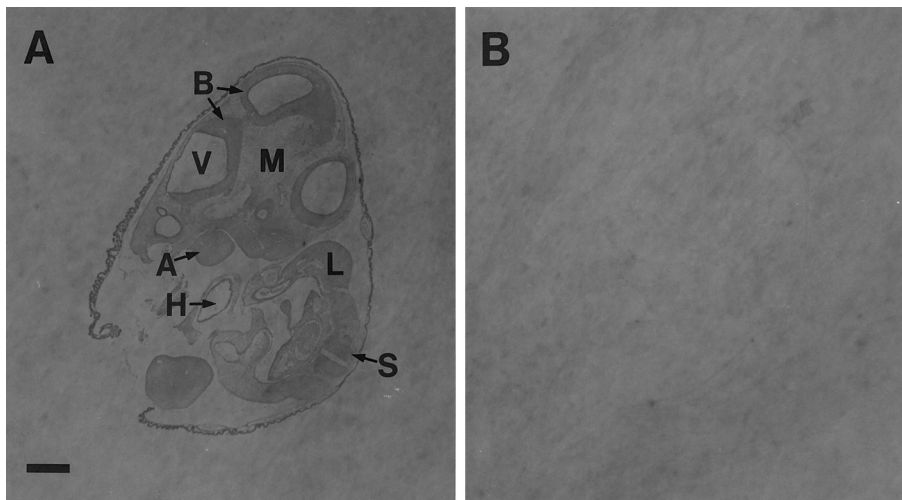


FIG. 2. Distribution of injected IgG in embryos. Detection of IgG by ICC in 8- $\mu$ m parasagittal sections. (A) E10.5 embryo injected with anti-PDGF-A IgG and collected 6 hr later. Anti-PDGF-A IgG immunoreactivity is detected throughout the embryo including the mesenchyme (M), branchial arches (A), heart (H), and limb (L), which express PDGF receptors, and in the brain (B) and spinal cord (S), which do not. Ventricle (V). (B) No background staining was observed in E10.5 embryos injected with saline and collected 6 hr later. Bar, 500  $\mu$ m.

be measured accurately. No difference was seen between goat and rabbit IgG-injected embryos so the data were pooled. IgG diffuses into the embryo rapidly, but peak titers (of about 230 ng/mg) are not achieved until approximately 6 hr after injection (Fig. 1). By 18 hr, concentrations have dropped significantly but IgG is still present and remains at approximately 50 ng/mg for at least 6 more hr (Fig. 1). This lower concentration of IgG does not represent a return to baseline since no IgG was detected in saline or uninjected controls. (Saline and uninjected controls were obtained from different litters than IgG-injected embryos, because IgG could be detected in uninjected littermates of IgG-injected embryos.)

Embryos injected as above were also examined by immunocytochemistry (ICC) to determine the distribution of injected IgG. IgG was detected in embryos at all ages examined and was distributed throughout the embryo for at least 18 hr after injection (data not shown). Similar results were obtained when anti-PDGF-A IgG was used. The antibody was distributed throughout the embryo, and for at least 6 hr, antibody distribution did not correlate with the distribution of  $\alpha$ PDGFR (Schatteman *et al.*, 1992) (Fig. 2A). The relative intensity of immunoreactivity was consistent with IgG titer data, although ICC was less sensitive than the ELISA. No reactivity was detected in saline-injected or uninjected controls (Fig. 2B).

### Characterization of Anti-PDGF-A IgG

To verify that the anti-PDGF-A IgG which was generated against human recombinant PDGF-A recognizes mouse PDGF-A, Western analysis was performed. Two bands were detected in embryo extracts representing proteins of molec-

ular weights of approximately 45 and 28 kDa (Fig. 3), similar to the molecular weights reported for immature (44 kDa) and mature (30–31 kDa) baboon PDGF-A (Kraiss *et al.*, 1993). No bands were detected in platelet-free preparations of adult red blood cells which do not contain PDGF (Fig. 3) or when a nonimmune IgG was used (data not shown).

