Insights into the development of molecular therapies for craniosynostosis

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For the past 2 decades, clinical and basic science researchers have gained significant insights into the molecular and genetic pathways associated with common forms of craniosynostosis. This has led to invaluable information for families and physicians in their attempts to understand the heterogeneity of craniosynostosis. Genetic mutations have been identified in the fibroblast growth factor receptors (FGFRs) as well as in other targets, including TWIST1, BMP, and RUNX2. Greater understanding of these and other pathways has led to the development of innovative approaches for applying medical therapies to the treatment of craniosynostosis, in particular by maintaining suture patency. In this article, the authors discuss the molecular pathophysiological mechanisms underlying various forms of craniosynostosis. They also highlight recent developments in the field of molecular craniosynostosis research with the hope of identifying targets for medical therapies that might augment the results of surgical intervention.

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IN 1993, Jabs et al.¹¹ were the first to identify a genetic cause for craniosynostosis with the *MSX2* gene mutation resulting in “Boston-type” craniosynostosis. Shortly thereafter, the role of fibroblast growth factor receptor (FGFR) in Crouzon syndrome was identified,³⁵ and transforming growth factor β (TGF β) was recognized as a powerful modulator of suture fusion.³³ From these early investigations, our understanding of the molecular pathways contributing to craniosynostosis has greatly expanded, and additional molecular and genetic pathways continue to be recognized.

Contemporary treatment of craniosynostosis has seen a number of changes in the past 30 years. There have been significant studies performed on the neurocognitive outcomes associated with craniosynostosis that have helped craniofacial surgeons identify the need for early intervention.⁴² Along these lines, minimally invasive endoscope-assisted surgery has been developed,¹⁵ which has helped to treat children at younger ages with fewer comorbidities

and costs associated with surgery.⁴ There remains, however, a 1%–2% mortality rate associated with open cranial vault remodeling and surgical approaches.³⁸ The advent of molecular treatments in other area of medicine has been based on genetic analysis of an individual patient's profile for a particular phenotype. These same principles may be applicable to the field of craniosynostosis.

It is possible that exploitation of this pharmacological approach will, in the future, yield therapeutic targets to prevent premature suture refusion following craniosynostosis surgery, particularly in children with syndromic craniosynostosis. Premature fusion of sutures following surgery may lead to suboptimal cosmetic results, decreased intracranial volumes, and ultimately, neurological comorbidities prompting a surgical revision.

In this article, we review the current literature on the molecular mechanisms related to the development of craniosynostosis and discuss the most promising candidates for translational therapies (Table 1).

ABBREVIATIONS BMP = bone morphogenetic protein; ERK1/2 = extracellular signal-related kinases 1 and 2; FGFR = fibroblast growth factor receptor; SNP = single nucleotide polymorphism; TGF β = transforming growth factor β .

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TABLE 1. Molecular genetics of craniosynostosis and potential translational approaches

Signaling Molecule	Type of Molecule	Associated Disorders	Translational Approaches	References
FGFR1–3/FGFs	Cell surface receptor/ligand	Apert, Crouzon, Pfeiffer, Jackson-Weiss, & Muenke syndromes; nonsyndromic craniosynostosis	Soluble FGFR2 receptor, small hairpin RNA interference, viral vector introduction of dominant-negative receptor	9, 23, 27, 35, 36, 40, 43, 50, 51, 53
TWIST1	Transcription factor	Saethre-Chotzen	Pending	6
TGFβ	Cytokine	None	Anti-TGFβ2 antibody	24, 30–33
RUNX2	Transcription factor	Nonsyndromic craniosynostosis	Pending	10, 46
MSX2	Transcription factor	Boston-type craniosynostosis	Pending	12
Ephrin-B1	Cytokine	Craniofrontonasal syndrome	Pending	44
Erf	Transcription factor	Single- & multisuture synostosis	Pending	45

Biology of the Mammalian Skull

In its most basic form, the mammalian skull is composed of 5 bones: the paired frontal bones, paired parietal bones, and the unpaired interparietal bone, which receives contributions from the squamous temporal bone and greater wing of the sphenoid.²⁵ Whereas the parietal bones are derived from the paraxial mesoderm, the frontal bones derive from the neural crest.^{5,14} The interparietal bone receives both mesoderm and neural crest contributions.

The cranial vault forms by intramembranous ossification, whereby the mesenchymal tissue between bony fronts is recruited to ossify. The sutures form as the margins of developing bones approximate, and constitute major growth centers of the skull vault.²⁷ The suture may be thought of as a complex of the osteogenic fronts of the calvarial bone plates, the mesenchyme spanning these 2 surfaces, the dura mater, and the pericranium (Fig. 1).²⁵ All parts of this complex have been shown to affect skull growth.²⁰ The coordinated growth of these centers is interdependent, and if a molecular abnormality occurs in their formation, craniosynostosis may result in premature skull fusion.

