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REVIEWS

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Cranial Sutures as Intramembranous Bone Growth Sites

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ABSTRACT **Intramembranous bone growth is achieved through bone formation within a periosteum or by bone formation at sutures. Sutures are formed during embryonic development at the sites of approximation of the membranous bones of the craniofacial skeleton. They serve as the major sites of bone expansion during postnatal craniofacial growth. For sutures to function as intramembranous bone growth sites, they need to remain in an unossified state, yet allow new bone to be formed at the edges of the overlapping bone fronts. This process relies on the production of sufficient new bone cells to be recruited into the bone fronts, while ensuring that the cells within the suture remain undifferentiated. Unlike endochondral growth plates, which expand through chondrocyte hypertrophy, sutures do not have intrinsic growth potential. Rather, they produce new bone at the sutural edges of the bone fronts in response to external stimuli, such as signals arising from the expanding neurocranium. This process allows growth of the cranial vault to be coordinated with growth of the neurocranium. Too little or delayed bone growth will result in wide-open fontanels and suture agenesis, whereas too much or accelerated bone growth will result in osseous obliteration of the sutures or craniosynostosis. Craniosynostosis in humans, suture fusion in animals, and induced suture obliteration in vitro has been associated with mutations or alterations in expression of several transcription factors, growth factors, and their receptors. Much of the data concerning signaling within sutures has been garnered from research on cranial sutures; hence, only the cranial sutures will be discussed in detail in this review. This review synthesizes classic descriptions of suture growth and pathology with modern molecular analysis of genetics and cell function in normal and abnormal suture morphogenesis and growth in a unifying hypothesis. At the same time, the reader is reminded of the importance of the suture as an intramembranous bone growth site.** **© 2000 Wiley-Liss, Inc.**

Key words: growth plate; craniosynostosis; cranial vault; TGF-β; FGF; MSX; TWIST; CBFA1; growth factor receptors; intramembranous bone; cell proliferation; cell differentiation; apoptosis; morphogenesis; development

INTRODUCTION

Morphogenesis of the bones of the cranial vault and facial complex is a lengthy developmental process initiated during early embryogenesis and completed during adulthood. In humans, the end point of cranial vault growth is determined upon fusion of associated bones in the third decade of life. However, in the facial complex, the bones remain separated by a fibrous union until the seventh or eighth decade of life. Although there is a large body of literature on the formation of the mandible and maxilla, relatively little is written specifically on the development of the cranial vault. Recent information on pathologic growth of the cranial vault has brought to the fore the importance of appropriate morphogenesis and continued growth of the bony vault. This review will critically focus on current but contrasting opinions of the embryology, development, and growth of the cranial bones. Described below are the sections into which this review is divided.

Embryology of the Craniofacial Complex: The embryonic development of bone blastemas is described briefly, along with their growth through classically described intramembranous bone formation at the expanding bone fronts. During mid to late gestation, the cranial bone fronts approximate one another and either abut or overlap, with the creation of a suture between them.

Cranial Suture Biology: Here, a brief definition of sutures is given, followed by a description of the role of

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various perisutural tissues in regulating formation of sutures and suture patency.

Role of Growth and Transcription Factors in Regulating Suture Patency: This section provides a detailed description of the localization of transcription factors, growth factors, and their receptors in developing, fully formed, and fusing sutures. This is followed by a description of the nature of known mutations associated with the numerous, severe, developmental disorders arising from premature osseous obliteration of sutures, i.e., craniosynostosis. The regulation of cell function by transcription factors, growth factors, and their receptors known to be associated with suture development and growth is then discussed.

Sutures as Intramembranous Bone Growth Sites: This section briefly describes and discusses the differences between intramembranous bone growth sites and endochondral bone growth centers. Our current understanding of suture biology is put into perspective by discussing increased recognition of its critical role as a bone growth site and the need for future research in this area.

Perspectives: This section summarizes the overall discussion and defines the controversies within the field. Some future research directions are indicated.

To avoid confusion when referring to gene activity (mRNA expression, gene mutation, transgenic allele) and protein expression in human versus other species, all references to genes and their products in this review will be made in upper case letters. An explanation in the text will indicate whether genes or their products are being referred to and whether human or animal studies are being quoted.

EMBRYOLOGY OF THE CRANIOFACIAL COMPLEX

At the end of gastrulation, three primary tissue layers — ectoderm, mesoderm, and endoderm — are present in the embryo. A fourth tissue layer becomes identifiable with the migration of the neural crest away from the closing and closed neural folds. Cranial neural crest migration in the mouse begins at embryonic day 8 (E8) and is completed after approximately 2 days (Slavkin, 1979). In humans, the equivalent time falls between E19 and E38 (Dixon, 1977). The migration of neural crest corresponds to around 5 days before the appearance of the mandibular bone matrix in mice (Dixon, 1977) and around 7 to 8 days before appearance of calvarial bone matrix (Lemire, 1986, 2000; Alberius and Friede, 1992). After migration of the neural crest, the head has mesenchyme originating from two sources, namely paraxial mesoderm and cranial neural crest (Noden, 1983).

Although the embryonic tissue of the facial bones is clearly recognized as neural crest in origin in all vertebrate species, including humans (Noden, 1983, 1986a; Couly and Le Douarin, 1985, 1987; Couly et al., 1992), the origin of the cranial vault bones remains contentious. By using a quail-chick chimera culture system, Couly and colleagues demonstrated the origin of the membranous bones of the skull (frontal, parietal, and squamosal), their intervening sutures, overlying dermis, and underlying dura mater to be from early migratory populations of cephalic neural crest cells (Couly et al., 1992, 1993). Noden (1986a,b), by using the same quail-chick chimera system and more recently by using viral transfection assays (personal communication, 2000), showed the intramembranous bones of the cranial vault, their overlying periosteum, and the intervening sutures to be paraxial mesoderm in origin, whereas the underlying dura mater was neural crest derived.

These distinctions become important when comparing intramembranous bone formation in the parietal region of the cranial vault to that in the maxilla, mandible, and frontal region of the facial skeleton. Classic experiments have shown that neural crest cells destined to become mandibular bone are induced to do so by the overlying epithelium, whereas mesenchyme from other sources and other embryonic regions is not inducible (Hall, 1981). The window of susceptibility to induction is relatively short. Once the induction has occurred, continued presence of the inducing tissue is no longer required. This is similar to the requirement of maxillary mesenchyme for presence of epithelium to promote osteogenesis (Tyler and Cobb, 1980). Development of the frontal bone likewise requires the inductive influence of ectoderm on the underlying neural crest cells to form bone (Tyler, 1983). All of these inductive influences occur between neural crest derived mesenchyme and overlying epithelium. The same inductive influences would be expected to occur in the parietal portion of the cranial vault, if the cranial vault were neural crest in origin as suggested by Couly et al. (1992, 1993). Because inductive influences were found in chick skull bones (Benoit and Schowing, 1970), it is likely that there is some neural crest contribution to the bones of the cranial vault, at least in birds. However, if there is limited or no neural crest contribution to cranial vault bones, alternate inductive influences may occur.