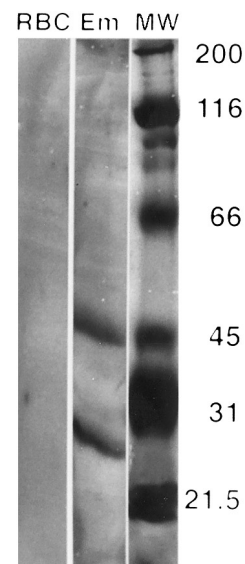


FIG. 3. Western blot analysis of mouse tissue and blood extracts with anti-PDGF-A IgG. (A) Molecular weight standards. (B) Extract of E14 whole mouse embryo. (C) Extract of platelet-free adult mouse red blood cells.

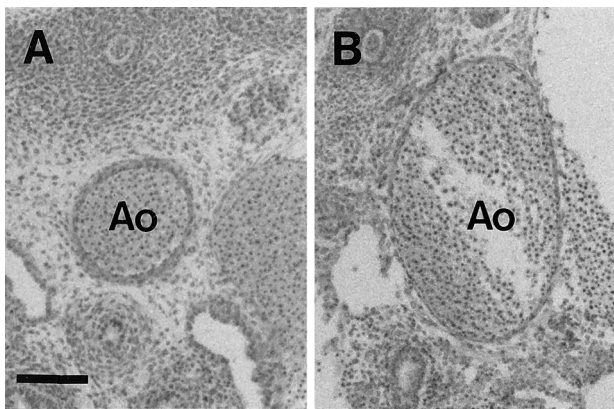


FIG. 4. Injection of anti-PDGF-A at E10.5 leads to aortic dilation. Hematoxylin- and eosin-stained 8- $\mu$ m transverse sections of E12.5 lower thoracic aorta. (A) Cross section through the largest aorta (Ao) of 11 rabbit IgG-injected embryos. (B) Cross section through the largest aorta of anti-PDGF-A IgG-treated embryos. The cross-sectional area of the aorta in B is three times that of the control aorta in A. Bar, 100  $\mu$ m.

#### *Injection of Anti-PDGF-A Antibody Results in Aortic and Veinous Dilation*

To perturb the interaction of PDGF-A with its receptor, anti-PDGF-A IgG was injected *in utero* into the decidua of mouse embryos at E8.5, E9.5, or E10.5, and the embryos were collected 48 hr later. The resorption rates of anti-PDGF-A- and nonimmune IgG (control)-injected embryos of 8 and 4% at E8.5 ( $P = .31$ ), 18 and 17% at E9.5 ( $P = .85$ ), and 15 and 20% at E10.5 ( $P = .26$ ), respectively, were not significantly different. Nonresorbed abnormal embryos were present in both anti-PDGF-A IgG- and control-injected litters as determined by gross visual observation. However, only grossly normal embryos were examined, because the abnormalities observed were similar between the two groups and were no more frequent in anti-PDGF-A IgG-injected embryos (4%) than in controls (8%) ( $P = .42$ ).

In anti-PDGF-A IgG-treated embryos injected at E8.5 and E9.5, the lower thoracic aorta appeared to be slightly inflated, although neither the abdominal aorta nor veins were noticeably dilated. Examination of anti-PDGF-A IgG-treated embryos injected at E10.5 revealed huge dilation of the aorta, as well as dilation of large veins although to a lesser extent. In 11 of 23 anti-PDGF-A IgG-injected embryos, the aortas examined were larger than the largest of 11 control aortas, and 17 anti-PDGF-A IgG-treated aortas were larger than the third largest control aorta. The largest anti-PDGF-A IgG-treated aorta (Fig. 4B) had a cross-sectional area three times greater than that of the largest control (Fig. 4A), and the mean cross-sectional area of anti-PDGF-A-treated embryos was significantly larger ( $3.3 \times 10^4 \mu\text{m}^2$ ) than that of controls ( $2.1 \times 10^4 \mu\text{m}^2$ ) ( $P = .01$ ). In most of the distended aortas, the vessel wall was thin relative to that of controls (Fig. 4).

