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Review Article

The Pathogenesis of Craniosynostosis in the Fetus

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Craniosynostosis occurs in approximately 1:2000 live births. It may affect the coronal, sagittal, metopic and lambdoid sutures in isolation or in combination. Although non-syndromic synostoses are more common, over 150 genetic syndromes have been identified. Recent advances in genetic mapping have linked chromosomal mutations with craniosynostotic syndromes. Despite the identification of these genetic mutations, the fundamental biomolecular mechanisms mediating cranial suture biology remain unknown. Today, many laboratories are investigating murine cranial suture biology as a model for human cranial suture development and fusion. Normal murine cranial suture biology is very complex, but evidence suggests that the dura mater provides the biomolecular blueprints (e.g. the soluble growth factors), which guide the fate of the pleuripotent osteogenic fronts. While our knowledge of these dura-derived signals has increased dramatically in the last decade, we have barely begun to understand the fundamental mechanisms that mediate cranial suture fusion or patency. Interestingly, recent advances in both premature human and programmed murine suture fusion have revealed unexpected results, and have generated more questions than answers.

Key Words: Cranial suture biology, craniofacial disorders, dura mater, osteoblast, craniosynostosis

INTRODUCTION

As the neural crest-derived cranial connective tissue framework begins to ossify, it forms calvarial plates with skull-based cartilaginous synchondroses and interplate cranial sutures. At parturition, these cranial sutures allow the calvarial bone plates to overlap and mold during passage through the birth canal. Postnatally, these sutures function as shock absorbers for childhood bumps and bruises, and serve as principal sites for rapid calvarial expansion.[1] In fact, coordinated allometric growth of the cranium is achieved through a complex series of tissue interactions between the brain, dura mater, suture mesenchyme and osteogenic fronts. These tissue interactions couple the expansion of the brain to the growth of the calvarial bone plates, in a direction perpendicular to the cranial sutures. Genetic or environmental perturbations of this complex system can lead to premature cranial suture fusion or craniosynostosis.

Craniosynostosis occurs in approximately 1: 2000 live births.[2] It may affect the coronal, sagittal, metopic and lambdoid sutures in isolation or in combination. Although non-syndromic synostoses are more common, more than 150 genetic syndromes have been identified.[3] Untreated, craniosynostosis can cause a characteristic dysmorphic cranial shape, midface hypoplasia, deafness, blindness, seizures, and mental retardation.[4] Almost all cases are identified postnatally, but ultrasonographic prenatal detection of iatrogenic ovine coronal synostosis and at least 8 cases of Apert syndrome have been reported.[5,6] Although we would NOT presently recommend *in utero* correction of prenatally diagnosed craniosynostosis, ultrasound-initiated, minimally invasive biologically based therapies may find appropriate application in this new millennium.

Recent advances in genetic mapping have linked chromosomal mutations with craniosynostotic syndromes. For example, studies have demonstrated that gain-of-function mutations in three of the four known fibroblast growth factor receptors (FGFRs) are associated with Apert, Crouzon, Jackson-Weiss and Pfeiffer syndromes.[7-11] Interestingly, these FGFR mutations are

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the most common genetic abnormalities associated with familial craniosynostotic syndromes. Additional syndromic mutations have been identified in *MSX*, *TWIST*, and GLI3.[12-18] Despite the identification of these genetic mutations, the fundamental biomolecular mechanisms mediating cranial suture biology remain unknown. Ultimately, the understanding and correction of congenital malformations, such as craniosynostosis, depends upon our knowledge of normal development and the deviation that has occurred. In this article, we present our hypotheses and current research on the molecular mechanisms of cranial suture biology.

THE CRANIOFACIAL TEAM AND THE SURGICAL APPROACH TO CRANIOSYNOSTOSIS

Each child with craniosynostosis should be seen by a craniofacial team, consisting of multiple specialists, including a pediatrician, plastic surgeon, neurosurgeon otolaryngologist, dentist, orthodontist, speech therapist, ophthalmologist, geneticist and a social worker. As part of a thorough evaluation, the child may require roentgenographic evaluation of the calvarium, face and orbits to rule out craniofacial dysostosis. The brain may need to be scanned for evidence of hydrocephalus or anatomic irregularity, and the cervical spine should be evaluated for roentgenographic abnormality or instability.[19,20] In addition, depending on the diagnosis, the child may require supplementary imagining such as a kidney ultrasound.

