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The platelet-derived growth factor signaling system in snapping turtle embryos, *Chelydra serpentina*: potential role in temperature-dependent sex determination and testis development

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Abstract

The platelet-derived growth factor (*Pdgf*) signaling system is known to play a significant role during embryonic and postnatal development of testes in mammals and birds. In contrast, genes that comprise the *Pdgf* system in reptiles have never been cloned or studied in any tissue, let alone developing gonads. To explore the potential role of PDGF ligands and their receptors during embryogenesis, we cloned cDNA fragments of *Pdgf-A*, *Pdgf-B*, and receptors *PdgfR-α* and *PdgfR-β* in the snapping turtle, a reptile with temperature-dependent sex determination (TSD). We then compared gene expression profiles in gonads from embryos incubated at a male-producing temperature to those from embryos at a female-producing temperature, as well as between hatchling testes and ovaries. Expression of *Pdgf-B* mRNA in embryonic gonads was significantly higher at a male temperature than at a female temperature, but there was no difference between hatchling testes and ovaries. This developmental pattern was reversed for *Pdgf-A* and *PdgfR-α* mRNA: expression of these genes did not differ in embryos, but diverged in hatchling testes and ovaries. Levels of *PdgfR-β* mRNA in embryonic gonads were not affected by temperature and did not differ between testes and ovaries. However, expression of both receptors increased at least an order of magnitude from the embryonic to the post-hatching period. Finally, we characterized expression of these genes in several other embryonic tissues. The brain, heart, and liver displayed unique expression patterns that distinguished these tissues from each other and from intestine, lung, and muscle. Incubation temperature had a significant effect on expression of *PdgfR-α* and *PdgfR-β* in the heart but not other tissues. Together, these findings demonstrate that temperature has tissue specific effects on the *Pdgf* system and suggest that *Pdgf* signaling is involved in sex determination and the ensuing differentiation of testes in the snapping turtle.

Keywords

gonad development; *Pdgf*; reptile; temperature-dependent sex determination

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INTRODUCTION

The platelet-derived growth factor (*Pdgf*) signaling system is composed of four ligands, PDGF-A, PDGF-B, PDGF-C, and PDGF-D, and two receptors, PDGFR- α and PDGFR- β . While the function of the first two ligands and receptors is well characterized, much less is known about PDGF-C, and PDGF-D. In general, PDGF-A and PDGF-B act as paracrine signals that induce key cellular processes including proliferation, migration, and differentiation (Heldin and Westermark, 1999; Hoch and Soriano, 2003). The ligands function as homodimers (PDGF-AA and PDGF-BB) and heterodimers (PDGF-AB) that bind to and activate receptors with an intracellular tyrosine kinase domain. Like their ligands, the transmembrane receptors form homodimers and heterodimers, which together confer some degree of signaling specificity. To be exact, PDGF-AA only activates PDGFR- α homodimers, PDGF-AB can activate PDGFR- α homodimers or PDGFR- $\alpha\beta$ heterodimers, while PDGF-BB activates all three receptor dimers. Moreover, the precise ligand-receptor combination has been shown to trigger slightly different intracellular signaling cascades (Heldin and Westermark, 1999).

As a consequence of these signaling differences and variation in *Pdgf* gene expression among tissues, knockout mice lacking individual components of the *Pdgf* system display distinct phenotypes (Klinghoffer et al., 2001). For example, *Pdgf-B* and *PdgfR- β* are required for normal development of the vasculature and kidneys: mice lacking functional copies of either of these genes die perinatally. Mice missing *Pdgf-A* die as a result of lung abnormalities, whereas *PdgfR- α* knockout mice have problems with myotome formation as well as craniofacial and central nervous system defects. Disruption of *Pdgf* signaling also has adverse effects on testis development. Studies in mammals and birds show the *Pdgf* system is involved in migration of cells from the mesonephros into the developing gonad, differentiation of Leydig and perimyooid cells, and vascularization of the testis (Gnessi et al., 1995; Gnessi et al., 2000; Chiarenza et al., 2000; Uzumcu et al., 2002; Brennan et al., 2003; Smith et al., 2005). Given the general importance of the *Pdgf* system for embryogenesis and the key role it plays during testis formation in other amniotes, we decided to characterize tissue specific patterns of expression for *Pdgf-A*, *Pdgf-B*, *PdgfR- α* , and *PdgfR- β* in snapping turtle embryos and to study the influence of incubation temperature on gene expression in developing gonads.

The snapping turtle differs from birds and mammals in that it has temperature-dependent sex determination (TSD) rather than genotypic sex determination (GSD). The TSD pattern in snapping turtles is female-male-female from low to high incubation temperatures (Yntema, 1976; Janzen, 1992; Rhen and Lang, 1998; Ewert et al., 2005). Nevertheless, the basic process of gonad development is similar in all amniotes, including turtles (Wibbels et al., 1991; Yao and Capel, 2005; Rhen, unpublished observations). The genital ridge initially forms as a thickening of the coelomic epithelium that overlies the mesonephros. This ridge grows and develops in an identical fashion in all embryos to form the bipotential gonad, which is composed of an inner medullary region and an outer cortex. Testes develop when the medulla grows, seminiferous tubules differentiate, and the cortex regresses. In contrast, ovaries develop when the medulla regresses and the cortex grows and differentiates. Ovarian development involves aggregation of germ cells in clusters and reorganization of germ and somatic cells into primordial follicles. In addition, several genes seem to play the same role in gonad development in all amniotes, including those species with TSD (Spotila et al. 1998; Western et al. 1999; Fleming et al. 1999; Western et al. 2000; Kettlewell et al. 2000; Valleley et al. 2001; Torres Maldonado et al. 2003; Murdock and Wibbels 2003^{a,b}; Schmahl et al. 2003; Shoemaker et al. 2007^{a,b}; Ramsey and Crews 2007^{a,b}; Rhen et al. 2007; Ramsey et al. 2007; Smith et al., 2008).

A defining event occurs when the bipotential gonads commit to develop as testes or ovaries, a process referred to as sex determination. The developmental window when temperature establishes organ fate is called the temperature sensitive period (TSP) and has been defined by shifting eggs between male- and female-producing temperatures at different developmental stages and examining hatchling sex ratios. The TSP comprises 20-35% of the total embryonic period in most TSD reptiles (Mrosovsky and Pieau, 1991; Lang and Andrews, 1994; Crews, 1996), with the notable exception of the snapping turtle. In our model organism, exposure of embryos to a female-producing temperature for 5-6 days (~8% of embryogenesis) is sufficient to induce ovary development in embryos otherwise incubated at a male-producing temperature (Yntema, 1979; Rhen, unpublished observations). This relatively short TSP allows us to focus on changes in gene expression during a discrete phase of development when the fate of the bipotential gonad is established.