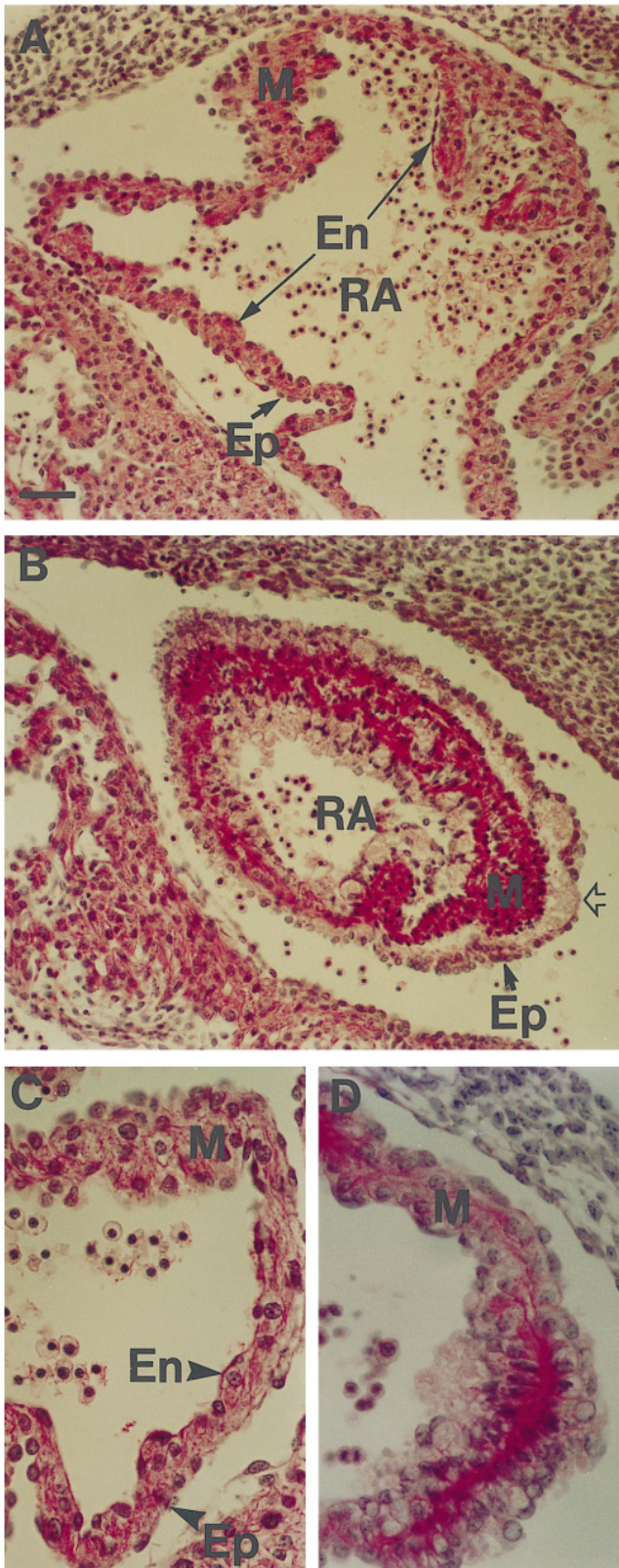
#### *Anti PDGF-A Injection Leads to Atrial and Ventricular Wall Abnormalities*