Even if neural crest cells do not contribute directly to the intramembranous bones of the cranial vault, they could do so indirectly by means of the dura mater, which undisputedly arises from neural crest cells. Yu et al. (1997) demonstrated that neonatal dura mater is capable of undergoing bone formation when transplanted underneath epithelium, but not when transplanted under deeper mesodermally derived tissue layers. These authors hypothesized that dura mater is capable of inducing bone formation in the presence of epithelium. Based on the experiments detailed above (Tyler and Cobb, 1980; Hall, 1981; Tyler, 1983), it is more likely that the neonatal epithelium induced bone formation by dura mater. Interestingly, these experiments seem to point to a longer inductive period than was described earlier.

This issue becomes less debatable if neural crest and mesoderm derived tissues are interchangeable. However, little is known about the ability of embryonic tissues to compensate for one another. For example, deficiencies in neural crest cells could occur due to insufficient migration or proliferation and mesodermal cells may make up for the deficiency in some manner. This was addressed by the recent work of Schneider (1999), that challenges current hypotheses disputing the ability of neural crest to replace mesodermal elements of the braincase. By using the chick-quail chimera system used by both Couly and Noden (Couly et al., 1992, 1993; Couly and Le Douarin, 1985, 1987; Noden, 1984, 1986a,b; 1991, 1992), Schneider (1999) demonstrated that changes in the allocation of migrating cells could enable a neural crest-derived skeletal element to replace a mesodermal portion of the braincase during evolution. In all likelihood, the major issue of these inductive influences is the presence of appropriate inductive tissues and the presence of sufficient tissue capable of responding to these inductive influences. This point of view is reinforced by the examples given by Hall and Miyake (2000) in their recent review, in which establishing condensation size is shown not only to affect the final size of the skeletal element, but also whether the element will form at all. Once condensations of cranial bones have appeared and ossification proceeds, the next important event occurs when the bones approximate one another and the formation of sutures is initiated. It is at this time that sutures will become the major sites of neonatal and postnatal intramembranous bone growth during rapid expansion of the neurocranium and development of the maxillary complex.

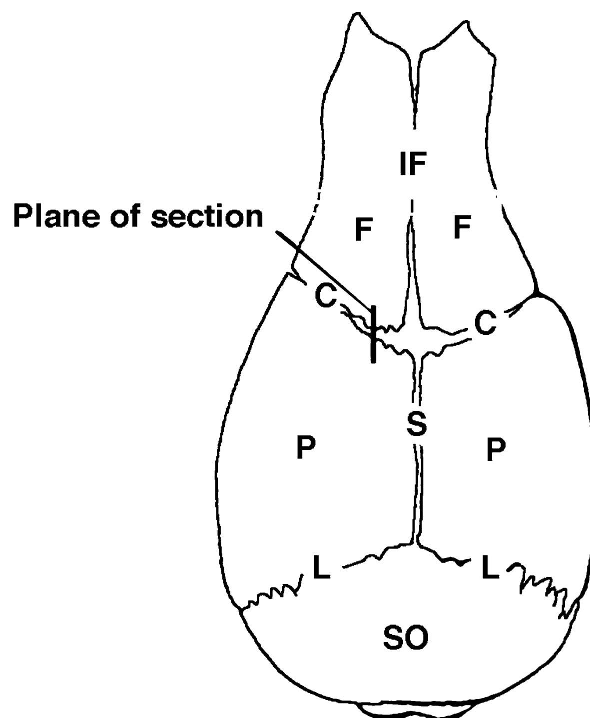

Fig. 1. Diagrammatic representation of a rat skull showing the location of the sutures (C, coronal, IF, interfrontal, S, sagittal, L, lambdoid) and the membranous bones (F, frontal bone, P, parietal bone, SO, supraoccipital bone) of the cranial vault. A black line indicates plane of section for the sutures in Figure 2. (Modified from Opperman et al., 1997, J Bone Miner Res 12:301–310 and printed with permission of the American Society for Bone and Mineral Research.)

CRANIAL SUTURE BIOLOGY

Definition of Sutures

Cranial vault sutures, the fibrous tissues uniting the bones of the skull, are the major sites of bone growth along the leading margins of the cranial bones during craniofacial development, especially during rapid expansion of the neurocranium (Baer, 1954). To function as bone growth sites, sutures need to remain patent, while allowing rapid bone formation at the edges of the bone fronts. Premature osseous obliteration of sutures (craniosynostosis) by fusion of bone fronts across the suture site, prevents further bone formation at this site. The loss of the sutural growth sites causes an inability to accommodate rapid, expansive growth of the neurocranium, leading to abnormal compensatory morphogenesis throughout the head (Enlow, 1986, 2000) and typically results in craniofacial dysmorphology.

To begin understanding the role of cranial sutures as intramembranous bone growth sites, it is necessary to establish where sutures occur, how they form, and what regulates their formation and maintenance. In humans, cranial vault sutures typically form with the interfrontal (metopic) suture between the frontal bones, the sagittal suture between the parietal bones, the paired coronal sutures between the two frontal and two parietal bones, the paired lambdoid sutures between the supraoccipital and parietal bones, and the squamosal sutures between the parietal, temporal, and sphenoid bones. This arrangement is very similar to the arrangement seen in other species such as rabbits, mice, and rats (Fig. 1), which have been used as research tools to examine suture biology and pathology. For the purposes of this review, the facial sutures have been omitted.

Regulation of Suture Morphogenesis

After induction of osteogenic potential, initiation of intramembranous bone formation proceeds through development of mesenchymal blastemas, the precursors of each of the bones of the cranial vault (Dixon, 1977; Alberius and Friede, 1992; Langille, 1994; Aubin and Liu, 1996). During this process, mesenchymal cells begin to differentiate and deposit extracellular matrix consisting primarily of type I and other collagens (van der Rest, 1991) as well as other bone-related proteins and proteoglycans (Cole and Hanley, 1991; Tracy and Mann, 1991), which are then mineralized. Intramembranous ossification proceeds radially from each of