We recently described temperature-dependent expression patterns for nine snapping turtle genes known to play a role in sex determination in mammals (Rhen et al., 2007). Six of these genes were strongly regulated by incubation temperature during the TSP and therefore appear to play a conserved role in sex determination in the snapping turtle. Studies in birds and mammals suggest the *Pdgf* system may also be a conserved module in the gene regulatory network for testis formation. In the current study, we cloned cDNA fragments of *Pdgf-A*, *Pdgf-B*, *PdgfR- α* , and *PdgfR- β* in the snapping turtle, compared nucleotide and predicted amino acid sequences to orthologs in other vertebrates, and examined expression profiles during the TSP of gonad development. We also studied expression of these genes in several other embryonic tissues and in testes and ovaries from hatchlings. Our results suggest the *Pdgf* system is involved in TSD and the differentiation of testes in snapping turtles.

MATERIALS AND METHODS

Egg collection

Snapping turtle embryos and hatchlings were treated according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota. We collected eggs within 24 hours of laying from turtle nests along the Clearwater and Mississippi Rivers in north-central Minnesota, USA (area bounded by the following coordinates: 47°15'N to 47°45'N and 95°05'W to 95°21'W). We transported eggs to the animal quarters in the Biology Department at the University of North Dakota and held them at ~20°C for less than one week. Eggs were washed and candled to separate fertile and infertile eggs. Approximately equal numbers of eggs from each clutch were assigned to one of two temperature treatments to control for genetic variation in thermal sensitivity (Rhen and Lang, 1998; Rhen et al., 2007). Eggs were placed in containers filled with moist vermiculite and randomly positioned within foam box incubators (Rhen and Lang, 1994). Extensive details concerning incubation regime, tissue collection, RNA extraction, and the measurement of mRNA levels have already been described (Rhen et al., 2007), so we briefly summarize our procedures below.

Egg incubation and tissue sampling

Eggs in one treatment group were incubated at a male-producing temperature (26.5°C) throughout development. Eggs in a second treatment group were incubated at 26.5°C for most of embryogenesis, but were exposed to a female-producing temperature (31°C) for 6 days starting at stage 16. This brief exposure to 31°C is sufficient to induce ovary development in all embryos (Rhen et al., 2007). We collected gonads from embryos incubated at 26.5°C and from clutch mates incubated at 26.5°C-31°C-26.5°C on days 2, 3, 4, and 5 of the temperature shift. This corresponds to the period when temperature establishes

the fate of the bipotential gonads (Rhen, unpublished results). Hereafter, we refer to the male-producing thermal regime as 26C and the female-producing thermal regime as 26-31-26C.

This experimental design has two advantages over incubating eggs at male- vs. female-producing temperatures throughout development. First, temperature has pervasive effects on reptiles beyond its impact on the gonads (Rhen and Lang, 2004). Thus, general temperature effects on traits like developmental rate could be confounded with specific effects involved in sex determination. By incubating embryos at the same temperature until the start of the TSP, we minimize these potentially confounding effects. Second, we can use a discrete temperature “pulse” to tease apart upstream events involved in TSD from downstream aspects of testis and ovary differentiation. For example, we see significant changes in gene expression as soon as 1-2 days after a temperature shift but there are no visible histological differences between incipient testes and ovaries until after the TSP (Rhen et al., 2007; Rhen, unpublished observations).

It is also important to point out that gonads were micro-dissected from the adrenal-kidney complex for cloning cDNA fragments and for gene expression analyses. This allows us to characterize gene expression exclusively in the gonads (Ramsey and Crews, 2007a). A subset of embryos was allowed to hatch to confirm that 26C produced males, that 26-31-26C produced females, and to compare gene expression in gonads from embryos and hatchlings. We also collected brain, heart, intestine, kidney, liver, lung, and skeletal muscle from a few embryos during the TSP to test for generalized temperature effects on *Pdgf* mRNA expression and tissue-specific expression patterns. All tissues were placed in RNAlater solution (Ambion, Austin TX) immediately after dissection and stored at -20°C until RNA was extracted.

RNA extraction, reverse transcription, and cDNA cloning

Total RNA was extracted from pairs of gonads from individual embryos or hatchlings as described (Rhen et al., 2007). Total RNA was treated with DNase to eliminate trace amounts of genomic DNA. Purified total RNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA from all samples had 260/280 absorbance ratios of 1.8-2.0 in elution buffer. Total RNA from hatchlings displayed discrete 18S and 28S rRNA bands when analyzed on agarose gels. Total RNA (100 ng) from each pair of gonads was used in a 20 µl reverse transcription reaction using the iScript cDNA Synthesis Kit, which contains a mix of oligo dT and random hexamers (BioRad, Hercules, CA).

We designed cloning primers to hybridize highly conserved regions of *Pdgf-A*, *Pdgf-B*, *PdgfR-α* and *PdgfR-β* coding sequences. We also used nested PCR primers to increase specificity for each gene. Fresh PCR products amplified from embryonic and/or hatchling cDNA were analyzed by gel electrophoresis. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Colonies were picked, cultured overnight in LB, and plasmids purified from 1 ml of liquid media using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Inserts were sequenced with M13 Forward and M13 Reverse primers and the BigDye® Terminator v3.1 reagents (Applied Biosystems, Foster City, CA). Sequences from multiple clones were aligned to generate consensus sequences, which were then used in nucleotide BLAST searches to retrieve orthologous sequences. Alignment of snapping turtle gene fragments with orthologs from other vertebrates was done with BioEdit software. MEGA4 software was used to produce neighbor-joining trees, which were used to infer phylogenetic relationships among snapping turtle genes and orthologs from other vertebrates. We used the online ExPASy tool (<http://ca.expasy.org/tools/dna.html>) for *in silico* translation of nucleotide sequences to examine conservation at the amino acid level.

Quantitative Real Time PCR—Real time PCR was used to measure expression of *Pdgf-A*, *Pdgf-B*, *PdgfR-α*, and *PdgfR-β* mRNA and 18S rRNA in pure gonads. In brief, each reaction tube contained 7.5 μl of 2X SYBER GreenER solution (Invitrogen), 200 nM of each forward and reverse primer (see Tables 1-4 for primer sequences), 5 μl of cDNA synthesized from gonads isolated from one individual (equivalent to 2.5 nanograms [ng] of total RNA), and water to bring the total reaction volume to 15 μl. Reactions were run on the Chromo4 Real-Time PCR System (BioRad, Hercules, CA). The thermal profile was 95°C for 10 min to activate the DNA polymerase followed by 40 cycles of two-step PCR (94°C for 15 sec and 60°C for 1 min).

