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TGFβ superfamily signaling in the neural crest lineage

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The neural crest cell (NCC) lineage is often referred to as the fourth germ layer in embryos, as its wide range of migration and early colonization of multiple tissues and organ systems throughout the developing body is astounding. Many human birth defects are thought to have their origins within the NCC lineage. Exciting recent conditional mouse targeting and transgenic combinatorial suppression approaches have revealed that the TGFβ superfamily is a key signaling pathway within the cardiac and cranial NCC subpopulations. Given the complexity of TGFβ superfamily signaling and that multiple ligand and receptor combinations have already been shown to be expressed within the NCC subpopulations, and the difficulty in transgenically targeting entire signaling cascades, we review several up-to-date transgenic approaches that are revealing unexpected consequences.

Introduction

NCCs are amazing pluripotent, highly migratory cells, which contribute to the development of multiple different tissues and organs including smooth muscle cells of the cardiac outflow tract, craniofacial bone and cartilage, pigmented cells in the skin, and peripheral and enteric neurons and glia.[1] Formation of NC is induced in dorsal ridges of the developing neural tube as a result of complex interactions between the neuronal and non-neuronal ectoderm and the underlying paraxial mesoderm.[2] Upon induction, NCCs undergo epithelial-to-mesenchymal transformation (EMT); they delaminate and migrate ventrolaterally to populate their target tissues.[3-6] Anterior NCCs from the axial level of the posterior forebrain (diencephalon) to the fifth somite pair populate cranial, facial and pharyngeal structures and are therefore called the cranial NCC.[5,7] Unlike NCCs from other axial levels, these cells are negative for expression of classical homeobox genes, and they have a unique capability to differentiate to connective tissues contributing to the formation of most of the craniofacial bones and cartilage.[8] They also play an important inductive role in development of pharyngeal organs including the thymus and the thyroid and para-thyroid glands.[9] A subpopulation of more caudally located NCCs, from the level of the mid otic vesicle to the level of the third somite pair, give rise to the cardiac NCC.[6,10] These cells migrate along the pharyngeal arch arteries of arches 3, 4 and 6, and contribute to the formation of the smooth muscle cell layer surrounding these vessels. Moreover, a subpopulation of cardiac NCCs migrates deeper towards the base of the aortic sac between the left fourth and sixth aortic arch arteries, where it subsequently contributes to the formation of the aortico-pulmonary septum and are critically required for normal cardiovascular morphogenesis.[11-13]

Understanding of a molecular control of processes that govern different stages of NCCs biology has been greatly facilitated by the development of innovative research tools. First chick-quail chimeras turned out to be highly productive in lineage-tracing studies,[14] and surgical ablation of the various NCC-containing chick embryo neural folds categorically demonstrated the requirement of the cranial and cardiac NCC subpopulations for appropriate cardiac outflow tract and

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craniofacial development.[10] Subsequently, the development of diverse transgenic technologies in mice has allowed a further refinement of both fate determination studies as well as examination of roles of individual genes in NCC induction, migration and differentiation. In this respect, the so-called *Cre-loxP* methodology has played an important role.[15] This method allows abrogation or activation of genes in any specific tissue or cell type. In addition, in conjunction with inducible transgenic techniques, such as doxycyclin or tamoxiphen-mediated transgene induction, exquisite spatiotemporal control of a desired genetic manipulation can be achieved.[16,17] One of the most potent tools to abrogate genes in NCCs is the *Wnt1-Cre* transgenic mouse line.[18] It has been very well validated and used in a large number of studies to analyze a gene function in NCCs. Among numerous key findings, these techniques have facilitated demonstration of an instrumental role of transforming growth factor beta (TGFβ) superfamily signaling in the development of neural crest-derived structures during mammalian embryogenesis.

