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Contribution of cranial neural crest cells to mouse skull development

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ABSTRACT The mammalian skull vault is a highly regulated structure that evolutionally protects brain growth during vertebrate development. It consists of several membrane bones with different tissue origins (*e.g.* neural crest-derived frontal bone and mesoderm-derived parietal bone). Although membrane bones are formed through intramembranous ossification, the neural crest-derived frontal bone has superior capabilities for osteoblast activities and bone regeneration via TGF, BMP, Wnt, and FGF signaling pathways. Neural crest (NC) cells are multipotent, and once induced, will follow specific paths to migrate to different locations of the body where they give rise to a diverse array of cell types and tissues. Recent studies using genetic mouse models have greatly advanced our knowledge of NC cell induction, proliferation, migration and differentiation. Perturbations or disruptions of neural crest patterning lead to severe developmental defects or diseases. This review summarizes recent discoveries including novel functions of genes or signaling molecules that are capable of governing developmental processes of neural crest patterning, which may function as a gene regulatory network in controlling skull development. The proposed regulatory network will be important to understand how the signaling pathways and genes converge to regulate osteoblast activities and bone formation, which will be beneficial for the potential identification of molecular targets to prevent or alleviate human diseases or disorders involving defective neural crest development.

KEY WORDS: *neural crest, frontal bone, parietal bone, skull vault, mesoderm*

Introduction

The mammalian skull vault is a highly regulated structure and exquisitely patterned during development. Several membrane bones occupy the skull vault, namely a pair of frontal and parietal bones and an unpaired inter-parietal bone. These bone elements have different tissue origins. Frontal bones derive from the cranial neural crest (CNC), and parietal bones stem from paraxial mesoderm-derived tissue (Jiang *et al.*, 2002) (Fig 1). The inter-parietal bone itself is of dual tissue origins, which is the evolutionary consequence of the fusion between the crest-derived "postparietals" and the mesoderm-derived "tabulars" (Koyabu *et al.*, 2012). The skull vault protects the brain and accommodates its growth. Although the tissue origins of the skull vault in mouse were identified late compared to that in birds through chick-quail chimaeras, this valuable model has brought up enough attentions to understand the genetic and molecular mechanism determining the regional differences within the mouse skull vault.

Osteogenic potential of neural crest-derived osteoblast *vs.* mesoderm-derived osteoblasts

The frontal bone exhibits distinct differences in osteogenic potential and regenerative capability depending on their origin (neural-crest vs. mesoderm-derived): 1) Osteoblasts from the neural crest-derived frontal bone are less differentiated, grow

Abbreviations used in this paper: BMP, bone morphogenetic protein; CNC, cranial neural crest cell; EMT, epithelial-mesenchyme transition; Fb, frontal bone; FGF, fibroblast growth factor; Pb, parietal bone.

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faster, and have a more rapid bone nodules formation compared to parietal bone-derived osteoblasts (Xu *et al.*, 2007). 2) A higher level of activation of the FGF signaling pathway is found in the frontal bone (Li *et al.*, 2010, Quarto *et al.*, 2009), which profoundly impacts calvarial regeneration (Behr *et al.*, 2010). 3) Active canonical Wnt signaling also contributes to intrinsic osteogenic potential and tissue regeneration in the neural crest-derived frontal bone (Quarto *et al.*, 2010). Enhanced activation of Wnt signaling in the parietal bone can mimic a neural crest origin bone, like the frontal bone (Li *et al.*, 2015). 4) Neural crest-derived osteoblasts have low apoptosis when stimulated by TGF-beta signaling (Senarath-Yapa *et al.*, 2013). 5) In humans, gene expression profiling within these two bone elements (Homayounfar *et al.*, 2015) indicates a broad spectrum of differentially expressed genes, but how and when they will initiate to orchestrate the features of skull vault with different origins remains elusive (Fig. 1).

Origin of neural crest cells and a genetic mouse model to label neural crest cells

Neural crest cells specify at the border of the neural plate and non-neural ectoderm after gastrulation. During neurulation, the borders of the neural plate begin to converge at the dorsal midline to form the neural tube. Subsequently, neural crest cells from the roof plate of the neural tube delaminate from the neuroepithelium and migrate into different locations of the body through a definable pathway towards their destinations (Chen *et al.*, 2017, Huang and Saint-Jeannet, 2004).

