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ADDENDUM

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SDF1-CXCR4 signaling: A new player involved in DiGeorge/22q11-deletion syndrome

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ABSTRACT

The DiGeorge/22q11-deletion syndrome (22q11DS), also known as velocardiofacial syndrome, is a congenital disease causing numerous structural and behavioral disorders, including cardiac outflow tract anomalies, craniofacial dysmorphogenesis, parathyroid and thymus hypoplasia, and mental disorders. It results from a unique chromosomal microdeletion on the 22q11.2 region in which the transcriptional activator TBX1 is decisive for the occurrence of the disease. During embryogenesis, *Tbx1* is required for patterning of pharyngeal region giving rise to structures of the face, neck and chest. Genetic and developmental studies demonstrated that the severity and variability of the syndrome are determined by *Tbx1* targets involved in pharyngeal neural crest cell migration and survival. Recently, we demonstrated that the chemokine *Sdf1/Cxcl12* and its receptor *Cxcr4* are genetically downstream of *Tbx1* during pharyngeal development and that reduction of CXCR4 signaling results in defects which recapitulate the major morphological anomalies of 22q11DS, supporting the possibility of a pivotal role for the SDF1/CXCR4 axis in its etiology.

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DiGeorge/22q11-deletion syndrome: Clinical features and genetic basis

DiGeorge syndrome (DGS), also referred to as velocardiofacial syndrome, is a relatively-frequent congenital disease with an incidence estimated to 1:2000/1:7000. It represents a typical example of multiple-anomaly syndromes covering nearly every organ and system. [1] Of the diverse clinical features found in DGS, those that have attracted the greatest attention are the developmental and behavioral disorders. More than 70% of patients with DGS exhibit conotruncal heart anomalies, including defective outflow septation, outflow misalignment or mispatterning of the great arteries, as observed in tetralogy of Fallot, persistent truncus arteriosus, and double-outlet right ventricle. Craniofacial anomalies also concur to a significant proportion of DGS cases, with a characteristic facial appearance and palatal/velopharyngeal dysfunction with or without cleft. Numerous other structural anomalies have been described in DGS, in particular mispositioning of the vascular system in the pharynx, neck and chest, and hypoparathyroidism, responsible for severe hypocalcemia. Likewise, due to thymic hypoplasia, immune disorders, such as chronic respiratory infections, are also relatively common although severe immunodeficiencies are the exception. Dysphagia is often a serious complication of DGS, but the exact physiological causes for this anomaly are still debated. Finally, numerous psychiatric disorders, especially psychosis, schizophrenia, mental retardation and speech and language impairment, have been associated with DGS.

The wide phenotypic spectrum (more than 180 clinical features both physical and behavioral) exhibited by patients with DGS has long hampered the precise diagnosis of the pathology and the elucidation of its etiology. In addition, no single clinical feature occurs in 100% of cases and there are no reported

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cases of the syndrome that has all or even most of the clinical findings. [1] To date, the only reliable criterion to diagnose DGS cases is the hemizygous chromosomal microdeletion within the 22q11.2 region, and it is now widely accepted that this pathology is primarily defined on this genetic basis. [1,2] This is why hereafter, this syndrome will be referred to as 22q11-deletion syndrome (22q11DS). The 22q11.2 region harbors about 30 to 40 different genes, and genetic studies in mice carrying deletions in the region of synteny to the 22q11.2 region (chromosome 16 in the *Df1*/+ and *LgDel*/+ mouse strains) helped to identify *Tbx1*, a member of the T-box family of transcription factors, as one of the major genes responsible for the abnormalities observed. [3] Moreover, *Tbx1*−/− mice recapitulate many of the clinical features of patients with 22q11DS, including cleft palate, absent or reduced parathyroid and thymus as well as malformed outflow tract. [4] In human, *TBX1* maps to the 22q11.2 region and its haploinsufficiency or sequence mutations have been found in patients with 22q11DS. [5] It should be emphasized though that many 22q11.2 deletion variants do not impact *TBX1* and that *TBX1* mutations in humans are primarily associated with cardiac manifestations, implying that *TBX1* alone cannot account for the complexity of the 22q11DS spectrum.

