RESEARCH COMMUNICATION

Inactivation of TGFβ signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome

Heiko Wurdak,[1,5] Lars M. Ittner,[2,5] Karl S. Lang,[3] Per Leveen,[4] Ueli Suter,[1] Jan A. Fischer,[2] Stefan Karlsson,[4] Walter Born,[2] and Lukas Sommer[1,6]

[1]Institute of Cell Biology, Department of Biology, Swiss Federal Institute of Technology, ETH-Hönggerberg, Zurich, CH-8093, Switzerland; [2]Research Laboratory for Calcium Metabolism, University Hospital Balgrist, University of Zurich, Zurich, CH-8008, Switzerland; [3]Institute of Experimental Immunology, University Hospital, University of Zurich, Zurich, CH-8091, Switzerland; [4]Departments for Molecular Medicine and Gene Therapy, Lund University, Malmö, S-22184, Sweden

Specific inactivation of TGFβ signaling in neural crest stem cells (NCSCs) results in cardiovascular defects and thymic, parathyroid, and craniofacial anomalies. All these malformations characterize DiGeorge syndrome, the most common microdeletion syndrome in humans. Consistent with a role of TGFβ in promoting non-neural lineages in NCSCs, mutant neural crest cells migrate into the pharyngeal apparatus but are unable to acquire non-neural cell fates. Moreover, in neural crest cells, TGFβ signaling is both sufficient and required for phosphorylation of CrkL, a signal adaptor protein implicated in the development of DiGeorge syndrome. Thus, TGFβ signal modulation in neural crest differentiation might play a crucial role in the etiology of DiGeorge syndrome.

Supplemental material is available at http://www.genesdev.org.

Received July 16, 2004; revised version accepted December 23, 2004.

During development, neural crest cells emerge from the dorsal part of the neural tube and emigrate to various locations within the embryo to generate most of the peripheral nervous system and a variety of other structures (Le Douarin and Dupin 2003). In particular, neural crest cells localized in the pharyngeal apparatus contribute to non-neural tissues, such as craniofacial bone and cartilage, thymus, parathyroid glands, and cardiac outflow tract and septum (Kirby and Waldo 1995; Jiang et al. 2000; Graham 2003). The function of neural crest cells in the generation of these tissues, however, has been debated (Graham 2003).

Formation of the pharyngeal apparatus involves complex interactions of neural crest, ectoderm, endoderm, and mesoderm whose development must be coordinated (Graham 2003). Significantly, alterations to the development of this region are often associated with congenital human birth defects such as DiGeorge or Velocardiofacial syndrome. DiGeorge syndrome is the most common microdeletion syndrome in humans, characterized by cardiovascular defects plus thymic, parathyroid, and craniofacial anomalies (Lindsay 2001; Vitelli and Baldini 2003). Approximately 80% of the patients carry a variably sized deletion on chromosome 22 (*del22q11*). Ablation of genes affected by the microdeletion indicated two pathophysiological mechanisms causing DiGeorge syndrome: Mutations of the transcription factor Tbx1 lead to disturbed pharyngeal arch patterning. Consequently, neural crest cells are unable to populate the pharyngeal apparatus (Jerome and Papaioannou 2001; Lindsay et al. 2001; Merscher et al. 2001). However, upon deletion of the gene encoding the signal adaptor protein CrkL, neural crest cells localize to the pharyngeal apparatus, but they do not form non-neural derivatives from this structure (Guris et al. 2001). In both cases, aberrant neural crest cell migration, survival, or differentiation might contribute to DiGeorge syndrome (Lindsay 2001; Vitelli and Baldini 2003). A direct role of neural crest cells in the development of the pharyngeal apparatus, however, was not demonstrated by the analysis of these mutants. Furthermore, the signals specifying non-neural fates from neural crest cells in the pharyngeal apparatus have not yet been identified.

Previously, cell culture experiments allowed the identification of several growth factors able to instruct migratory and post-migratory neural crest cells to adopt specific lineages (Le Douarin and Dupin 2003; Lee et al. 2004). One of these factors is transforming growth factor (TGF) β, which elicits multiple responses in cultured neural crest stem cells (NCSCs). Depending on the cellular context, it can promote the generation of non-neural smooth-muscle-like cells or autonomic neurons, or induce apoptosis (Shah et al. 1996; Hagedorn et al. 1999, 2000). Here we investigated the role of TGFβ signaling in NCSCs and their derivatives in vivo, with an emphasis on migration, non-neural fate decision, and differentiation processes of neural crest cells in the pharyngeal apparatus.