Cell fate mapping using a NC-specific promoter has facilitated genetic labeling of NC cells and their derivatives. Multiple Cre transgenic mouse lines have been generated using a NC marker gene promoter, e.g., *Wnt1-Cre*, *P0-Cre*, *Dhh-Cre*, *Pax3-Cre*, *HtPA-Cre*, *Sox10-Cre*, *Sox10ER(T2) CreER(T2)*, *Mef2c-F10N-Cre*. Among them, *Wnt1-Cre* and *P0-Cre* lines have been widely used. The *Wnt1-Cre* model is currently the gold standard for NC lineage. However, *Wnt1-Cre*-labeled cells in the neural tube, which are not neural crest-derived, and *Wnt1-Cre* transgene causes ectopic activation of Wnt signaling, which induces defective midbrain development (Lewis *et al.*, 2013). For *P0-Cre* line, it has been challenging to specifically label early neural crest cells (Yamauchi *et al.*, 1999). Our recent evidence shows that the *P0-Cre* transgenic model specifically labels neural crest cells at an early stage. We also found a profound and unrecognized difference in the Cre distribution within the midbrain labeled by *Wnt1-Cre* compared to an extensive labeling within the hindbrain by *P0-Cre (Chen et al., 2017)* (Fig. 2). This may be the reason why different conclusions have been made using *P0-Cre* and *Wnt1-Cre* in labeling CNC (Barriga *et al.*, 2015).

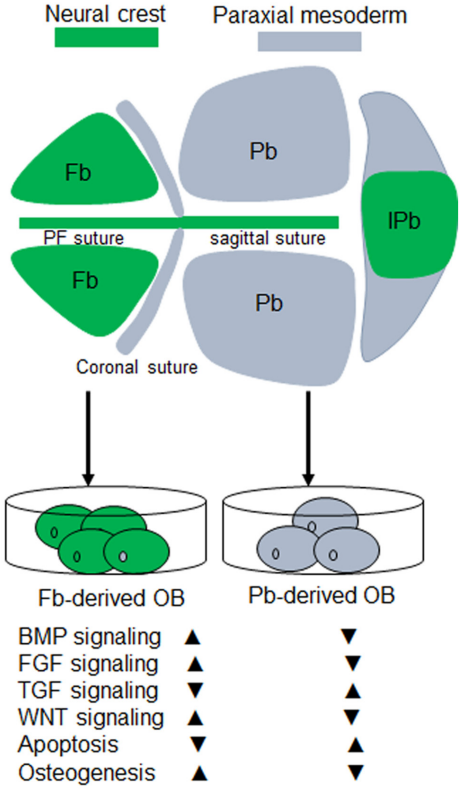

Fig. 1. Diagram of tissue origins in the skull vault and distinct features of tissue-derived osteoblasts activities. *Frontal bone-derived osteoblasts showed higher activities of several signaling pathway including BMP, FGF and Wnt, but lower activities of TGF signaling and apoptosis compared to parietal bone-derived osteoblasts. Fb, frontal bone; Pb, parietal bone; OB, osteoblast; IPb, interparietal bone; PF, parietofrontal.*

Neural crest patterning during skull development

A gene regulatory network has been potentially revealed in NC patterning, and a diverse set of interacting signals, transcription factors, and downstream effector genes are involved in the different stages of NC development (Meulemans and Bronner-Fraser, 2004). Genetic approaches in human, fish, chicken, and mice have shown that some genes that specify the complex morphology of the vertebrate skull are similar and therefore a precise shape and position of every skull vault has developed in the vertebrate. Transgenic mouse models provide a useful tool for genetic manipulation of neural-crest-derived components during embryogenesis. We here try to summarize recent findings that associated with skull development and then propose a model for how potential gene regulatory network may function in mouse skull vault (Fig. 3).

Neural fold specification and neural crest induction

Neural tube closure and neural crest delamination are transient and dynamic. How these critical steps orchestrate timely and spatially are very intriguing. Although the detailed mechanism is not clear, recent findings provide valuable information to better understand their regulations and functions. Wnt signaling, BMP signaling, and SHH signaling pathways are induced early in neural fold specification. Sensing the gradients of Shh signaling is important for neural tube closure (Balaskas *et al.*, 2012), and Shh acts as a survival factor to mediate CDO (cell-adhesion molecule-related/downregulated by oncogenes) which is a pro-apoptotic in the developing neural tube (Delloye-Bourgeois *et al.*, 2014). FGF3 regulates BMP signaling in the neuroepithelium, which in turn regulates neural tube closure (Anderson *et al.*, 2016). In addition, Geminin is required for neural tube patterning (Patterson *et al.*, 2014), and Lbx1 promotes neural tube closure (Kruger *et al.*, 2002), while Pax-3 and Pax-7 genes specify dorsal fate in the vertebrate neural tube (Wada *et al.*, 1997).

Specification of the neural tube is essential for the induction of the neural crest. Several neural crest specifiers have been identified to be critical in neural crest cell induction, such as the ZIC family, TFAP2, Msx1/2, SOX9/10, Snail1/2, Pax3/7, and Myc (Rogers *et al*., 2012, Stuhlmiller and Garcia-Castro, 2012). In a recent publication visualizing the gene expression pattern in the frontal and parietal compartment in humans showed that only TFAP2 is highly expressed in the neural crest-derived frontal bone (Homayounfar *et al*., 2015). Our recent data shows that both TFAP2 and SOX10 are highly expressed in the neural crest-derived frontal bone tissue in mouse. This suggests a distinct regulation of different specifiers in neural crest-derived tissues in a species-dependent manner, such as in chicken, SOX10 and SOX9 are regarded as the earliest neural crest-specifying gene (Betancur *et al*., 2010). However, how these neural crest specifier genes control or guide the cell behavior towards skull development are not clear. One possible explanation is that they co-occupy the promoters of critical regulatory genes related with craniofacial development (Miranda *et al*., 2017).