Development of the pharyngeal region: A crosstalk between several embryonic tissues deregulated in 22q11DS

How can TBX1 mutation result in such a diversity of phenotypes and extreme variability in their occurrence? Part of the answer lies in the embryological origin of the tissues affected. Indeed, all the cardiac, facial, thymic and parathyroid defects concern a single broad region, termed pharyngeal arches, that is situated ventrally in the anterior part of the embryo. These transient structures are bilateral, segmented structures that develop during the initial steps of cephalic development to form, at the level of the hindbrain, 6 arches and pouches in a cranial-to-caudal order. Later during development, pharyngeal arches undergo extensive structural reorganizations and are at the origin of most tissues and structures of the face, neck, and chest, including the upper and lower jaws, the pharynx as well as components of the cardiac outflow tract and cephalic vasculature. Early during their development, *Tbx1* is prominently expressed in pharyngeal arches and *Tbx1*−/− mice display severe pharyngeal defects. [4,6] Furthermore, *Tbx1* is required at different times, in different tissues and at different levels to pattern all pharyngeal arch-derived structures. [7-11]

Pharyngeal arches are of a mixed embryonic origin, with contributions of the 3 primary germ layers (ectoderm, endoderm, mesoderm) along with neural crest (NC) cells, thus raising the question of the identity of cells affected in DGS. While the pharyngeal endoderm provides precursors to craniofacial organs, the mesodermal contingent of the pharyngeal arches gives rise to facial and neck muscles as well as to most of the cardiac and vascular systems. Regarding NC cells, after migrating from the dorsal hindbrain toward the arches where they form the mesenchyme surrounding the arch arteries and the core mesoderm, they contribute to the skeletal structures of the face and, in the neck and chest, to the conotruncus, the parathyroid, part of the thymus and the peripheral nervous system. Quite remarkably, most tissues that are affected in the face and neck of patients with 22q11DS comprise at least partly NC-derived cells. Genetic studies in mouse provided evidence that defects in NC development can result in malformations reminiscent of 22q11DS. [12] However, it was the classical embryology techniques developed in avians that clearly demonstrated that 22q11DS results primarily from defective development of NC cells, making this pathology one of the most severe among the numerous neurocristopathies. Specific ablation of the NC population that invade the first 6 pharyngeal arches (hereafter called pharyngeal NC) was found to phenocopy many of the cardiocraniofacial anomalies found in patients with DGS. [13]

Tbx1 orchestrates tissue interactions during pharyngeal development

Intriguingly, at least in chick and mouse, *Tbx1* transcript are not found in NC cells themselves in pharyngeal arches. Rather, *Tbx1* is expressed dynamically in the pharyngeal ectoderm and endoderm as well as in the mesoderm.[8,14] In addition, loss of *Tbx1* impairs NC cell migration and differentiation,[15-17] indicating that it acts on NC development in a non-autonomous fashion. Since then, many studies focused on the signals elicited by *Tbx1* that mediate interactions between pharyngeal NC cells and their surrounding tissues to uncover the molecular mechanisms underlying DGS.