Results and Discussion

To address the role of TGFβ signaling in neural crest development in vivo, we have used the *cre/loxP* system to conditionally inactivate the *TGFβ receptor type II* (*TβRII*) gene essential for TGFβ signal transduction (Leveen et al. 2002). *Wnt1-Cre*-mediated recombination of a floxed allele of *TβRII* resulted in loss of TβRII protein in neural crest cells isolated from mutant embryos and in mutant neural crest derivatives in vivo (Fig. 1), consistent with the previously reported activity of *wnt1-Cre* in virtually all neural crest cells (in addition to its expression in the mid/hindbrain area) (Brault et al. 2001; Lee et al. 2004). At embryonic day 10.5 (E10.5), mutant embryos did not exhibit obvious malformations, and

[*Keywords*: Neural crest; TGFβ; CrkL; Src kinase; fate decision; pharyngeal apparatus; DiGeorge syndrome]
[5]These authors contributed equally to this work.
[6]Corresponding author.
E-MAIL lukas.sommer@cell.biol.ethz.ch; FAX 41-1-633-10-69.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.317405.

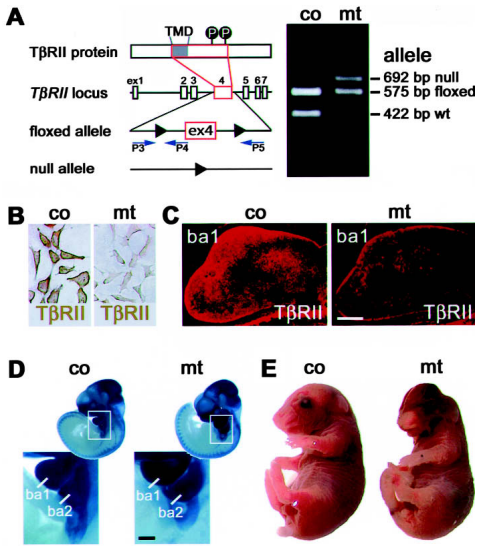

Figure 1. Neural crest-specific conditional ablation of *TβRII*. (*A*) Exon 4 (ex4) of the *TβRII* locus, encoding the transmembrane domain (TMD) and the intracellular phosphorylation sites (P) of the TβRII protein, is flanked by loxP-sites and deleted in NCSCs upon breeding with *wnt1-Cre* mice. Identification of the *floxed TβRII* allele in control (co) and of the recombined *TβRII*-null allele in mutant (mt) animals by PCR with indicated primers P3, P4, and P5 (Leveen et al. 2002). (*B*,*C*) Presence and absence of TβRII (brown/red) in primary NCSC explants (*B*), and in neural crest cells populating the branchial arch 1 (ba1) in control and mutant embryos at E11.5 (*C*). (*D*) Normal distribution of βGal-expressing neural crest cells in the pharyngeal apparatus of E10.5 mutant mice, as assessed by in vivo fate mapping. Branchial arch 1 (ba1) and 2 (ba2) in enlarged areas marked by boxes. (*E*) Overall appearance of control (co) and mutant (mt) mice at E18.5. Note the craniofacial anomalies in the mutant. Bars, 200 µm.

TβRII-deficient neural crest cells migrated correctly into the pharyngeal apparatus, as assessed by in vivo fate mapping using the *ROSA26* Cre reporter mouse line (Fig. 1D; Jiang et al. 2000; Hari et al. 2002). Mutants were recovered at the expected Mendelian frequency until E18.5, but they died perinatally, displaying multiple developmental defects including mid/hindbrain abnormalities not covered in this study (Fig. 1E). Control littermates carrying a nonrecombined *TβRII* allele showed no anomalies.

Malformations of cranial bones and cartilage as well as cleft palate were found in all mutant mice examined at E18 (Fig. 2A,B), as described (Ito et al. 2003). As cleft palate together with craniofacial dysmorphism are typical features of DiGeorge syndrome in humans, we examined mutant mice at different developmental stages for other defects characterizing DiGeorge syndrome. Patients suffering from DiGeorge syndrome have absent or hypoplastic parathyroid and thymus glands (Kirby and Waldo 1995). Similarly, in all *TβRII*-mutant mice at E18, staining of serial cross-sections of the neck for parathyroid hormone indicated either hypoplastic (three of five) or undetectable (two of five) parathyroid glands (Fig. 2C). Likewise, the size of the thymus glands was reduced in mutant mice at E18 (58% ± 7% of control size; $n = 8$; $p < 0.01$). In normal mouse development, mesenchymal derivatives of neural crest cells are transiently detectable in the cortical region of the developing thymus and parathyroid glands (Fig. 2D; Jiang et al. 2000). Moreover, elimination of cephalic premigratory neural crest in chicken and absence of neural crest in the pharyngeal apparatus of transgenic mice results in hypoplastic parathyroid and thymic glands (Kirby and Waldo 1995; Vitelli and Baldini 2003). This suggests that development of pharyngeal arch-derived glands might depend on interactions between neural crest-derived cells and pharyngeal endoderm. Consistent with the hypothesis that neural crest cells are important for proper thymus gland formation, neural crest-specific inactivation of TGFβ signaling resulted in a reduction of neural crest cells found by in vivo fate mapping in the cortex of the developing thymus at E13.5 (Fig. 2D).