Epithelial-mesenchyme transition (EMT) for neural crests

When the identities of neural crest cells are established, neural crest cells will undergo dynamic transformation from epithelium to mesenchyme. Cell-cell junctions and potential space for cell-cell contacts are required for the transition. Some genes are crucially involved in the regulations of junctions: FoxD3 and Snail downregulate expressions of the molecules associated with epithelial cells

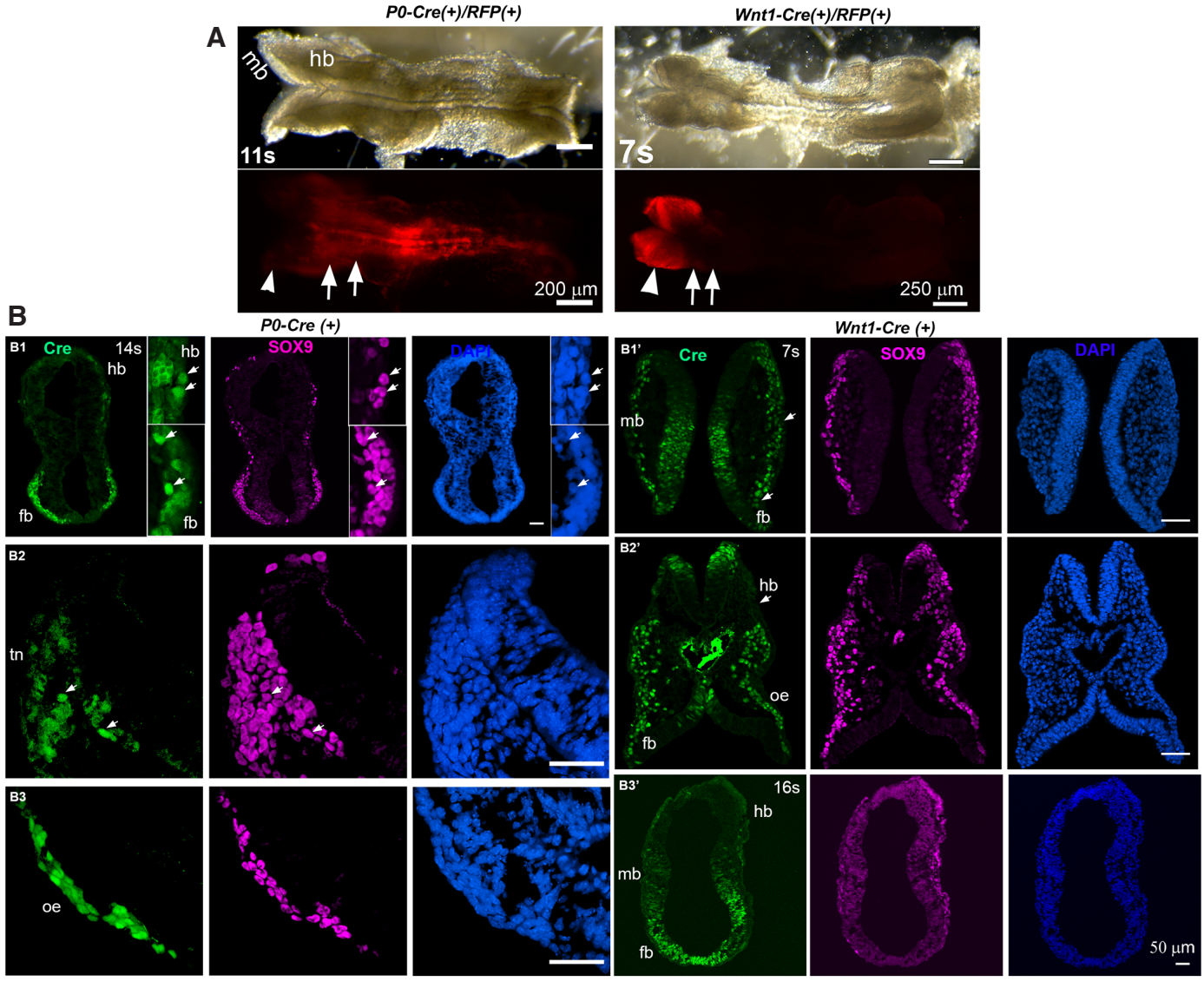

Fig. 2. Differences in neural crest cell labeling using *P0-Cre* and *Wnt1-Cre* at E8.5 *(modified from our publication, Chen* et al., *2017).* **(A)** P0-Cre/RFP *labels the hindbrain region (arrow) but not midbrain region (arrowhead) at 11 somite at E8.5, while* Wnt1-Cre/RFP *labels midbrain region (arrowhead) but spares the hindbrain region (arrow).* **(B)** P0-Cre *embryos showed abundant Cre immunostaining signals in hindbrain (hb) and forebrain region (fb) at 14 somite (B1), post-migratory NC cells in trigeminal neural crest (tn, B2) at posterior hindbrain level, and in the optic eminence (oe, B3), in anterior hindbrain (hb, B3).* Wnt1-Cre *activities were extensive at 7-somite stage (B1'-2'), in the pre- and post-migratory neural crest cells in midbrain (mb, B1') and forebrain (fb, B2'-3') regions, optic eminence (oe, B2') but sparse in hindbrain (hb, B2'). Scale bars: 250* μm *in (A) and 50* μm *in (B).*

such as N-cad and E-cad or Cad6B, while upregulate mesenchymal migratory proteins such as Cad7. Cad6B inhibits beta-catenin signaling and affects NC delamination (Rabadan *et al.*, 2016). Snail downregulates tight junctions to allow for the upregulation of gap junctions (Taneyhill *et al.*, 2007). p53 is reported to regulate EMT, and p53 mutants display broad craniofacial defects in skeletal bone (Rinon *et al.*, 2011). GTPases (Faure and Fort, 2015) and Sox2 (Mandalos *et al.*, 2014) function during epithelial to mesenchymal transitions. Besides, ERK-dependent epigenetic signaling results in a gene expression program which is essential for driving EMT (Navandar *et al.*, 2017). Interestedly, some miRNAs are also functional to the developmental EMT (Banerjee *et al.*, 2016). However, how the direction of delaminating neural crest is determined and then follows a certain pathway into different locations of the body remains to be understood.

Neural crest proliferation and apoptosis