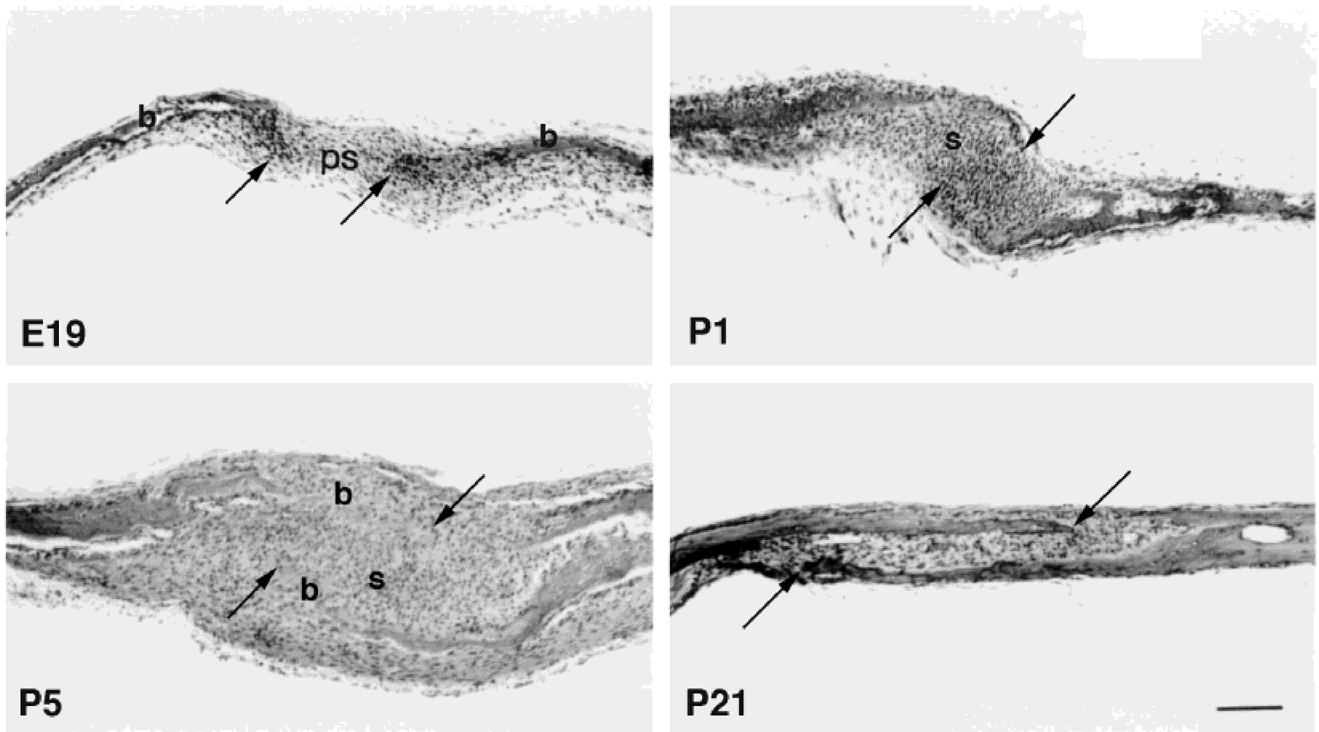

Fig. 2. Photomicrographs of hematoxylin and eosin stained histologic sections through normal rat coronal sutures, showing various stages of morphogenesis. Tissues are oriented with periosteal surface at the top and dura mater underneath. Arrows indicate the leading edges of the bone fronts (b). E19, embryonic day 19 suture showing frontal and parietal bones (b) widely separated by presumptive suture (ps) mesenchyme. Note highly cellular nature of the sutural edges of the bone fronts (arrows). P1, postnatal day 1 suture showing overlapping frontal and parietal bones separated by a highly cellular suture blastema (s). P5, postnatal day 5 suture showing gradual thickening of the bones on either side of the suture and increased overlap of the bone fronts compared with P1. P21, postnatal day 21 sutures showing dramatic remodeling of the suture matrix to a narrow strip of fibrous, cellular material separating the overlapping frontal and parietal bones. (Modeled after Opperman et al. 1993. Dev Dyn 198:312–322.) Scale bar = 100 μm.

these foci (Alberius and Friede, 1992). The borders of each cranial bone are initially widely separated due to rapid expansion of the neurocranium (Kokich, 1986; Cohen, 2000). However, as ossification proceeds and neural growth abates, the bone fronts approximate one another and suture formation is initiated as the bone fronts abut or overlap one another, with fontanels representing the unossified regions of confluence of more than two cranial vault bones.

During morphogenesis of the rat coronal suture, the approaching frontal and parietal bone fronts of E19 calvaria are separated by presumptive suture (ps) matrix. The tips of the two bone fronts contain large numbers of osteoprogenitor cells and large cuboidal osteoblasts (arrows, Fig. 2). By P1, 72 hours later, the two bone fronts overlap one another and a highly cellular suture matrix is seen separating the bone fronts. Although a distinct fibrous periosteal layer is seen around the approximating bone fronts of facial bones (Pritchard et al., 1956), no such intervening layers are seen in the developing rat coronal suture or in the developing mouse suture (Johansen and Hall, 1982). During rapid expansion of the neurocranium, the suture remains highly cellular, but as cranial expansion slows by P21, the number of cells lining the bone fronts declines and the suture narrows (Fig. 2). Histologically, these events are remarkably similar to those occurring during development of the human cranial vault (Markens, 1975). During development of the sutures, the growing and expanding bone fronts both invade and recruit the intervening mesenchymal tissue into the advancing edges of the bone fronts. During this process, the mesenchyme becomes separated by the intervening bones into an outer ectoperiosteal layer and an inner dura mater (Kokich, 1986).

It is currently unclear which tissues and signaling factors are responsible for induction of suture formation. Although the dura mater is not necessary to induce initial overlap of the bone fronts during coronal suture development (Fig. 3A), its presence is required for initial stabilization of the suture (Fig. 3B; Opperman et al., 1993). The midline sutures (sagittal, interfrontal) are butt sutures, which do not overlap, whereas the transversely situated sutures (lambdoid, coronal) do overlap. It is currently believed that the approximating bone fronts set up a gradient of growth factor signaling between them, which initiates suture formation (Opperman et al., 1993; Roth et al., 1996).