Normal atrial wall organization with clear delineation between the epicardium, myocardium, and endocardium was observed in 54 of 55 control IgG-injected and 14 of 14 uninjected embryos (Figs. 5A and 5C). However, in 10 of 34 (29%) E8.5, 3 of 18 (17%) E9.5, and 5 of 32 (16%) E10.5 anti-PDGF-A IgG-injected embryos, small or large patches of severe myocardial disorganization were observed. In severely affected embryos of all three ages, the epicardium was lifted away from the myocardium and the interleaving space was filled with cells and/or extracellular matrix (Fig. 5B). In other areas the epicardium appeared to be absent (Fig. 5B). The myocardium was thickened, probably by a combination of hypertrophy, hyperplasia, and excessive extracellular matrix production. In some cases cells and extracellular matrix nearly filled the atrial cavity (Fig. 5B). In most regions of myocardial hypertrophy it was unclear whether an endocardium was present since cells lining the atrial lumen were hypertrophic and resembled neither myocardial nor endocardial cells (Fig. 5D). In some areas where the endocardium appeared to be present, as with the epicardium, it was lifted from the myocardium by interleaving cells and extracellular matrix material (Fig. 5B).

Most commonly the atria were affected, but in 12 of 84 (14%) ( $P < .001$ ) embryos the ventricular wall was also abnormal. In most of these embryos the ventricular wall abnormalities were mild and were manifest as a thickening of the ventricular wall, but in 4 (5%) ( $P = .04$ ) embryos, the ventricular abnormalities were more severe, such that the ventricular cavity was nearly filled with cells and extracellular matrix material (Figs. 6B and 6D). The wall myocardium was thickened, but whether this was due to hyperplasia, hypertrophy, or extracellular matrix abnormalities was unclear. Unlike the atria, the filling of the ventricles appeared to be due primarily to hypertrabeculation rather than a simple thickening of the ventricular wall (Figs. 6B and 6D). The epicardium was typically intact although slightly lifted from the myocardium (Fig. 6B). Normally at E12.5, some portions of the epicardium covering the ventricles is loosely adherent while in other parts it is tightly adhered (or at least closely apposed) to the myocardium. However, even in regions where the myocardium appeared normal, the regions of adherent epicardium were smaller and the extracellular space between the epicardium and myocardium in nonadherent regions was typically wider than in controls (data not shown). Ventricles of all injected (55) and uninjected (14) controls appeared normal (Figs. 6A and 6C).

#### *Subpericardial Blood and Other Abnormalities Are Observed in Anti-PDGF-A-Treated Embryos*

Small amounts of subpericardial blood, possibly the result of breakage of small blood vessels, were occasionally observed in both anti-PDGF-A IgG- and control-injected embryos at all ages, although blood was more common in anti-



PDGF-A IgG-treated embryos. However, of 21 anti-PDGF-A IgG-treated embryos injected at E10.5, subpericardial blood was seen in 3 at the time of dissection, and large amounts of blood were seen in 5 more by histological analysis (Fig. 7B), but large amounts of blood were not seen in controls (Fig. 7A) ( $P < .001$ ). No broken blood vessels were apparent in these embryos, but we routinely observed tiny holes in the heart wall in the atrioventricular ring of the heart in anti-PDGF-A IgG-treated embryos, which were rarely present in control embryos. Further, two anti-PDGF-A IgG-treated embryos injected at E10.5 had large transmural perforations of the heart wall in this region (Fig. 8) that could be seen in many histological sections.

Holes in the anterior thoracic wall were present in anti-PDGF-A IgG-treated embryos injected at E10.5. Holes were found in 5 of 23 anti-PDGF-A IgG-injected embryos, and an additional 5 had a thinned wall relative to controls (data not shown). In the one embryo with a large hole, the heart was malpositioned, having rotated toward the right side (data not shown).

## DISCUSSION

$\alpha$ PDGFR, the receptor subunit that mediates biological activity of PDGF-A, is found in both neural crest and mesodermal components of the cardiovascular system during development.  $\alpha$ PDGFR expressing neural crest derivatives include the spiral septum of the outflow tract, and the thoracic aorta, while mesodermal derivatives include the atrioventricular septal cushions, primitive trabeculae of the heart, portions of the myocardium and epicardium, and the mesenchyme from which blood vessels condense (Morrison-Graham *et al.*, 1992; Schatteman *et al.*, 1992, 1995). No expression is detected in the endocardium at any stage of development. Table 1 summarizes the abnormalities we