Prenatal testing is available for the FGFR-related craniosynostotic syndromes. When one parent is affected, the risk that the child will inherit the disease-causing mutation is 50%. If a FGFR mutation has been identified in a parent, prenatal testing can be carried out on fetal DNA obtained through amniocentesis or chorionic villus sampling. In low-risk pregnancies, in which an abnormal skull shape is detected on prenatal ultrasound, DNA analysis could also be performed. Currently, we would not recommend this approach, however, because the positive predictive value of this test is exceeding low and even if a FGFR mutation were identified, the prognosis could not be determined without postnatal clinical findings.

The optimal timing for the surgical treatment of craniosynostosis is still controversial. Many surgeons prefer to operate early to capitalize on the ameliorating effects of brain growth on skull shape. If there is evidence of increased intracranial pressure (e.g. bulging fontanels, progressive optic atrophy, seizures, or multiple-suture synostosis) most surgeons intervene at the earliest opportunity. There is a 7% chance of increased intracranial pressure when one suture is affected and when multiple sutures are involved the risk increases.[21] For example, in cases of multiple suture synostosis the incidence of increased intracranial pressure can be as high as 62%.[22]

The goals of therapy are to provide adequate intracranial volume, in order to allow for brain development, and to create an aesthetically normal skull shape. Surgical treatment dates from the late nineteenth century, when the first techniques were aimed solely at correcting the functional aspects of this deformity. The earliest technique, linear craniectomy and fragmentation of the cranial vault, is still occasionally used today by some surgeons for particularly profound deformities. This method provides temporary brain and eye protection until a more definitive craniofacial procedure can be undertaken. The next advancement, simple craniectomy, was unfortunately accompanied by a high rate of re-ossification and gave only modest results, unless mobilization of the orbits, midface and cranium was performed concurrently. Today's surgical interventions can be divided into three procedures: 1) suture release, cranial vault decompression, and upper orbital reshaping and advancement in infancy (6 to 12 months); 2) operations to correct midface deformities in childhood (6 to 12 years); and 3) orthognathic surgery in adolescence (14 to 18 years). The exact timing and sequence of each of the aforementioned surgical procedures is dependent on both the functional and the psychological needs of the patient.

ETIOPATHOGENIC THEORIES

The cranial suture complex can be divided into

four principal components: 1) the dura mater underlying the suture; 2) the osteogenic fronts of the calvarial bone plates; 3) the intervening cranial suture mesenchyme; and 4) the overlying pericranium. Although numerous theories have been proposed, three anatomically based etiopathogenic perspectives (i.e. osteogenic fronts, suture mesenchyme, and dura mater) have dominated the study of craniosynostosis. In 1851, Rudolph Virchow, in his paper on cretinism and pathologic brain malformation, suggested that cranial suture fate was independent of the neurocranial environment.[23] Virchow presumed that the osteogenic fronts of the calvarial suture possessed an autonomous capacity (i.e. independent of interactions with the dura mater or brain) to fuse or remain patent. Furthermore, he noted that premature fusion inhibited growth perpendicular to the synostosed suture, resulting in a characteristic calvarial dysmorphology. In 1920, Park and Powers postulated that craniosynostosis was caused by a primary defect in the cranial suture mesenchyme.[24] They alleged that an embryologic defect caused a lack of growth in the mesenchyme and this led to premature fusion. Finally, in 1959, Moss hypothesized that the dura mater acted as a conduit for cranial base biomechanical forces.[1] Accordingly, transmitted tension from one or more of the five spatially malpositioned basicranial points of dural attachment (i.e. the crista galli, the crest of the otic capsules bilaterally, and the superior edge of the lesser wings of the sphenoid bilaterally) was believed to alter normal cranial suture physiology.

Contemporary molecular biologic research has fundamentally reformed our understanding of cranial suture biology. A substantial body of recent experimental evidence suggests that, at least in murine models of normal suture fusion, the regional cranial suture-associated dura mater is responsible for determining the fate of the overlying cranial suture.[25-29] *In vitro* and *in vivo* experiments have demonstrated that the subjacent dura mater interacts with the cranial suture complex by temporally and spatially supplying osteoinductive growth factors (e.g. TGF-βs or FGF-2) and cellular elements (e.g. osteoblast-like cells) to the overlying osteogenic fronts and suture mesenchyme.[25,26,28-39] Concurrent analysis of human syndromic and nonsyndromic craniosynostoses indirectly supports this hypothesis.[40] For example, researchers have now identified gene mutations in multiple, highly conserved, cranial suture growth factor-mediated signaling pathways that lead to craniosynostosis.[41] Dura mater-directed cytokine control of murine cranial suture fate marks a recent paradigm shift in cranial suture etiopathogenesis.