The TGFβ superfamily includes Bone morphogenetic proteins (Bmps), Growth and differentiation factors (Gdfs), Activins, Nodal, Müllerian inhibitory factor and TGFβs.[19] These secreted growth factors signal via heterotetrameric receptor complexes composed of two type-II and two type-I receptors. Upon ligand binding, the type-II receptor, which is a constitutively active Ser/Thr kinase, transphosphorylates the type-I receptor in the intracellular GS domain (rich in glycine and serine). This event leads to activation of Ser/Thr kinase activity of the type-I receptor, which in turn leads to phosphorylation of cytoplasmic signal transducers, the receptor-mediated Smads (or rSmads). TGFβ signaling is mediated via rSmads 2 and/or 3, while Bmps signal mostly via rSmads 1, 5 and 8. Phosphorylated rSmads form a complex with a common Smad (coSmad), Smad4, which is shared by both TGFβ and Bmp pathways. rSmad/coSmad complexes then accumulate to the nucleus, where they act as transcriptional co-regulators. In addition to rSmads and coSmad, there also are two inhibitory Smads, i.e., Smad6 and 7. Smad7 competes with rSmads by binding to the activated type I receptor, while Smad6 forms a complex with coSmad preventing the function of rSmads.[20-23] In addition to Smad-mediated signaling, ligands in the TGFβ superfamily may signal via Smad-independent pathways leading to activation of a number of different signal transducers, e.g., small Rho-related GTPases, Map kinases (p38 and Jnk), Ikk and PI3-kinase.[24] Interestingly, it seems that some of these so called non-canonical (Smad-independent) signaling processes are not dependent on the kinase activity of the type-I receptor.[25]

NC-Specific TGFβ Receptor Mutants

TGFβs signal via heteromeric complexes composed of two type-II receptors and two type-I receptors. Genes encoding either the TGFβ type-II (*TGFbr2*) and type-I receptors (*TGFbr1* or *Alk5*) have been abrogated in NCCs using the *Wnt1-Cre* driver line[26-30] (summarized in **Table 1**). NC-specific *TGFbr2/Wnt1-Cre* mutants displayed severe craniofacial and cardiac phenotypes. At birth their calvaria were rudimentary, they showed cleft palate and mandibular hypoplasia.[27] More detailed studies revealed that while *TGFbr2*-deficient NCCs migrated normally, postmigratory NCCs lacking *TGFbr2* failed to proliferate normally suggesting that TGFβ signaling via *TGFbr2* is required for appropriate control of a size of the post-migratory NCCs pool. Moreover, *TGFbr2/Wnt1-Cre* mutants displayed interrupted aortic arch and common arterial trunk (also known as persistent truncus arteriosus), which results from the failed septation between the aorta and pulmonary trunk.[28] However, both the pharyngeal organ development and smooth muscle cell differentiation were not affected in *TGFbr2/Wnt1-Cre* mutants.

Based on the established roles of TGFβ type-II and type-I receptors in TGFβ signal transduction, one would have assumed that neural crest-specific abrogation of the gene encoding TGFβ type-I receptor (*TGFbr1 or Alk5)* using the same *Wnt1-Cre* driver line would have resulted in identical embryonal phenotypes with those seen in *TGFbr2/Wnt1-Cre* mutants. However, this turned out not to be the case.[29,30] While superficially *TGFbr2/Wnt1-Cre* and *Alk5/Wnt1-Cre* mutants carried significant phenotypic similarities, e.g., rudimentary calvaria, small mandible, cleft palate and common arterial trunk, the detailed examination revealed that *Alk5/Wnt1-Cre* mutant phenotypes were consistently more severe than those of corresponding *TGFbr2* mutants: the calvaria were even smaller and less well developed, the snout showed less ossification, the mandible was smaller and palatal shelves were more rudimentary. Similarly, the common arterial trunk phenotype was different. Rather than showing interruption of the aortic arch (PTA type A4 according to van Praagh classification), *Alk5/Wnt1-Cre* mutants displayed a single vessel (PTA type A2) accompanied with severe shortening of structures derived from the aortic sac. However, both mutants displayed severe dilatation of vascular structures at sites where the underlying smooth muscle cell layer was of NC origin, which resulted from a poorly organized vascular elastic matrix in late-stage embryos.[31] Moreover, the pharyngeal organ migration failed in *Alk5/Wnt1-Cre* mutants, while in corresponding *TGFbr2* mutants, the pharyngeal organs migrated normally.[27,30] Unlike in *TGFbr2/Wnt1-Cre* mutants, intense post-migratory NCC apoptosis could be seen in *Alk5/Wnt1-Cre* mutants, which will likely explain at least some of the more severe phenotypes seen in *Alk5* mutants when compared to *TGFbr2* mutants. Based on the differences between *Alk5/Wnt1-Cre* and *TGFbr2/Wnt1-Cre* mutants, it was concluded that in NCCs the Alk5 type-I receptor may act in conjunction with type-II receptors other than TGFβRII or that alternatively not all the TGFβ signals are mediated via TGFβRII.[30]