FIG. 5. Anti-PDGF-A injection leads to atrial abnormalities. 8- $\mu$ m hematoxylin- and eosin-stained sections of atria from embryos injected at E10.5 and collected at E12.5 with rabbit IgG (A, C)- and anti-PDGF-A IgG-injected (B, D) embryos. (A) The epicardium (Ep), myocardium (M), and endocardium (En) of the atrial wall can be seen surrounding a large open right atrial cavity (RA). (B) A thick eosinophilic myocardium can be seen surrounding a small right atrial cavity. The epicardium is generally intact, but is pulled away from the myocardium, and in some regions the epicardium may be absent (open arrowhead). Hypertrophic cells line the atrial cavity, but it is unclear whether these are endocardial or myocardial cells. (C) Continuous layers of epicardium and endocardium can be seen ensheathing the myocardium. (D) Normal looking atrial wall can be seen adjacent to a region of hypertrophy. In the hypertrophic region a characteristic band of eosinophilic material splits what appear to be two layers of myocytes. The myocytes are surrounded both lumenally and abluenally by large indistinct cells that do not resemble either normal epi- or endocardial cells. Bars: A and B, 50  $\mu$ m; C and D, 25  $\mu$ m.

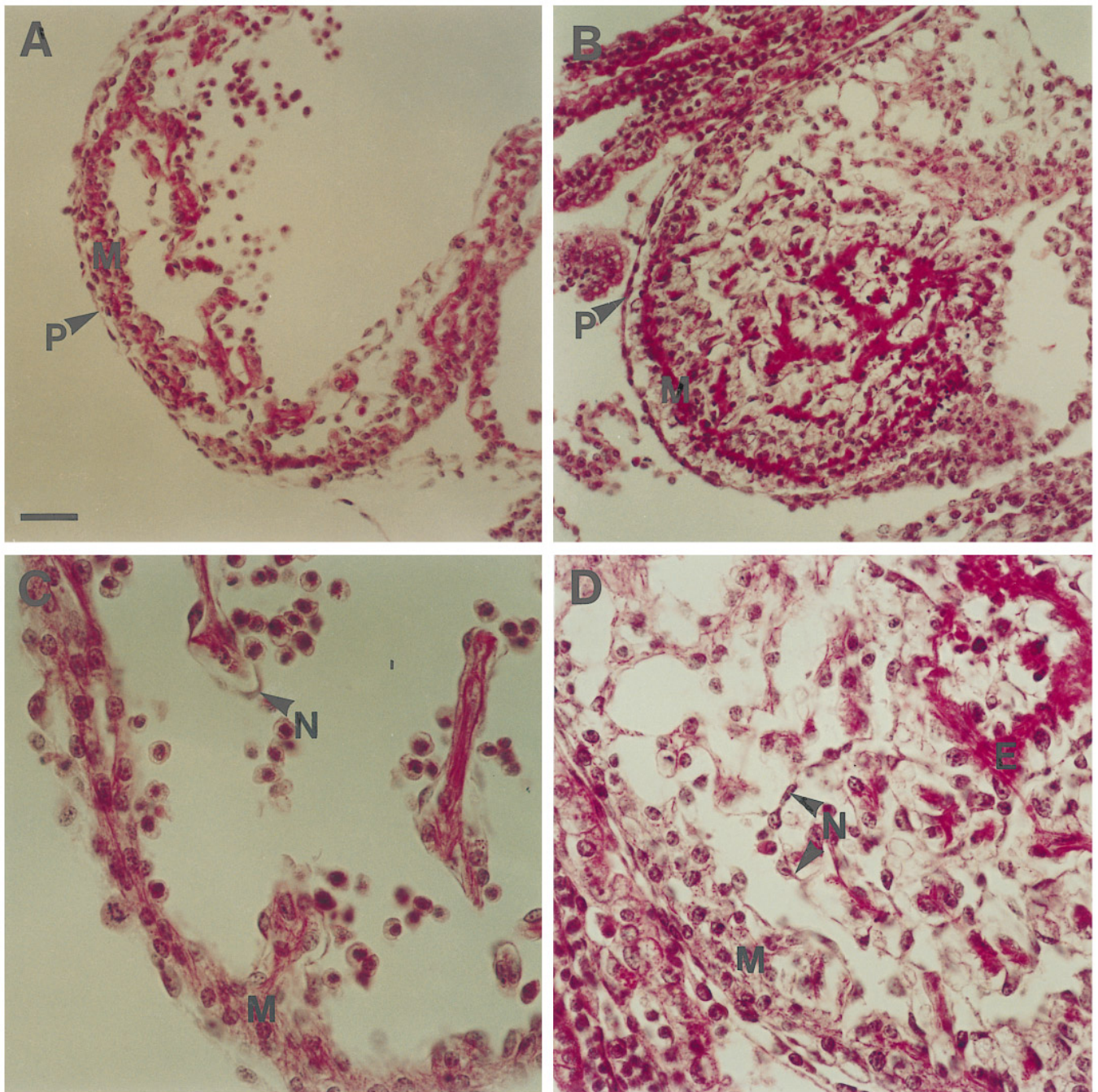