THE DURA MATER CONTROLS MURINE CRANIAL SUTURE FATE

There are seven murine cranial sutures; predictably, one fuses while the others remain patent. By manipulating the cranial sutures with respect to the underlying dura mater, we learned that murine cranial suture fate is programmed by dura-derived paracrine signals. Although the suture complex includes the pericranium and the intercalary mesenchyme, it appears that neither plays a critical role in cranial suture fate. The following series of experiments provide compelling evidence that supports the essential role of the dura mater in murine cranial suture biology.

The temporal and spatial sequence of cranial suture fusion

In order to understand the molecular mechanisms mediating craniosynostosis, our laboratory and others have investigated normal suture fusion. Despite the obvious differences in human and rodent craniofacial characteristics, there is an astounding conservation of the molecular specification and assembly of the embryonic cranial structures.[42] Exploiting this conservation, we have investigated murine cranial suture development as a model of human development. By serially sectioning murine cranial sutures, our laboratory and others have demonstrated that the posterior frontal (PF) suture fuses in anterior to posterior and endocranial to ectocranial directions from postnatal days 12 - 22 in the rat and 25 - 45 in the mouse, while all other sutures, including the coronal (COR) and sagittal (SAG), remain patent.[31,33,43] Interestingly, by comparative study the murine PF suture seems analogous to the

human metopic suture. The divergent fates of these murine sutures auspiciously enabled researchers to compare and contrast the natural biology of fusing and patent sutures.

Dura mater-suture communication guides cranial suture fusion

Opperman et al. and other investigators examined the role of the dura mater during murine cranial suture morphogenesis.[44-46] By heterotopically transplanting embryonic or neonatal rat COR sutures into the mid-parietal bones of syngeneic adults, the authors liberated the nascent sutures from cranial-based biomechanical forces.[46] They demonstrated that heterotopic COR suture developmental morphogenesis was independent of the subjacent dura mater (i.e. heterotopic COR sutures developed normally with or without the underlying dura mater). However, COR suture fate was fundamentally governed by the presence of the dura mater (i.e. COR sutures transplanted without dura aberrantly fused, while COR sutures transplanted with intact dura remained normally patent). They obtained similar results in an *in vitro* embryonic organ culture model.[27]

In 1996, Roth et al. investigated postnatal dura mater-suture communication by studying the effects of PF cranial suture separation from the subjacent dura mater using an intervening impermeable silicone membrane.[25] In their experiment, 6 day-old Sprague-Dawley rats were divided into four groups. The control animals had no operation. Experimental animals underwent craniotomy alone, PF dural elevation only or silicone membrane interposition between the PF suture and the underlying dura. As expected, the unoperated animals and the animals that underwent craniotomy alone, demonstrated normal PF suture fusion. Animals that underwent PF dural reflection alone initiated delayed suture fusion on postnatal day 22 and completed ossification by postnatal day 30. However, the PF suture of experimental animals with dura mater-suture silicone separation remained patent through the period of predicted suture fusion (i.e. postnatal days 12 - 22), and these animals did not initiate PF suture fusion until postnatal day 30.

Taken together, the above studies suggest that postnatal dura mater plays a fundamental role in controlling murine cranial suture fate. Furthermore, these results lead naturally to the hypothesis that the PF dura mater secretes soluble factors that diffuse into the overlying cranial suture, and initiate fusion. In other words, the regionally specialized PF dura mater may provide the osteoinductive signaling (e.g. the soluble growth factors) necessary to recruit and stimulate osteoblasts, from the osteogenic fronts or the suture mesenchyme, to lay down the osteoid and extracellular matrix proteins necessary for ossification. Furthermore, the PF dura mater is capable of precisely regulating suture fusion in the anterior-posterior and endocranial-ectocranial directions. In contrast, the programmed SAG dura mater may provide the biomolecular signals necessary to maintain overlying suture complex patency or it may provide an inadequate level of osteogenic cytokines necessary for suture fusion. In order to address these hypotheses, we began to explore the regional specialization of the suture-associated dura mater.