NC-Specific Bmp Receptor Mutants

Similar to TGFβs, Bmps signal via heteromeric receptor complexes composed of two type-II Bmps receptors, and two type-I receptors. NCC-specific *Bmpr2/Wnt1-Cre* mutants display remarkably mild craniofacial and cardiac phenotypes.

Table 1. Neural crest-restricted TGFβ superfamily transgenic mouse lines and their resultant phenotypes

Mutation	Lethal	Migration	NCC Apop	Facial defects	Calvaria defects	OFT	PAA defects	Pharyngeal organ defects	Myocardial wall defects	Defects in OFT elasto-genesis	Defects in SNS
TGFbr2/Wnt1-Cre[27,28,31]	Birth	Normal	-	Yes, CP	Major	CAT, Type 4	-	No	No	Yes	N/A
Alk5/Wnt1-Cre[29,30]	Birth	Normal	+	Yes, Midfacial cleft, CP	Major	CAT, Type 2	+	Yes	No	Yes	N/A
Bmpr2/Wnt1-Cre[32]	Birth	Normal	-	N/A	N/A	DORV	-	No	No	No	N/A
Alk3/Wnt1-Cre[33,34]	E12.0	Normal	+	N/A	N/A	CAT	N/A	N/A	Yes	N/A	Yes
Alk2/Wnt1-Cre[36,37]	E14-birth	Affected (proximal OFT)	-	Yes, CP	Minor	CAT, Type 2	+	No	No	No	N/A
Smad4/Wnt1-Cre[35,38-41]	E12.5	Affected (proximal OFT)	+	Yes, Midfacial cleft	Major	CAT	-	N/A	Yes	N/A	N/A
Trigenic *Smad7*[42] (induced at E7.5)	Birth	Affected (proximal OFT)	+	Yes	Major	CAT, Type 2	-	No	No	No	N/A

OFT, outflow tract; CP, cleft palate; CAT, common arterial trunk; DORV, double outflow tract right ventricle; SNS, sympathetic nervous system; N/A, not available.

Only abnormal positioning of the aorta was reported in reference 32. This will likely reflect the fact that in addition to BmpRII, Bmps can also signal via Activin type-II receptors A and B.

In contrast to the *Bmpr2/Wnt1-Cre* mutants, NCC-specific *Bmpr1a (Alk3)* mutants displayed very severe phenotypes and died around embryonal days 11.5–12.0.[33,34] NCCs deficient in *Alk3* showed normal specification and migrated normally. Moreover, their initial differentiation appeared normal. However, the mutants displayed a common arterial trunk and thin ventricular wall.[33] It also was shown that NCC-specific *Alk3* mutants had hypoplastic outflow tract cushions, which resulted in a reversed diastolic arterial blood flow.[34] These authors suggested that the primary defect in *Alk3* mutant embryos was the defective proliferation of NC-derived cushion mesenchymal cells. It was not shown whether the involvement of Bmp signaling via Alk3 in regulation of cell proliferation was direct and whether non cell-autonomous effects would play a role, particularly in the proximal cushions, which are not colonized by the cardiac NC. Subsequent studies have demonstrated that Bmp signaling via Alk3 is required for appropriate formation, growth and differentiation of the sympathetic ganglia.[35] While proliferation and differentiation of the sympathetic ganglia were mediated by the Smad-dependent signaling, survival of sympathetic nervous system precursors was controlled by Smad-independent mechanisms. Interestingly, administration of the β-adrenergic agonist isoproterenol rescued the embryonic lethal phenotype of *Alk3/Wnt1-Cre* mutants demonstrating that the reason for the embryonic death was norepinephrine insufficiency.[35]