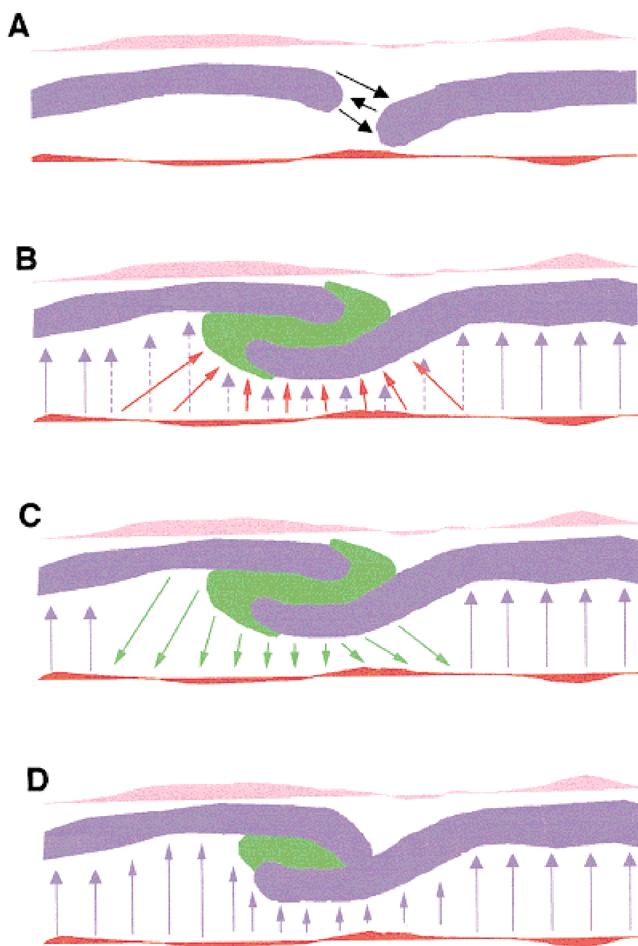

Fig. 3. Diagrammatic representation of various stages of suture morphogenesis (A–C) and suture fusion (D). **A:** Inductive signals (arrows) arising from the approaching bone fronts (blue), allow the bone fronts to deflect away from each other or butt up against one another, without obliterating the suture. These signals are independent of signals from dura mater or periosteum. **B:** Once the bone fronts have overlapped one another, a signal (red arrows) arising from the dura mater maintains the presence of the newly formed suture (green). Osteogenic signals (blue arrows) from the dura mater cause the bones to become thickened by depositing and mineralizing new osteoid on the periosteal surface. These osteogenic signals may be continuous along the dura mater before formation of the suture (dotted blue arrows). **C:** Once the suture is stabilized, it signals (green arrows) the local underlying dura mater not to produce osteogenic signals. **D:** In the absence of osteoinhibitory signals from the suture, the underlying dura mater remains continuously osteogenic (blue arrows), overriding signals within the suture and resulting in osseous obliteration of the suture. Periosteum is colored pink.

However, it is currently unknown how this occurs or whether this signaling regulates the type of suture that will appear.

Regulation of Suture Patency

In the rat, all cranial vault sutures with the exception of the posterior interfrontal suture remain patent for the life of the animal. In humans, the interfrontal (metopic) suture fuses between the second and fifth year after birth, with approximately 10% of the population having metopic sutures remaining patent (Kokich, 1986). Early attempts to culture sutures to examine factors regulating suture patency failed, probably because calvaria were dissected at 37°C and the resulting hypoxia produced cartilage at the suture sites (Markens and Taverne, 1978). However, in later attempts, transplants of E19 calvaria into parietal bone defects in adult rats resulted in normal coronal suture development (Opperman et al., 1993). Also in this study, removal of fetal dura mater before transplant initially resulted in normal overlap of the bone fronts (Fig. 3A). In the absence of dura mater, however, the newly formed sutures were unable to sustain themselves and became obliterated by bone. When these experiments were repeated by using an in vitro organ culture system, similar results were obtained (Opperman et al., 1995). Furthermore, the more fully developed coronal sutures of P1 calvaria were found to be able to sustain themselves in culture even in the absence of dura mater. These results indicated that dura mater is permissive for suture formation, but that an inductive stimulus from dura mater is required during suture formation before the suture is able to maintain itself (Fig. 3B). A similar inductive event was noted to be required for mouse suture development, which also showed postnatal independence from continued presence of dura mater (Kim et al., 1998). In experiments where the ectoperiosteal layer was removed, it was found that the periosteum was not required for maintenance of suture patency (Opperman et al., 1994). The role of these tissues is different due to two alternate possibilities, depending on the source of the mesenchyme originating the tissue. One possibility is that the dura mater is strictly neural crest derived and that periosteum has some contribution from paraxial mesoderm as suggested by Noden (1986a); hence, their role in regulating suture morphogenesis is different. The other possibility is that all subepidermal cranial vault tissues are neural crest in origin (Couly et al., 1992, 1993) and the role of the tissues becomes altered as their association with one another changes, i.e., ectoperiosteum becomes associated with forming bone and dermis, whereas dura becomes associated with forming bone and brain. It should be noted that the facial sutures, which appear very similar to cranial vault sutures in both morphology and function, do not have contact with an underlying dura mater. It is likely that tissues surrounding the facial sutures regulate the sutures in a similar manner to the dura, but which tissues provide the signals have not been identified.

Studies on the normally fusing posterior interfrontal suture in postnatal animals (Roth et al., 1996) demonstrated that inhibiting contact between the suture and underlying dura mater led to delayed fusion of this suture. When posterior interfrontal sutures were cultured in the presence of dura mater, they fused as seen in vivo. However, when these sutures were cultured without their dura mater, they remained patent in