The dura mater acts as a regionally specialized endogenous tissue engineer

In order to investigate the regional specialization of the dura mater, Levine et al. rotated the PF and SAG sutures with respect to the underlying dura.[26] Six day-old Sprague-Dawley rats were divided into two groups. The control group underwent a rectangular craniotomy from the lambdoidal suture to the jugum limitans inclusive of the PF and SAG sutures. The calvarium was separated from the underlying dura mater and then placed back on the dura mater in its original orientation. The experimental animals underwent the same procedure except that the excised strip craniotomy was rotated 180 degrees about the mid-sagittal axis. This rotation placed the PF suture over the SAG dura mater and the SAG suture over the PF dura mater. Control animals demonstrated normal suture physiology: the SAG suture remained patent while the PF suture completed normal anterior-posterior and endocranial-ectocranial ossification. In marked contrast, the PF suture (overlying the sagittal dura mater) of experimental animals remained patent while

the SAG suture (overlying the posterior frontal dura mater) fused. Furthermore, the rotated SAG suture appeared to follow an anatomic anterior-posterior (overlying SAG suture posterior-anterior) and endocranial-ectocranial pattern of ossification.

Bradley et al. corroborated Levine's results with an *in vitro* mouse cranial suture organ culture system.[30,32] Bradley's *in vitro* rotational and translocational cranial suture data reemphasized the regional specialization of the underlying PF and SAG dura mater. Moreover, the data implied that dura mater-suture communication, at least during postnatal murine development, is not dependent on tensional forces or distant endocrine hormones.

Characterizing isolated PF and SAG-derived dural cells

In order to explore the regional differences between dural cells, Mehrara et al. isolated the PF and SAG sutures of 6 day-old Sprague-Dawley rats.[36] The underlying suture-associated dura mater was dissected free of the overlying suture complex, and individual PF and SAG dural cell lines were established. When compared to PF dural cells, first-passage SAG suture-derived dural cells demonstrated decreased cellular contact inhibition and significantly increased rates of cellular proliferation. In contrast, PF dural cells expressed more than twice as much alkaline phosphatase activity and collagen I protein. The PF and SAG dural cells both possessed the capacity to form bone nodules. Collectively, these data demonstrate that phenotypic differences exist between early-passage dural cells derived from fusing and patent sutures. The formation of bone nodules suggests that both PF and SAG dura mater contain a population of osteoblast-like cells; however, elevated collagen I protein expression and alkaline phosphatase activity in PF dural cells suggest that the PF dura may contain more mature osteoblast-like cells. Cellular maturation and differentiation of PF dural osteoblast-like cells may be responsible for their decreased cellular proliferation and enhanced contact inhibition.

In summary, the phenotypic differences identified in suture-specific dural cells, in conjunction with the translocational and rotational suture data, support the hypothesis that the murine dura mater is regionally differentiated and provides developmental signals to the overlying murine suture complex.

The pericranium and cranial suture mesenchyme do not control cranial suture fate

Moss was the first to investigate the role of the pericranium in suture fusion.[47] Stripping the pericranium from neonatal rat calvaria, Moss observed normal PF suture fusion and COR suture patency. Opperman et al. added to Moss's findings by demonstrating that removal of the pericranium did not affect heterotopically transplanted fetal or neonatal suture fate.[48]

By analyzing the gene expression within the suture complex, Spector et al. demonstrated that, like the pericranium, the intercalary suture mesenchyme appears not to participate in osteoinductive signaling; instead, it remains primed, awaiting molecular instructions from the underlying dura mater.[49] In order to demonstrate this, 6 day-old Sprague-Dawley rat calvaria were harvested and the subjacent dura mater and overlying pericranium removed. The isolated PF and SAG sutures were separated and either snap-frozen and homogenized or digested with collagenase and used to establish early-passage suture-derived cell lines. Extracellular matrix protein and growth factor mRNA expression were compared in these snap-frozen (*in vivo*) PF and SAG sutures. Identical analysis was performed in the established *in vitro* PF and SAG suture-derived cells. Snap- frozen PF sutures were found to express significantly more collagen I α I, collagen III and osteocalcin transcript than SAG sutures. In contrast, the level of TGF-β1 mRNA was equal in the snap-frozen PF and SAG suture complexes. These initial results implied that the pre-fusing PF suture complex does not intrinsically express critically important osteoinductive cytokines. Instead, the PF suture mesenchyme appears to upregulate osteoid and ECM gene expression in response to inductive dura-derived signals.

Collectively, this series of experiments supports the theory that neither the pericranium nor the intercalary suture mesenchyme appears to guide cranial suture fate in murine models of normal

suture fusion. Instead, the evidence suggests that the osteogenic machinery within the isolated cranial suture complex remains primed, awaiting osteoinductive molecular instructions from the underlying dura mater. The following series of experiments investigate the details of the developmental mechanisms governing dura mater-suture interactions.