FIG. 6. Anti-PDGF-A injection leads to ventricular abnormalities. 8- $\mu$ m hematoxylin- and eosin-stained sections of atria from embryos injected at E10.5 with rabbit IgG (A, C) or anti-PDGF-A IgG (B, D) and collected at E12.5. (A) Open ventricular cavity with normal trabeculation and association of epicardium (P) with the myocardium (M). (B) Ventricle with an intact epicardium (P) surrounding the eosinophilic ventricular wall myocardium (M). The space separating the myocardium and epicardium is larger than that seen in the ventricle in A. (C) High-power view of ventricular wall and trabeculae surrounded by endocardium (N). (D) High-power view of ventricle. No well-formed trabeculae can be seen, although what appear to be endocardial cells (N) are present. Eosinophilic extracellular matrix material (E) can also be seen. Bars: A and B, 50  $\mu$ m; C and D, 25  $\mu$ m.

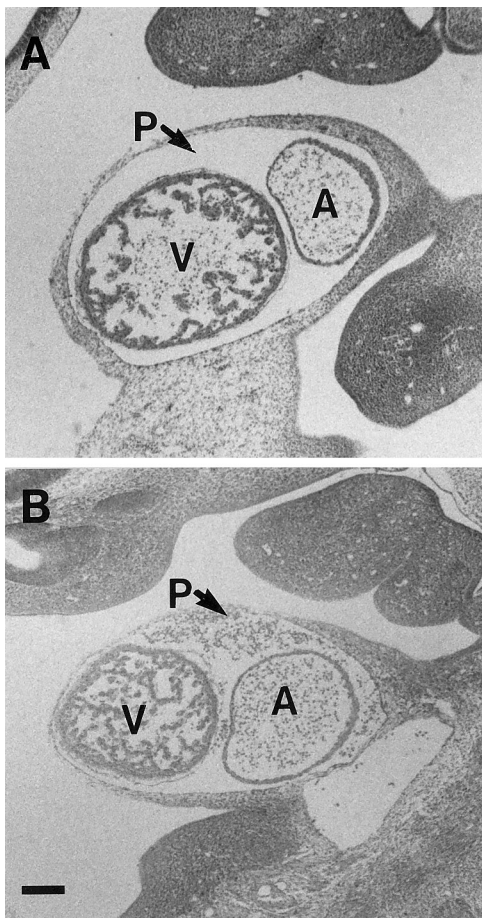


FIG. 7. Subpericardial blood is present in anti-PDGGF-A-treated embryos. Parasagittal hematoxylin- and eosin-stained 8- $\mu$ m sections of E12.5 heart and pericardial cavity from rabbit IgG (A) and anti-PDGGF-A IgG-injected (B) embryos. (A) No blood is present within the subpericardial space (P) surrounding the ventricle (V) and atrium (A). (B) Large amounts of subpericardial blood are present in the pericardial space. Bar, 150  $\mu$ m.

observed in the cardiovascular system when the PDGF-A/ $\alpha$ PDGFR system was temporarily disrupted during embryogenesis.