The essential role of *Tbx1* in pharynx morphogenesis relies on its ability to interact with crucial signaling pathways during development, such as the fibroblast growth factor (FGF), hedgehog and retinoic acid.[2] Among them, FGF8 emerged as a key player for mediating *Tbx1* effect on pharyngeal NC cells. *Fgf8* is downregulated in the pharyngeal endoderm of *Tbx1* mutants and mice heterozygous for both *Fgf8* and *Tbx1* have a greater penetrance of pharyngeal arch defects than *Tbx1* heterozygotes.[18] In addition, in *Fgf8*-hypomorphic mice, pharyngeal NC cells undergo massive cell death in the pharynx, suggesting that FGF8 plays a major role in maintaining NC cell survival during the late phases of arch colonization.[19,20] Further experiments performed in chick demonstrated that FGF8 is chemotactic for NC cells migrating to the heart, [21] therefore raising the intriguing possibility that the pharyngeal NC defects observed in DGS may not be solely due to defective survival signals in the arches, but also to misregulation at earlier stages during their dorsoventral migration from the hindbrain to the arches. However, *Fgf8* defects cannot by far account for the whole range of NC defects as it was shown that only cells that populate the heart (i.e. originating from the caudal hindbrain at the level of rhombomere 6-7 and invading archs 3-6) respond to FGF8 while those populating arch 2 from rhombomere 4 respond to VEGF. [21,22] If chemotaxis is truly involved in 22q11DS occurrence, then another factor attracting indistinctly all pharyngeal NC cells into arches 1-6 is to be identified. Beside FGF8 and VEGF, NC cells have been previously shown to respond to a variety of chemoattractants, including FGF2, semaphorins, and the Stromal-Derived Factor-1 (SDF1). [23]

SDF1 chemokine signaling is required for pharyngeal NC development

SDF1 (also named CXCL12), a member of the chemokine family that is widely expressed during embryonic development, attracted our attention for several reasons. It is capable of driving oriented migration of various NC cell populations, either truncal or cranial, through binding to its CXCR4 receptor. [23,24] Loss of *Sdf1* or *Cxcr4* in mouse embryo causes ventricular septal defects similar to those observed in 22q11DS. [25,26] Interestingly, signaling by CXCR4 is deficient in brain cortical interneurons of the *LgDel*/+ and *Df1*/+ mouse strains, resulting in their abnormal migration, and this is believed to constitute the basis of the mental and behavioral disorders shown by patients with 22q11DS.[27,28] Finally, in silico analysis revealed the presence of putative TBX1-binding sites in the *Cxcr4* promoter. [29] We thus analyzed whether CXCR4 signaling could drive oriented migration of pharyngeal NC cells and investigated whether its misregulation could ultimately cause cardiocraniofacial defects associated with 22q11DS. [30,31]

In a first study, we focused on NC cells that migrate from the posterior hindbrain to populate the heart and termed cardiac NC using the chick embryo as a model, taking advantage that in this species, the spatiotemporal course of NC cell migration has been fully documented and can be easily and specifically manipulated owing to numerous available molecular tools. [30] We found that cardiac NC cells express the CXCR4 receptor during their initial migration from the hindbrain to pharyngeal arches 3-6, and that *Sdf1* exhibits a complementary pattern in the ectoderm along their migration route. We then performed functional experiments using electroporation in premigratory cardiac NC cells either of a miRNA, to knockdown *Cxcr4* expression, or of a dominant-negative form of *Cxcr4* in which the chemotactic, but not the survival, response to SDF1 was abolished. We also challenged cardiac NC cells with *Sdf1* ectopically expressed in the neural tube. *Cxcr4* loss-of function caused delayed migration and enhanced death (when miRNA to Cxcr4 was applied) of cardiac NC cells, whereas *Sdf1* misexpression resulted in diversion of cardiac NC cells away from their normal migration pathway, confirming our hypothesis that SDF1 acts as a chemoattractant for cardiac NC. Finally, alterations of SDF1 signaling during migration led invariably at late stages of development to various cardiovascular defects commonly found in 22q11DS. These data therefore identify *Sdf1* and its receptor *Cxcr4* as candidate genes responsible for the cardiovascular congenital malformations of 22q11DS.