The main cause of mortality in DiGeorge patients is congenital heart defects such as a single truncus arteriosus and a ventricular septal defect (VSD) (Kirby and Waldo 1995). Aberrant development of cardiac neural crest that normally contributes to the heart and its outflow tract is thought to cause these defects. This assumption is based on genetic manipulations in mouse models and on cardiac neural crest ablation in chicken, which leads to a truncus arteriosus and a VSD (Kirby and Waldo 1995; Vitelli and Baldini 2003). Recently, neural crest-specific inactivation of the type I bone morphogenic protein (BMP) receptor Alk2 resulted in cardiac outflow tract malformations (but not in other tissue anomalies also associated with DiGeorge syndrome), indicating a role of TGFβ family factors in cardiovascular development (Kaartinen et al. 2004). To determine whether the prenatal lethality in the *TβRII*-mutant mice might result from heart defects, we examined the hearts of mutant mice at E18 histologically and by intracardial ink injection. All mutant hearts ($n = 18$) displayed a truncus arteriosus together with a VSD (Fig. 3). In addition, we found abnormal patterning of the arteries arising from the aortic arch, as also found in DiGeorge syndrome patients (Fig. 3D; Kirby and Waldo 1995). Overall, these heart defects led to functional right-sided heart

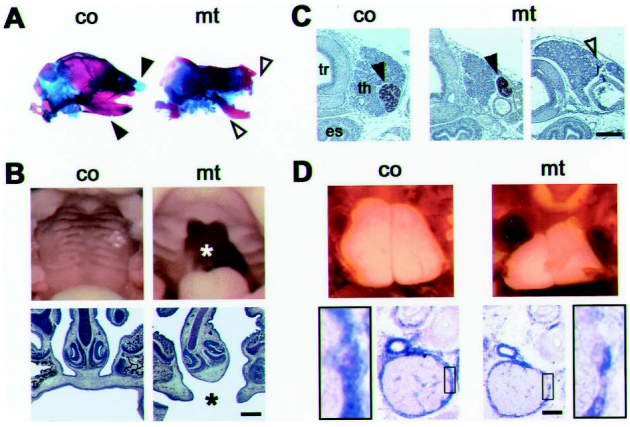

Figure 2. Features of DiGeorge syndrome in *TβRII* mutant mice. (*A*) Hypoplastic or absent bone (red) and cartilage (blue) structures (open arrowheads) in mutant (mt) mice at E18. (co) Control. (*B*) Cleft palate (*) in macroscopic view (*top*) and on frontal sections (*bottom*) in mutant (mt) mice at E18. (*C*) Parathyroid glands stained for parathyroid hormone (brown; arrowhead) were hypoplastic or undetectable in mutant (mt) embryos at E18 (open arrowhead). (tr) Trachea; (es) esophagus; (th) thyroid gland. (*D*) Hypoplastic thymus in mutant (mt) mice at E18 (*top*), correlating with fewer cortical βGal-expressing neural crest cells (blue) compared to control (co) at E13.5 (*bottom*). Bars: *B*,*C*, 200 µm; *D*, 100 µm.

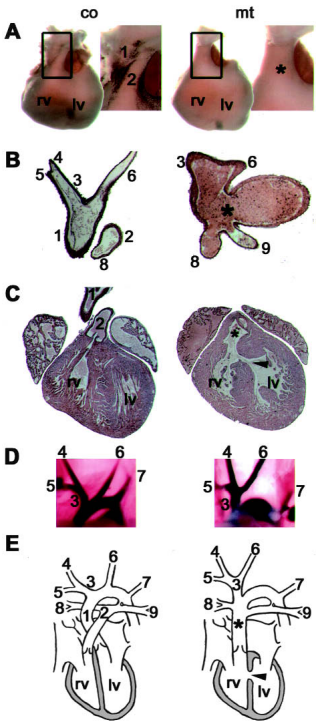

Figure 3. Malformations in the heart of *TβRII* mutant mice. (*A*) Ventricular heart at E18 with detailed view of the paired outflow tract in control (co) and of the truncus arteriosus (*) in mutant (mt) mice. (rv) Right ventricle; (lv) left ventricle; (1) aorta; (2) pulmonary trunk. (*B*) Frontal section of the normal outflow tract and the truncus arteriosus (*). (*C*) Frontal section of the heart in control and mutant with defective septum between right ventricle (rv) and left ventricle (lv; arrowhead). (*D*) Abnormal branching of the left carotid artery from the brachiocephalic trunk in mutant mice visualized by intracardial ink injection. (*E*) Schematic view of abnormal branching and heart defects in the mutant compared to wild-type anatomy. The mutant's left carotid artery (6) arises from the brachiocephalic trunk (3), and the pulmonary arteries (8, 9) originate in the truncus arteriosus (*). (4) Right carotid artery; (5) right subclavian artery; (7) left subclavian artery.

failure with venous congestion, resulting in a vasodilatation of the jugular veins (data not shown), a characteristic clinical feature of patients with severe cardiac insufficiency.