In addition to Alk3, Bmps can also signal via Bmp type-I receptors Bmpr1b (Alk6) and Acvr1a (Alk2). While Alk6 is not required for any major non-redundant functions in NCCs, Alk2 seems to mediate several functions, some of which are overlapping with Alk3 while others seem to be unique for Alk2.[36,37] Like in other NC-specific TGFβ superfamily receptor mutants, the overall migration of NCCs in *Alk2/Wnt1-Cre* mutants was unaffected. The mutants displayed a shortened snout, low hanging ears, hypoplastic frontal bones and a small mandible. A rate of cell proliferation in Meckel's cartilage, which functions as a template for the developing mandible, was attenuated in *Alk2/Wnt1-Cre* mutants. Moreover, they displayed defective palatogenesis, which was due to a failure in palatal shelf elevation.[36]

Alk2/Wnt1-Cre mutants also displayed a spectrum of cardiac and vascular abnormalities.[37] They showed common arterial trunk (Type 2) and abnormal patterning of the pharyngeal arch arteries. Unlike in TGFβ mutants, which showed a seemingly normal cardiac NCC migration, in *Alk2/Wnt1-Cre* mutants, NCCs failed to enter into the proximal OFT, which likely contributes to the development of the common arterial trunk phenotype. Moreover, in *Alk2/Wnt1-Cre* mutants, smooth muscle cells surrounding the pharyngeal arch arteries failed to differentiate appropriately, which subsequently led to regression of both the third, and particularly the sixth arch arteries. About 50% of the *Alk2/Wnt1-Cre* embryos died between embryonal days 14 and 16, while the rest succumbed soon after birth. Reasons behind the gestational death are currently not known. However, since NC-specific *Alk2* and *Alk3* mutants often display similar, albeit not identical, developmental phenotypes without detectable functional redundancy, it is possible that defects in

the sympathetic ganglia could explain the partial embryonic lethality in *Alk2/Wnt1-Cre* mutants as well.

NC-Specific Smad4 Mutants

Smad4, the only known mammalian co-Smad, has traditionally been thought to transduce all the Smad-dependent (canonical) TGFβ superfamily signals. Several studies have described abrogation of *Smad4* in NCCs.[38-41] In *Smad4/Wnt1-Cre* mutants, initial specification and migration of NCCs seemed to be unaffected. However, *Smad4/Wnt1-Cre* mutants died around E12.5 and displayed several craniofacial and cardiac defects. Both frontonasal processes and the mandibular arch were hypoplastic and failed to fuse in the midline, the trigeminal ganglia were hypoplastic and ectomesenchymal patterning in the first pharyngeal arch was altered. Moreover, *Smad4/Wnt1-Cre* mutants displayed common arterial trunk, hypoplastic OFT cushions, and like in *Alk2/Wnt1-Cre* mutants, NCCs defective in *Smad4* failed to migrate to the proximal OFT. Patterning of pharyngeal arch arteries was normal, and no differences in smooth muscle cell differentiation were reported. However, *Smad4/Wnt1-Cre* mutants displayed a similar increase in apoptosis of post-migratory NCCs as seen in *Alk5/Wnt1-Cre* mutants and the thin myocardium as seen in *Alk3/Wnt1*-Cre mutants. Since the embryonic death coincides with the thin myocardium (both *Smad4* and *Alk3* mutants) and defective sympathetic nervous system (*Alk3*) mutants, it is likely that the myocardial defects are secondary to insufficient noradrenergic differentiation of sympathetic neurons. Taken together, phenotypic comparison between different NCC-specific TGFβ superfamily receptor mutants and corresponding *Smad4* mutants demonstrates that most of the TGFβ and BMP signaling processes in the NC are mediated via the Smad-dependent (canonical) signal transduction pathway.