Standard curves were used to quantify mRNA expression. Real time PCR primers were used to amplify gene fragments of interest from embryonic cDNA. We gel purified PCR products and measured DNA concentration using a Nanodrop ND-1000 spectrophotometer. Purified PCR products were then diluted to produce a series of standards. PCR products were added to reaction tubes in the following amounts: 5,000,000 attograms (ag = 10⁻¹⁸ gram)/tube, 500,000 ag/tube, 50,000 ag/tube, 5,000 ag/tube, 500 ag/tube, 50 ag/tube, 5 ag/tube, and 0.5 ag/tube. Standard curves produced in this manner allowed rigorous quantification of gene expression differences across 8 orders of magnitude. Furthermore, all of our samples fell within the log-linear range of the standard curve (i.e., all samples had threshold cycles above the detection limit of the assay). Standard curves were used to estimate the amount of mRNA in attograms per 2.5 ng of total RNA from a pair of gonads. More precisely, we estimate how much cDNA was synthesized from mRNA for a given gene in 2.5 ng of total RNA. This procedure was also used to measure gene expression in other tissues.

Control reactions verified that we measured cDNA synthesized from mRNA. First, there was no amplification when reverse transcriptase was not added to total RNA (no RT control). This demonstrates that input RNA was free of genomic DNA. Moreover, there was no detectable amplification of PCR products when RNA template was not added to RT reactions (no template control). This indicates that there was no contamination from exogenous RNA. Finally, there was no amplification in tubes with water added rather than template, indicating that our reagents were pure and that there was no cross contamination from any other DNA source (i.e., carryover PCR products). Finally, efficiencies of real time PCR reactions were estimated from the slope of our standard curves (efficiencies ranged from 93% to 98%: *Pdgf-A* = 95.0%, *Pdgf-B* = 97.0%, *PdgfR-α* = 93.1%, and *PdgfR-β* = 98.0%). A melting temperature analysis was added at the end of each real time PCR to verify that a single product was amplified for each gene analyzed.

Statistical Analysis

We used JMP 5.0.1.2 software for all statistical analyses (SAS Institute, Cary, NC). We analyzed patterns of *Pdgf* gene expression in embryonic gonads using three-way ANOVA: clutch, temperature treatment, sampling day, and interactions among these variables were included in the model. We used Ct values for 18S rRNA as a covariate to control for potential variation in the quality of input RNA as well as variation in the efficiency of the reverse transcription reaction. We also used expression levels for each *Pdgf* gene as a covariate for expression of other *Pdgf* genes. For instance, we used expression levels for *Pdgf-B*, *PdgfR-α*, and *PdgfR-β* as covariates for *Pdgf-A* expression. This was done to test the hypothesis that genes in the *Pdgf* system influence expression of other genes in the system via feedback loops. Given significant main effects or interactions between independent variables, we used the Dunn-Sidak method to correct for multiple comparisons among treatment groups. The nominal significance level was calculated as $\alpha' = 1 - (1 - \alpha)^{1/k}$, where k is the number of comparisons for an experiment wise $\alpha = 0.05$. Simple t-tests were used to compare expression between hatchling testes and ovaries. Sample sizes for experimental

groups are shown in each figure. Finally, we used discriminant function analysis to characterize tissue specific patterns of *Pdgf* gene expression. Discriminant analysis is conceptually related to multivariate ANOVA and is used to determine which variables distinguish two or more naturally occurring groups (Sokal and Rohlf, 1981). In our case, these groups are different embryonic tissues. The canonical variable, also called a variate, is a linear combination of the original variables (mRNA levels for *Pdgf-A*, *Pdgf-B*, *PdgfR- α* , and *PdgfR- β*). The canonical plot shows canonical variate for each sample and the mean for each canonical variate in the two multivariate dimensions that best separate the tissues.

RESULTS

Snapping turtle *Pdgf* sequences and phylogenetic analysis of vertebrate *Pdgf* orthologs

We used reverse transcription and nested PCR to clone fragments of *Pdgf-A*, *Pdgf-B*, *PdgfR- α* , and *PdgfR- β* cDNA from the snapping turtle (see Tables 1-4). We used BLAST to retrieve orthologous sequences from several amniotes and from frogs (or zebrafish) for sequence comparisons. All four snapping turtle genes showed greater similarity to chicken orthologs than to orthologs from other vertebrates. This relationship between turtle and bird orthologs had moderate to high bootstrap support. Snapping turtle *Pdgf-A* displayed 85% nucleotide identity and 95% amino acid identity with chicken *Pdgf-A* (Table 1). Snapping turtle *Pdgf-B* displayed 79% nucleotide identity and 78% amino acid identity with chicken *Pdgf-B* (Table 2). *PdgfR- α* from the snapping turtle displayed 87% nucleotide identity and 92% amino acid identity with chicken *PdgfR- α* (Table 3). Snapping turtle *PdgfR- β* displayed 85% nucleotide identity and 91% amino acid identity with chicken *PdgfR- β* (Table 4).

We constructed neighbor-joining trees, which are shown in Figure 1. Putative relationships among placental mammals, marsupials, monotremes, and the turtle/bird clade were much less consistent than the relationship between turtles and birds. The platypus and opossum *Pdgf-A* gene grouped with turtle and chicken rather than other mammals (Figure 1A). In contrast, all mammalian *Pdgf-B* genes formed a single clade distinct from the turtle/bird clade (Figure 1B). Although *PdgfR- α* genes in mammals formed a single group, relationships among placental mammals, marsupials, and monotremes did not have strong bootstrap support (Figure 1C). Finally, the *PdgfR- β* gene in the turtle/bird clade grouped with placental mammals, while the platypus *PdgfR- β* gene was as distinct from other amniotes as the zebrafish ortholog (Figure 1D).

Gene expression in embryonic gonads during the thermosensitive period

Temperature had no effect on *Pdgf-A* mRNA expression in embryonic gonads ($F_{[1,53]} = 0.21$, $p = 0.65$; Figure 2). *Pdgf-A* mRNA expression varied with sampling day ($F_{[3,53]} = 3.35$, $p = 0.03$) and the interaction between clutch and day during the TSP ($F_{[3,53]} = 2.32$, $p = 0.05$). Expression of *Pdgf-A* mRNA increased significantly from day 3 to day 5 after the temperature shift (Figure 2). No other independent variables affected *Pdgf-A* mRNA expression during the TSP. However, there was a significant difference in *Pdgf-A* mRNA levels between hatchling males and females, with expression nearly three fold higher in testes than in ovaries ($F_{[1,15]} = 6.35$, $p = 0.02$; Figure 2).

Expression of *Pdgf-B* mRNA was strongly influenced by incubation temperature during the TSP ($F_{[1,53]} = 21.8$, $p < 0.0001$). *Pdgf-B* mRNA levels were two fold higher in gonads from embryos at 26C than in gonads from embryos at 26-31-26C on days 3, 4, and 5 of the temperature shift (Figure 3). Levels of 18S rRNA were a significant covariate ($F_{[1,53]} = 3.94$, $p = 0.05$). No other independent variables influenced *Pdgf-B* mRNA abundance during the TSP. Despite the incubation temperature effect on embryonic gonads, *Pdgf-B* mRNA

expression did not differ between hatchling testes and ovaries ($F_{[1,16]} = 0.58$, $p = 0.46$; Figure 3).