The Role of FGFR/FGF

Mutations in FGFRs, particularly FGFR2, have been linked to 5 of the most common craniosynostosis syndromes: Apert, Pfeiffer, Jackson-Weiss, Crouzon, and

Muenke (Fig. 2). This has resulted in the FGFRs being the most studied proteins in the pathogenesis of craniosynostosis. The FGFR is composed of 3 extracellular immunoglobulin-like domains, 1 transmembrane domain, and 1 cytoplasmic tyrosine kinase domain.¹³ There are 4 human isoforms that interact with at least 22 FGFs.^{17,34} Of these, only FGFR1, 2, and 3 have been shown to contribute to the development of craniosynostosis. The FGFR1, 2, and 3 molecules have additional isoforms that are predominantly expressed in epithelial and mesenchymal tissues.^{4,17,29} Activation of these receptors results in dimerization and autophosphorylation, affecting multiple downstream pathways, including protein kinase C, Src, and canonical Wnt signaling.³⁴

Most observed FGFR mutations affect the ligand-

FIG. 1. Diagram of the biology of the human skull demonstrating the 2 osteogenic fronts with intervening mesenchyme, underlying dura, and overlying pericranium. Interactions between various molecular components regulate cranial suture development. EFNB = Ephrin-B1.

FIG. 2. Illustrative 3D head CT scans in 2 patients who had craniosynostosis with genetic syndromes. A: Frontal view demonstrating bicoronal synostosis in a patient affected by Muenke syndrome. B: Axial view of the same patient demonstrating brachycephaly. C: Frontal view demonstrating unicoronal synostosis in a patient affected by Saethre-Chotzen syndrome. D: Axial view of the same patient.

binding domain, although a few affect the tyrosine kinase domain.⁴⁹ These result in a gain of function through increasing ligand affinity, decreasing receptor specificity, or increasing intrinsic receptor activity.⁴⁹ Several point mutations in the FGFRs have been identified. Mutations in FGFR2 (S252T and P253R) account for a majority of all Apert syndrome cases,⁵¹ whereas multiple different cysteine substitutions in this receptor are associated with Crouzon syndrome.^{35,43} Mutations in FGFR1 (P252R) are associated with Pfeiffer syndrome,³⁶ and changes in FGFR3 (P250R) are found in Muenke syndrome.²⁷

In a recent series of 284 children with craniosynostosis, the FGFR3 (P250R) mutation accounted for 24% of all genetic craniosynostoses and 5% of the entire cohort.⁵⁰ Others have reported this mutation in 31% of patients with nonsyndromic coronal craniosynostosis.²³ The FGFR2 mutations (S252T and P253R) have also been reported in children with nonsyndromic craniosynostosis.⁵⁰

Several translational animal models have been developed to study the effect of various FGFR mutations. Deletion of both FGFR1 and FGFR2 are lethal.²⁵ Mice with gain-of-function mutation FGFR2 (Cys342Tyr) demonstrate a phenotype similar to Crouzon syndrome, with a shortened face, protruding eyes, and premature fusion of the coronal sutures.⁷ Bone marrow samples from this mouse model demonstrate proliferation of osteoprogenitor cells, suggesting the importance of FGFR2 in the early stages of osteocyte formation. A mouse model of Pfeiffer syndrome carrying the FGFR1 (P252R) mutation has also been developed.⁵⁴ These mice exhibit premature sagittal and coronal suture fusion, midface hypoplasia, and facial asymmetry. Increased bone mineralization and elevated markers of bone formation are seen in these mice, suggesting that the FGFR1 plays a more important role in osteoblast differentiation.

Of all the FGF ligands, FGF2 seems to be most involved in suture fusion.²⁰ Increases in FGF2 mRNA in translational models are seen in the dura mater underlying fused frontal sutures. Overexpression of FGF2 using a viral vector also causes premature fusion.²⁶

Interference or downregulation of FGF at the suture interface is the most promising candidate for medical treatments of craniosynostosis. In an effort to inhibit FGF2 ligand signaling and maintain coronal suture patency in a model of Apert syndrome, Yokota et al. recently reported the capacity of a soluble FGFR2-impregnated nanogel to inhibit the actions of multiple ligands at the suture interface.⁵³ Dominant-negative FGFRs, whose functional capacity are diminished, have been introduced in utero into animal models of craniosynostosis, and subsequent postnatal fusion of the cranial sutures were prevented.⁹ Additional forms of molecular FGF manipulation have also shown therapeutic potential through the use of RNA interference to prevent suture fusion in models of Apert syndrome,⁴⁰ as well as treatment with MAP kinase inhibitors that inhibit premature suture fusion.