Using the same experimental approach, we then investigated whether this SDF1-mediated chemotactic mechanism applies to the other NC populations migrating more anteriorly through pharyngeal arches 1-2. [31] We found that, both in chick and mouse, *Cxcr4* and *Sdf1* exhibit the same dynamic spatiotemporal expression patterns as more caudally and that reduction of CXCR4 signaling causes early misrouting of pharyngeal NC cells and, later, dramatic

morphological alterations in the mandibular skeleton, thymus and cranial sensory ganglia. Thus, the SDF1-CXCR4 axis fulfills almost all the requirements for being implicated in the morphological disorders observed in patients with 22q11DS. Moreover, we showed that, in the mouse embryo, *Tbx1* is expressed in the same regions of the ectoderm and pharyngeal endoderm as *Sdf1* during initial migration of NC cells under the ectoderm. In addition, we found a strong reduction of both *Sdf1* and *Cxcr4* expressions in the pharyngeal arches of *Tbx1* mouse mutants compared to wild-type embryos. Our data therefore indicate that in mouse, *Cxcr4* and *Sdf1* are downstream of *Tbx1* in the genetic cascade regulating pharyngeal arch development and suggest a model in which *Tbx1* may regulate *Sdf1* expression in the lateral ectoderm and pharyngeal endoderm and control the chemotactic guidance of pharyngeal NC cells toward the pharyngeal arches. This view is fully consistent with the recent findings showing functional defects of CXCR4 signaling in the brain of the *LgDel/+* and *Df/+1* mice. [27,28]

How Tbx1 *and SDF1 signaling interact during pharyngeal development?*

A question remains as to how *Tbx1* regulates *Sdf1* and *Cxcr4* expressions in cells. Although bioinformatic analyses in mouse identified putative binding sites for TBX1 in the *Cxcr4* promoter, [29] their exclusive expression patterns in distinct compartments of the pharyngeal arches (the ectoderm and endoderm for *Tbx1* and migrating NC cells for *Cxcr4*) clearly exclude a direct regulation. Conversely, the coincident expression of *Sdf1* and *Tbx1* in the lateral ectoderm and in the pharyngeal endoderm would rather be in favor of a possible direct regulation. However, if it exists, such a mechanism would be only transient and require additional co-factors as both genes exhibit poorly-overlapping expression patterns once NC cells colonize the arches. Moreover, previous studies failed to detect difference in the relative expression of *Sdf1*/*Cxcl12* in *Tbx1−/−* versus *Tbx1+/+* cells. [32] Therefore, more complex relationships are likely to occur between *Tbx1, Sdf1* and its *Cxcr4* receptor. A possible mechanism is that these genes are under the control of a common set of transcriptional regulators. For example, *Tbx1* expression in the pharyngeal endoderm and mesoderm has been demonstrated to be regulated by the FOX transcription factors *Foxa2, Foxc1* and *Foxc2*, [33] while genomic analyses revealed conserved binding sites for *Foxa2* and *Foxc2* in the proximal enhancer region of the *Cxcr4* gene. [34] Alternatively, more indirect mechanisms may also operate. Thus, we found that the expression domain of *Sdf1* in the ectoderm becomes gradually restricted with the lateral progression of the cardiac NC stream toward the pharyngeal arches, [30] suggesting that NC cells themselves provide the cues for repressing *Sdf1* expression during migration. However, we also showed that if NC cells are prevented to migrate laterally along the ectoderm, *Sdf1* becomes downregulated, indicating that its expression requires positive signals emanating from NC cells. Conversely, forced overexpression of *Sdf1* is able to induce *Cxcr4* expression. These observations suggest that *Sdf1* and *Cxcr4* cross-regulate each other in neighboring cells by a yet-undefined mechanism. This process may therefore explain why in *Tbx1−/−* embryos, expression of both *Sdf1* and *Cxcr4* would be down-regulated although they are not coexpressed in the same cell populations. Such a mechanism is believed to ensure a fast and robust regulation of chemotactic guidance of NC cells. Of interest, a similar loop of regulation has been proposed for FGF8 and its receptor during their migration toward the heart. [21]

SDF1-CXCR4 signaling: A central role in 22q11DS defects?

