Molecular and cellular bases of syndromic craniosynostoses

Jacky Bonaventure and Vincent El Ghouzzi

Premature fusion of cranial sutures underlies the clinical condition of 'craniosynostosis', a common human disorder that occurs in both nonsyndromic and syndromic forms. The subgroup of syndromic craniosynostoses usually associates limb abnormalities and facial dysmorphism to skull distortion. Over the past decade, some of the genes causing these phenotypes have been identified. Among these, the gene encoding FGFR2, one of four members of the fibroblast growth factor receptor (FGFR) family, has been shown to account for several severe conditions including Apert, Pfeiffer, Crouzon, Beare–Stevenson and Jackson–Weiss syndromes. Two other FGFRs, FGFR1 and FGFR3, also account for craniosynostoses of variable severity [Pfeiffer, Crouzon with acanthosis nigricans (a pre-malignant skin disorder), and Muenke syndromes]. By contrast, Saethre–Chotzen syndrome and craniosynostosis (Boston-type) arise from mutations in the Twist and muscle segment homeobox 2 (MSX2) transcription factors, respectively. Whereas most FGFR mutations are likely to cause ligand-independent activation of the receptor, leading to an upregulation of signaling pathways, mutations in the basic helix–loop–helix (bHLH) transcription factor Twist appear to induce loss of protein function. This review will summarise and discuss some of the cellular and molecular mechanisms involved in normal and abnormal craniofacial development, focusing on the possible interactions between the different factors controlling membranous ossification.

Formation of the skeleton in vertebrates, including humans, requires two distinct types of process: endochondral ossification, involving the conversion of an initial cartilage template into bone; and membranous ossification, involving development of a membrane of vascularised primitive mesenchymal tissue into bone without prior formation of cartilage. Whereas the axial

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(vertebrae) and appendicular (limb) skeletons form almost exclusively through endochondral ossification, cranial skeletogenesis is a more sophisticated process and can involve both types of ossification. Cranial bones expand during normal development but do not fuse, giving rise to a functional structure called a suture that is able to accommodate brain growth. Premature fusion of one or more of the sutures results in cranial deformations that define a group of skeletal disorders known as the craniosynostoses (Ref. 1). The past few years have seen the identification of mutations that cause most of the major craniosynostosis syndromes. Some of the mechanisms involved in the process of craniosynostosis formation are progressively being unravelled (Ref. 2), providing clues for elucidating the developmental pathway of suture biogenesis. This review will focus on the molecular and cellular defects that lead to premature fusion of sutures and their associated phenotypes.

Formation of the human skull

The skeleton of the human head consists of 22 separate bones and comprises the viscerocranium (jaws and other branchial arch derivatives) and the neurocranium (skull vault and base). The neural crest cell origins of the viscerocranium and the anterior skull base have been firmly established (Ref. 3). During embryonic development, these cells migrate from the lateral edges of the dorsal neural tube and give rise to bones of the skull base that pre-form in cartilage and ossify by the process of endochondral ossification. There are four primary areas in the neurocranium: the occipital, orbitotemporal, otic and ethmoidal regions. The occipital and otic regions are chordal and the orbitotemporal and ethmoidal regions are prechordal, reflecting the extent to which the notochord is involved (Ref. 4).

The membrane bones that roof the skull are flat bones. The skull vault (dermatocranium) consists of a pair of frontal bones, a pair of parietal bones and one occipital bone (Fig. 1a). These bones are not preformed in cartilage but ossify from several ossification centres directly from ectomesenchyme, giving rise to the cranial vault (calvaria). The bones do not complete their growth during human foetal life and, at 18 weeks of gestation, mineralising bone fronts meet but do not fuse (Ref. 4). The soft fibrous sutures that join them permit the vault to deform and the skull to enlarge by appositional growth at the suture and deposition of premineralised matrix (osteoid) along the suture margins. Six sutures are present in the human skull, two coronal, one sagittal, two lambdoid and one metopic (Ref. 1; Fig.1a).