Taken together, our findings show that mice with a mutation of *TβRII* in NCSCs develop all the morphological features of DiGeorge syndrome. The question arises, whether these developmental defects are due to impaired migration, programmed cell death, and/or a failure in fate decision or differentiation of neural crest cells. In vivo fate mapping revealed that, as in the control, mutant neural crest cells were able to populate the pharyngeal apparatus and the forming aorto-pulmonary septum at E10.5 (Figs. 1, 4). Likewise, expression of the NCSC-marker Sox10 (Paratore et al. 2001) and migration were not affected in *TβRII*-deficient neural crest cells emanating from neural tubes in explant cultures (data not shown). As in the control, neural crest-derived cells in the pharyngeal apparatus of mutant mice lost sox10 expression while expressing the neural crest markers pax3 and ehand (data not shown). In addition, we did not observe differences in the number of apoptotic cells in the pharyngeal apparatus of control and mutant mice at E10.5 (data not shown).

Our data indicate that neural crest cell differentiation in the pharyngeal apparatus rather than migration or cell survival is defective in the absence of TGFβ signaling. To test this hypothesis, we analyzed the expression of smooth muscle α-actin in neural crest derivatives, since TGFβ is known to induce a smooth-muscle-like cell fate in NCSCs in vitro (Shah et al. 1996). While in the control at E10.5, βGal-expressing neural crest derivatives in the prospective aorto-pulmonary septum stained for smooth muscle α-actin, *TβRII*-mutant neural crest cells in this region did not contribute to the development of the smooth musculature (Fig. 4B). We therefore suggest that the non-neural, smooth muscle α-actin-positive cells (which have also been termed myofibroblasts and smooth muscle-like cells) (Hagedorn et al. 1999; Morrison et al. 1999) generated from TGFβ-treated NCSCs in culture indeed represent smooth muscle cells. At later developmental stages, neural crest cells that failed to adopt a smooth muscle cell fate underwent apoptotic cell death (data not shown). The absence of neural crest-derived smooth muscle cells in mutants explains the defective separation of the aorta from the pulmonary trunk, leading inevitably to a truncus arteriosus (Fig. 3).

The transcription factor Sox9 is required for the specification of an osteochondroprogenitor cell from neural crest cells, and conditional inactivation of *sox9* in NCSCs results in craniofacial anomalies (Mori-Akiyama et al. 2003). During normal development sox9 is expressed in neural crest cells in the first and second pharyngeal arches, which give rise to the craniofacial skeleton. In contrast, sox9 expression was markedly reduced in *TβRII*-mutant neural crest cells populating the first and second pharyngeal arches (Fig. 4A). These data indicate that neural crest cells are unable to acquire an osteochondroprogenitor cell fate in the absence of TGFβ signaling, which leads to the malformations of cranial bones and cartilage observed in *TβRII*-mutant mice (Fig. 2A).

The microdeletion on chromosome 22 (*del22q11*) found in most of the DiGeorge syndrome patients encompasses ~30 genes but does not include genes encod-

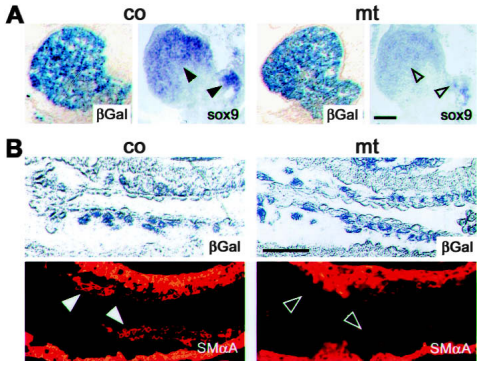

Figure 4. *TβRII*-mutant neural crest cells fail to acquire non-neural fates. (*A*) Equal distribution of βGal-expressing neural crest cells (blue) in the first and second branchial arch of control (co) and *TβRII*-mutant (mt) mice at E10.5. Presence (arrowheads) and absence (open arrowheads) of sox9, required for normal craniofacial bone and cartilage development, in branchial arches. (*B*) Presence of βGal-expressing neural crest cells (blue) in the developing aorto-pulmonary septum of mutant embryos at E10.5. Mutant neural crest cells fail to differentiate into smooth muscle α-actin (SMαA)-expressing cells (red) forming the septum. Bars, 100 μM.

ing either TGFβ receptors or its ligands (Lindsay 2001; Vitelli and Baldini 2003). Thus, gene products eliminated by *del22q11* may functionally interact with TGFβ signaling in neural crest cells. For instance, orthologs of *PCQAP*, a gene within the microdeleted region (Berti et al. 2001), have been shown to modulate TGFβ signaling (Kato et al. 2002), although the role of *PCQAP* in the development of the pharyngeal apparatus is unknown. Inactivation of two of the genes deleted in *del22q11*, *Tbx1* and *Crkol*, phenocopies symptoms of DiGeorge syndrome (Lindsay 2001; Vitelli and Baldini 2003). In *Tbx1* mutants, early steps in pharyngeal arch patterning are disrupted, which, as a secondary effect, interferes with neural crest migration into the pharyngeal arch complex (Vitelli et al. 2002; Vitelli and Baldini 2003). Similarly, manipulation of genes that genetically interact with *Tbx1*, such as *Fgf8* and *Vegf*, affect migration and/or survival of neural crest cells into the pharyngeal apparatus (Vitelli and Baldini 2003). Inactivation of any one of *Tbx1*, *Fgf8*, or *Vegf* results in abnormal patterning of the aortic arch arteries, leading to conotruncal heart defects (Vitelli and Baldini 2003). In contrast, we found that *Tbx1* expression was not altered in mice carrying a neural crest-specific mutation of *TβRII* (data not shown) and that the development of DiGeorge-like syndrome in these mice is due to a failure in non-neural fate acquisition and differentiation rather than in migration or survival of mutant neural crest cells. Moreover, intracardial ink injection at E10.5 revealed normal formation of the aortic arch arteries in *TβRII*-mutant mice (data not shown). Thus, although we cannot exclude it at present, the differences in phenotypes do not support an association between a *Tbx1* genetic pathway and TGFβ signaling in neural crest cells.

