PDGF function in diverse neural crest cell populations

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Introduction

Platelet derived growth factors (PDGF) were originally identified as potent mitogens for vascular smooth muscle cells (VSMC). The PDGF ligands exert their function by causing dimerization and activation of the PDGF receptors. Ligand binding leads to phosphorylation of these receptor tyrosine kinases on tyrosine residues in their cytoplasmic domains. This, in turn, results in activation of a multitude of intracellular signaling cascades. The outcomes of these signaling events are diverse and include proliferation, migration, matrix deposition, survival and epithelial to mesenchymal transition. In vivo experiments suggest that the mechanism of action of PDGF in neural crest remains controversial. In this review, we examine the current knowledge of PDGF function during neural crest cell (NCC) development, focusing on its role in the formation of different neural crest-derived tissues and the implications for PDGF receptors in NCC-related human birth defects.

The PDGF receptors can function as either homo- or heterodimers, and each of the ligands binds the two receptors with different affinities. PDGFAA and PDGFCC exclusively bind and activate PDGFRαα, while PDGFDD preferentially activates PDGFRββ. PDGFBB can bind and activate either receptor homodimer (PDGFRαα or PDGFRββ) and PDGFBB and PDGFAB activate the heterodimer (PDGFRαβ). This review discusses the known roles of PDGF signaling in neural crest development. For a list of these PDGF-related NCC phenotypes, see Table 1. We focus on the function of PDGFs in distinct neural crest cell (NCC) populations and the potential mechanisms underlying the observed NCC abnormalities in genetically manipulated animals. Finally, we summarize the connections between human disease and PDGF signaling in NCCs.

Cranial Neural Crest Cells

NCCs are a migratory group of cells that delaminate from the embryonic dorsal neural tube. They are commonly divided into four populations that give rise to a diverse array of cell types: cranial, cardiac, trunk and sacral NCCs. The cranial NCCs form craniofacial cartilage, bone, sensory neurons, odontoblasts, connective tissue and pericytes. PDGFRα expression has been reported in Xenopus and mouse premigratory and migratory neural crest as well as many cranial NCC derivatives. The two PDGFRα-specific ligands (PDGFαA and PDGFCC) are expressed discretely in the epithelial lining of the branchial arches and oral and nasal cavities. PDGFα expression intensifies in the medial seam epithelia (oral epithelia covering the palate) just prior to palatal fusion, foreshadowing a requirement in palatal development. Expression of PDGFβ, PDGFβ and PDGFβ has not been reported during cranial NCC development, but PDGFβ and PDGFβ are expressed in endothelial cells and some pericytes in the head, respectively.

Palate and cranial bones. The first identified mutation of a PDGF receptor was the spontaneously occurring Patch (Ph) mutant mouse that was originally studied because of white patches of hair on its trunk region. This PDGFRα allele is a large genomic deletion that contains the PDGFRα genomic region and cKit transcriptional regulatory domains. Analysis of mice homozygous for this PDGFRα mutant allele (Ph/Ph) suggested a plethora of embryonic phenotypes including some that mimic phenotypes observed when NCC development is...
Table 1. Summary of PDGF mutant NCC phenotypes