Temperature had no influence on *Pdgfr-α* mRNA expression in embryonic gonads ($F_{[1,53]} = 0.92$, $p = 0.34$; Figure 4). However, *Pdgfr-α* mRNA expression was affected by clutch ($F_{[2,52]} = 9.7$, $p = 0.0003$) and the interaction between clutch and temperature during the TSP ($F_{[2,52]} = 3.7$, $p = 0.03$). No other variables influenced *Pdgfr-α* mRNA levels during the TSP. Expression of *Pdgfr-α* mRNA diverged in male and female hatchlings, with roughly three fold higher expression in testes than ovaries ($F_{[1,16]} = 5.1$, $p = 0.04$). Moreover, expression of *Pdgfr-α* mRNA was ten fold higher in hatchling testes than in embryonic gonads, but only three fold higher in hatchling ovaries than in embryonic gonads (Figure 4).

Temperature had no effect on *Pdgfr-β* mRNA expression during the TSP ($F_{[1,53]} = 0.09$, $p = 0.77$; Figure 5). However, expression of *Pdgfr-β* mRNA was affected by clutch ($F_{[2,52]} = 4.0$, $p = 0.03$) and the interaction between clutch and temperature approached significance ($F_{[2,52]} = 3.0$, $p = 0.06$). Levels of *Pdgfr-α* mRNA were a significant covariate for *Pdgfr-β* ($F_{[1,51]} = 5.9$, $p = 0.02$). No other variables influenced *Pdgfr-β* mRNA levels during the TSP. Expression of *Pdgfr-β* mRNA did not differ between testes and ovaries at hatch ($F_{[1,15]} = 0.89$, $p = 0.36$; Figure 5). Although there were no differences between incubation temperatures or sexes, *Pdgfr-β* mRNA levels increased dramatically (~30 fold) from the embryonic to the post-hatching period.

Pdgf genes display tissue specific expression patterns

Embryonic tissues were reliably classified (i.e., 83% of samples) by their *Pdgf* expression profiles using discriminant analysis (Pillai's Trace = 2.03; approximate F-statistic = 3.51; df numerator = 20, df denominator = 68; $p < 0.0001$). A plot of the first versus second canonical variables for each sample and 95% confidence ellipses for each tissue are shown in Figure 6. The first and second canonical variables together accounted for 99.1% of the variance in gene expression among tissues (Table 5). Brain, heart, and liver samples were correctly identified in every instance by discriminant analysis. In contrast, one lung sample was misclassified as intestine and one intestine sample as lung. Two muscle samples were misclassified as intestine and lung. In summary, the heart was distinguished from all other tissues by slightly higher *Pdgfr-A* and *Pdgfr-B* expression and extremely low *Pdgfr-β* expression (i.e., along the first canonical axis in Figure 6). The brain and liver also differed from each other along the first canonical axis: the brain had higher *Pdgfr-A* and *Pdgfr-B* and lower *Pdgfr-β* expression than the liver (Figure 6). In addition, the brain and liver differed from all other tissues along the second canonical axis, which reflected lower *Pdgfr-α* expression in comparison to heart, intestine, lung, and muscle (Figure 6). Although sample sizes were small, the temperature effect on *Pdgfr-α* expression in the heart approached significance: expression was higher at 26C than 26-31-26C (student's t test = 3.85, df = 2, $p = 0.06$). Temperature had a significant effect on *Pdgfr-β* expression in the heart: expression was higher at 26C than 26-31-26C (student's t test = 4.12, df = 2, $p = 0.05$). In contrast, temperature had no influence on *Pdgf* gene expression in any other tissue.

DISCUSSION

We report partial cDNA sequences for *Pdgfr-A*, *Pdgfr-B*, *Pdgfr-α*, and *Pdgfr-β* and characterize expression of these genes for the first time in a reptile. We found tissue specific patterns of *Pdgf* expression in snapping turtle embryos, as well as temperature-induced variation in *Pdgf* expression in developing gonads. In particular, expression of *Pdgfr-B* mRNA in embryonic gonads was higher at a male-producing temperature than at a female-producing temperature. This effect occurred when gonads commit to testicular vs. ovarian

fate, which suggests that *Pdgf* signaling may be involved in temperature-dependent sex determination. Our findings in hatchlings further implicate *Pdgf* signaling in the differentiation of snapping turtle testes: expression of *Pdgf-A* and *PdgfR- α* mRNA was significantly higher in testes than in ovaries. In contrast, *PdgfR- β* mRNA expression did not differ between embryonic gonads at different temperatures or between hatchling testes and ovaries.

These results, in conjunction with studies in birds and mammals, suggest the *Pdgf* system plays a conserved, but complex, role in testis development in amniotes. For example, mouse *PdgfR- α* have pleiotropic defects, including disruption of male-typical embryos lacking vasculature, fewer testis cords, and drastically reduced numbers of Leydig cells (Brennan et al., 2003). Nevertheless, embryonic mice lacking *Pdgf-A* develop normal testes with fetal Leydig cells that secrete testosterone (Gnessi et al., 2000). Hence, another ligand besides PDGF-A must signal via PDGFR- α during early testis development. A conserved sex difference in *Pdgf-B* expression in embryonic gonads in mice (Puglianiello et al., 2004) and in snapping turtles (this study) points to PDGF-B as a likely candidate for activation of PDGFR- α . Indeed, addition of PDGF-B to serum free media induces formation of cords in embryonic mouse testes grown in culture (Ricci et al., 2004). The latter study also found that PDGF-B induces migration of mesonephric cells and proliferation of somatic cells within the nascent testis. Mesonephric precursors give rise to endothelial and interstitial cells, but not to myoid cells, in the mammalian testis (Wilhelm et al., 2007; Cool et al., 2008). While sex-specific movement of cells from the mesonephros into the gonad is conserved in mammals and birds, mesonephric cells do not appear to migrate into embryonic gonads in turtles (Yao et al., 2004; Smith et al., 2005; Yao and Capel, 2005). However, studies in turtles are not yet definitive with regard to this question.

Research in knockout mice indicates a developmental transition from PDGF-B signaling in embryos to PDGF-A signaling after birth. Although *Pdgf-A* is not needed for the initial formation or function of testes in embryos, adult Leydig cells fail to develop in *Pdgf-A* deficient mice (Gnessi et al., 2000). In wild-type mice, fetal Leydig cells regress after birth and are functionally replaced by a new cohort of Leydig cells that differentiate from distinct precursors at puberty. Failure to induce proliferation and differentiation of adult Leydig cell precursors results in reduced testis size and androgen titers. In short, PDGF-B signaling is sufficient for development of fetal Leydig cells, whereas differentiation of adult Leydig cells requires PDGF-A signaling. Our findings in the snapping turtle suggest a similar ontogenetic shift in signaling between embryonic and post-embryonic testes. While *Pdgf-B* expression was higher in embryonic gonads at a male-producing temperature, there was no difference in *Pdgf-B* expression between hatchling testes and ovaries. In contrast, expression of *Pdgf-A* and *PdgfR- α* did not differ in embryonic gonads at male- vs. female-producing temperatures, but was higher in hatchling testes than ovaries. While these results are suggestive, experimental manipulations will be required to test the hypothesis that *Pdgf-B* plays a key role in fetal Leydig cell development and that *Pdgf-A* is required for adult Leydig cell differentiation in the snapping turtle. Expression patterns for *Pdgf-A* and *PdgfR- α* in snapping turtle embryos were different than those reported in mouse and chicken embryos, where expression was higher in testes than in ovaries (Brennan et al., 2003; Smith et al., 2005). It is currently unclear whether this species difference has any functional consequences.