Ablation of the *del22q11* gene *Crkol* in mice, however, generates a phenotype reminiscent of that obtained by TGFβ signal inactivation in NCSCs, although with a lower penetrance (Guris et al. 2001). While pharyngeal arch patterning is normal, *Crkol*-mutant mice develop cardiovascular and mild craniofacial anomalies, as well as hypoplastic thymic and parathyroid glands. These deficiencies conceivably result from a post-migratory defect of neural crest cells lacking *Crkol* (Guris et al. 2001). This is analogous to the deficiencies observed in mice with *TβRII*-mutant neural crest cells, which also result from post-migratory defects (Figs. 4, 5). The gene product of *Crkol*, CrkL, belongs to the Crk family of adaptor proteins that are involved in different signaling pathways (Feller 2001). In particular, CrkII—which shares 60% identical residues with CrkL—mediates oncogenic transformation induced by epidermal growth factor and TGFβ (Kizaka-Kondoh et al. 1996), and overexpression of a dominant negative mutant of CrkII prevents TGFβ-induced cell differentiation in vitro (Ota et al. 1998). Therefore, CrkL might be involved in TGFβ-dependent differentiation of neural crest cells. To address this hypothesis, we first assessed the status of CrkL phosphorylation during development of the pharyngeal apparatus. Tyrosine-phosphorylated CrkL was readily detectable in several structures of the wild-type pharyngeal apparatus at E10, including the pharyngeal arches 1 and 2 and the prospective aorto-pulmonary septum (Fig. 5A; Supplementary Fig. 1). However, phosphorylation of CrkL was restricted to a narrow, early time window during development of the pharyngeal apparatus and was no longer observed from E11.5 onward (Supplementary Fig. 1). Intriguingly, CrkL phosphorylation was impaired in *TβRII*-deficient neural crest cells in the pharyngeal apparatus at E10, concomitant with absence of TGFβ-dependent phosphorylation of Smad2 in the mutant (Fig. 5A; Supplementary Fig. 1). Moreover, TGFβ signaling promoted CrkL phosphorylation in an NCSC line that gives rise to smooth-muscle-like cells in response to TGFβ (Fig. 5B,C; Sommer et al. 1995; Chen and Lechleider 2004). This demonstrates that CrkL phos-