In a pathology such as 22q11DS presenting a large spectrum of phenotypes with a variable incidence, it is of importance to determine whether candidate genes can explain the vast majority of the clinical features of the disease or only a few of them, thereby accounting for its diversity. While there is no doubt that *Tbx1* is an essential gene in 22q11DS as its inactivation in mouse and haploinsufficiency in human cause a large array of velocardiofacial defects, the question is of a great relevance for its putative transcriptional downstream targets. As discussed above, *Fgf8* has been found to drive chemotactic migration of NC cells to pharyngeal arches 3-6 but not of those invading arch 2. [21] In agreement with this observation, conditional deletion of *Fgf8* in the arches results primarily in anomalies in the cardiac outflow tract, the parathyroid and the thymus, all derived from arches 3-6, [35] indicating that it is not involved in the occurrence of facial defects observed in 22q11DS. Moreover, targeted

manipulation of *Fgf8* expression in the *Tbx1*-positive cells in the mouse embryo was found to cause skeletal abnormalities and to alter patterning of the aortic arches but not the outflow tract, suggesting that *Tbx1* and *Fgf8* play independent roles in regulating outflow tract development. [36] Thus, *Fgf8* may account mostly for the variable penetrance of the cardiovascular and glandular defects in 22q11DS and not for its overall phenotype. Likewise, the homeobox-containing transcription factor, *Gbx2*, another downstream effector of *Tbx1*, has been shown to be required in the pharyngeal ectoderm to drive NC cell migration into pharyngeal arch 3-6 via the Slit/Robo signaling pathway. [16] However, as for *Fgf8, Gbx2* function is restricted to arch 3-6 and does not extend to more anterior regions, thereby accounting only partially for 22q11DS defects.

In striking contrast, our studies suggest that, unlike *Fgf8* and *Gbx2*, the *Sdf1-Cxcr4* signaling axis may play a broad role in governing migration and survival of most if not all NC cells invading pharyngeal arches 1-6. Accordingly, inhibition of CXCR4 activity or alterations in *Sdf1* expression pattern both lead to a large spectrum of abnormalities in the cardiovascular, skeletal, neuronal and glandular derivatives of pharyngeal NC. [30] Moreover, we also observed considerable mispatterning of cranial sensory ganglia in embryos in which CXCR4 activity was abolished with variable phenotypes, such as missing, fused or misrouted cranial nerves, as also reported in the *LgDel/+* mouse. [37] Importantly, these anomalies did not result from a direct effect on NC cells at the origin of the cranial sensory ganglia and nerves (these cells do not respond to SDF1 signals), but merely from the misrouting of pharyngeal NC cells (which do rely on SDF1 for oriented migration) to aberrant locations. Thus, the implication of the SDF1-CXCR4 signaling pathway in the occurrence of the diverse clinical features of 22q11DS may extend well beyond the sole NC derivatives in the pharyngeal arches and might concern tissues that do not rely directly on *Tbx1* expression (Fig. 1). Given that CXCR4 signaling has also been shown to be defective in the brain of mouse models of