The patchiness of atrial and ventricular wall abnormalities in the anti-PDGGF-A IgG-injected embryos is consistent with the pattern of  $\alpha$ PDGFR expression which is also patchy in the myocardium and epicardium (Schatteman *et al.*, 1992, 1995). Whether the abnormalities observed in the heart wall are due to the effects of PDGF deprivation on the epicardium or myocardium or both could not be determined. However, since the endocardium does not express detectable  $\alpha$ PDGFR at any stage of development (Schatteman *et al.*, 1995), the lack or hypertrophy of endocardium in regions of myocardial thickening is clearly a nonautonomous event and is probably due to aberrant myocardial signaling. Ventricular wall thickening and hypertrabeculation

were less common and less extensive than atrial thickening and were observed only in embryos exhibiting severe atrial wall abnormalities. This suggests that the atrial wall is more sensitive to PDGF-A deprivation. Further, it appears that the heart wall may be more sensitive to anti-PDGGF-A IgG-induced defects at E8.5 than at E9.5 or E10.5 since atrial thickening was found in 29% of the younger embryos compared to 16% in the older embryos ( $P = .04$ ).

Also consistent with the pattern of  $\alpha$ PDGFR distribution is the finding of small myocardial perforations in the atrial-ventricular ring, which expresses high levels of  $\alpha$ PDGFR during development (Schatteman *et al.*, 1995). In this region the myocardium which surrounds the atrioventricular cushions is typically thin in all embryos. For this reason, small holes in the myocardial wall could be created as a by-product of tissue sectioning. However, although we routinely observed holes in this region in anti-PDGGF-A IgG-treated embryos, they were rarely present in control embryos and when present they were small. Further, in two anti-PDGGF-A IgG-treated embryos, the perforations were large and persisted through many sections.

We have previously shown extravascular blood to be common in *Patch* embryos, so it was not surprising that subpericardial blood was found in anti-PDGGF-A IgG-treated embryos. However, in *Patch* embryos, embryonic bleeding was attributed to vascular rupture which was a consistent finding, but in anti-PDGGF-A IgG-injected embryos, no major vessel ruptures were apparent. Thus, while the small amount of blood observed in embryos treated at E8.5 could be explained by the undetected rupture of small blood vessels, leakage through the myocardium is a more plausible explanation for the large amounts of subpericardial blood observed in embryos injected at E10.5. This is further supported by the fact that extravascular blood was not observed throughout the embryos, but was confined to the pericardium. Leakage may also be occurring in regions of myocardial hypertrophy where the normal myocardial organization is disrupted. Interestingly, anti-PDGGF-A IgG may induce vascular rupture at later stages of development, since in preliminary studies hematomas and purpura are present on most embryos treated with anti-PDGGF-A IgG at E12.5 (Schatteman, unpublished). Perhaps the initially slight dilation of the aorta that increases as development progresses is an early indicator of the dependence of blood vessels on PDGF-A for normal development, but that dependence is not critical until later in development. Indeed, embryos that lack PDGF-B do not develop hemorrhages until just prior to birth (Leveen *et al.*, 1994).

The finding of an open thoracic cavity or thinned thoracic wall is consistent with the phenotype of *Ph/Ph* mice in which the thoracic wall is consistently open or thin. What was surprising, however, was that although no thinning was apparent in embryos treated at either E8.5 or E9.5, embryos treated at E10.5 had thinned or open walls. Further, preliminary analysis of embryos injected at E12.5 showed no thoracic wall abnormalities (Schatteman, unpublished). Thus, the dependence on PDGF-A for thoracic wall formation may be specific for one