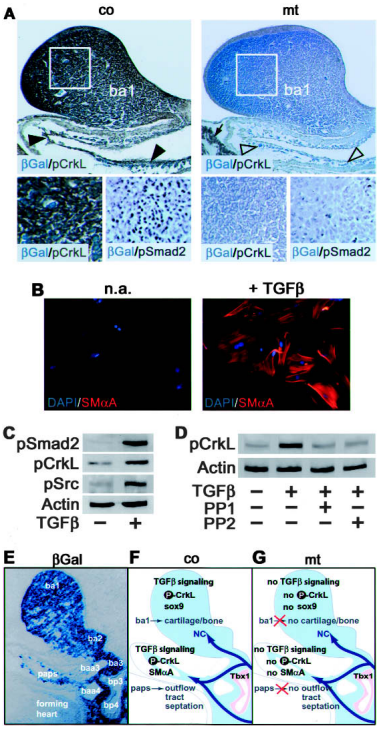

Figure 5. TGFβ-dependent CrkL phosphorylation and neural crest differentiation. (*A*) Mutant (mt) neural crest cells expressing βGal (blue) in branchial arch 1 (ba1), branchial arch 2 (Supplementary Fig. 1), and the developing aorto-pulmonary septum (arrowheads) at E10 show strongly reduced phosphorylation of CrkL (pCrkL; brown; gross and detailed view) and lack nuclear phospho-Smad2 (brown; detailed view) compared with control (co). Note comparable staining intensity for phosphorylated CrkL in βGal-negative tissue (arrow) in mutant (mt) and control (co). (*B*) Immortalized NCSCs (Monc1 cells) express smooth muscle α-actin (SMαA) upon treatment with TGFβ, while untreated (n.a.) cells are SMαA-negative. (*C*) Western blot analysis reveals increased Smad2, CrkL, and Src phosphorylation by TGFβ in Monc1 cells. (*D*) CrkL phosphorylation in TGFβ-treated Monc1 cells is reduced to levels of untreated cells in the presence of Src kinase inhibitors PP1 and PP2, respectively. (*E*) βGal-expressing neural crest cells populate the pharyngeal apparatus (saggital section). (ba1–3) Branchial arches 1–3; (bp3/4) branchial pouches 3/4; (baa3/4) branchial arch arteries 3/4; (paps) prospective aorto-pulmonary septum. (*F*) Neural crest cells (NC; blue) present in the pharyngeal apparatus require TGFβ-signaling for CrkL phosphorylation and for expression of the non-neural markers sox9 in the first branchial arch and SMαA in the forming septum of the heart outflow tract. (*G*) Similar to neural crest cells in *Crkol*-mutant mice, *TβRII*-deficient neural crest cells migrate normally into the pharyngeal apparatus. Here, the signal adaptor protein CrkL fails to be phosphorylated in response to TGFβ, and mutant neural crest cells fail to express sox9 and SMαA. Tbx1 expression in the branchial pouch endoderm and early pharyngeal arch patterning is not affected. The failure of mutant neural crest cells to acquire non-neural fates in the early pharyngeal apparatus impairs the development of tissues derived from the pharyngeal apparatus and leads to a DiGeorge-like phenotype.

phorylation is downstream of TGFβ signaling in neural crest cells. Thus, CrkL might mediate TGFβ-dependent non-neural fate acquisition of neural crest cells at early stages of pharyngeal apparatus development. Conceivably, at later developmental stages TGFβ signaling might have CrkL-independent activities in neural crest-derived structures, as suggested by the milder phenotype of *Crkol*-mutant mice (Guris et al. 2001) as compared to the mutant described here.

TGFβ elicits a wide range of cellular processes, involving activation and cross-talk of multiple signaling pathways (Derynck and Zhang 2003). For instance, TGFβ signaling has been reported to induce phosphorylation and activation of Src tyrosine kinase regulating TGFβ-dependent apoptosis, cell migration, or tumor cell invasiveness (Fukuda et al. 1998; Kim and Joo 2002; Park et al. 2004; Tanaka et al. 2004). Interestingly, a recent report identified CrkL as a downstream signaling component of Src kinase in integrin-induced migration (Li et al. 2003). Together, these studies point to a potential association between TGFβ, Src, and CrkL signaling. In agreement with this hypothesis, levels of phosphorylated active Src were increased by TGFβ treatment of an NCSC line, concomitant with increased phosphorylation of CrkL (Fig. 5C). Importantly, the pharmacological Src inhibitors PP1 and PP2 reduced CrkL phosphorylation in TGFβ-treated neural crest cells to levels of untreated cells (Fig. 5D). These data indicate that Src kinase activity is involved in TGFβ-dependent phosphorylation of CrkL, possibly linking TGFβ signaling to the etiology of DiGeorge syndrome in humans.

In conclusion, we propose that TGFβ isoforms expressed in the pharyngeal apparatus (Millan et al. 1991) promote non-neural fate decisions in post-migratory neural crest cells, allowing these cells to contribute to multiple craniofacial, cardiovascular, and glandular structures (Fig. 5E–G). Accordingly, inactivation of TGFβ signaling in neural crest cells prevents development of these structures and recapitulates all morphological features of DiGeorge syndrome.

Materials and methods

Generation of mutant animals

Animals homozygous for the *TβRII* floxed allele (Leveen et al. 2002) were mated with *wnt1-Cre* mice heterozygous for the floxed allele (Hari et al. 2002). For in vivo fate mapping of NCSCs, *wnt1-Cre* mice heterozygous for the *TβRII* floxed allele were mated with animals homozygous for the *TβRII* floxed allele and carrying a *ROSA26* Cre reporter (R26R) allele, which expresses β-galactosidase (βGal) upon Cre-mediated recombination (Soriano 1999). Genotyping was performed as described (Hari et al. 2002; Leveen et al. 2002).

Staining procedures

In situ hybridization with digoxigenin-labeled riboprobes and detection of LacZ-reporter gene expression was performed as described (Hari et al. 2002). Whole-mount X-Gal-stained embryos were paraffin-embedded and sectioned. Paraffin sections (7 µm) were stained with goat anti-PTH antibodies using the Vectastain method (VectorLab). Specificity of the antibody was tested by saturation with PTH(1–34). Anti-smooth muscle α-actin (Clone 1A4; Sigma) and anti-TβRII antibodies (C-16; Santa Cruz Biotechnology Inc.) were visualized by Alexa546-linked secondary antibodies (Abcam Inc.) or by the Vectastain method using NovaRed as a substrate (VectorLab), and phospho-Smad2 and phospho-CrkL (Cell Signaling Technology, Inc.) by Metal Enhanced DAB (Pierce). Standard protocols were used for bone and cartilage staining with alizarin S red and alcian blue and for staining by van Kossa's method and haematoxylin/eosin. TUNEL assays on paraffin-embedded tissue sections were performed following the producer's guidelines (Roche Diagnostics). Each marker was analyzed on at least three embryos per stage. Intracardial Indian ink injection was carried out as reported (Jerome and Papaioannou 2001).