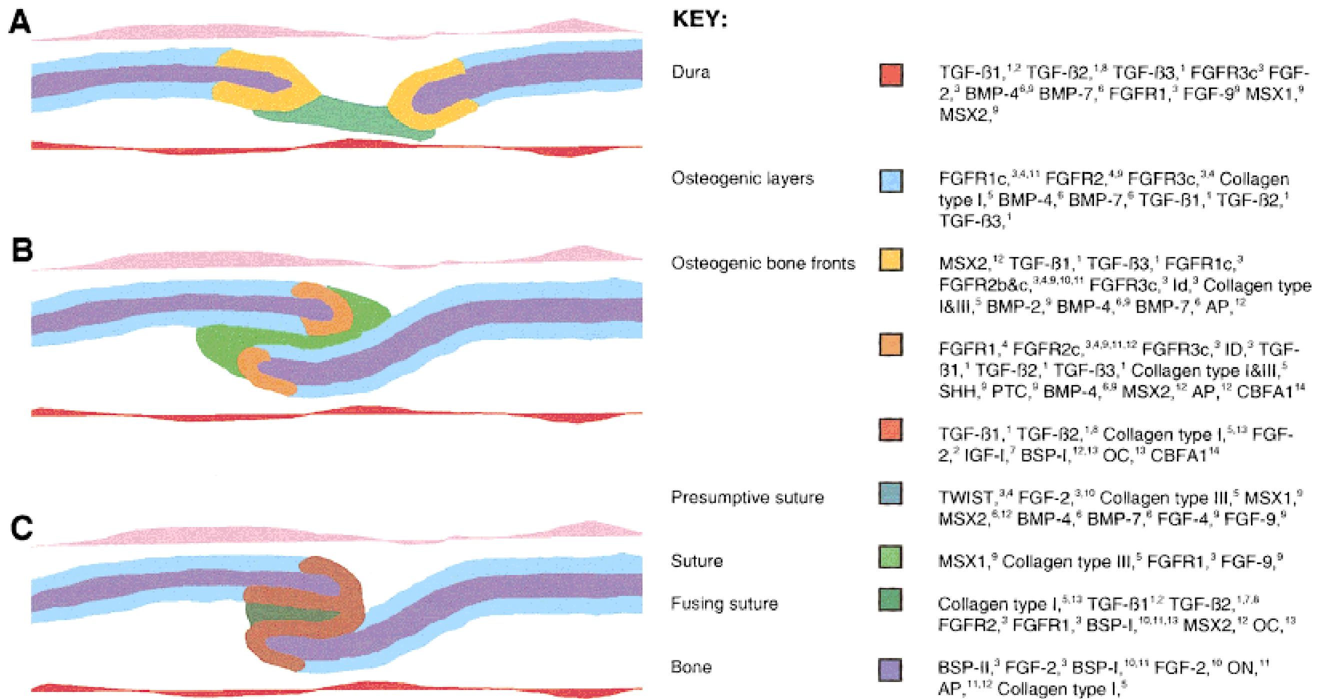

Fig. 4. Diagrammatic representation of a presumptive suture (**A**), a fully formed suture (**B**), and a fusing suture (**C**). The color-coded regions of the sutures (green), osteogenic bone fronts (orange), and bone (blue) are given in the accompanying key. Periosteum is colored pink. The key lists the growth and transcription factors, receptors, and extracellular matrix components known to be present in each of the stages of suture morphogenesis presented. ([1]Opperman et al., 1997, [2]Most et al., 1998, [3]Rice et al., 2000, [4]Johnson et al., 2000, [5]Marks et al., 1999, [6]Rice et al., 1999, [7]Roth et al., 1997a, [8]Roth et al., 1997b, [9]Kim et al., 1998, [10]Iseki et al., 1997, [11]Iseki et al., 1999, [12]Liu et al., 1999, [13]Lemmonier et al., 2000, [14]Zhou et al., 2000.)

culture indefinitely (Bradley et al., 1996). These studies demonstrated in an alternate way that once the sutures have been formed, they are capable of maintaining themselves independent of the presence of dura mater. However, these data support the hypothesis that the dura mater underlying the suture is altered once the suture is formed. This idea is supported by the findings of Levine et al. (1998) that the dura mater showed regional differences in its ability to regulate suture patency. This was done by rotating dura mater normally underlying patent anterior interfrontal sutures so that it came to underlie normally fusing posterior interfrontal sutures. The posterior interfrontal sutures now remained open, whereas the anterior interfrontal sutures fused, indicating that the dura mater underlying the anterior interfrontal suture became imprinted with a signal preventing osteogenic signaling from this site (Fig. 3C).

This imprinting is necessary because fetal and infant dura mater is highly osteogenic, being capable of completely re-ossifying the cranial vault after craniectomy (Drake et al., 1993; Hobar et al., 1993, 1996). However, the dura mater underlying the sutures does not form bone, as demonstrated both clinically and experimentally by the re-growth of skulls complete with sutures from infant dura mater (Mabbutt and Kokich, 1979; Mabbutt et al., 1979; Drake et al., 1993). Failure of the suture to imprint the dura mater with a signal inhibitory to bone formation would result in absence or obliteration of sutures as described above by Levine et al. (1998) (Fig. 3D). This was also seen clinically in failure of sutures to re-grow at sites where sutures initially failed to form (Drake et al., 1993). Interestingly, dura mater transplanted from beneath normally patent sutures to sites of sutural obliteration in a naturally occurring craniosynostotic rabbit model was able to keep these sites from re-obliteration (Mooney et al., 2000). A lack of imprinting may be responsible for posterior interfrontal suture obliteration in the presence of dura mater in the rat and mouse (Bradley et al., 1996; Roth et al., 1996) and for normal obliteration of the metopic suture in humans. The nature of this imprinting signal, along with the signaling involved in the suture's ability to function as a bone growth site, is currently being elucidated.

THE ROLE OF GROWTH AND TRANSCRIPTION FACTORS IN REGULATING SUTURE PATENCY

In experiments in which dura mater was physically removed from calvaria and then co-cultured with the calvaria, it was demonstrated that dura mater secreted soluble, heparin-binding factors that were required for maintaining suture patency (Opperman et al., 1996).

An effective way to begin to understand the regulatory mechanisms that maintain sutures in their unossified state, while allowing them to function as bone growth sites, is to look at systems in which these regulatory mechanisms are interrupted. It is now known that several growth factor receptor and transcription factor mutations are associated with craniosynostosis and that interaction of these factors with one another and with a host of other growth factors is necessary for maintenance of normal sutures.

Several recent reviews and a new book have very eloquently described the clinical pathologies associated with the known mutations (Webster and Donoghue, 1997; Wilkie, 1997; Elmslie and Reardon, 1998; Jabs, 1998; Nuckolls et al., 1999; Sperber, 1999; Cohen, 2000). These reviews give excellent descriptions of the types of mutations that occur and describe how some mutations appear to manifest very different phenotypes. Typically, the nature of the mutations is not completely described. For example, mutations of FGF receptor and MSX2 genes generally are described as activating or gain of function mutations, whereas mutations in TWIST are considered as loss of function; but the "function" that is affected is often not clear.

Many recent studies have begun to look at how gene products regulate cell function within the cranial suture tissues. Although some of these do attempt to examine the complex interaction of factors regulating suture morphogenesis and patency, many ignore that setting up and maintaining a patent suture is only the first step in establishing an intramembranous bone growth site. The remainder of this review will elucidate the specific roles of known factors involved in regulating cell function at the cranial suture site, keeping in mind its role in intramembranous bone formation. This information will then be synthesized into a unifying hypothesis of the mechanisms by which bone growth at the suture are regulated to allow rapid expansion of the cranial vault without overgrowth of the bone resulting in osseous obliteration of the suture.

Localization of Growth and Transcription Factors and Their Receptors Within Sutural and Perisutural Tissues

It is essential to look at the distribution and localization of factors known to be involved in suture morphogenesis to begin to understand how suture morphogenesis is regulated and to explore what goes wrong during premature fusion of sutures (Fig. 4). It is clear that before suture formation, factors known to be involved in epithelio-mesenchymal signaling, such as BMP-4, BMP-7, FGF-9, MSX1 and MSX2, as well as TWIST, are present in the presumptive sutural mesenchyme, the underlying dura and the approaching bone fronts (Fig. 4A, shades of green, red, and shades of orange, respectively). Also present are FGF receptors 1–3, with FGFR2 absent from the suture and dura, but highly expressed in the approaching bone fronts. TGF-β1, 2, and 3 are present in the dura and approaching bone fronts, but are absent from the suture mesenchyme.

During the process of approximation and overlap of the bone fronts (Fig. 4B), factors involved in pattern formation, such as SHH, PTC, and ID are present, whereas TWIST and MSX2 are down-regulated. At the same time, differential distribution of extracellular matrix components can be seen, with CBFA1, osteopontin (BSP-I), bone sialoprotein (BSP-II), type I collagen, osteonectin (ON), and alkaline phosphatase (AP) found in the bone and type I collagen and type III collagens being expressed in the bone fronts, along with FGFR1-3 and TGF-β1, 2, and 3. The suture matrix shows mostly type III collagen expression and still expresses FGF-9, MSX1, and begins to express FGFR1.

To characterize a fusing suture (Fig. 4C), data were taken from both normally fusing sutures, such as the interfrontal suture of rats and mice and from abnormally fusing sutures either experimentally induced or as a product of activity of mutant genes. Factors associated with bone formation, such as type I collagen, TGF-β1, TGF-β2, FGFR1, FGFR2, and BSP-I are up-regulated in the suture matrix, whereas CBFA1, FGF-2, and IGF-1 become expressed in the bone fronts. MSX1, ID, SHH, PTC, and FGF-9 are all down-regulated and in the completely fused suture, the suture is indistinguishable from bone.