NC-Specific TGFβ Suppression and Activation Mutants

Taking advantage of Smad7's ability to act as a negative regulator of TGFβ superfamily signaling and *Cre-loxP* technology, a novel three-component triple transgenic system was recently generated to examine the combinatorial effects of simultaneous suppression of TGFβ/BMP signaling within the *Wnt1-Cre* marked NCC lineage.[42] When Smad7 was induced via doxycycline within the NCC lineages at pre-migratory/EMT stages, craniofacial, pharyngeal arch and cardiac OFT septation defects resulted. Significantly, while initial cranial and cardiac NC emigration and migration were unaffected despite significantly suppressed phosphorylation levels of both Smad1/5/8 and Smad2/3 in vivo, increased cell death was observed in pharyngeal arches and facial mesenchyme, coincident with differentiation of the NCC. While many of the phenotypes of the NC-specific trigenic *Smad7* mice are in a complete agreement with phenotypes of the corresponding Bmp and TGFbeta receptor mutants, there also are some differences. Most notably, the trigenic *Smad7* mice (induction at E7.5) do not show defects in the myocardium, OFT elastogenesis or pharyngeal organs, and they survive until the birth (**Table 1**). The reasons for these differences are currently not known. Perhaps, the transgene induction at E7.5 is not early enough, maybe the initial concentration of Smad7 is not sufficiently high to inhibit all the signaling aspects in pre/early migratory NCCs, or alternatively, it may be that simultaneous suppression of both TGFβ and Bmp signaling is less harmful than loss of individual TGFβ superfamily signaling components. Interestingly, induction of Smad7 in post-migratory NCC resulted in interventricular septal chamber, septation defects but no craniofacial abnormalities, suggesting that TGFβ superfamily signaling is essential for cardiac NCC at post-migratory stages but not during cranial NCC differentiation.[42] Given the almost complete absence of experimental data to address the role of the cardiac NCC within the heart itself, these spatiotemporally inducible transgenic approaches are bound to lead to new insights as to the requirement of TGFβ superfamily and NCC post-colonization of target tissues. Similarly, the dedicated Bmp antagonist Noggin has been elegantly employed as a suppression tool to probe the restricted differentiation capabilities of the most caudal trunk NCC subpoulation.[43] More recently, transgenic expression of constitutively active forms of Alk3 and Alk6 as well as Noggin have been used to drive excessive and reduced Bmp signaling within the developing palate, demonstrating that restriction of Bmp signaling is as important as stimulation in normal craniofacial development.[44] Use of these spatiotemporally regulatable suppression and induction systems will likely play an increasingly important role in further detailed analysis of TGFβ superfamily signaling in the NCC development.

Future Directions

As the TGFβ superfamily is known to be required for normal tissue development and homeostasis, and aberrant TGFβ expression and signaling have been implicated in numerous NC-related disease states, more than a few transgenic approaches have been used to molecularly probe its requirement and function. Several different reporter mouse lines have been generated to detect activation of either TGFβ or Bmp-responsive reporter genes.[45-47] While these mouse lines can be used to provide valuable information about activation of canonical BMP and TGFβ pathways in defined time points during development of NCC-derived structures, advanced dynamic information can also be obtained using corresponding zebrafish reporter lines.[48] Systemic deletion of individual TGFβ members has revealed the initial unique requirement of several of family members, but usually these mouse mutants are either lethal very early in development or appear largely unaffected. The effects of genetic redundancy, parallel pathways, synergist properties and largely unresolved crosstalk between individual family members and other signaling pathways,[49] is thought to underlie some of the absence of phenotype and perplexing (and on occasion contradictory) results. *Cre-loxP* conditional targeting of single and double TGFβ ligands and receptors has dramatically increased our understanding of their lineage-specific roles and circumvented early lethality. However, despite their obvious power and appeal, there are disadvantages to using conditional targeting strategies. Most critically, the lack of

availability of enough precise lineage and subpopulation-restricted promoters to drive site-specific Cre and Flp recombinases and the possibility of incomplete recombination are important confounding caveats. The advent of temporal mutagenesis via tamoxifen induction of recombinase-estrogen receptor fusion proteins is extending our ability to perform more defined and postnatal conditional targeting. Recently a Cre-like recombinase, Dre, has been added to the molecular toolbox,[50] increasing the likelihood of future combinatorial lineage-restricted targeting in compound mice models. The addition of doxycyclin-inducible combined TGFβ superfamily suppressors and signaling pathway activators such as the Smad7/Noggin and constitutively active receptor approaches only adds to the exciting array of transgenic tools to enable upcoming precise manipulation of the TGFβ superfamily within the NCC sublineages at different stages of morphogenesis and homeostasis.

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