Neural crest specifiers are important in cell proliferation. Proto-oncogene c-myc deficiency driven by *Wnt1-Cre* in the neural crest results in viable adult mice with defective frontal bone (Wei *et al.*, 2007). Low expression of TFAP2 leads to reduced cell proliferation (Pfisterer *et al.*, 2002). In SOX10 mutant mice, apoptosis increased in the sites of early neural crest cell development (Kapur, 1999). This well explains that higher expression levels of TFAP2 and SOX10 within frontal bone in mouse may contribute to a high potential for proliferation of neural crest-derived frontal bone. In addition, mouse Foxd3 is essential in maintaining the proliferating and self-renewing population of progenitor cells for neural crest (Hanna *et al.*, 2002). Proliferation and apoptosis is evolutionarily balanced during neural crest development, such as apoptosis in the odd-numbered rhombomeres is needed to eliminate the migration of r3 and r5 crest into first and third arches and therefore avoid extra neural crest-derived muscle attachment at these sites (Ellies *et al.*, 2002).

TGF and WNT signaling pathways are also involved in neural crest proliferation and apoptosis. Deletion of adenomatous polyposis coli (APC), which downregulates Wnt signaling, leads to massive apoptosis of cranial neural crest cells which further results in craniofacial defects (Hasegawa *et al.*, 2002). TGF-beta-activated kinase 1 (Tak1)-deficient mutants display a round skull, hypoplastic maxilla and mandible (Yumoto *et al.*, 2013). Moreover, the Hippo signaling pathway is well known for its role in cell proliferation and growth. Conditional deletion of Yap and Taz in the CNC, two components for Hippo signaling pathway, using *Wnt1-Cre* and *Wnt1-(Cre2SOR)* result in enlarged CNC and reduced proliferation in the branchial arch mesenchyme (Wang *et al.*, 2016). Rho kinase (Rock) in mouse is crucial for the survival of NCC to form the craniofacial region (Phillips *et al.*, 2012).

Some genes and transcriptional factors specifically regulate neural crest proliferation and apoptosis: Prtg-deficient (protogenin protein) mice show malformation of bones due to increased apoptosis of rostral CNC (Wang *et al.*, 2013). Polycystin-1 (Pkd1) is required for the proliferation of subpopulations of cranial osteochondroprogenitor cells of both mesodermal and neural crest origin during the growth of the skull (Kolpakova-Hart *et al.*, 2008). Deficiencies of Msx1 and Msx2, homeodomain transcription factor, result in defective patterning and survival of the cranial neural crest (Ishii *et al.*, 2005). TALE-class homeodomain transcription factors Meis and Pbx have specific functions in embryogenesis. Conditional inactivation of Meis2 using crest-specific AP2alpha-IRES-Cre mouse displays perturbed development of the craniofacial skeleton with severe anomalies in cranial bones (Machon *et al.*, 2015). Furthermore, gap junctions are also necessary for the survival of neural crest cells.