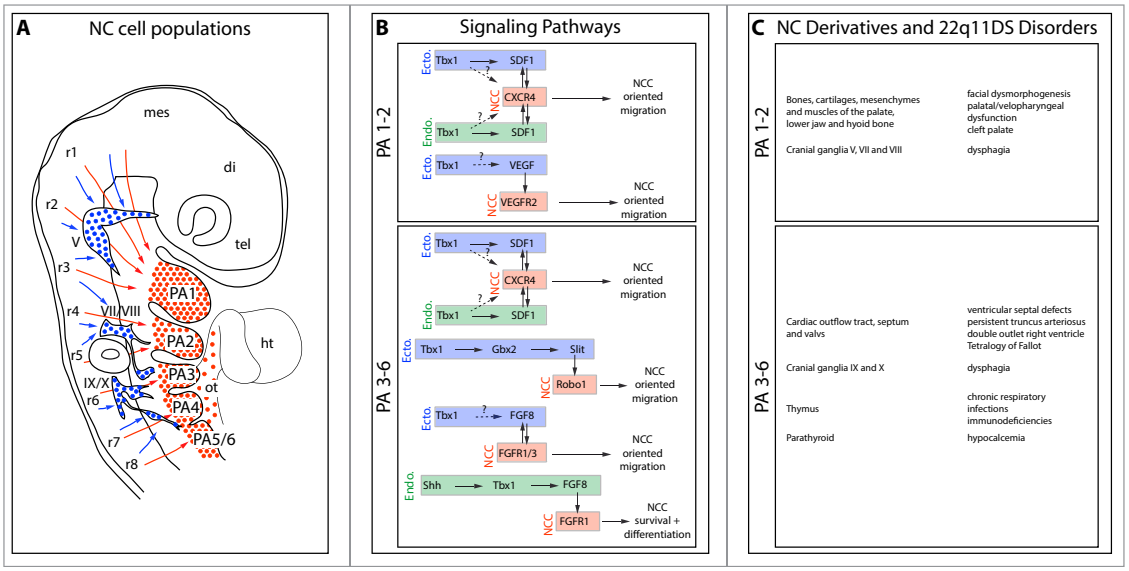

Figure 1. The SDF1/CXCR4 signaling pathway: A new player involved in DiGeorge/22q11-deletion syndrome malformations. A. Schematic representation of the different NC cell populations that colonize the pharyngeal arches, the cardiac outflow tract and the cranial ganglia. NC cells that express the CXCR4 receptor and respond to SDF1 signals allowing them to migrate along the superficial ectoderm and populate the arches and the cardiac outflow tract are indicated in red. Those which do not express CXCR4 and migrate ventrally to contribute to the cranial sensory ganglia are in blue. Arrows indicate the level of origin of NC cells along the rostro-caudal axis. tel, telencephalon; di, diencephalon; mes, mesencephalon; r1-r8, rhombomeres 1-8; V, trigeminal ganglion; VII/VIII, facial and vestibuloacoustic ganglia; IX/X, glossopharyngeal and vagal ganglia; PA1-6, pharyngeal arches 1-6; ht, heart; ot, outflow tract. B. Putative signaling pathways regulating migration, survival and differentiation of NC cells in PA1-2 and PA3-6, respectively. The tissular origin of the signal (endodermal or ectodermal) is indicated. Single arrows with plain line represent demonstrated (direct or indirect) regulations and double arrows indicate reciprocal regulations. Arrows with dashed line and associated with question marks represent hypothetical regulatory pathways. C. Lists of the main NC derivatives generated in PA1-2 and PA3-6, respectively, and of the corresponding disorders commonly observed in 22q11DS. Neural defects consecutive to CXCR4 signaling defects and causing mental retardation and other psychiatric disorders are not listed because, to our knowledge, they are not directly related with anomalies in pharyngeal NC cells.

DGS, [27,28] we propose that, although the 22q11.2 region deleted in patients harbors neither gene, the SDF1-CXCR4 signaling axis may play a pivotal role in 22q11DS etiology and account for its neural, behavioral, cardiovascular, immune, endocrine and facial aspects.

In conclusion, our study illustrates the great utility and versatility of the avian system to investigate the function of candidate genes for complex human congenital diseases and to analyze in detail the developmental processes responsible for their occurrence. More specifically in the case of 22q11DS, the use of the chick embryo allowed establishment of the precise origin of the NC cell populations that populate the branchial arches and their contribution to the facial, pharyngeal and cardiac tissues, [13] to demonstrate the importance of guidance systems in the segregation of the different NC populations that arise at cranial levels and in their patterning into various derivatives, [22,30], [31] and to characterize the implication of several genes located in the 22q11.2 region, such as *HIRA, Ufd1l*, and *DGRC6*, in the conotruncal anomalies observed in 22q11DS. [38-40]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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