| Gene            | Phenotype                     |
|-----------------|-------------------------------|
| Patch           | Cleft palate<sup>1,4,22,26</sup> |
|                 | Melanocyte deficiencies<sup>22,26,35</sup> |
|                 | VSD<sup>24,26,43</sup> |
|                 | PTA<sup>42,26-43</sup> |
|                 | Aortic arch abnormalities<sup>42,43</sup> |
|                 | Thymic hypoplasia<sup>14,26</sup> |
|                 | Cleft palate<sup>28,32,34</sup> |
|                 | Cranial bone defects<sup>28</sup> |
|                 | Melanocyte deficiencies<sup>28</sup> |
|                 | Dental abnormalities<sup>32</sup> |
|                 | Cleft palate<sup>35</sup> |
|                 | Melanocyte deficiencies<sup>35</sup> |
|                 | Cleft palate<sup>39</sup> |
|                 | Cranial bone defects<sup>29</sup> |
|                 | Thymic hypoplasia<sup>29</sup> |
|                 | Aortic arch abnormalities<sup>29</sup> |
|                 | VSD<sup>33</sup> |
|                 | VSD<sup>44,50</sup> |
|                 | None detected<sup>44</sup> |
|                 | Cleft palate<sup>45</sup> |
|                 | Thymic hypoplasia<sup>45</sup> |
|                 | Aortic arch abnormalities<sup>45</sup> |
| PDGFRα<sup>loxP/loxP</sup> | None detected<sup>46</sup> |
|                 | Cleft palate<sup>46</sup> |
|                 | Thymic hypoplasia<sup>46</sup> |
|                 | Aortic arch abnormalities<sup>46</sup> |
| PDGFRα<sup>-/-</sup>; PDGFRβ<sup>-/-</sup> | Cleft palate<sup>47</sup> |
| PDGFRα<sup>-/-</sup> | VSD<sup>48</sup> |
| PDGFRβ<sup>-/-</sup> | None reported<sup>49</sup> |
| PDGFC | Cleft palate<sup>50</sup> |
| PDGFD | Not determined<sup>51</sup> |

VSD, ventricular septal defect.

Disrupted<sup>22,24,25</sup>. Most of the NCC phenotypes were attributed to loss of PDGFα because the only NCC phenotype observed in eKit mutant animals was a melanocyte defect.<sup>24,25</sup>

While the only other overt NCC phenotypes exhibited by Ph/+ mice were a shortened mandible and wider cranial vault,<sup>22</sup> examination of embryos homozygous for Ph or various PDGFα-null alleles exhibited additional severe craniofacial phenotypes. The most obvious was the complete clefting of the frontonasal process and palate.<sup>14,22,27,28</sup> Other affected bones included the basi-sphenoid, presphenoid, alisphenoid, hyoid bone and the thyroid cartilage. Interestingly, the disrupted bones were derived from different arch regions. In addition, some derivatives of the same arch formed normally in the mutants. This observation suggests that PDGFα does not affect all of the NCC derivatives within a given arch region and that there are specific subpopulations of NCCs that require PDGFα signaling.

Attempts to identify the direct cellular requirement for PDGFα signaling in cranial NCCs have not been conclusive. Initial analyses of Ph homozygous mutants suggested a potential migratory or extracellular matrix deposition defect that led to the craniofacial disruption,<sup>26</sup> although reagents to clearly track these specific defects were unavailable at the time. Another potential mechanism was reduced MMP2 activity resulting in decreased migration of NCCs. This idea was further supported by the observation that stimulation of branchial arch cells with PDGF ligands resulted in increased levels of MMP2.<sup>27</sup> Additional genetic manipulations of PDGFα revealed other mechanisms for PDGFα activity. Embryos with an engineered null allele of PDGFα had increased apoptosis along cranial neural crest migration routes, suggesting a role for this receptor in survival of this cell population.<sup>28</sup> Later analysis of embryos lacking PDGFα exclusively in the NCC lineage did not reveal a significant amount of apoptosis in the cranial NCC population.<sup>29</sup> These analyses were accomplished using animals containing a PDGFα Δsp-Δp-null allele and a Wnt1Cre transgene, which causes recombination early in most NCC populations.<sup>30,31</sup> Further analysis is required to create a consensus of how and when PDGFα is functioning in these NCC.

A variety of different PDGFα mutants exhibit a cleft palate with high penetrance.<sup>14,22,27,28</sup> The palate forms from migrating NCCs in association with the pharyngeal ectoderm. These bilateral maxillary processes grow vertically along the developing tongue and then rapidly elevate to a horizontal position above the tongue. Examination of PDGFα-null embryos revealed a defect in the extension and elevation stage.<sup>32</sup> Further analysis showed no difference in proliferation or apoptosis rates in the developing palatal mesenchyme and when placed in culture, PDGFα<sup>-/-</sup> palatal shelves were capable of fusion.<sup>32</sup> These results point to a failure in the extension of the palatal mesenchyme, but the PDGF-driven process behind this defect is unclear. One proposed mechanism is that PDGFα is required for expression of molecules directing matrix reorganization, such as MMP-2, and that loss of this remodeling leads to a failure in shelf elevation.<sup>32</sup>