Two large membrane-covered fontanelles occupy the areas where more than two cranial vault bones meet. Anatomically, the suture is a simple structure comprising two plates of bone separated by a narrow space containing immature, rapidly dividing osteogenic stem cells, a proportion of which are recruited to differentiate into osteoblasts and make new bone (Fig. 1b). Evidence also exists that the dura mater [the outermost membranous tissue of the three membranes (meninges) covering the brain and spinal cord] underlying the suture is required for the maintenance of sutural growth (Ref. 5). The embryonic origin of the skull vault is still debated. One group (Ref. 3) described it as being entirely derived from the neural crest in avians, whereas others have suggested that it is of mixed neural crest and mesodermal origin (Refs 6, 7). The recent engineering of transgenic mice expressing a neural crest cell lineage marker (Ref. 8) has shown that, while frontal bones are derived from the neural crest, parietal bones have a mesodermal origin but are separated by a tongue of neural crest cells. Consequently, coronal and sagittal sutures in mammals are formed at a mesoderm–neural crest interface.

Craniosynostosis syndromes

Clinical aspects

Craniosynostoses, the early fusion of skull sutures, are clinically heterogeneous conditions (Ref. 1). Although the different sutures look anatomically equivalent, subtle differences between them probably exist given the significant variations in the incidence of synostoses between craniosynostotic patients (Ref. 9). Early sutural synostosis leads to skull distortion and is often associated with high intracranial pressure, impaired blood flow, altered vision and hearing performances, and adverse psychological effects including mental retardation (Ref. 10). Diagnosis of craniosynostosis first relies on cranial or craniofacial dysmorphism. However, all skull distortions should not be referred to as craniosynostoses since a large number of cranial distortions are not associated with suture fusion. Appropriate diagnosis of sutural synostosis thus requires confirmation by radiological examination of the skull vault and base. In some syndromic forms, limb abnormalities can also be helpful for diagnosis. A group of clinically related

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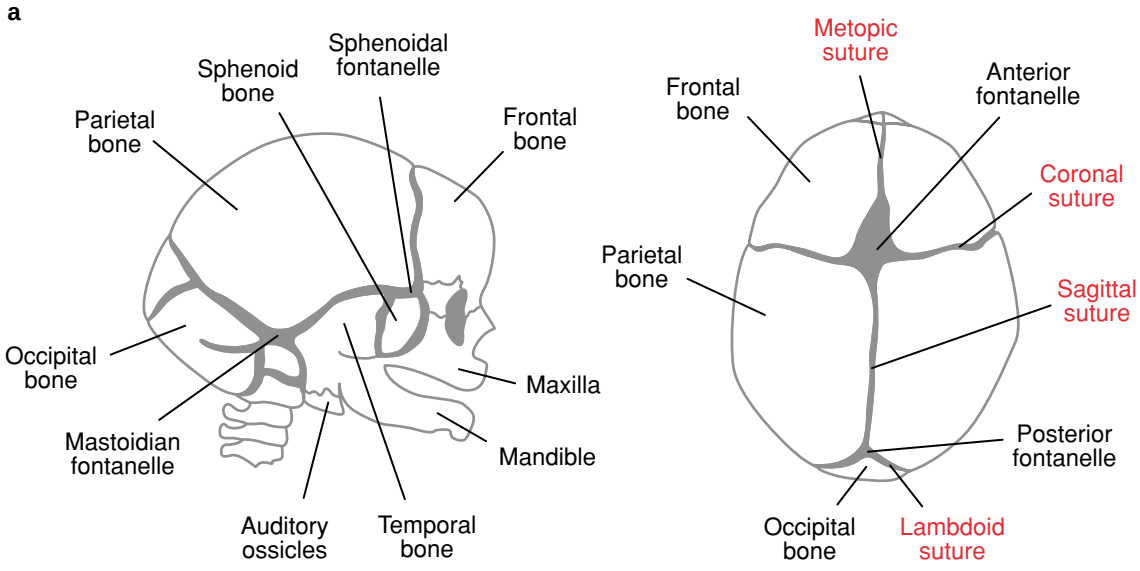

Structure of the human skull at birth and histological characteristics of normal and prematurely fused sutures