The Role of TWIST1

A transcription factor important for mesodermal patterning of the calvaria, *TWIST1* is located on chromosome

7.² The gene was so named because fly embryos lacking this factor die with a twisted appearance.⁴¹ The *TWIST1* gene is mutated in the majority of patients with Saethre-Chotzen syndrome.⁶ These patients demonstrate coronal craniosynostosis, facial asymmetry, prominent ear crus, and distal limb abnormalities (Fig. 2).⁶ Mutations of *TWIST1* also have been documented in patients with nonsyndromic craniosynostosis.^{39,50}

The *TWIST1* protein is expressed in the osteoprogenitor cells within the coronal and sagittal sutures and is thought to be involved in osteoblast proliferation and differentiation.¹⁶ Unlike FGFR mutations, the mutations in this gene are generally missense or nonsense, resulting in loss of function.²⁰ Most if not all identified patients to date have been haploinsufficient.

In mice, homozygous deletion of *TWIST1* is lethal.²⁸ Disruption of *TWIST1* expression is thought to cause pathological conditions by interfering with osteoblast differentiation, at least partially by disrupting the *RUNX2* pathway.²⁰ The notch ligand *Jagged1* also appears to be downstream of *TWIST1*, because *TWIST1* mutants demonstrate decreased *Jagged1* expression.⁵² Like *TWIST1*, *Jagged1* localizes to mesoderm-derived cells that lie along the osteogenic-nonosteogenic boundary of the coronal suture. Conditional knockout of this protein leads to osteogenic differentiation and coronal craniosynostosis.⁵² Potential therapeutic approaches for *TWIST*-associated craniosynostosis may target delivery of the normal *TWIST1* or *Jagged1* protein to the cranial sutures.

The Role of TGFβ

Although not associated with a known syndrome, TGFβ plays an integral role in cranial suture fusion. Analysis of tissue from normal and synostotic sutures of human infants undergoing cranial vault remodeling for single-suture synostosis demonstrated elevations of TGFβ immunoreactivity in the abnormal fused suture.³⁷ Extracellular signal-related kinases 1 and 2 (ERK1/2) are important downstream actors of TGFβ2, because application of an ERK1/2 inhibitor disrupts TGFβ-related suture fusion as well as ERK1/2 expression and phosphorylation.³¹

A series of studies by Opperman et al. demonstrated that TGFβ1, 2, and 3 are expressed in developing sutures and dura.³³ The TGFβ2 molecule seems to promote suture fusion, whereas TGFβ3 promotes patency.³¹ Capitalizing on the therapeutic potential of TGFβ, this group demonstrated that application of antibodies to TGFβ2 prevented normal molecular signaling and resulted in suture patency.^{30,32} Other studies have revealed that treatment of suturectomy sites in rabbits with anti-TGFβ2 antibodies can maintain suture patency. These also illustrate that greater intracranial volumes can be achieved several months after surgical suturectomy, suggesting a potential translational use in humans.²⁴

The Role of BMP

A recent study of 130 infants with nonsyndromic sagittal synostosis highlights the role of bone morphogenetic proteins (BMPs) in craniosynostosis. The genomes of these patients were analyzed and compared with 450 con-

trols to identify single nucleotide polymorphisms (SNPs) of interest. The most significantly linked SNPs were located in a region downstream of *BMP2*, possibly a regulatory protein. In this study, BBS9, a component of a protein aggregation important for moving cargo molecules in and out of cilia, was also linked to craniosynostosis.¹⁸ Older studies have demonstrated disproportionately high expression of the BMP antagonist *Noggin* in patent sutures, with overexpression of this molecule resulting in failure of sutures to close.⁴⁸ Treatment with *Noggin* or a similar BMP antagonist may also be critical to maintain suture patency following surgery.

The Role of RUNX2

The *RUNX2* gene and its associated protein are master regulators of osteoblast differentiation and function.⁸ The *RUNX2* gene is a downstream target of FGF, BMP, and TWIST1 proteins (Fig. 3). The *Runx2*^{-/-} mice do not develop mature osteoblasts or bone,¹⁹ whereas premature activation of *RUNX2* by insertion of the gene downstream of the paired related homeobox1 (*Prrx1*) promoter is sufficient to cause calvarial intramembranous bone formation.²² Although no syndrome has been linked to this gene, there have been reports of a triplication and quadruplication of this gene resulting in dolichocephaly⁴⁶ and

pan-suture craniosynostosis,¹⁰ respectively. Its therapeutic potential is currently being investigated.