CANDIDATE DURA MATER-DERIVED GROWTH FACTORS

While the dura mater, independent of cranial base forces, appeared critical in determining sutural fate, the precise nature of the dura mater-suture paracrine interactions remained unknown. We used a candidate gene approach to identify the cytokines that participate in dura mater-suture communication. Using immunolocalization and *in situ* hybridization techniques, we identified osteogenic factors in the PF dura mater. Furthermore, by modulating the expression of these candidate cytokines, we changed the fate of murine sutures.

The insulin-like growth factors

Thaller et al. first demonstrated that systemic administration of insulin-like growth factor I (IGF-I) promoted healing of critical-size calvarial defects and caused premature anterior frontal suture fusion in Sprague-Dawley rats. Bradley et al. subsequently examined the temporal and spatial expression patterns of IGF-I and IGF-II during normal cranial suture fusion by harvesting rat calvaria from gestational day 16 through postnatal day 80.[50-53] Interestingly, during this study, Bradley et al. demonstrated that IGF-I and IGF-II mRNAs and proteins were almost exclusively expressed in the fusing PF dura mater and the suture mesenchyme, respectively.[53] Moreover, the transcripts and proteins appeared prior to the onset of PF suture fusion (postnatal days 2-10) and persisted until the suture had completed fusion (postnatal day 30). The authors also discovered that osteoblasts in the PF suture complex appeared to co-localize IGF proteins and osteocalcin, a marker of the mature osteoblast phenotype, suggesting that dura-derived IGF-I and IGF-II were acting on the overlying osteoblasts to increase their rate of differentiation and bone formation. The effects of IGFs on bone formation is well known, but it was surprising to find that the PF dura mater was acting as an endogenous source of IGF proteins. However, the role of IGFs in premature cranial suture fusion remains to be determined.

The transforming growth factor-betas

The mammalian TGF-β family consists of three closely related isoforms: TGF-β1, β2 and β3. The TGF-βs are potent growth regulatory molecules that, depending on the state of cellular differentiation, stimulate osteoblast proliferation and induce the synthesis of collagen, osteocalcin and other extracellular matrix proteins.[54-58] In addition, TGF-βs inhibit extracellular matrix degradation by inhibiting osteoclast activity and down regulating the expression of tissue metalloproteinases.[59-63]

Using *in situ* hybridization, investigators identified TGF-β1 mRNA production in the PF dura mater and demonstrated a significant increase in TGF-β1 transcription prior to and during overlying PF suture fusion.[64,65] In marked contrast, the dura mater underlying the patent SAG suture expressed little TGF-β1 transcript throughout the period of predicted suture fusion (i.e. 12-22 days). Immunohistochemistry revealed that elevated TGF-β1 transcripts in the PF dura were translated into protein.[33,34] We hypothesize that this protein diffuses into the suture mesenchyme of the pre-fusing and fusing PF suture. Additional work immunolocalized TGF-β receptors I and II (TGF-βRI and II) to the osteoblasts of the PF osteogenic front and the dura mater subjacent to the actively fusing PF suture. These data suggest that TGF-β1 may act in a paracrine fashion to drive osteoblast differentiation and suture fusion.[37]

In order to further investigate this hypothesis, we injected the PF dural tissues of 24 day-old CD-1 mice with a recombinant, replication-deficient adenovirus, containing the cDNA of a truncated form of the type II TGF-β receptor (Ad-TTR). The overexpression of this dominant-negative receptor bound TGF-β (all isoforms),

and prevented the transphosphorylation and activation of the type I TGF-β receptor. Failure of the dominant-negative type II receptor to activate the type I receptor prevented the initiation of the intracellular SMAD signaling pathways. The PF dura mater of CD-1 mice was transfected with this Ad-TTR construct, placed in organ culture, and examined 30 days later. Interestingly, transfection with the Ad-TTR adenovirus significantly inhibited PF suture fusion [unpublished data]. This observation provided important evidence, and implicated a role for the TGF-βs in cranial suture fusion.

An analysis of human, nonsyndromic, unicoronal craniosynostotic sutures recapitulated the differential pattern of TGF-β expression found in the murine models.[66] Immunohistochemical analysis of these clinical specimens demonstrated a marked increase in the expression of TGF-β1 and β2 ligands in the osteogenic front of prematurely fusing sutures. In contrast, the patent sutures expressed minimal TGF-β1 or β2. Interestingly, TGF-β3 protein immunolocalization was limited to the sutural margin of the patent sutures. It is believed that this restricted pattern of protein expression links TGF-β3 to the suppression of osteogenesis and maintenance of suture patency.[35]

In summary, the human craniosynostotic findings, in combination with the murine data, may implicate TGF-β signaling in the regulation of suture fate. In addition, a similar pattern of TGF-β expression in rats and humans supports the supposition that programmed PF suture fusion in murine models and premature suture closure in man share, at least in part, evolutionarily conserved molecular mechanisms. Additional work is necessary to clarify the complex roles of TGF-βs in cranial suture biology.