Neural crest migration

Upon the emigration of neural crest cells from the neural tube, Noggin and Chordin are involved in this process (Anderson *et al.*, 2006). During closure of the neural tube, transcription factor AP2 is prominently expressed in migrating NC from the neural folds,

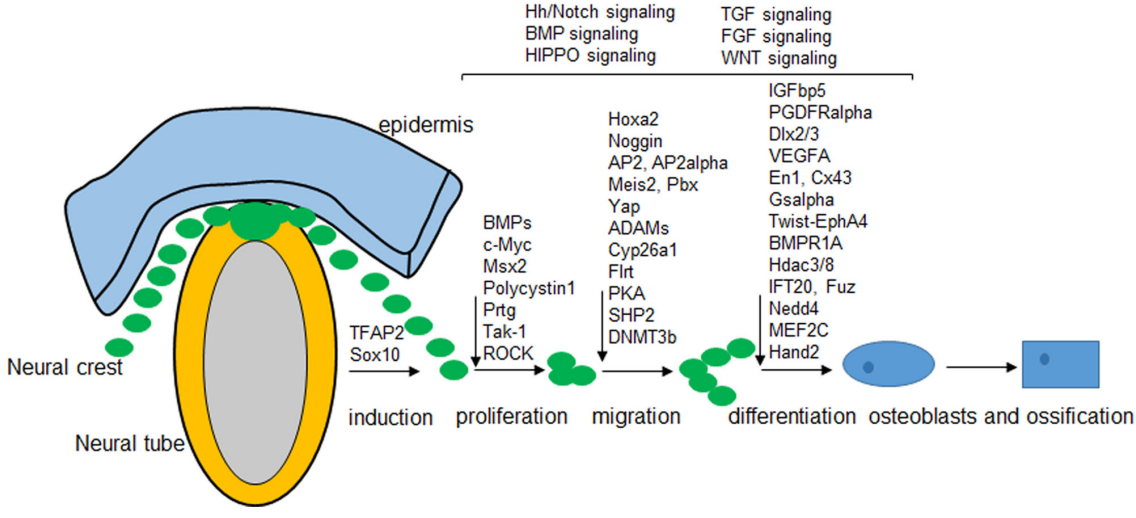

Fig. 3. The proposed gene regulatory network that may be involved in the regulation from neural crest patterning to skull development. *Neural crests form at the dorsal part during the closure of the neural tube. Specified neural crest cells delaminate, proliferate, migrate and differentiate into mesenchyme where they give rise to osteoblasts during skull formation. Identified signaling pathways and genes that function throughout the neural crest patterning are marked.*

and later expressed in the regulatory regions (Enkhmandakh and Bayarsaihan, 2015), where it acts as a non-autonomous factor to induce skeletogenesis. AP2 mutant mice are severely defective in face and skull bone.

Following migration, SOX10 and SOX9 are immediately expressed in migrating neural crest cells in mouse, which is different from that in chicken where ETS1 and SOX9 are pan-neural crest regulators of the migratory CNC (Simoes-Costa *et al.*, 2014). Flrt2 and Flrt3, members of the Fibronectin Leucine-Rich Transmembrane (Flrt) gene family, mediate CNC migration during craniofacial development (Gong *et al.*, 2009). Upon the production of migratory cranial neural crest cells, CYP26A1 and CYP26C1 (Uehara *et al.*, 2007) and transmembrane metalloproteases (ADAMs) (Cousin *et al.*, 2011) act as positive regulators, for example, preventing the shuttle of the cleaved cytoplasmic domain of ADAM13 into the nucleus inhibits CNC cell migration *in vivo* (Cousin *et al.*, 2011). ADAM13 functions in an autonomous manner to trigger CNC migration during skull development (Cousin *et al.*, 2012).

Ablation of PTPN11 in premigratory neural crest cells, which encodes the protein tyrosine phosphatase (SHP2), contributes to profound skull deficits (Nakamura *et al.*, 2009). A subpopulations of postmigratory CNC have roles in patterning distinct derivatives, such as Hoxa2 (Tavella and Bobola, 2010) acts as a selector gene for patterning of branchial arch structures, and cAMP-dependent protein kinase (protein kinase A (PKA)) has strict regulation on the derivative patterning (Jones *et al.*, 2010). How these genes and signaling pathways work dynamically and directionally in neural crest migration are intriguing.

Osteogenesis in neural crest

Some key transcriptional factors play essential roles during osteogenesis in the neural crest: Firstly, Msx2 drives CNC differentiation into the skeletal system. Msx2 mutants are defective in skeletogenic mesenchyme cells. Overexpression of Msx2 in mice leads to the growth of parietal bone into the sagittal suture. Neural crest-specific removal of Msx2 results in a larger defect in the frontal bone (Roybal *et al.*, 2010). An interaction between Msx2 and Twist is needed during the differentiation and proliferation of skeletogenic mesenchyme and formation of skull vault (Bildsoe *et al.*, 2009).