The general requirement for PDGFα signaling in the developing palate is underscored by phenotypes of PDGF ligand null and PDGFα hypomorphic alleles. PDGFA and PDGFC, ligands specific for PDGFα, are both expressed in the epithelium of the palatal shelf, mirroring PDGFα expression in the mesenchyme.<sup>16,17</sup> However, PDGFC<sup>-/-</sup>, but not PDGFA<sup>-/-</sup>, animals recapitulated a cleft palate phenotype identical to PDGFα-null mutants, suggesting a non-redundant role for PDGFC in palatal development. Further studies identified a unique requirement for phosphatidylinositol-3 kinase (PI3K) signaling downstream of PDGFα signaling. Signaling point mutants of PDGFα lacking activation of either PI3K, Src or multiple downstream pathways demonstrated that loss of PDGFα-dependent PI3K activation was the essential signaling pathway for palate development.

Loss of Src signaling had no obvious effect on NCC development.<sup>33</sup>

Modulation of PDGF receptor expression, without direct genetic manipulation, also results in cranial NCC defects. In zebrafish, microRNA 140 (Miri140) is expressed in skeletal precursors and when activity of this microRNA was disrupted, cranial facial defects were observed, including cleft palate.<sup>34</sup> The authors identified that overexpression of Mir140 negatively regulated PDGFα protein levels, mimicking zebrafish with a disruption in PDGFα expression. The loss of PDGFα protein...
led to defects in NCC reaching the oral ectoderm. Interestingly, loss of Mirn140 function resulted in increased PDGFRα protein, but defects in the palate were still observed. The defect was attributed to an inability of PDGFRα-expressing cells to leave a PDGFA ligand-rich region and complete migration into the oral ectoderm.

With the multitude of other defects present in PDGFRα-null embryos, one question was whether the observed NCC abnormalities were due to a cell-autonomous defect specifically in NCCs or if the cleft palate was caused indirectly due to disruption of other tissues. This is an especially interesting point because it has been suggested that the PDGFRα-expressing portion of the cranial mesenchyme is derived from a precursor population distinct from the NCC population. This population of cells was termed the ectomesenchyme. Chimeric analysis with PDGFRα+ embryonic stem (ES) cells revealed an almost 100% exclusion of mutant cells from neural crest-derived head and branchial arch mesenchyme suggesting a cell-autonomous requirement for PDGFRα within the NCC-derived population. Further investigation of PDGFRα contribution to NCC lineages was accomplished using mice lacking PDGFRα in the Wnt1-expressing NCC population. These animals survived until birth but exhibited respiratory problems consistent with a failure to separate the oral and nasal cavities. All of these NCC mutants demonstrated clefting of the primary palate. These data point to a key role for PDGFRα in cranial NCC populations but do not exclude an additional role for the PDGFRβ in other cranial cell populations.

Despite the expression of PDGFRβ in migratory cranial NCC (unpublished observation, MDT), PDGFRα seems to be the PDGF receptor involved in NCC development. This reliance on PDGFRα signaling may occur because the ligands that are predominantly expressed in cranial NCC tissues are PDGFA and PDGFC. Nonetheless, several lines of evidence suggest that PDGFRβ may also play a minor role in the development of cranial NCC-derived tissues. The first evidence is that NCC-specific deletion of both receptors causes more severe and penetrant bone abnormalities than loss of PDGFRα in NCC. For example, development of the hyoid bone and the basiphenoïd bone is significantly more affected in embryos lacking both PDGFR receptors in NCC than PDGFRα NCC mutants (unpublished observation, Michelle D. Tallquist). A supportive role for PDGFRβ signaling in cranial NCC development was further suggested when hypomorphic signaling mutants of PDGFRα and PDGFRβ were generated. In this study, it was found that a hypomorphic mutation in PDGFRα did not exhibit the complete range of NCC phenotypes normally associated with PDGFRα-null embryos. Only when embryos also expressed a similar hypomorphic PDGFRβ allele were significant cranial facial abnormalities reported.39