The fibroblast growth factors

The FGFs are a highly conserved family of at least 19 closely related monomeric peptides. These growth factors act in concert with heparin sulfate-containing proteoglycans to modulate cell migration, angiogenesis, bone development and repair, and epithelial-mesenchymal interactions.[67-71] FGF-2 is the most abundant ligand and has been shown to stimulate osteoblast proliferation and enhance bone formation *in vivo* and *in vitro*.[72-74] FGF-2 expression is elevated during fracture healing and exogenously applied FGF-2 accelerates osteogenesis in critical size bone defects and fracture sites.[75-77] Furthermore, the FGF-2 signaling cascade augments the expression of TGF-β and its myriad of pro-osteogenic effects.[78]

Mehrara et al. and Most et al. spatially and temporally localized the expression of FGF-2 mRNA during rat calvarial morphogenesis and PF suture fusion.[64,65] *In situ* hybridization revealed an abundance of FGF-2 transcript in the PF dura mater prior to and during PF suture fusion. In contrast, there was a paucity of FGF-2 mRNA in the SAG dura mater throughout the period of predicted suture fusion.[64] Furthermore, immunohistochemistry demonstrated marked increases of FGF-2 protein production in the osteogenic front of the pre-fusing and fusing PF suture.[79] In addition to the experimental evidence supporting its regulatory roles in osteogenesis and epithelial-mesenchymal interactions, the spatial and temporal expression data strongly implicate FGF-2 in the regulation of calvarial bone induction.

Iseki et al. investigated the different roles of FGFR1, FGFR2, and FGFR3 during mouse calvarial development.[80,81] The authors showed that FGFR2 expression coincided with areas of rapid cellular proliferation, but that it was absent from domains of osteoblast differentiation. In contrast, FGFR1 expression was associated with osteoblast differentiation. Finally, FGFR3 was expressed in both the osteogenic and chondrogenic regions of the skeleton, including the thin plate of cartilage that underlies part of the coronal suture, suggesting a cooperative role between FGFR2 and FGFR3 signaling in osteogenic cell proliferation.

These results raise a paradox. If FGFR2 and FGFR3 are expressed by immature osteoblasts, why do activating mutations apparently cause differentiation, ultimately leading to bone formation and craniosynostosis? To address this question, Iseki et al. implanted FGF-2 soaked beads over the coronal suture, thus disrupting the normal suture biology and inducing the ectopic expression of osteopontin (a marker of osteoblast differentiation).[80] FGFR2 expression was absent from the area immediately underlying the bead, but was apparent as a ring surrounding its

margins. This finding suggested that excessive FGF-2 signaling resulted in osteogenic differentiation and reciprocal FGFR2 down regulation. This finding is important because it implies that differential FGF signal intensity may have qualitatively distinct cellular consequences. Further investigation has revealed that some FGFR2 mutations (e.g. Ser 252Trp and Pro253Arg) increase the affinity of the mutant receptor for specific ligands, which leads to excessive signaling and increased osteoblast differentiation and bone matrix formation, under conditions where the availability of FGF ligand is limited.[82,83]

Since *in situ* hybridization and immunohistochemistry spatially and temporally implicated FGF-2 in cranial suture physiology, Greenwald et al. attempted to reverse the fate of murine cranial sutures by manipulating FGF-biologic activity.[84] Using replication-deficient adenoviruses encoding a truncated form of FGFR1 (Ad-FTR) or a secreted form of FGF-2 (Ad-FGF-2), the authors were able to abrogate or increase FGF-biologic activity in the dura mater underlying a fusing or patent suture, respectively. Greenwald et al. demonstrated that *in utero* Ad-FTR infection of the dural tissues underlying the PF cranial suture inhibited programmed posinatal cranial suture fusion, while *in utero* Ad-FGF-2 infection of the dural tissues underlying the coronal suture resulted in posinatal pathologic fusion of this normally patent suture. Using a variety of *in vitro* analyses, the authors demonstrated that the reversal of fate was mediated via alterations in cellular proliferation, extracellular matrix molecule gene expression, and TGF-β1 synthesis. These data provide direct support for the hypothesis that FGF-biologic activity is a critical regulator of both programmed and pathologic cranial suture fusion.