Furthermore, the homeodomain family of transcription factors regulate the development of CNC-derived craniofacial skeleton. Dlx2 is expressed in the epithelium and mesenchyme cells within branchial arches. Deletion of Dlx2 leads to skeletal anomalies (McKeown *et al.*, 2005). Mice lacking Dlx3 exhibit alteration of calvaria (Duverger *et al.*, 2013). Dlx5 and Dlx6 function during craniofacial development, and MEF2C is required for the expressions of Dlx5 and Dlx6. Transcription factor gene Hand2 is expressed in neural crest-derived mesenchyme cells during the branchial arches (Ruest *et al.*, 2003), suggesting its role in craniofacial development.

Moreover, Connexin43 (Cx43) is required for neural crest cell patterning and ossification. Cx43 mutants show delayed ossification of the cranial vault (Lecanda *et al.*, 2000). Loss of Gsalpha, G-protein alpha-subunit, in neural crest cells does not affect CNC migration or cell proliferation, but significantly accelerates osteochondrogenic differentiation (Lei *et al.*, 2016). Conditionally overexpression of autoactivated platelet-derived growth factor receptor alpha (PDGFRalpha) in neural crest cells enhanced proliferation of osteoprogenitors and accelerated ossification of osteoblasts (Moenning *et al.*, 2009).

Signaling pathways have been extensively studied during the osteogenesis in the neural crest. FGF signaling plays an essential role in the skeletogenic differentiation from the cranial neural crest. Conditional overexpression of the FGFR2 S252W mutation in neural crest-derived tissues causes a more severe craniofacial phenotype (Heuze *et al.*, 2014). Simultaneously, activation of FGFR2 (S252W) is sufficient to cause craniosynostosis (Holmes and Basilico, 2012). Interestingly, a novel skull defect with only a single bone pair is found in Fuz mutant mice and this phenotype can be rescued by loss of one allele of Fgf8 (Tabler *et al.*, 2016). The receptor of FGF signaling regulates homeoprotein engrailed 1 (EN1) during osteogenic differentiation (Deckelbaum *et al.*, 2012). Moreover, Hedgehog signaling (Hh) is also critical for neural crest patterning: Shh promotes survival of neural crest cells to colonize in the first branchial arch (Delloye-Bourgeois *et al.*, 2014). Disruption of Hh signaling leads to abnormal neural crest development, resulting in malformed skull base. Removing Hh-responsiveness specifically in neural crest cells leads to the absence of many CNC-derived skeletal components (Jeong *et al.*, 2004).

BMP and TGF signaling pathway are also important in osteoblast differentiation and bone formation (Chen *et al.*, 2012). BMPs ligands such as Bmp2 and Bmp7 are required in frontal bone primordium. Inactivation of Bmp2 and Bmp7 leads to multiple defective CNC-derived skeletal elements (Bonilla-Claudio *et al.*, 2012). Neural crest-specific deletion of Acvr1, a type I receptor for BMPs, induces craniofacial defects, and deletion of another type I receptor, Bmpr1a, causes mid-gestation lethality (Stottmann *et al.*, 2004). Constitutive overexpression of Bmpr1a in neural crest cells leads to premature suture fusion in mice (Komatsu *et al.*, 2013). TGF-beta signaling regulates the fate of neural crest cells. Mice with neural crest-specific deletion of TGF-beta receptor 2 (Tgfbr2) show craniofacial skeletal malformations (Ho *et al.*, 2015). TGF-beta mediates Msx2 expression during skull development. Msx2 is regulated by BMP-Smad signaling pathway, and Twist1 is a downstream target of Wnt signaling. In short, these interactions suggest integrated functions of the different signaling pathways during osteogenesis in the neural crest.

Epigenetic regulation

Histone deacetylases (Hdacs), transcriptional repressors, displays essential roles in neural crest cell patterning. Hdac8 represses a subset of transcription factors in CNC, such as Otx2 and Lhx1, and thereby specifically influences the patterning of the skull. Hdac8 mutant mice are perinatally lethal due to skull instability (Haberland *et al.*, 2009). Hdac3 is needed for neural crest cells during craniofacial development. Conditionally knockout Hdac3 in the neural crest reveals fully penetrant craniofacial abnormalities and upregulates Msx1, Msx2, and Bmp4 expression in the mesenchyme, suggesting that Hdac3 serves as a critical regulator for craniofacial morphogenesis (Singh *et al.*, 2013). In addition, mutation of *de novo* DNA methyltransferase DNMT3b leads to defects in neural crest-derived craniofacial skeleton (Jacques-Fricke *et al.*, 2012).