Salivary gland and dentition. The salivary gland is another cranial NCC-derived structure recently shown to require PDGF signaling during development. Wnt1Cre lineage tracing, which permits indelible tagging of NCCs, showed that the mesenchymal compartment of the developing salivary gland is derived from neural crest progenitors. PDGFRα and PDGFRβ are both expressed in the submandibular gland (SMG) mesenchyme and PDGFA is expressed in the SMG epithelium. Activation or inhibition of PDGF signaling in an ex vivo organ culture system, illustrated a positive role for PDGF signaling in inducing SMG branching morphogenesis. Despite the mesenchymal specific expression of the receptors, PDGF activation also induced epithelial cell proliferation. Analysis of other signaling components revealed that this effect was mediated by inducing FGF ligand expression in the mesenchyme that initiated paracrine communication with the epithelium.36 While no defects in salivary gland morphogenesis have been reported in PDGFRα receptor mutant animals, careful examination of the SGM is needed. Because both PDGF receptors are expressed in this tissue, it may even be necessary to generate animals lacking both PDGFRα and PDGFRβ to reveal a phenotype.

Similar to the epithelial expression of PDGFA in the SMG epithelium, PDGFA is also expressed in the oral epithelium and adjacent dental lamina.32,37 PDGFRα expression is maintained in the cranial NCC-derived dental mesenchyme. Initial studies using exogenous PDGF in an ex vivo mandibular explant revealed accelerated tooth development and increased dental mesenchyme proliferation. Examination of PDGFRα-null embryos revealed no changes in cell proliferation or survival in the dental epithelium or cranial NCC-derived mesenchyme, but early lethality prevented analysis of tooth development at later time points. Therefore, mandibular explants were placed under the kidney capsule to study later developmental time points. Analysis revealed a disorganized alignment of odontoblasts in the developing dental cusp, which led to smaller tooth size and failure to form mature, well-defined occlusal cusps of the first molar.39

**Cardiac Neural Crest**

Cardiac NCCs originate from the neural tube between the caudal hindbrain and the third somite to form the aorticopulmonary septum of the heart.38,39 They contribute to vascular smooth muscle cells of the aortic arch, the ventricular and aorticopulmonary septa and both semilunar and AV valves. Disruption of cardiac NCCs commonly leads to heart deformations including persistent truncus arteriosus (PTA), ventricular septal defects (VSD) and aortic arch abnormalities.42 Both PDGF receptors are expressed in the migrating cardiac NCC and their expression segregates after arrival at the developing outflow tract.43,45 Similar to the expression of the developing palate, all four PDGF ligands are present in the developing heart with expression of PDGFA in the epicardium, PDGFB in endothelial cells,45,47 PDGFC in cardiomyocytes47 and PDGFD in the epicardium and myocardium.48

Initial examination of Ph/Ph embryos revealed a dilated pericardium with a VSD and PTA.44,45,46 However, this phenotype was only 79% penetrant.26 Because very few PDGFRα-null embryos survive until late gestation, the range and cause of cardiac NCC defects have been difficult to ascertain. The use of NCC-specific PDGF receptor mutants has been one way to focus on cardiac NCC defects. In PDGFRα NCC mutants, greater than 50% of the animals exhibited pharyngeal arch defects such
as PTA, membranous VSD and ectopic origin of the right subclavian artery (REO).29 The incomplete penetrance of cardiac defects in PDGFRα mutants suggests that there might be compensation from other signaling pathways. Interestingly, examination of PDGFβ ligand knockout animals also revealed cardiac defects including VSDs.30

Like PDGFRα, PDGFRβ is expressed in many mesenchymal cells throughout the embryo including cardiac NCCs.44,52 Once cardiac NCCs reach the outflow tract a segregation of PDGF receptor expression occurs, where PDGFRα is associated with the tunica adventitia and mesenchyme throughout the aortic arch and PDGFRβ is expressed predominantly in the neural crest and non-neural crest-derived vascular smooth muscle cell population.44 80% of global PDGFRβ-null animals had some form of cardiac defect including a thin myocardium and VSD, but no PTA or REO was detected.44,50 However, when both PDGFRα and PDGFRβ were deleted in NCCs, PTA and VSD occurred with 100% penetrance, suggesting that within cardiac NCC the two receptors might have overlapping functions. Interestingly, no VSDs were observed in embryos with NCC deletion of PDGFRβ.44 An explanation for this discrepancy is that possibly a non-NCC population could be the cause of the VSD in PDGFRβ mutant embryos or that PDGFB may be the essential ligand for PDGFRα-dependent ventricular septum formation. Similar to the palate, no difference in proliferation or apoptosis was detected in the heart of NCC-specific PDGFR receptor mutants, but a reduction in cells migrating into the conotruncal region was noted.44