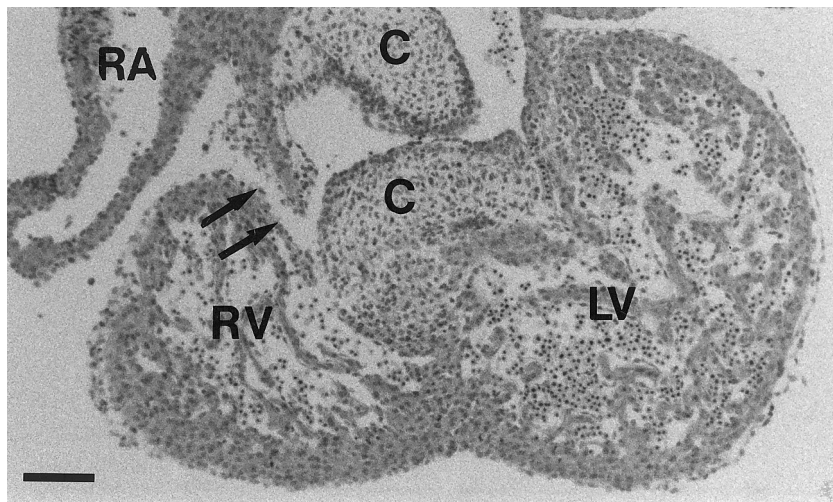


FIG. 8. Injection of anti-PDGF-A induces myocardial perforations. Hematoxylin- and eosin-stained 8- $\mu$ m oblique section of E12.5 heart injected with anti-PDGF-A IgG. A patent transmural channel (arrows) can be seen in the right ventricular (RV) wall in the atrioventricular ring near the atrioventricular cushions (C). Right atrium (RA). Left ventricle (LV). Bar, 100  $\mu$ m.

stage of development. Additionally, since the thoracic wall is closed in early stage embryos, the finding that anti-PDGF-A IgG treatment led to an open wall suggests that PDGF-A may be controlling cell adhesion processes.

All of the abnormalities discussed above are associated with mesodermally derived tissues. Notably, even dilation of the aorta was confined to the lower thoracic and abdominal (mesodermally derived) portion of the vessel and was not seen in

the upper (neural crest-derived) thoracic aorta. Many of the observed defects were identical to those seen in *Patch* mice. However, in *Patch* mice, in addition to a broad spectrum of mesodermally associated abnormalities, embryos are severely dysmorphic in structures derived from the neural crest. The lack of a neural crest-associated phenotype may indicate that the mesoderm is more susceptible to disruption of PDGF-A-mediated signaling than is the neural crest at the time points tested. Either antibody titers may have been insufficient to affect the neural crest or the disruption of PDGF-A signaling was not long enough to cause other phenotypic changes. It is also possible that the embryo requires only that either PDGF-A or PDGF-B be present, not both.

The findings here demonstrate for the first time that PDGF-A is required for normal embryonic development. Disruption of PDGF-A ligand-receptor interactions results in a different but overlapping array of embryonic abnormalities, depending on the embryonic age at which the disruption occurs. By injecting antibodies *in utero* we have been able to obtain information about the temporal requirements of the embryo for PDGF-A that could not have been ascertained from the analysis of knockout mice. We believe that this is a powerful technique that can be applied to many systems to screen for developmental abnormalities, perhaps as a precursor to doing costly time consuming knockouts. Further, it can complement knockout mice by helping to define critical developmental periods and processes that require the knocked out factor.

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TABLE 1  
Phenotypic Abnormalities Observed in Anti-PDGF-A-Injected Embryos

Defect	Age	Affected	$P^a$
Resorption	E8.5	8%	0.31
	E9.5	18%	0.85
	E10.5	14%	0.26
Aortic inflation	E8.5	±	
	E9.5	±	
	E10.5	48%	<.001
Thickened heart wall	E8.5	29%	0.02
	E9.5	17%	0.08
	E10.5	16%	0.007
Subpericardial blood	E8.5	±	
	E9.5	±	
	E10.5	38%	<.001
Heart wall perforations	E8.5	—	
	E10.5	>7%	<.14
Thoracic wall thinning	E8.5	—	
	E9.5	—	
	E10.5	48%	<.001

<sup>a</sup> Comparison to injected controls.

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