The Nature of Mutations Associated With Abnormal Suture Development and Growth

Although the first mutation to be associated with craniosynostosis was found in the MSX2 gene (Jabs et al., 1993), subsequent mutations were identified in the FGF receptor genes (Jabs et al., 1994; Muenke et al., 1994; Reardon et al., 1994; Shiang et al., 1994). Boston-type craniosynostosis is the only craniosynostotic syndrome associated with mutations in the gene for MSX2 (Jabs et al., 1993). MSX2 mutation enhances DNA binding of the MSX2 protein to its DNA binding sequence (Ma et al., 1996). This produces an autosomal dominant defect resulting in fused cranial sutures, a finding supported by studies in which overexpression of MSX2 results in suture fusion (Liu et al., 1995, 1999).

The converse of premature fusion occurs with functional haploinsufficiency of MSX2 in humans, resulting in delayed suture formation and wide-open fontanels (Wilkie et al., 2000). MSX2-deficient mice showed similar delayed suture formation and marked endochondral growth plate defects, with narrowing of the cranial base synchondroses and long bone growth plates and reduced cancellous bone and cortical bone thickness. This finding is not surprising, because MSX2 abrogates cellular responsiveness to FGF-2 (Newberry et al., 1997a,1997b). It is tempting to speculate that the cranial vault defect seen in MSX2 haploinsufficiency is due mainly to a shortening of the cranial base synchondroses. The open fontanels would then be a result of rapid expansion of mesenchymal tissue within the su-

ture to compensate for failure of growth in the cranial base and to accommodate the growing brain. This is best demonstrated in hydrocephalus, for which abnormal expansion of the neurocranium is accommodated by rapid expansion of the bones of the cranial vault (Schendel and Shuer, 1994).

Mutations in FGFR2 associated with the Crouzon phenotype result in disulfide-bonded receptor dimers that are constitutively activated, leading to increased kinase activity (Galvin et al., 1996). In contrast to these mutations, the mutations associated with Apert and Jackson-Weiss phenotype exhibit a selective decrease in FGF-2 dissociation kinetics from mutant FGFR (Anderson et al., 1998). All mutations result in fusion of cranial vault sutures and mid-face hypoplasia, with Crouzon syndrome also exhibiting shortening of the cranial base. A similar phenotype is noted in the *Bey* mutant mouse, in which an insertional mutation at the FGF-3/FGF-4 locus produces a mouse with facial shortening, increased interorbital distance, and premature craniosynostosis (Carlton et al., 1998). Expression of both FGF-3 and FGF-4 is up-regulated in this mutant mouse, indicating that locally elevated levels of growth factors can produce similar phenotypes to those seen with mutant growth factor receptors. Interestingly, Coffin and co-workers (1995) showed that transgenic mice over-expressing FGF-2 exhibit markedly shortened long bones associated with growth plate defects, as well as "calvaria enlarged over the occipital bones," which is usually associated with either cranial base shortening or with suture fusion. However, these authors did not report on whether the sutures were patent and did not report looking at the cranial base. This phenotype appears to be similar to the cranial bossing seen in achondroplasia, in which mutations result in FGFR3 receptor dimerization and ligand-independent stimulation of kinase activity. This produces inappropriate cartilage growth plate differentiation, with abnormally shortened long bones and cranial base (Webster and Donoghue, 1996). These data were supported by the findings of Naski et al. (1998), who showed that mice with an activated FGFR3 transgene have shortened long bones and cranial occipital bossing.

This role for FGFR3 in cartilage growth plate development was confirmed by knockout experiments (Deng et al., 1996), in which disruption of FGFR3 expression led to prolonged endochondral bone growth and concomitant elongated long bones. However, no abnormalities in the intramembranous bones of the craniofacial skeleton were found, indicating that osteoblast function appeared unaffected by the presence or absence of FGFR3. Muenke craniosynostosis, which is the result of a P250R mutation in FGFR3, does present with unilateral or bilateral coronal craniosynostosis (Muenke et al., 1997). However, it is unclear whether this is a consequence of a primary defect at the suture or is secondarily induced by changes in the cranial base.

Recently, (Zhou et al., 2000) demonstrated that mice carrying a P250R substitution in FGFR1, orthologous to the Pfeiffer mutation in humans, exhibited fusion of interfrontal, sagittal and coronal sutures. This mutation resulted in transiently increased cell proliferation and dramatically increased CBFA1 expression at the suture site. CBFA1 is a protein required for activation of osteoblast differentiation. Analysis of the cranial base revealed no premature closure of the synchondroses, supporting the idea that craniosynostosis can develop as a primary defect, without involvement from the cranial base (Moss, 1960; Smith and Tondury, 1978). These data contradict the long-stated hypothesis that the cranial base is the primary site of defects in human craniosynostosis (Cohen, 1993; Moss, 1959).

Role of Transcription Factors, Growth Factors, and Their Receptors in Regulating Cell Proliferation and Cell Differentiation Within Sutures

The cell assay system used to establish that Crouzon mutations result in constitutively activated receptors also showed increased numbers of foci of transformed cells, suggesting that the mutations result in increased cell proliferation (Galvin et al., 1996). However, little is known about how the mutation affects cell proliferation or cell differentiation. In experiments in which osteoblasts were isolated from Apert patients and cultured, Fragale et al. (1999) demonstrated that these osteoblasts showed low levels of cell proliferation accompanied by elevated markers for differentiation, such as AP, BSP-I, and BSP-II. This was similar to the findings of De Pollak et al. (1996), who cultured cells isolated from patients with nonsyndromic craniosynostosis. Experiments by Lomri et al. (1998) demonstrated that osteoblasts with FGFR2 mutations resulting in the Apert phenotype appeared to have normal proliferative responses to the addition of FGF-2. However, these cells showed accelerated differentiation and bone formation in the presence of FGF-2.

Increased presence of FGF-2 has been demonstrated to be associated with both normal (Most et al., 1998) and induced (Iseki et al., 1997) suture closure. Iseki et al. (1999) found that FGF-2-induced suture closure was associated with a localized decrease in cell proliferation, a change from FGFR2 to FGFR1 expression by osteoblasts in the bone fronts, and increased expression of BSP-I, a marker for osteoblast differentiation. These findings were confirmed by Lemonnier et al. (2000), who showed that the Apert S252W mutation did not alter cell proliferation, but up-regulated collagen type I, osteocalcin (OC), and BSP-I, associated with protein kinase C-independent down-regulation of FGFR2. Importantly, it has been noted that immature osteoblasts respond to FGF-2 by proliferating, whereas differentiating osteoblasts respond by becoming apoptotic (Mansukhani et al., 2000). Osteoblasts transfected with Apert S252W or Crouzon C342Y mutant FGFR2 showed inhibited differentiation, with dramat-