**Trunk and Enteric Neural Crest**

Trunk NCCs can differentiate into a wide variety of cell types. These include neurons and glia of the dorsal root and sympathetic ganglion, Schwann cells, adrenal chromaffin cells and pigment cells.52 Additionally, when this cell population is disrupted, it affects thyroid and thymic formation.53 Investigation of NCC-derived neuronal cells in PDGFRα mutants revealed no obvious disruptions in this cell population as judged by staining for early neuronal markers.26,28 While PDGF signaling does not appear to affect neuronal or neuroendocrine cells, it has been implicated in thymus and melanocyte development.

Mice harboring the Ph/Ph mutation have thymic defects ranging from complete absence of the thymus to reduced size in one of the thymic lobes26 and mice with NCC deletion of PDGFRα revealed a low penetrance of thymic hypoplasia.29 More recently, explants of PDGFRαβ-thymic tissue showed a severe loss of thymic mass due to decreased mesenchymal cell proliferation.29 PDGFRβ mutants have no reported thymic defects. By contrast, in mice with NCC deletion of PDGFRα and PDGFRβ thymic absence or hypoplasia was observed consistently.44 These data suggest that combined PDGF receptor signaling may be important during thymus development. Because only a small component of the thymus is neural crest derived, it is likely that inductive signals from PDGF receptor-expressing cells is required for adequate thymic development and that loss of this signaling results in hypoplasia.

The Ph mutation was originally identified as a dominant pigment phenotype.22 Because of its linkage to the kit locus, which encodes a well-known regulator of melanocyte development, there has always been a question of whether the melanocyte defect could be attributed to loss of PDGFRα or if it is due to aberrant kit expression. Support for the latter mechanism occurred when it was found that melanocytes formed in Ph/Ph mutant embryos and, a significant increase in kit receptor expression was observed, reportedly due to loss of cis-regulatory elements.55 Furthermore, the Ph+/+ pigmentation phenotype is not recapitulated in mice heterozygous for null PDGFRα alleles and PDGFRαβ-embryos have similar numbers of TRP-2+ cells (melanocyte precursors) at E10.5.78

Although these data point to overexpression of the kit receptor as the primary melanocyte defect in Ph mice, there is a hint that PDGFRα may also play a direct role in some aspect of melanocyte development. This suggestion comes from the observation that when ES cells are heterozygous for a PDGFRα-null allele, coat color contribution by ES cell-derived melanocytes was significantly reduced.28 From this single result, one could argue that these targeted mutant alleles also lead to a disruption of kit expression. This possibility becomes unlikely because chimeras generated from ES cells with signaling point mutant alleles containing similar targeted genomic arrangements also demonstrate a melanocyte phenotype. The failure to generate ES cell-derived melanocytes was dictated by the severity of the PDGFRα disruption. When a wild type or mild hypomorphic allele (a mutation in the Src binding sites) of the PDGFRα was introduced into ES cells by a targeted mutation, coat color contribution could attain 80–90%. By contrast, when severely hypomorphic alleles were targeted into the ES cells, coat color contribution was never greater than 40%.33 These results suggest that in a competitive environment, such as a chimeric animal, melanocytes or melanocyte precursors with disrupted PDGFRα function fail to compete successfully with melanocytes possessing normal PDGFRα signaling.

**Disease Relevance**

Many of the NCC phenotypes observed in PDGF ligand and PDGF receptor mutant mice are similar to observed human birth defects. Studying these disease models could lead to a better understanding of the etiology, progression and treatment of these anomalies. Below, we will focus on the relationship between human diseases caused by NCC defects and their relationship to PDGF signaling.