Figure 1. Structure of the human skull at birth and histological characteristics of normal and prematurely fused sutures. (a) Schematic representation of the human skull shown from the side (left) and the roof (right). The skull vault consists of a pair of frontal bones, a pair of parietal bones and one occipital bone. The frontal bones are derived from the neural crest, whereas the parietal bones are of mesodermal origin. Six sutures are present: two coronal (between the frontal and parietal bones), one sagittal (between the parietal bones), two lambdoid (between the parietal and occipital bones) and one metopic (between the frontal bones). Anterior, posterior, mastoidian and sphenoidal fontanelles occupy the area where more than two cranial bones meet. Mandible, maxilla and auditory ossicles (malleus, incus and stapes) are branchial arch derivatives. (b) Histological characteristics of coronal sutures from a control foetus (left) showing the gap between the osteogenic fronts, and a 24-week-old foetus with Apert syndrome (right), showing an abundant mineralised bone trabecula in the prematurely fused suture. Abbreviations: Fb, frontal bone; Me, mesenchyme; Of, osteogenic front; Pb, parietal bone; Tb, bone trabecula **(fig001jbp)**.

craniosynostosis syndromes including Apert, Crouzon, Pfeiffer, Jackson–Weiss, Saethre–Chotzen, craniosynostosis (Boston-type) and Muenke syndromes has received particular attention during the past decade (Table 1). The clinical features of these will be briefly mentioned below and the reader is referred to Ref. 1 for further details.

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Table 1. Main syndromic craniosynostoses and their frequency (tab001jbp)

Syndrome	Year disease described	Frequency[a]	Chromosomal location	Mutated gene[b]	OMIM entry	Refs
Crouzon	1912	1/50000	10q26	FGFR2 FGFR3[c]	123500	43, 56
Apert	1906	1/60000	10q26	FGFR2	101200	46
Pfeiffer	1964	1/100000	10q26 8p11	FGFR2 FGFR1	101600	47, 21, 53
Jackson–Weiss	1976	NA	10q26	FGFR2	123150	48
Craniosynostosis (Boston-type)	1993	NA	5q34	MSX2	123101	23
Beare–Stevenson	1969	NA	10q26	FGFR2	123790	49
Muenke syndrome	1996	1/50000	4p16	FGFR3	602849	57, 16
Saethre–Chotzen	1931	1/100000	7p21	TWIST	101400	28, 29

[a] Frequencies are based on all births. The frequency of three rare forms of syndromic craniosynostoses is not available (NA).
[b] FGFR1,-2 and -3 are members of the fibroblast growth factor receptor family.
[c] Causes Crouzon with acanthosis nigricans (a pre-malignant skin disorder with hyperkeratosis and hyperpigmentation).

Apert syndrome is the most severe of the craniosynostoses. The condition is characterised by increased head height, flat face, feet and hand syndactylies (cutaneous and bony fusion of the digits) and central nervous system defects often resulting in mental retardation (Refs 11, 12).

Patients with Crouzon, Pfeiffer or Jackson–Weiss syndromes all exhibit similar facial abnormalities with prominent eyes (ocular proptosis); however, although there are no syndactylies of the upper or lower extremities in Crouzon syndrome, hand and feet anomalies consisting of broad and short thumbs and toes characterise Pfeiffer syndrome (Ref. 13). Jackson–Weiss syndrome is defined by the presence of radiographic abnormalities of the feet showing broad great toes with medial deviation and tarsal–metatarsal coalescence, without hand abnormalities (Ref. 14).

Saethre–Chotzen syndrome is characterised by facial asymmetry with eyelid dropping (ptosis), ear anomalies, cutaneous syndactyly and broad or bifid toes. Originally described in a three-generation family, craniosynostosis (Boston-type) is characterised by skull malformations including frontal bossing and clover-leaf skull. No hand or foot anomalies are visible (Ref. 15). The recently delineated Muenke syndrome associates unilateral or bilateral fusion of coronal sutures with bulging of temporal fossae (Ref. 16). Mild limb abnormalities such as fusion of carpal bones are not constant among patients (Ref. 17).