Another important question is whether the *Pdgf* system is involved in the development of other organs in turtle embryos. We observed tissue-specific expression patterns, which imply unique roles for different components of the *Pdgf* system in different tissues (Heldin and Westermark, 1999; Klinghoffer et al., 2001; Hoch and Soriano, 2003). The heart, for instance, differed from all other tissues examined in that expression of *Pdgf-A* and *Pdgf-B*

was slightly higher than average whereas *Pdgfr-β* expression was extremely low. The latter result is consistent with reports that *Pdgfr-β* expression is faint in cardiac muscle later in mouse development (Betsholtz et al., 2001). Nevertheless, these observations are surprising because *Pdgfr-β* knockout mice have dilated hearts, hypotrophic cardiac muscle, and defects in the ventricular septum (Betsholtz et al., 2001). This discrepancy may be biologically irrelevant if low levels of PDGFR-β are sufficient for normal cardiovascular development or if PDGFR-β expression is higher at earlier developmental stages (i.e., when precursors commit to develop as cardiac muscle). In any event, the heart was the only tissue (besides the gonads) in which temperature influenced gene expression. Expression of *Pdgfr-α* and *Pdgfr-β* was higher in hearts from embryos incubating at a male temperature vs. a female temperature. The functional significance of these differences is not clear, but they are associated with variation in physiology: heart rate is much lower in embryos at the male-producing temperature than in embryos at a female-producing temperature (Dane Crossley, personal communication).

Expression patterns in the liver were the opposite of expression patterns in the heart. While the liver had average *Pdgfr-β* expression, it had the lowest *Pdgfr-A*, *Pdgfr-B*, and *Pdgfr-α* expression of any tissue we studied. Information on *Pdgfr* signaling in the liver is limited to studies showing that *Pdgfr-B* can stimulate migration and proliferation of hepatic fibroblasts *in vitro* and that over expression of *Pdgfr-B* in the liver of mice causes fibrosis (Tangkijvanich et al., 2002; Czochra et al., 2006). The brain also displayed a unique expression pattern with slightly higher *Pdgfr-A* and *Pdgfr-B* and lower *Pdgfr-β* expression than the liver. Expression of *Pdgfr-α* in the brain was also relatively low. In contrast, *Pdgfr-α* expression was highest in intestine, lung, and muscle of snapping turtle embryos. These results are consistent with the function of *Pdgfr-A* and *Pdgfr-α* in formation of intestinal villi and lung alveoli and *Pdgfr-α* in myotomes in mice (Betsholtz, 2004). In summary, our expression data suggest the *Pdgfr* signaling system plays a conserved role in the developing testis as well as in the development of several other organs in the snapping turtle.

Finally, the *Pdgfr* sequences we obtained have some bearing on phylogenetic relationships among amniotes. Neighbor joining trees for all four genes place turtles as a sister group to birds, rather than in a more basal position as sister group to birds and mammals. This result is consistent with recent analyses based on morphology and molecular sequences in extant species as well as the fossil record (Meyer and Zardoya, 2003). Unfortunately, *Pdgfr* sequences are not yet available from any other reptile, which would allow us to infer evolutionary relationships among turtles, birds, and various diapsids (i.e., crocodilians, lizards, snakes, and the tuatara). Given the apparent conservation of function for the *Pdgfr* signaling system across great evolutionary distances, sequence data from *Pdgfr* orthologs in other reptiles could help resolve controversy surrounding the placement of turtles within the amniote tree (Meyer and Zardoya, 2003).

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Figure 1. Neighbor joining trees obtained by analysis of partial cDNA sequences from (A) *Pdgf-A*, (B) *Pdgf-B*, (C) *PdgfR-α*, and (D) *PdgfR-β* genes in various amniotes. Trees were rooted using sequences from non-amniotes (frogs or zebrafish). Bootstrap percentages obtained after 1,000 replicates are shown above selected branches. Scale bar indicates the number of nucleotide changes per site.

Figure 2.

Platelet-derived growth factor A (*Pdgf-A*) expression during the thermosensitive period of gonad development and in hatchling testes and ovaries in the snapping turtle. Levels of *Pdgf-A* mRNA are least squares means (± 1 SE) for each temperature regime and time point. The amount of mRNA is measured in attograms (ag) per 2.5 nanograms (ng) of total RNA from a pair of gonads. The arrow indicates a significant difference in *Pdgf-A* expression between hatchling testes and ovaries.

Figure 3.

Platelet-derived growth factor B (*Pdgf-B*) expression during the thermosensitive period of gonad development and in hatchling testes and ovaries in the snapping turtle. Levels of *Pdgf-B* mRNA are least squares means (± 1 SE) for each temperature regime and time point. The amount of mRNA is measured in attograms (ag) per 2.5 nanograms (ng) of total RNA from a pair of gonads. Arrows indicate significant differences in *Pdgf-B* expression between temperatures at the indicated time points.

Figure 4.

Platelet-derived growth factor receptor alpha (*PdgfR-α*) expression during the thermosensitive period of gonad development and in hatchling testes and ovaries in the snapping turtle. Levels of *PdgfR-α* mRNA are least squares means (± 1 SE) for each temperature regime and time point. The amount of mRNA is measured in attograms (ag) per 2.5 nanograms (ng) of total RNA from a pair of gonads. The arrow indicates a significant difference in *PdgfR-α* expression between hatchling testes and ovaries.

Figure 5.

Platelet-derived growth factor receptor beta (*Pdgfr* β) expression during the thermosensitive period of gonad development and in hatchling testes and ovaries in the snapping turtle.

Levels of *Pdgfr* β mRNA are least squares means ($\pm$ 1 SE) for each temperature regime and time point. The amount of mRNA is measured in attograms (ag) per 2.5 nanograms (ng) of total RNA from a pair of gonads. There were no differences in *Pdgfr* β expression between temperatures or between hatchling testes and ovaries.

Figure 6.

Scatter plot of canonical scores for *Pdgf* gene expression in various tissues. Canonical scores were calculated using discriminant analysis as described in the text and summarized in Table 5. The first canonical variable is the X axis and the second canonical variable is the Y axis. The 95% confidence ellipses are shown for each tissue.