CRANIOSYNOSTOTIC SYNDROMES

FGFR mutations

The four FGFRs are split cytoplasmic tyrosine kinase transmembrane receptors.[69,85-87] The extracellular domain contains three immunoglobulin-like domains (IgI-IgIII).[88,89] Ligand binding occurs at the second and third immunoglobulin-like domains, causing dimerization, tyrosine kinase transphosphorylation and a cascade of intracellular signals.[90,91] The FGF/FGFR system achieves its specificity through temporal and spatial variations in expression patterns. Mutations in FGFR1, FGFR2, and FGFR3 have been associated with a variety of craniosynostosis syndromic phenotypes.[7-11] However, to date, there is no evidence that FGFR4 is involved in craniofacial or skeletal disorders.[92]

There are at least six FGFR-related craniosynostosis syndromes (Apert, Crouzon, Jackson-Weiss, Pfeiffer, Muenke, and Beare-Stevenson Syndrome) that are characterized by brachycephaly or kleeblattschadel deformities, distinctive facial features, and variable hand and foot findings. Mutations of equivalent amino acids in the IgII/IgIII linker region of FGFR1-3 paralogues are associated with different syndromes, suggesting that all three genes play essential, but specific roles in calvarial development: (1) the Pro252Arg mutation of FGFR1 causes a mild form of Pfeiffer syndrome, (2) the Pro253Arg mutation of FGFR2 causes Apert syndrome and (3) the Pro250Arg mutation of FGFR3 is associated with Muenke syndrome. Interestingly, identical mutations have been observed in Crouzon, Pfeiffer, and Jackson-Weiss patients, suggesting that unlinked modifier genes or epigenetic factors play a role in determining the final phenotype.[2,93-96]

To date, all persons with FGFR-related craniosynostosis syndromes are heterozygotes for these mutations. The homozygous phenotype has not been identified and may well be lethal. Interestingly, all six syndromes share premature cranial suture fusion, but show variable involvement and severity of syndactly symphalangism in the hands and feet. It is interesting to speculate that craniosynostosis and syndactly symphalangism result from inadequate apoptosis or inappropriate cellular differentiation in specific interskeletal areas. Furthermore, the specificity of the FGFR-mutation phenotypes may require FGF-mediated regulation of interskeletal tissue by some unique and hitherto unexpected, biological pathway. New findings from clinical molecular genetics have raised important issues concerning FGF receptor function and skeletal biology. The challenge now

is to complete the connections between our knowledge of the altered genotypes and the molecular basis of the altered phenotypes.

MSX mutations

In 1993, Warman et al. described 19 affected individuals from a five-generation pedigree afflicted with an autosomal dominant craniosynostosis syndrome.[97] This Boston family could be sub-classified into four cranial-shape phenotypic variants: 1) eight members had fronto-orbial recession, 2) seven members manifested turribrachycephaly, 3) two members had kleeblattschadel, and 4) two members manifested only frontal bossing. Additional findings included short first metatarsals, headaches, seizures, myopia, and other visual deficits.

Shortly after the initial report, Jabs et al. identified a Pro148His substitution in the *MSX2* gene of this affected family.[12] This mutation conferred enhanced DNA binding affinity and reduced ligand dissociation, suggesting that the substitution of a histidine for a proline, in the highly conserved protein-DNA binding motif, created a gain-of-function mutation.[98,99] Interestingly, the overexpression of *Msx2* prevented osteoblast differentiation and mineralization of the extracellular matrix.[100] Transgenic models indicated that mice overexpressing the *Msx2* mutation have a complex dose-dependent phenotypic expressivity. For example, when a Pro7His *Msx2* gene was expressed under the control of the weak murine *Msx2* promoter, the numbers of proliferative and early-stage osteoblastic cells were increased in the osteogenic fronts.[101] These mice did not develop craniosynostosis, but displayed enhanced calvarial bone growth and narrowing of the cranial sutures. However, when the *Msx2* gene was widely overexpressed under the control of the strong cytomegalovirus (CMV) or the tissue inhibitor of metalloprotease (TIMP-1) promoters, the mice exhibited enhanced calvarial bone growth and craniosynostosis.[99]

Tremendous speculation surrounds the mechanism of *Msx2*-mediated craniosynostosis. However, most researchers hypothesize that two growth factor-mediated signaling pathways modulate *Msx2* expressivity. First, *Msx2* expression in the oral epithelium and in the hindbrain can be induced by the implantation of beads soaked in BMP-2 or BMP-4.[102,103] This suggests that BMPs may drive osteoblast proliferation through *Msx2* signaling pathways. Second, Crouzon, Jackson-Weiss, Apert and Pfeiffer syndromes are caused by mutations in the extracellular domain of the FGFR2 gene. Moreover, FGFR2 is co-expressed with *Msx2* in various sites in the developing mouse embryo.[104,105] A direct role for FGFR in the determination of the competence of a tissue to respond to a signaling molecule has been established by experiments in *Xenopus laevis*, which showed that a dominant negative mutant FGFR2 interferes with the *Msx2*-dependent inductive processes that determine posterior mesodermal fate.[106] Thus, FGFR2 and *Msx2* may function together, perhaps in the same epistatic pathway, to regulate the temporal sequence of suture development and thus to coordinate the growth of the skull with that of the brain.