Other Signaling Molecules

The *MSX2* gene mutation in Boston-type craniosynostosis, a syndrome now recognized in 2 families,¹² was the first genetically linked craniosynostosis to be recognized.¹¹ The *MSX2* gene is part of the homeobox-containing gene family and encodes a transcription factor that is important for pattern formation during development. The single-base mutation underlying Boston-type craniosynostosis increases the binding affinity of *MSX2*, and accelerates suture formation.¹ Transgenic mice expressing this mutation likewise express a craniosynostotic phenotype, reflecting the role of this protein in suture development.²¹ Despite the early identification of this gene, its role in craniosynostosis has been limited.

Ephrin-B1 (*Efnb1*), a membrane-anchored ligand for Eph receptor tyrosine kinase, has recently been linked to craniofrontonasal syndrome, an X-linked syndrome resulting in coronal craniosynostosis and marked hypertelorism, which is more pronounced in heterozygous females than males (Fig. 4).⁴⁴ Multiple mutations from 24 different affected females were identified. In situ hybridization localizes *EFNB1* mRNA to the developing murine

FIG. 3. Illustration of various molecular signaling pathways involved in craniosynostosis. Shown are the relationships between FGFRs, TGFβ2, BMP, and downstream signaling molecules TWIST1, ERK1/2, ERF, and RUNX2. Activating mutations of FGFRs may result in overactivity of RUNX2 as well as activation of ERK1/2. Similarly, TGFβ2 activation results in ERK1/2-mediated suture fusion. Activation of ERK1/2 leads to inhibition of ERF, an inhibitor of RUNX2. The RUNX2 molecule may also be activated by BMP, and inhibited by TWIST1. Overactivation of RUNX2 leads to premature suture fusion.⁸

FIG. 4. Illustrative 3D head CT scans in a female patient with craniofrontonasal syndrome. **Left:** Frontal view revealing frontonasal abnormalities with hypertelorism. **Right:** Axial view revealing bilateral coronal craniosynostosis with brachycephaly.

coronal suture, suggesting a role for this protein in boundary formation.

Recent work has demonstrated that the *Erf* gene, whose protein is an inhibitory transcription factor and binding target of ERK1/2, may also be a cause of craniosynostosis (Fig. 3).⁴⁵ Multiple mutations in the *Erf* gene have been demonstrated in a small group of patients exhibiting both single- and multisuture synostosis. These patients harbor monoallelic mutations, suggesting that the pathological mechanism is haploinsufficiency. In mice, *Erf* heterozygotes are normal, and *Erf*^{-/-} knockout mice are not viable, but mice engineered with a conditional knockout resulting in decreased *Erf* transcription demonstrate a domed head shape with multisuture synostosis.

Discussion

While the aforementioned molecular pathways are yielding innovative candidate therapies for forms of genetic craniosynostosis, the biology of nonsyndromic craniosynostosis, the most common form, remains elusive. Larger genome-wide studies of children affected by these forms of craniosynostosis will probably reveal new molecular targets. In addition, recent advances in cell biology have identified primary cilia as a signaling center coordinating the various molecular pathways responsible for craniofacial formation.³ Sonic hedgehog, Wnt, and FGF signaling are coordinated through primary cilia, and translational models have increasingly identified these cellular structures as potential therapeutic targets for various cranial pathological entities.⁴⁷

Further exploration of the *RUNX2*, *TWIST1*, and ERK1/2 pathways is also likely to improve our understanding of both normal and pathological suture biology. Application of this knowledge will lead to the development of therapeutic agents that may eventually augment surgery for patients with craniosynostosis. The future of craniofacial surgery will be integrally involved in the development of medical therapies preventing premature bone fusion. Preventing or controlling bone fusion rates with medical treatments delivered locally and at the time of surgery may allow full cranial vault expansion along normal developmental timelines. The goal of such inter-

ventions will be to reduce reoperation rates for cranial vault expansion. Agents that are to be used in a clinical setting must exhibit a low-risk safety profile, be capable of targeted delivery, and yield lasting effects throughout calvarial development.

Conclusions

Surgical treatment remains critical in the clinical care and outcome of patients affected by craniosynostosis; however, given the significant advances in our understanding of the biomolecular etiologies of suture development, there is the potential for innovative methods to treat and/or prevent craniosynostosis. Fibroblast growth factor signaling and its role in the pathogenesis of craniosynostosis currently presents the most promising target for pharmacological manipulation of the craniosynostosis phenotype. The FGFRs remain the most important molecules in the pathological development of craniosynostosis, with mutations found in both syndromic and nonsyndromic patients alike. The *TWIST1* and *RUNX2* signaling pathways have emerged as important factors in suture formation, and will probably continue to yield information about the biology of suture fusion. With the expanded knowledge of the molecular mechanisms underlying premature suture fusion, the next generation of targeted molecular therapies in the treatment of craniosynostosis is on the horizon.

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