Table 1

Alignment of snapping turtle, chicken (AB 031022.1), short-tailed opossum (XM 001367261), platypus (XM 001511830), mouse (BC 003817.1), human (X 03795.1), and African clawed frog (NM 001087835) Pdgfr-A sequences. Identities with the snapping turtle sequence are shown with dots, while nucleotide differences are indicated with the base. Gaps are indicated with a dash. Primer sequences for real time PCR are underlined.

snapping turtle	1	CCCTGAGGGATGGTA-CTGAAGTGCCAGCCACTGGAGCGCGGCGGAGCTCCGCCACAGCAGCCTTGCTCTGTTGTCGCCCAAGTGTCTTAGACCG
Chicken	267	T.T.TGC..GG.A.....C.T.C.....T.T.C.....T.C.....TCCC.C...AT.TA....CAC.....
Short-tailed opossum	51	T.T.TGC.....C.T.A.....TA.....TC...G.TCCCAC..A.T.TA....CACCC.GA..
Platypus	296	G...ACT..GCCAC.G...GCC.CTGGCT.TC.AAT.....T.T.CG..GA-GA.TG...TG...TAGAACCCCAA.GAT...C.CC.GGA...GGC
Mouse	81	T.T.T.G.C...A...C...T...G...CG.C.C.G...C...CTGC..A..TT.C..C.C.CG.GA..
Human	287	A.....TT.T.G.C...A...A.C...T...G...G.C.C.C.G...C...CC.C..A..AG.C.C.C.C..CG.GA..
African clawed frog	412	T...CTCC..G..T.A...G..T.T.CG..TA.TGCA.A.G..G.TGTCA.T.A..AGA..TC.CAGC..GA..
snapping turtle		CGATGAGG-ACCTGGGCTTGCAATTTGCTGATAGGTTTTCG-T-----ACCTTCCCTTTGCCCTT-----TCCTGAGGAAGCTGAAATCCCCCAGG
Chicken		G.....G.....G.....GT.....A...C-----CTGC.....G..C.G.....G...
Short-tailed opossum		T..TG.....C...G..G.A-----GT..A.....T.C.....C.....G...
Platypus		TG..TTCT.T.CA.TA.GAA..GCA..ACT.A.G.CA..A.GGGAAGGT.CT.A.G.A.T..CTGATTGGGATCC.....C..G.....G..
Mouse		C.GC....CC.C.C.GCG.A-----CG.CA...C-----GG.C.....C.G.A....G..
Human		T...C.GC....CC.C.C.GCG.A-----CG.CA..TTC-----GG.C...C..G.....GC.
African clawed frog		A.....T.....G..C...C..A.CG.C...-GCTCTT...GT.TCC.T.A-----GGGC...A..G.....
snapping turtle		AGCTAATCAGAGAGACTGGCTCAGACGAAATCCACAGCATCCGCGATTTCAGCGCCTCTGGAGATTGATTCCG-----ATATGACGATGTTTCAGA
Chicken		...G...C.G...G.....G.....C.....C...TAGG---C.T.....
Short-tailed opossum		.G.G.....G..TA...CC.....A.....TAGG---C..G.....
Platypus		...G...G.....G.....CC.....A.....TAGG---C.A.....
Mouse		.T.G...C.G...GA.TC.G.....G..CC.C..A..T...A..C...TAGG---GGC...G...CC.TG..
Human		.G.G.....G...C.G...TC.G.....G..CC.C..A.....A..C...TAGG---GAG...G...TC.TG..
African clawed frog		...G...G.....G...A..CC.....T.....C.....TAGGAGG.GGC..G...C...T.C
snapping turtle		AACAAACTTGAGATCTCA-----TAGTGTTCATCTGCTAAACATGTTCAAGAGAATCACCCCTGTACCATTTCGCAGAAAAAGAACGATTGAGGAAGCC
Chicken		G.....G..T-----G.....C.GT...G.....G...
Short-tailed opossum		G.....C..A.G.....TG.....G..C.C.....G..G..A..G.GT.....A.A.....T.....G...
Platypus		C.....A.A.C.CGG...C...C...A..GAGA...C..A..A.....T.....
Mouse		G...G.C...G.C...-G.GTCC..G.CAT..C...G.CC...G.GG...G.....G..G...T.....
Human		C..C.G.C.....G.....CG.G..C..G.CA..G...G.CC...G.GG.CC.G.....G..G..G.....C.....T
African clawed frog		G.C..A.A...A..AAACACGACT.C..CAAA..GG.TG..G.C..A..G.G.T.C.T..G.....TG...A...A

snapping turtle	ATTCTGCGCTGTGCAAAACAAAGAACTGTTATATACGAGATACCTCGGAGTCAGATCGATCCACATCCGCTAACTTCCTTGATATGGCCACCGTGCGTGG	578
Chicken	.C.G..T.....C.G..G..T.....T.....C.G.C.....G.....	
Short-tailed opossum	G.....T..T.....G....G....T..T.....C.....T.....G..G..C.....C.....C...T...	
Platypus	T.....G..G..A....T.....T.....G..G..C..T.....	
Mouse	C..A..T....G..C..G..G..C..T.....C..G..G..C.....G..C.....C...C..A..T....	
Human	G.C..C..T.....G..C..G..G..C..T.....T.....G....G....C....C.....C.....	
African clawed frog	G.C...TA...T.....T...C..A..T....A....T..C..T..T...C...T..A.....	
snapping turtle	<u>AGGTGAAACGTTGTACTGGCTGTTGCAACACCAGCAGCGTGAAATGCCAGCGTCA</u> CGGATACATCACAGAAAGTGTC <u>AAGGTGGCAAAAAGTGGA</u>	578
Chicken	G..C..C.....C.....T.....C.C.....	835
Short-tailed opossum	C...A..C..A....C..T..T....T...C.....A..T.....C..C.....C.....	620
Platypus	C.....C.....C.....T.....	702
Mouse	G..C..C.....T.....C..G....T.....G..C..C..C.C.....C.....	649
Human	C..C..C.....C.....G....T..C..G.....C..C..CG..C..C...C.C.C.....C..G.....	855
African clawed frog	A..C..G..A..C.....T..C..G.....A..A....C....G.....A....	898

Table 2

Alignment of snapping turtle, chicken (AB 031025.1), short-tailed opossum (XM 001367235), platypus (XM 001473288), Human (CU 013426.1), and African clawed frog (NM 001094466) Pdgf-B sequences. Identities with the snapping turtle sequence are shown with dots, while nucleotide differences are indicated with the base. Gaps are indicated with a dash. Primer sequences for real time PCR are underlined.