In 2000, Wilkie et al. identified a *MSX2* functional haploinsufficiency in three unrelated families with enlarged parietal foramina.[107] *Msx2* haploinsufficient mice had similarly persistent calvarial foramen.[108] The authors hypothesized that defective proliferation of osteoprogenitors at the osteogenic fronts caused the defect in skull ossification. These findings were fascinating because they helped to formulate a hypothetical mechanism for *Msx2* function. Collectively, these results indicate that high levels of *Msx2* expression can impede the final phases of osteoblastic differentiation, and that insufficient *Msx2* expression leads to osteoprogenitor burn-out and the cessation of bone growth.

In summary, we postulate that Boston-type *MSX2* gain-of-function mutation may transiently retard osteogenic cell differentiation within the cranial suture, and that his leads to an increased pool of proliferative cells that remain primed and ready to receive differentiation signals (e.g. BMPs and FGF-2) from the subjacent dura mater. Ultimately, normal growth factor signaling to a supplementary population of osteoblasts may create a pathologic increase in cranial suture bone deposition, causing premature fusion. Further studies are needed to test this hypothesis.

TWIST mutations

Although Brueton et al. mapped Saethre-Chotzen syndrome to chromosome 7p21 in 1992, the mutated basic helix-loop-helix containing gene, *TWIST*, was not cloned until 1997.[16-18,109] More than 50 nonsense, deletion, and missense mutations have now been identified.[110] These mutations are hypothesized to create a haploinsufficient phenotype resulting in premature osteoblast maturation and cranial suture fusion. Recently, novel FGFR2 and FGFR3 mutations were discovered in a subset of Saethre-Chotzen patients.[18] This subset of patients provides interesting insight into the molecular mechanism mediating the cranial suture biology of Saethre-Chotzen syndrome. For example, *TWIST* haploinsufficiency may cause the upregulation of FGFR expression. Alternatively, *TWIST* haploinsufficiency may mediate an alteration in the ratio of FGFR isotype expression (e.g. increased FGFR1/FGFR2) leading to premature osteoblast differentiation. More work is needed to clarify these hypotheses. Eventually, expression assays of *TWIST* and FGFRs in craniosynostosis syndrome patients may decipher the complex interactions between these genes and the developmental pathways involved in craniofacial and limb development in humans.

GLI3 mutations

Greig cephalopolysyndactyly syndrome, characterized by variety of craniofacial and limb anomalies, is associated with point mutations, deletions, and translocations within the centrally located zinc finger domain of the GLI3 gene.[13-15] It has been hypothesized that these mutations cause GLI3 functional haploinsufficiency leading to unopposed GLI1 signaling and increased transcription of the SHH target genes (e.g. BMP-2, BMP-4, and *MSX2*).[111,112] The coexistence of craniofacial and limb anomalies in Greig syndrome is fully compatible with the temporal and spatial diversity of SHH expression during calvarial and limb development.[113]

Therefore, GLI3 mutations may not only impair DNA-binding, but also transcriptional activity resulting in a variety of syndromic findings. Interestingly, the phenotypic-genotypic correlation in Greig syndrome seems to be complicated by phenotypic variability, in the same way that the mouse extra toes mutation (Xt), a deletion of part of Gli3, causes considerable phenotypic heterogeneity of the affected feet.[114] Ultimately, we may find that the phenotypic variability in Greig syndrome is a result of mutant GLI3 modifying interactions with paralogues of the SHH signaling cascade.

CONCLUSIONS

Normal cranial suture biology is very complex and seems to require a coordinated cascade of molecular signals from the underlying dura mater. While our knowledge of these dura-derived signals has increased dramatically over the last decade, we have barely begun to understand the fundamental mechanisms that mediate cranial suture fusion or patency. Interestingly, recent advances in both premature human and programmed murine suture fusion have revealed unexpected results, and have created more questions than answers. Ultimately, by understanding the mechanisms that mediate cranial suture biology, we may be able to intelligently develop targeted strategies to treat or reverse prematurely fusing sutures.

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