Cell culture experiments

Neural tube explant cultures were done as described (Lee et al. 2004). Monc1 cells were cultured and treated with TGFβ (5 ng/mL) for 15 min at 37°C (Chen and Lechleider 2004). To inhibit Src kinases, PP1 or PP2 (Calbiochem) was added to the medium 40 min prior to TGFβ treatment. Western blot analysis of Monc1 cell extracts was carried out as described (Koller et al. 2004). Primary antibodies were against Actin (Chemicon Inc.), phospho-Smad2 (S465/467; Cell Signaling Technology, Inc.), phospho-Src (Y416; Cell Signaling Technology, Inc.), and phospho-CrkL (Y207; Cell Signaling Technology, Inc.). Immunocytochemistry of Monc1 cells treated with TGFβ for 3 d was done as described (Lee et al. 2004).

Acknowledgments

We thank B. Langsam, H.R. Sommer, and A. Wiesner for technical assistance; A. McMahon and P. Soriano for providing transgenic animals; and R. Zinkernagel, J.B. Relvas, and N. Mantei for helpful discussions. This work was supported by the Swiss National Science Foundation, by the National Center of Competence in Research "Neural Plasticity and Repair," by the Swiss Federal Institute of Technology, and by the Roche Research Foundation.

References

Berti, L., Mittler, G., Przemeck, G.K., Stelzer, G., Gunzler, B., Amati, F., Conti, E., Dallapiccola, B., Hrabe de Angelis, M., Novelli, G., et al. 2001. Isolation and characterization of a novel gene from the DiGeorge chromosomal region that encodes for a mediator subunit. *Genomics* **74:** 320–332.

Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D.H., McMahon, A.P., Sommer, L., Boussida, O., and Kemler, R. 2001. Inactivation of the β-*catenin* gene by *Wnt1-Cre*-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128:** 1253–1264.

Chen, S. and Lechleider, R.J. 2004. Transforming growth factor-β induced differentiation of smooth muscle from a neural crest stem cell line. *Circ. Res.* **94:** 1195–1202.

Derynck, R. and Zhang, Y.E. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425:** 577–584.

Feller, S.M. 2001. Crk family adaptors—Signalling complex formation and biological roles. *Oncogene* **20:** 6348–6371.

Fukuda, K., Kawata, S., Tamura, S., Matsuda, Y., Inui, Y., Igura, T., Inoue, S., Kudara, T., and Matsuzawa, Y. 1998. Altered regulation of Src tyrosine kinase by transforming growth factor β1 in a human hepatoma cell line. *Hepatology* **28:** 796–804.

Graham, A. 2003. Development of the pharyngeal arches. *Am. J. Med. Genet.* **119A:** 251–256.

Guris, D.L., Fantes, J., Tara, D., Druker, B.J., and Imamoto, A. 2001. Mice lacking the homologue of the human 22q11.2 gene CRKL phenocopy neurocristopathies of DiGeorge syndrome. *Nat. Genet.* **27:** 293–298.

Hagedorn, L., Suter, U., and Sommer, L. 1999. P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-β family factors. *Development* **126:** 3781–3794.

Hagedorn, L., Floris, J., Suter, U., and Sommer, L. 2000. Autonomic neurogenesis and apoptosis are alternative fates of progenitor cell communities induced by TGFβ. *Dev. Biol.* **228:** 57–72.

Hari, L., Brault, V., Kléber, M., Lee, H.Y., Ille, F., Leimeroth, R., Paratore, C., Suter, U., Kemler, R., and Sommer, L. 2002. Lineage-specific requirements of β-catenin in neural crest development. *J. Cell Biol.* **159:** 867–880.

Ito, Y., Yeo, J.Y., Chytil, A., Han, J., Bringas Jr., P., Nakajima, A., Shuler, C.F., Moses, H.L., and Chai, Y. 2003. Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. *Development* **130:** 5269–5280.

Jerome, L.A. and Papaioannou, V.E. 2001. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat. Genet.* **27:** 286–291.

Jiang, X., Rowitch, D.H., Soriano, P., McMahon, A.P., and Sucov, H.M. 2000. Fate of the mammalian cardiac neural crest. *Development* **127:** 1607–1616.

Kaartinen, V., Dudas, M., Nagy, A., Sridurongrit, S., Lu, M.M., and Epstein, J.A. 2004. Cardiac outflow tract defects in mice lacking ALK2 in neural crest cells. *Development* **131:** 3481–3490.

Kato, Y., Habas, R., Katsuyama, Y., Naar, A.M., and He, X. 2002. A component of the ARC/Mediator complex required for TGF β/Nodal signalling. *Nature* **418:** 641–646.

Kim, J.T. and Joo, C.K. 2002. Involvement of cell–cell interactions in the rapid stimulation of Cas tyrosine phosphorylation and Src kinase activity by transforming growth factor-β 1. *J. Biol. Chem.* **277:** 31938–31948.