Snapping turtle	1	GGGGACCCCATACCCGAAGACATTACAAAGATAATGAGTGACAGCTCGGTGCGCTCCAGGAGTGACCTCCAGCGTGCCTGCAGATAGACTCCCGTAGAAG
Chicken	162	T....TG...TT.G...G...C.....TC.....G.....G.
Short-tailed opossum	124	T.A.G.G.C.TG..C.GC.C..C.GCC...C.....TC.....ACCTG..TG.....G.
Platypus		
Mouse	64	T....T..T.G.AC.G.TG.A..GC...C..CA..C.A.C.....TTTGA..T..T....CCTG....C.G.....T.
Human	83	T....T....G.GC...TG...GC.....CA...A.C...TTTGA...T...A..CCTG....CGG...C..G..G.
African clawed frog	181	.A..T....T.A.G.G.T...AG..CTCA..GG.AG.T..CACT...TTTCA..AT.A.G.A.G.TT.....T.....T.
Snapping turtle		AGGATGGCTCCAAATTGGATCTGAACTCAACCGAGGCCATCCTG-----GTC--AAACTTGCTCGAGGGAGACGAAGTCTAG-----TGGTCGA
Chicken		...CA...TGCC...C.TG...T...T...G...AAAACCATGT...C.T...A...G.....C..TATGCTC...CA.C
Short-tailed opossum		.A...TGG.AG.AG...G...T.TG..T.G.TGA..TTCCCGAGCGA.C.GGT.GT.C.CT.C.G.AA..GA.G.ACT...CTGCAG..ACT.T
Platypus		
Mouse		.A.....GG.TG.GC...CT...ATG.....A.CT.C.GAGTCGA..TGG...G.CAT.....GA.G..C....GGTCCC...CA.C
Human		.A.....GG..G.G...C.....ATG...CT...CT...GAGGCGA.CTGG.G.G...G...T.A..A.G..C..G.GTTCCC..AC.AT
African clawed frog		.A..-GA.A.T.A..C..GATTAATGA.TA..AAGT..GCCACGAGGAATTCC.GT.A..T.C.G.TC.TT..C..CT.....
Snapping turtle		TGCTGAGCCAGCAGTACTCCAGAAATGCAAGACACGACGAGGAGTCTTTGAAATCTCCCGAGGATGGTAGATTCCACCAATGCCAACTTTGTGTGTGG
Chicken		A.A.....T.C..C..G.....T...G.....TGA.....G..CAG.....C.....
Short-tailed opossum		G.....CAGA.T.T.G.....GA...C...G...G.....C.CCA.C.CCG...C....CC.....
Platypus	205	..G..C..GA.T.C.....C.....G..C..G.....C..C.C..G..CC.....C.....CC.....
Mouse		A.A...T..T..A...C..G...G..C..A...G..CC.G...G...AC.CAC..CG...C....CC.....
Human		G..CAGA...C..G...G..C..C...G..C..G.....C..C.CA...CCG...C....CC.....
African clawed frog		..A.A.A..C..C..T...G...AC.C..CGTC..A..G...G..T..T..C.AGT.A.G..CC...T....T.....C.....
Snapping turtle		CCGCCGTGTGTGGAGGTGACGCGGTGCTCCGGCTGCTGCAACAAC
Chicken		.A..C..C.....
Short-tailed opossum		T..C.....C..T.....
Platypus		C..C....T.A.C.T..T.....
Mouse		C.....C.....T..
Human		C.....C.....
African clawed frog		.C.....C.A..T..A.A...T..G.....TGTT
		330
		500
		468
		343
		408
		427
		506

Table 3

Alignment of snapping turtle, chicken (NM 204749.1), short tailed opossum (XM 001363192), platypus (XM 001514868.1), mouse (BC 053036.1), human (M 21574.1), and African clawed frog (NM 001087084) Pdgfr- α sequences. Identities with the snapping turtle sequence are shown with dots, while nucleotide differences are indicated with the base. Gaps are indicated with a dash. Primer sequences for real time PCR are underlined.

Snapping turtle	1	CTAAATACCTCTGACATCCAGAGATCAATGTATGATCGGCTCTTCATATAAGAAAAATCTGTGTGAGAACAGAGTCAAAAAACCTGCTTTCTGATGA
Chicken	2270	T..T.....TG.A.....A.....G.....T.....T.....A.....T.....A.....
Short tailed opossum	2249	T..A..T.....A.....G.....A.....C.....G.....A.....C.....T.....C.....A.....
Platypus	2828	.C..G.....G.....C..CC..CAC.....G..C..C..C.....G..G..C.....G..CT..T.....CGG.....C..G..G..C..
Mouse	2451	GC...C.....A..C..C..C.....G.....CA..CT..CT.....C.....G..C..
Human	2394	T..C.....C..C.....T..A..C.....G.....A.....T..CT.....C.....A.....
African clawed frog	2462	.C..G..T..A..T.....CT.....C..T.....A..G..C.....G..GC..T..A-----T.....G.....T..A..CT..G.....
Snapping turtle		CAATTCTGACAGCCTCAGTTTGCTGGATTACTAAGCTTCATGTACCAAGTTGCACGGGGGATGGAGTTCTTGGCTTCTAAAAAATTGTGTGCACCGTGAC
Chicken		GG...G..GG..T..A..CC.A.....G.....CC...G.....A.....A.....C.....A.....
Short tailed opossum		T..G..A..AG..T.....C..CT.....C..G.....CC..T.....A..C..A..A.....T.....C.....C.....T
Platypus		TGG...G..AG...TG.CC.....CC.G..G.....T.CC.....G..GT..T..C.....C..A.....G.....C..C..C.....G..T
Mouse		TG.C..C..GG..T..G..C.....C..TG.....CC..T.....T..A..A.....A..T.....G.....C.....G...
Human		T..C..A..AG...T..C..AT.....GT..G.....CC..T.....C..A..A.....T.....A.....C.....T
African clawed frog		TGGA..TC..GG..T..G.C.A.G.A.....G..C..T..CA.....A.....C.....A.....C.....A..T..G..T
Snapping turtle		TTGGCAGCTCGTAATGTCTTCTGGCTCAAGGAAAAATTGTGAAGATCTGTGACTTTGTCTGGCTAGAGACATCATGATGGATTCCAACCTA
chicken	293	C.....C.....C.....GT.....
Short tailed opossum	2561	C...T.....C.....T.....C.....C.....
Platypus	2540	C...T..G..G..C.....G.....G..G..C.....C.....C.....G.....G.....G.....C..C.....
Mouse	3119	C...T..C..C..C.....C.....G.....T.....C.....C.....C.....
Human	2742	C...T.....C..C.....A.....C.....C.....C.....G.....
African clawed frog	2685	A.....AT..A..A..T..G.....A.....C.....A..G.....C.....
	2747	

Alignment of snapping turtle, chicken (XM 001233829), platypus (XM 001510374), mouse (BC 055311.1), human (NM 002609.3), and zebrafish (XM_682875.3) Pdgfr- β sequences. Identities with the snapping turtle sequence are shown with dots, while nucleotide differences are indicated with the base. Gaps are indicated with a dash. Primer sequences for real time PCR are underlined.