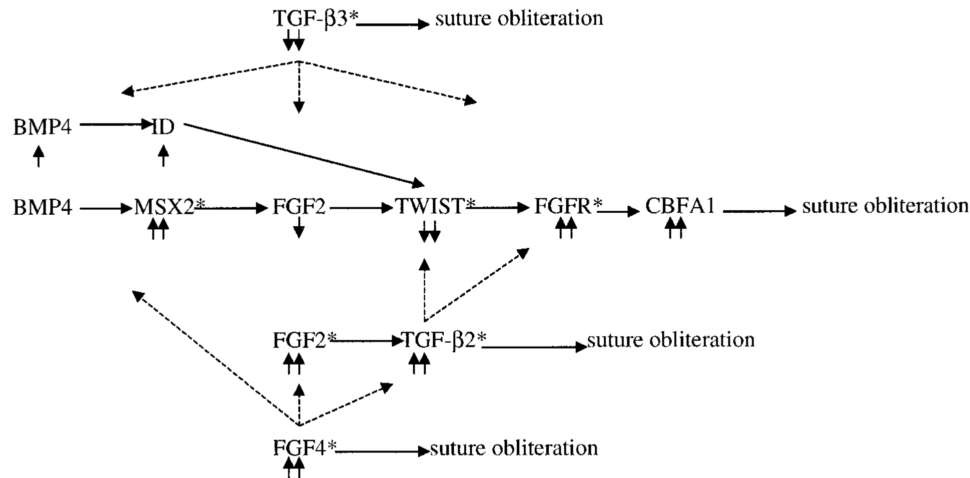

Fig. 5. Schematic representation of the known (solid long arrows) and some potentially interesting unknown (dashed arrows) associations between factors linked with craniosynostosis. Numbers of short arrows show degree of up-regulation (↑) or down-regulation (↓) of each factor known to result in craniosynostosis. Asterisks show factors that are known to result in craniosynostosis when perturbed independently of upstream regulators. Factors shown more than once show alternate pathways or pathways where possible links are not yet known.

ically elevated levels of apoptosis (Mansukhani et al., 2000), suggesting that the mutation likely affects maturation of osteoblastic cells at the suture and that apoptosis may be a feature of craniosynostosis.

In contrast to the pathology of these FGFR mutations, the P250R mutation in FGFR1, producing Pfeiffer phenotype when introduced in mice, results in transiently increased levels of cell proliferation before visible suture fusion (Zhou et al., 2000). This increase in cell proliferation is accompanied by increased expression of CBFA1, BSP-I, and OC. Zhou et al. (2000) demonstrated that CBFA1 is downstream of FGFR1 and suggest that mutated FGFR1 causes up-regulation of CBFA1, which activates expression of its target genes, BSP-I and OC. These authors found no changes in expression of STAT1, STAT5a, STAT5b, or MSX2, supporting that MSX2 is likely not downstream of FGF. Another mutation that results in premature suture fusion preceded by a transient increase in proliferative activity of the osteoblastic cells lining the osteogenic bone fronts (Liu et al., 1999) is one occurring in MSX2. MSX2 is expressed mainly in the suture mesenchyme, where it apparently binds to the promoter region of collagen type I and of OC (Towler et al., 1994; Dodig et al., 1996), inhibiting their transcription. Although it is counterintuitive that MSX2 inhibits osteoblast differentiation, it may be that MSX2 allows cells to go through additional proliferative cycles, increasing the total number of cells ultimately available for differentiation. Conversely, mice deficient for the MSX2 gene show wide open fontanels in the cranial vault, similar to the defect seen in the human condition induced by MSX2 haploinsufficiency (Satokata et al., 2000; Wilkie et al., 2000). This finding could be a result of reduction of numbers of cells available to become osteoblasts, due to reduced cell proliferation.

TWIST is a transcription factor specifically associated with Saethre-Chotzen syndrome (el Ghouzzi et al., 1997, 1999). In contrast to mutations in the MSX2 gene, for which overexpression of MSX2 results in craniosynostosis, mutations in the TWIST gene produce haploinsufficiency, resulting in craniosynostosis. This haploinsufficiency can result from several mutations, with insertion of premature stop codons, frameshifts, and nonsense mutations all resulting in a lack of functional protein product being translated (Paznekas et al., 1998; el Ghouzzi et al., 1999). TWIST is found in developing suture mesenchyme and has been shown to decrease osteoblast differentiation and FGFR2 in sutures (Johnson et al., 2000; Rice et al., 2000). FGF-2 induces up-regulation of TWIST expression (Johnson et al., 2000; Rice et al., 2000), which could be associated with normal suture development. However, because MSX2 decreases FGF-2 effects, it is possible that MSX2 overexpression or MSX2 mutations result indirectly in decreased TWIST expression by down-regulating FGF-2, producing the equivalent effect of TWIST haploinsufficiency and resulting in suture fusion (Fig. 5).

It is known that FGF-2 levels are highly elevated in fusing sutures (Most et al., 1998) and that addition of FGF-2 to sutures will induce fusion (Iseki et al., 1997). Interestingly, normal human osteoblasts increase TGF-β2 production during prolonged exposure to FGF-2, accompanied by increased OC production and matrix mineralization (Debiais et al., 1998). Recent studies demonstrated that TGF-β2 induced suture obliteration in cultured fetal rat calvaria accompanied by elevated levels of cell proliferation (Opperman et al., 2000) similar to that seen when dura mater was removed from calvaria before culture (Opperman et al., 1998). However, rescue of sutures from obliteration was achieved by removal of TGF-β2 activity with neu-

tralizing antibodies, without altering cell proliferation. Rescue of coronal sutures from obliteration was also achieved by addition of TGF-β3 to cultured calvaria, with increased concentrations of TGF-β3 resulting in decreased levels of cell proliferation (Opperman et al., 2000). In converse experiments, where sutural obliteration was induced by removal of TGF-β3 activity by neutralizing antibodies, it was found that sutural obliteration was preceded by elevated levels of cell proliferation.

These data provide good evidence for abnormally elevated cell numbers being a contributory factor to premature obliteration of cranial sutures. Support for the hypothesis that cell proliferation is associated with premature suture closure is provided by evidence that addition of FGF-4 to cultured fetal mouse calvaria induces premature suture fusion associated with elevated levels of cell proliferation (Kim et al., 1998), as did overexpression of MSX2 (Liu et al., 1999). Recent data provide several good lines of evidence that MSX2 and TGF-βs regulate suture patency in part through regulating cell proliferation. However, it is apparent that most of the FGFR mutations do not affect proliferative activity, but rather alter cell differentiation. Increased cell proliferation at the suture, contributing more cells to the bone cell lineage and accelerated osteoblast differentiation both result in increased bone formation. Therefore, up-regulation of either would be sufficient to induce premature suture obliteration.