There are several congenital anomalies caused by defects in NCC, including DiGeorge syndrome (DGS), cleft lip/palate, VSD, persistent truncus arteriosus (PTA) and cardiac outflow tract malformations. Some of these defects can be traced to the failure of NCC to migrate through the pharyngeal arches. As described above, these defects are also observed in PDGF signaling mutant mice, but the mechanisms causing these phenotypes are unclear and warrant further investigation.

Recently, PDGF signaling has been linked with cleft palate/lip in humans. As described in the mouse, loss of PDGFC expression
leads to cleft palate\textsuperscript{16} and in humans a SNP (rs28999109) in the regulatory region of \textit{PDGFC} was highly associated with cleft palate.\textsuperscript{38} Further analysis of this regulatory region revealed that these differences in the \textit{PDGFC} promoter lead to a decrease in expression using reporter assays to examine gene expression.\textsuperscript{34} This association is the first described linkage to a human birth defect with reduced PDGF signaling and hints at another etiology for cleft palate in human populations.

Recently, evidence has also associated PDGFC expression with birth defects caused by the teratogen retinoic acid. Exogenous addition of either retinoic acid or a PDGF neutralizing antibody resulted in defects in pharyngeal arch derivatives that gave rise to the developing palate. Interestingly, retinoic acid treatment also reduced expression of PDGFR\(\alpha\).\textsuperscript{57} Therefore, the modulation of PDGFR\(\alpha\) signaling during human development may be important for proper cleft palate formation similar to the genetic mutations described in the mouse.

Congenital heart defects are one of the most common birth defects. Those associated with NCCs range from atrial and VSD to complex outflow tract anomalies. There are many etiologies attributed to development of these defects, but there are few studies linking PDGF with human aortic arch defects. However, a recent study identified an association of PDGFR\(\alpha\) in humans with a defect in pulmonary vein patterning. Mutations in the \textit{PDGFR\(\alpha\)} gene in humans resulted in total anomalous pulmonary venous return (TAPVR), which causes oxygenated blood to return to the systemic venous system.\textsuperscript{38} Subsequent analysis confirmed similar defects in animals with reduced or absent PDGF signaling. The developmental origin of the pulmonary vein is controversial, but there is some suggestion that NCCs can contribute to adventitia in the venous poles of the heart (including the pulmonary vein) and dorsal mesocardium.\textsuperscript{59,60} Interestingly, the transgenic \textit{Cre} mouse line used to generate the TAPVR mouse model expresses \textit{Cre} recombine in developing NCCs.\textsuperscript{51}

Therefore, there is the possibility that proper pulmonary vein patterning might depend on PDGFR\(\alpha\) signaling in NCCs. The limited number of reports connecting PDGF signaling with human aortic arch defects might be due to the multifactorial nature of the disease. Further genetic linkage studies may reveal a role for PDGFs in human congenital heart disease.

**Conclusion**

We have summarized the roles of PDGF signaling during NCC development, but many questions remain about the mechanisms behind the various phenotypes observed upon disruption of PDGF signaling. Many of the NCC defects caused by inactivation of individual PDGF receptors or ligands show incomplete penetrance. In several instances combined disruption of both PDGF receptors increased the phenotype frequency to almost 100\%, suggesting partial redundancy within the PDGF receptor family. Therefore, there might be phenotypes in other neural crest-derived tissues that are not apparent when only one receptor is deleted. To fully investigate the mechanisms behind the phenotypes, further use of combined PDGF receptor deletion is necessary.

Considering PDGF's established role as a mitogen and survival factor,\textsuperscript{2,6,3} it is surprising that embryos lacking PDGF receptors in NCCs have no significant alteration in either of these processes. PDGF signaling directs a diverse array of cellular responses, and one of these may be disrupted in PDGF mutant mice. Examples of known PDGF downstream actions are matrix deposition and remodeling,\textsuperscript{7,34} migration,\textsuperscript{16} and cilia function.\textsuperscript{66} On the other hand, there may be a novel role for PDGF in this diverse cell population. Hopefully, future studies will reveal more details regarding the NCC requirement for PDGF signaling.

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