Table 4

Snapping turtle	1	ATGTCTGAGCTGAAGATCATGAGCCACCTGGGGCTCACCTARACATTGTCAACTTGTCTGGAGCCTGCACAAAAGGAGGCCCCCATCTATATCATAACGG
Chicken	1933	A.....C.....C.....G.....G.....C.....G.....C.....C.....
Platypus	2461	G..AT.....C.....G..T..C.....C.....G.....C.....TAGAC..CCCC..GCCT..CC
Mouse	2386	C.....T.....T..T..A..C.....G.....G.....C.....G.....G.....C.....G.....C.....C.....
Human	2414	G.....T.....T.....C.....G.....G.....C.....T.....G.....G.....C.....A.....C.....T.....
Zebrafish	1882	A..C.....T..T.....T.....A..C.....C.....C.....G.....C.....CA.....T..GT..A.....TGG..G..A..
Snapping turtle		AGTATTGCCCGCTATGGAGACCTCGTGACTAC-TGCTCCGGAACAAG-CACACCTTCCTCCAGTACTACACCGAGAAA---GCAAGGCGAG--AGATGG
Chicken		C.....T.....G.....G.....C.....A.....C.....G.....C.....TGG.....G.....CC..CG..G..GCA
Platypus		CACCC.C..AGCCC..GAC..TC..C..T..C..CC..T..GGG..G..G..AG..G..GAG..T.....T..G..G....AG.GGGCGT..TCCC.CCA--GCCCCA
Mouse		A..C.....A..C..T..T..G.....C.....A.....A.....T..G.....CGAC..T..A..C..GCATT..T..CC..CCA--GTGCT.
Human		C.....C.....G.....C.....A.....C.....A.....G.....C.....C.....T.....C.....GGGC..CC..CCA--GCGC..
Zebrafish		A..C.....A.....T..G.....C.....A..A..A.....A.....G.....A.....T..G.....T.....AACCA..TC..C..ATGGC..CTC
Snapping turtle		AGGTGTATGGGAATGCC---TCCGAAGRGACAAGGTGTACAGCCTCGCTCTCTCTGTCTGAAAGCGATGGGGGCTACATGGATATGAGCAAGGATGA
Chicken		C.....A.....--AT..A..G.....CC..C..G..T..ACT.....G.....G.....C.....
Platypus		GCAGC.CCC..TG.CCTTCTCC..CTCCT..CTGTC..C--G.....A..A..A..GC..G..CT..G.....C.....C.....
Mouse		C..C..C..A..C..C..CTGCA..TG...TT.TCCC.ACC.....A..TTGAA..C..GA...GG..G..T..C..T.....
Human		C..C..CA..C.....TCTGC...TT...CT.CCCC.CC.....AT..TG.....GAC..GG..G.....C..T.....C.....C.....
Zebrafish		A..C..GCAACG..CAG--TGA..A..TG..A.....AGAGTAT..TG..G..GGCAG...GT..T.....A.....T.....C.....C.....A..C.....
Snapping turtle		CTCCTTGGACTACGTTCCCATGCTGACATGAAGGAGAGATCAAGTATGCTGACATTGAGTCCTCCCAATTACGGCACCCCTACGAGCTGGACAGCTA-
Chicken		G..TC...T..T..G.....T..AG.....C.....C.....T..T..C..T.....A.....
Platypus		AG.....G.....CTGAG.....G.....C.....C..C..A.....C.....TG.....T..CAGTT..A.CCT
Mouse		A..TAA..T.....G.....TG.....A.....C.....A..C..A.....C.....GC..ATGG...T..T..TAACT..TGT..CC-
Human		G..GG...T..G.....CTG.....A.....CG.....A.....A.....C.....C.....ATGG...T.....TAACT..GTTC-
Zebrafish		GCAGACT..A.....C.....G.....CAG..GC..CGC..ACACT.....C.....CC..C..G..ACCC...AGT..T..G.....C.....A.....T...-
Snapping turtle		-----TTCCCCCTCAGCTCCCGAAAGGACGACCGAGTGACCTGTATAATGAGTCCCGCTCCTCAGCTACACAGACCTGGTTGGGTTTAGTTTCC
Chicken		-----A.....G.....A.....G.....A.....A.....A.....T..A.....TG...T.....G..C..C..C.....
Platypus		GCAGACCCCATGGGC..A...T..G....CTT..A..G..CC.....G..C.....T..C.....TG..T.....G..C.....A..
Mouse		-----A..TG..C..T-----CT..T..C..CC..T..A..C..C..C..A..AGG.....C..G..C..C..C..A..

Human	-----C..TG..C.T.-----G.....CTG....CA..TT....C..C....T..AG.G..A.....TG....C..G..C..C..C.A..
Zebrafish	-----CCAGGAGCA..G.---C..GT..T.ATTCTCTGG.C..C.G..T..T..G.T.....T..T.T.....T.....A..
Snapping turtle	AGGTGGCCCAACGGGATGGAGTTCTCGCGTCCAAAAACCTGTGTGCATCGAGACCTGGCAGCAAGGAACGTCCTCATCTGCGAGGGGGAAGCTGGTGAAGAT
Chicken	T.....T.....G..T.....T.....T.....T..C.....
Platypus	.A.....T..C..A.....C.....G....C..C..C..T....T..C.....G..G.....A...C....
Mouse	.A.....C.....C..T.A..C..T..G.....T..C....T..G..C....T..G.....C.....C....
Human	T..C.....T..G..C....G....C..C..CA.....G..T.....G....T.A..C.....C....
Zebrafish	C.A..C.....G..C..A..G....C.....T....C....A..T....G....T.A..C..A...CC....
Snapping turtle	CTGTGACTTTGGCCTGGCCAGAGATATCATGAGGGATTCCAACTACATCTCCAAAGGCAGCACCTTCCTGCCTTTGAAATGGATGGCCCCCAGAGAGCATC
680	
Chicken	T....A...C.....T.....T....CC.T..G.....
2613	
Platypus	TC.G..C.....A..C.....G.....TC.G..T...C.CCTC.CCTCCTT..CT---T..
3146	
Mouse	C.....TC...C.....C..A.....C...G.....
3066	
Human	TC...C....C..C..G..T.....TT....A..G....T..G.....
3094	
Zebrafish	...C.....C.....CAC..CAA.....T..T...C...G.....G..C.....
2556	

Results for discriminant analysis of *Pdgf* gene expression in various embryonic tissues. Four canonical variables (CV) are shown with their eigenvectors, eigenvalues, and the percentage of variation explained for expression of *Pdgf-A*, *Pdgf-B*, *PdgfR-α*, and *PdgfR-β*mRNA.

Table 5

	Eigenvector				Eigenvalue	%Variance
	<i>Pdgf-A</i>	<i>Pdgf-B</i>	<i>PdgfR-α</i>	<i>PdgfR-β</i>		
CV 1	-2.68	-1.22	-4.37	13.05	28.50	85.9
CV 2	1.28	-2.17	4.3	0.79	4.40	13.2
CV 3	4.22	2.84	-3.61	2.31	0.23	0.7
CV 4	-3.69	4.82	0.13	0.87	0.07	0.2