Ubiquitination

Wwp2 E3 ubiquitin ligase works with paired-like homeobox transcription factor Goosecoid (Gsc) during craniofacial development. Gsc is activated by the APC (Cdh1) E3 ubiquitin ligase. Conditional deletion of neural crest-specific Cdh1 gene causes bone malformation that is similar to Wwp2-deficient mice with a

domed skull displaying a short snout and a twisted nasal bone (Shao *et al.*, 2016). Mice with specific ablation of another E3 ubiquitin ligase, Nedd4, in neural crest cells or osteoblasts exhibit profound craniofacial defects with marked reduction of cranial bone (Wiszniak *et al.*, 2016).

Primary cilium-related regulation

The primary cilium is a cellular microtubule-based organelle that is important for cell proliferation, differentiation, survival, and homeostasis. Intraflagellar transport (IFT) functions in assembling primary cilia, such as IFT20 and IFT88. Deletion of IFT20 in neural crest cells leads to skeletal dysplasia, such as osteopenia in the facial region (Noda *et al.*, 2016). Deletion of IFT88 results in a decrease in neural crest cell proliferation at early stages (Tian *et al.*, 2017). Polycystin 2 (Pkd2) localized in primary cilia, and conditional deletion of Pkd2 in neural crest-derived cells causes malformed skull shapes (Khonsari *et al.*, 2013). Kif3a is the motor protein within primary cilia. Conditional deletion of Kif3a in neural crest cells has a dramatic effect on intramembranous ossification, and Shh signaling is disrupted in Kif3a-deficient neural crest-derived mesenchymal cells (Kolpakova-Hart *et al.*, 2007). However, ciliary proteins EVC and EVC2, which are regarded as causative genes and having an important role in transduction of Hedgehog signaling, do not show observed defects in skull development but defects in incisor growth using neural crest-specific mouse model (Zhang *et al.*, 2015).

Human diseases or disorders from defective neural crest patterning

Disruption or perturbation of the patterning on neural crest cells leads to defective organs and tissues. Some human diseases or disorders stem from abnormal neural crest patterning (Table 1). Treacher Collins syndrome is an autosomal dominant congenital disorder which is characterized by craniofacial deformities, such as facial bones. The prevalence of this disease is about 1:10,000-50,000 individuals. Loss of TCOF1 encoding TREACLE protein affects the craniofacial skeleton (van Gijn *et al.*, 2013). Premature fusion of the calvarial bones leads to craniosynostosis, a relatively common pediatric disease with occurrence in 1:2500 births. A variety of genetic lesions can result in craniosynostosis, such as gain-of-function of FGFR1-3 and loss-of-function of TWIST1 (Holmes and Basilico, 2012). Disruption of cranial neural crest migration and survival can cause human disease, such as mutation of MID1 gene results in X-linked Opitz syndrome (XLOS) with an estimated prevalence of 1:50, 000-100, 000 male individuals and the patients exhibit midline malformation with distinct craniofacial abnormalities such as wide-spaced eyes (Latta and Golding, 2012). Disrupted migration of neural crest cells with mutation of ephrin-B1 gene (EFNB1) lead to craniofrontonasal syndrome (CFNS), which is very rare inherited X-linked disorders with estimated birth prevalence of 1:120,000. Multiple defects were observed including asymmetry, midline defects, skeletal abnormalities, and unusual head shape (Wieacker and Wieland, 2005). A novel mutation in Zfhx1b gene in neural crest precursor cells in mouse can lead to Mowat-Wilson syndrome, a genetic disorder with distinct facial features and an unusual small head (Van de Putte *et al.*, 2007). Abnormal development of neural crest cells can result in a genetic disorder called Axenfeld-Rieger syndrome (ARS). ARS occurrence is about 1:200,000 individuals and eye development exhibits major disorder. PITX2 and FOXC1 are the leading causes of the disease.

Magnetic resonance imaging is a valuable tool to show multiple defects including the defective skull base (Whitehead *et al.*, 2013). SHP2 contributes to higher occurrence of Noonan syndrome disease, a genetic disorder characterizing by defective cardiac and craniofacial developments with estimated prevalence of 1:1000-1:2500 live births. SHP2 plays an essential role for CNC normal migration and differentiation into diverse lineages of the heart and skull (Nakamura *et al.*, 2009). However, the symptoms improve with age and most adults suffering NS do not need special medical care. Mutations from several genes have been associated with NS disease such as in KRAS, RAF1 and SOS1RIT1 (van Trier *et al.*, 2016).

Neural crest cells and regenerative medicine

Defective neural crest patterning results in severe birth defects, which may require a comprehensive surgery and rehabilitation at tremendous economic burden. Findings based on genetic mouse models are valuable to understand the regulation of genes and signaling pathway during skull development. This will potentially help to determine a target molecule that promotes the differentiation of neural crest cells to osteoblasts while maintains the superior osteogenic potential and bone regeneration potential compared to mesoderm-derived bone tissues, which are important for regenerative medicine in clinic.