Transcription Factor and Growth Factor Regulation of Apoptosis in the Suture

Because osteoblasts transfected with either Apert or Crouzon FGFR2 mutant genes exhibit dramatically elevated levels of apoptosis (Mansukhani et al., 2000), it is likely that apoptosis is a feature of craniosynostosis. Rice et al. (1999) showed that apoptosis occurs during normal suture morphogenesis in the cells lining the bone fronts and particularly at the leading edges of the overlapping bones within the suture. They proposed that apoptosis is a part of normal suture development and suggested that increased apoptosis could be associated with delayed suture closure, as occurs in cleidocranial dysplasia, whereas decreased apoptosis could result in premature suture fusion. That apoptosis slowed bone formation at the suture was confirmed by data showing that increased numbers of apoptotic cells were present in sutures both during normal suture maintenance and during rescue of sutures from obliteration with TGF-β3 (Opperman et al., 2000). Furthermore, low numbers of apoptotic cells were found in sutures induced to fuse by removal of dura mater or by addition of TGF-β2 (Opperman et al., 2000).

Furtwangler et al. (1985) hypothesized that apoptotic cells should be found at the edges of bone fronts that become too closely approximated, thereby preventing sutural obliteration. This idea was supported by the finding that abundant apoptotic cells were present along the bone fronts of sutures not undergoing fusion (Opperman et al., 2000). However, this was not sufficient to prevent obliteration, because apoptotic cells were also found along the bone fronts of fusing sutures. Current evidence indicates that to maintain suture patency, a critical number of cells within the sutural matrix must become apoptotic. If this does not happen, the number of cells within the suture will exceed critical density, triggering cell differentiation, ultimately leading to bony obliteration of the suture. This concept is supported by the data of Frenkel et al. (1990, 1992) who showed that using in vitro cell culture techniques that increased cell density is associated with differentiation.

SUTURES AS INTRAMEMBRANOUS BONE GROWTH SITES

Before discussing bone growth from the suture, it is important to understand the difference between a bone growth center and a bone growth site (Baume, 1961; Enlow, 1986, 2000). A bone *growth center*, as in the cartilaginous growth plates of long bones, has intrinsic growth potential and tissue-separating capabilities. The most common example of a bone growth center is the epiphysis of a growing long bone, which lengthens the long bones through cartilage proliferation and hypertrophy. The newly lengthened region is then stabilized and strengthened by replacement with bone. Bone *growth sites* on the other hand are secondary, adaptive regions at which bone remodeling takes place, without a cartilaginous intermediate. Bone growth sites remain dormant until stimulated to make bone by some external signal. In the cranial vault, the stimulus arises primarily from the expanding brain (Baer, 1954; Moss, 1954), sending signals by means of the dura mater (Kokich, 1986; Cohen, 2000). As the brain expands and the cranial base synchondroses (cartilaginous bone growth plates) lengthen, the sutures respond by adding intramembranous bone at the edges of the bone fronts, such that the sutures remain approximately the same width and the cranial vault increases in size to accommodate the enlarging brain.

For sutures to function as intramembranous bone growth sites, they need to remain in an unossified state, yet allow new bone to be formed at the edges of the overlapping bone fronts. This process relies on the production of sufficient new bone cells to be recruited into the bone fronts, while ensuring that the cells remaining in the suture remain undifferentiated. Interestingly, the dividing cells within the suture do not lie in the center of the suture itself but are within the osteogenic layer of cells lining the bone fronts (Pritchard et al., 1956; Opperman et al. 1998; Iseki et al., 1999). This introduces the interesting possibility that the suture cells themselves are recruited from the dividing cell population lining the bone fronts. Another possibility is that suture cells are long-lived. However, the levels of apoptosis noted within the suture would indicate that this is not the case (Opperman et al., 2000). During normal suture development, cells within

the osteogenic layer expressing FGFR2 divide (Iseki et al., 1999) and, although some of these cells divide further, others begin differentiating. The differentiating cells change expression from FGFR2 to FGFR1, which up-regulates CBFA1 expression (Zhou et al. 2000), and cells then begin to make bone-related proteins, such as collagen type I, BSP-I, and OC (Liu et al., 1999; Marks et al., 1999; Lemonnier et al., 2000), or become apoptotic (Opperman et al., 2000). This process is accompanied by cells within the suture matrix itself also becoming apoptotic (Opperman et al., 2000), presumably to keep the number of cells within the suture matrix from becoming elevated. This balance between cell proliferation, differentiation, and apoptosis is recognized in submandibular cells (Hoffman et al., 1996), osteoblast cells (Du et al., 2000; Mansukhani et al., 2000), and in developing chorionic villi (Qumsiyeh et al., 2000).

Craniosynostosis may result from perturbation of any of these cell functions. It will occur from mutations in MSX2 or growth factor induced increases in cell proliferation along the osteogenic bone fronts (Iseki et al., 1997; Kim et al., 1998; Liu et al., 1999; Opperman et al., 2000). This is likely accompanied by reductions in the number of apoptotic cells present in the suture (Opperman et al., 2000). The activating mutations in FGFR genes result in suture fusion, associated not with elevated cell proliferation, but with accelerated cell differentiation, both at the osteogenic fronts and also within the suture matrix (Iseki et al., 1997, 1999; Liu et al., 1999; Marks et al., 1999; Lemonnier et al., 2000).

PERSPECTIVES

Maintenance of suture patency depends on regulating a complex array of factors, that may work within the same pathways or independently of one another (Fig. 5). It is now known that MSX2 expression is regulated by BMP-4 (Kim et al., 1998) and that these factors regulate FGF-2 mediated reactions, including TWIST expression (Newberry et al., 1998) and TGF-β2 production (Debiais et al., 1998). TWIST in turn regulates FGFR expression (Johnson et al., 2000; Rice et al., 2000). Several of these factors have no known mutations associated with craniosynostosis. However, it is important to understand how their expression within the suture is regulated to get a full appreciation of how membranous bone growth occurs at the suture while keeping the suture in an unossified state. What makes this difficult is the lack of knowledge about expression levels of these factors during "normal" suture morphogenesis and growth and what constitutes sufficiently altered levels to result in suture obliteration. This process seems to be no less complicated than the regulation of bone growth occurring at the endochondral growth plate, although it is currently far less understood and studied.

Although it is important to understand mechanisms regulating both normal and abnormal cranial suture morphogenesis and growth, it is critical to begin understanding how morphogenesis and growth of facial sutures are regulated. Unlike cranial sutures, these sutures do not have an underlying dura mater or brain and, in many cases, are associated with cartilage. It will be interesting to establish whether the cartilage and perichondrium regulate bone formation at the facial sutures as hypothesized by Scott (1953a,b), in a similar manner to the brain and dura mater in the cranial vault sutures. Alternatively, completely different mechanisms may be at work in the facial sutures. Related to this, it is important to establish what kind of signals are generated by the expanding brain that regulate bone formation at the suture site. Furthermore, it is necessary to establish what role the cranial base synchondroses play in regulating bone growth at the suture sites. Another question relates to what the equivalent signals are that regulate bone formation at facial sutures. This area of research remains controversial, because it is not clear whether biochemical or biomechanical signals (or a combination of both) are responsible for inducing bone formation at the suture sites.

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