Kirby, M.L. and Waldo, K.L. 1995. Neural crest and cardiovascular patterning. *Circ. Res.* **77:** 211–215.

Kizaka-Kondoh, S., Matsuda, M., and Okayama, H. 1996. CrkII signals from epidermal growth factor receptor to Ras. *Proc. Natl. Acad. Sci.* **93:** 12177–12182.

Koller, D., Ittner, L.M., Muff, R., Husmann, K., Fischer, J.A., and Born, W. 2004. Selective inactivation of adrenomedullin over calcitonin gene-related peptide receptor function by the deletion of amino acids 14–20 of the mouse calcitonin-like receptor. *J. Biol. Chem.* **279:** 20387–20391.

Le Douarin, N.M. and Dupin, E. 2003. Multipotentiality of the neural crest. *Curr. Opin. Genet. Dev.* **13:** 529–536.

Lee, H.Y., Kleber, M., Hari, L., Brault, V., Suter, U., Taketo, M.M., Kemler, R., and Sommer, L. 2004. Instructive role of Wnt/β-catenin in sensory fate specification in neural crest stem cells. *Science* **303:** 1020–1023.

Leveen, P., Larsson, J., Ehinger, M., Cilio, C.M., Sundler, M., Sjostrand, L.J., Holmdahl, R., and Karlsson, S. 2002. Induced disruption of the transforming growth factor β type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood* **100:** 560–568.

Li, L., Guris, D.L., Okura, M., and Imamoto, A. 2003. Translocation of CrkL to focal adhesions mediates integrin-induced migration downstream of Src family kinases. *Mol. Cell. Biol.* **23:** 2883–2892.

Lindsay, E.A. 2001. Chromosomal microdeletions: Dissecting del22q11 syndrome. *Nat. Rev. Genet.* **2:** 858–868.

Lindsay, E.A., Vitelli, F., Su, H., Morishima, M., Huynh, T., Pramparo, T., Jurecic, V., Ogunrinu, G., Sutherland, H.F., Scambler, P.J., et al. 2001. Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature* **410:** 97–101.

Merscher, S., Funke, B., Epstein, J.A., Heyer, J., Puech, A., Lu, M.M., Xavier, R.J., Demay, M.B., Russell, R.G., Factor, S., et al. 2001. TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome. *Cell* **104:** 619–629.

Millan, F.A., Denhez, F., Kondaiah, P., and Akhurst, R.J. 1991. Embryonic gene expression patterns of TGF β 1, β 2 and β 3 suggest different developmental functions in vivo. *Development* **111:** 131–143.

Mori-Akiyama, Y., Akiyama, H., Rowitch, D.H., and de Crombrugghe, B. 2003. Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc. Natl. Acad. Sci.* **100:** 9360–9365.

Morrison, S.J., White, P.M., Zock, C., and Anderson, D.J. 1999. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96:** 737–749.

Ota, S., Kizaka-Kondoh, S., Hashimoto, Y., Nishihara, H., Nagashima, K., Kurata, T., Okayama, H., and Matsuda, M. 1998. Constitutive association of EGF receptor with the CrkII-23 mutant that inhibits transformation of NRK cells by EGF and TGF-β. *Cell Signal* **10:** 283–290.

Paratore, C., Goerich, D.E., Suter, U., Wegner, M., and Sommer, L. 2001. Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* **128:** 3949–3961.

Park, S.S., Eom, Y.W., Kim, E.H., Lee, J.H., Min, D.S., Kim, S., Kim, S.J., and Choi, K.S. 2004. Involvement of c-Src kinase in the regulation of TGF-β1-induced apoptosis. *Oncogene* **23:** 6272–6281.

Shah, N., Groves, A., and Anderson, D.J. 1996. Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* **85:** 331–343.

Sommer, L., Shah, N., Rao, M., and Anderson, D.J. 1995. The cellular function of MASH1 in autonomic neurogenesis. *Neuron* **15:** 1245–1258.

Soriano, P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21:** 70–71.

Tanaka, Y., Kobayashi, H., Suzuki, M., Kanayama, N., and Terao, T. 2004. Transforming growth factor-β1-dependent urokinase up-regulation and promotion of invasion are involved in Src-MAPK-dependent signaling in human ovarian cancer cells. *J. Biol. Chem.* **279:** 8567–8576.

Vitelli, F. and Baldini, A. 2003. Generating and modifying DiGeorge syndrome-like phenotypes in model organisms: Is there a common genetic pathway? *Trends Genet.* **19:** 588–593.

Vitelli, F., Morishima, M., Taddei, I., Lindsay, E.A., and Baldini, A. 2002. Tbx1 mutation causes multiple cardiovascular defects and disrupts neural crest and cranial nerve migratory pathways. *Hum. Mol. Genet.* **11:** 915–922.

Inactivation of TGFβ signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome

Heiko Wurdak, Lars M. Ittner, Karl S. Lang, et al.

Genes Dev. 2005, **19:**
Access the most recent version at doi:10.1101/gad.317405