Endogenous calvarial regeneration may be a promising solution for craniofacial reconstruction (Senarath-Yapa *et al.*, 2013), and a molecule or agent will be found to sufficiently drive endogenous bone formation. Similarly, fibronectin, which is involved in mediating differentiation of the skull and migration of neural crest cells, works as a carrier for BMPs and used as an essential component in stem cell technology associated with craniofacial

TABLE 1

HUMAN DISEASES OR DISORDERS FROM DEFECTIVE NEURAL CREST PATTERNING

Genes	Disease	Prevalence	Some symptoms	Reference
TCOF1	Treacher Collins syndrome	1:10,000-50,000	Craniofacial skeleton	(van Gijn *et al.*, 2013)
FGFR1-3, Twist1	craniosynostosis	1:2500	Premature fusion of skull	(Holmes and Basilico, 2012)
MID1	X-linked Opitz syndrome	1: 50,000-1:100,000	Craniofacial abnormalities	(Latta and Golding, 2012)
EFNB1	craniofrontonasal syndrome	1:120,000	Asymmetry, midline defects, skeletal abnormalities	(Wieacker and Wieland, 2005)
Zfhx1b	Mowat-Wilson syndrome	Not sure	Facial defects, unusual small head	(Van de Putte *et al.*, 2007)
PITX2, FOXC1	Axenfeld-Rieger syndrome	1:200,000	Defective eye, defective skull base	(Whitehead *et al.*, 2013)
PTPN11, KRAS, RAF1	Noonan syndrome	1:1000-1:2500 live births	Defective cardiac and craniofacial developments	(van Trier *et al.*, 2016)

surgery (Al-Qattan *et al*., 2014, Brunskill *et al*., 2014).

Engineering cranial neural crest cells is also a tool to treat diseases. Patient-derived CNC cells can manipulate bone formation *in vitro*, which can be used for transplantation. CNC have been successfully differentiated into osteoblasts *in vitro*, e.g. in a 3D bioengineering microenvironment where neural crest cells are encapsulated in gelatin-based photo-cross-linkable hydrogels and cultured for about 3 weeks to efficiently drive differentiation (Namkoong *et al*., 2016). In addition, CNCs supplemented with BMP4 in the culture media are capable of differentiating into osteocytes and chondrocytes (Mimura *et al*., 2016). Mouse embryonic stem cells can be selectively differentiated into cranial NC stem cells, which can further differentiate into other cell types including osteoblasts (Minamino *et al*., 2015). IPSCs are another useful tool for genetic manipulation of certain cell types *in vitro*. Skin-derived precursors can be differentiated into the neural crest lineage, which can be subsequently propagated and directed towards the mesenchymal lineages, such as osteogenic cells (Kang *et al*., 2011). Collectively, these examples demonstrate that neural crest cells are an accessible and potentially autologous source for tissue engineering and bone repair. Further researches will assure that the engineered neural crest cells can be delivered in a safe way, homed sufficiently, and functioned properly *in vivo*.

Summary

Researches understanding neural crest pattering have made great progress in recent years, which lead to a better understanding about its patterning, development, and regulation. Yet, new technologies are needed that can be widely used to further our understanding, especially under the serious reality that more than 30% of birth defects are involved in defective a head or face, and the craniofacial abnormalities are a primary cause of infant mortality. Translating novel findings into the clinic remains to be shown in the future. Timely diagnosis, disease prevention and treatment options are unsolved issues. Major progress in culturing cranial neural crest cells have been made, so that differentiation of neural crest cells into different cell types is invaluable for cell therapy or tissue repair in clinic in the coming future.

We here review the most recent findings regarding neural crest patterning and its potential relationship to the development of skull. We propose that a gene regulatory network is essential to maintain the superior regenerative capacity in neural crest-derived frontal bone. Visualizing the regulatory network in neural crest patterning during skull development will be important to understand how signaling pathways are coordinated to regulate osteoblast activities. This evolutional model of the skull vault involving dual tissue origins provides some cues that need to be dissected, which in turn will be beneficial to not only the regulation of bone biology but also their regenerative applications in clinic.

Conflict of interest

The Authors declare no conflict of interest.

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Int. J. Dev. Biol. (2009) 53: 69-79
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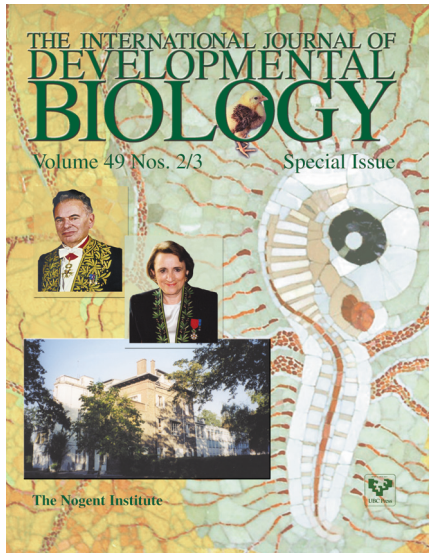

5 yr ISI Impact Factor (2013) = 2.879