Histological aspects

Histologically, the normal suture in a human foetus and a newborn consists of two unfused adjacent bone edges surrounded by pre-osteoblasts and mesenchymal cells in the cellular outlayer (Ref. 18; Fig. 1b). Close examination of the developing foetal mouse calvaria (skull vault) have shown that sutures form initially by a wedge-shaped proliferation of cells at the periphery of the

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extending bone fields called the osteogenic fronts. These structures are likely to govern morphogenetic determination of sutural architecture (Ref. 1). They either overlap each other with an intervening zone of immature fibrous connective tissue, leading to an overlapping suture (coronal suture), or they approximate in the same plane, giving rise to an 'end-to-end' type of suture (sagittal, metopic sutures). Depending on the overlapping or 'end-to-end' sutural initiation, two types of architectural forms of craniosynostosis occur: either with ridging or no ridging. In foetal Apert coronal sutures, large bony trabeculae showing active osteogenesis in the sub-periosteal area have been described (Ref. 18; Fig. 1b). Pre-osteoblasts present along the bone margins were found to have synthesised abundant amounts of bone matrix leading to higher amounts of trabecular bone than in age-matched controls (Ref. 18).

Genetic and molecular aspects

Abnormal sutural fusion can be secondary to extrinsic foetal factors such as intrauterine constraint (Ref. 19) or drug administration during pregnancy (Ref. 20) but, in several instances, a family history and/or the presence of associated limb abnormalities is suggestive of a genetically determined condition. So far, more than 100 entities (i.e. syndromic craniosynostoses with specific features) have been clinically delineated but, until 1993, their molecular bases remained unknown. Although craniosynostoses are usually sporadic, at least 8% of all craniosynostoses including syndromic and nonsyndromic forms are familial (Ref. 21). The suspected autosomal dominant inheritance of the most common craniosynostosis syndromes has been demonstrated through the identification of heterozygous gene mutations causing these conditions (Table 1) and, at present, causative mutations can be detected in more than 20% of craniosynostosis cases. Some of the mechanisms involved in the process of accelerated sutural fusion are progressively being deciphered (Ref. 2), as discussed below.

Molecular mechanisms underlying craniosynostosis

MSX2/Twist-associated craniosynostosis

The muscle segment homeobox 2 (MSX2) homeotic gene belongs to a family of three transcription factors that play important roles in the control of organogenesis (Ref. 22). The gene encoding MSX2 was the first to be identified as accounting for an autosomal dominant craniosynostosis (Boston-type) (Ref. 23), but no additional cases have been reported since. All affected members carried a P148H missense mutation in the MSX2 homeodomain that stabilises its binding to a target DNA sequence, thus inducing gain-of-function of the transcription factor (Ref. 24).

Twist is a transcription factor with a basic helix–loop–helix (bHLH) domain that dimerises to form a complex with DNA-binding capacities (Fig. 2). The human gene encoding H-TWIST maps to chromosome 7p21 (Ref. 25). On the basis of the Saethre–Chotzen-like phenotype of mice heterozygous for a Twist-null allele (Ref. 26) and the mapping of Saethre–Chotzen syndrome to chromosome 7p21 (Ref. 27), the human gene was screened for mutations. Heterozygous mutations including nonsense, missense, in-frame insertions and small deletions were identified (Refs 28, 29). Most mutations were expected to produce premature termination of the protein, suggesting that haploinsufficiency accounted for the phenotype. Detection in some cases of chromosome 7p microdeletions involving H-TWIST further supported this assertion (Ref. 30). Unexpectedly, in one patient, the translocation breakpoint was mapped 5 kb 3' from the gene encoding H-TWIST, indicating that sequences downstream of the gene could modulate its expression (Ref. 31).

Remarkably, all mutations identified to date affect the bHLH domain (Ref. 32). Although the in vivo partners for Twist are still elusive, it is able to form stable heterodimers with E12 (another bHLH transcription factor, encoded by the *E2A* gene) that efficiently bind to the E box sequence CATATG (Refs 33, 34). While nonsense mutations or deletions creating premature termination codons resulted in rapidly degraded shortened proteins, missense mutations affecting the helical regions hampered heterodimer formation (Ref. 33). Mutations in the basic or the loop region were shown to abolish protein–DNA binding (Fig. 2), resulting in a loss of protein function (Ref. 34). Interestingly, recent results in the nematode *Caenorhabditis elegans* suggest that certain mutations in the basic domain of Ce-Twist (the *C. elegans* homologue of H-TWIST) could exert a dominant-negative effect on protein function (Ref. 35). Nevertheless, data obtained with the vertebrate Twist protein indicate that

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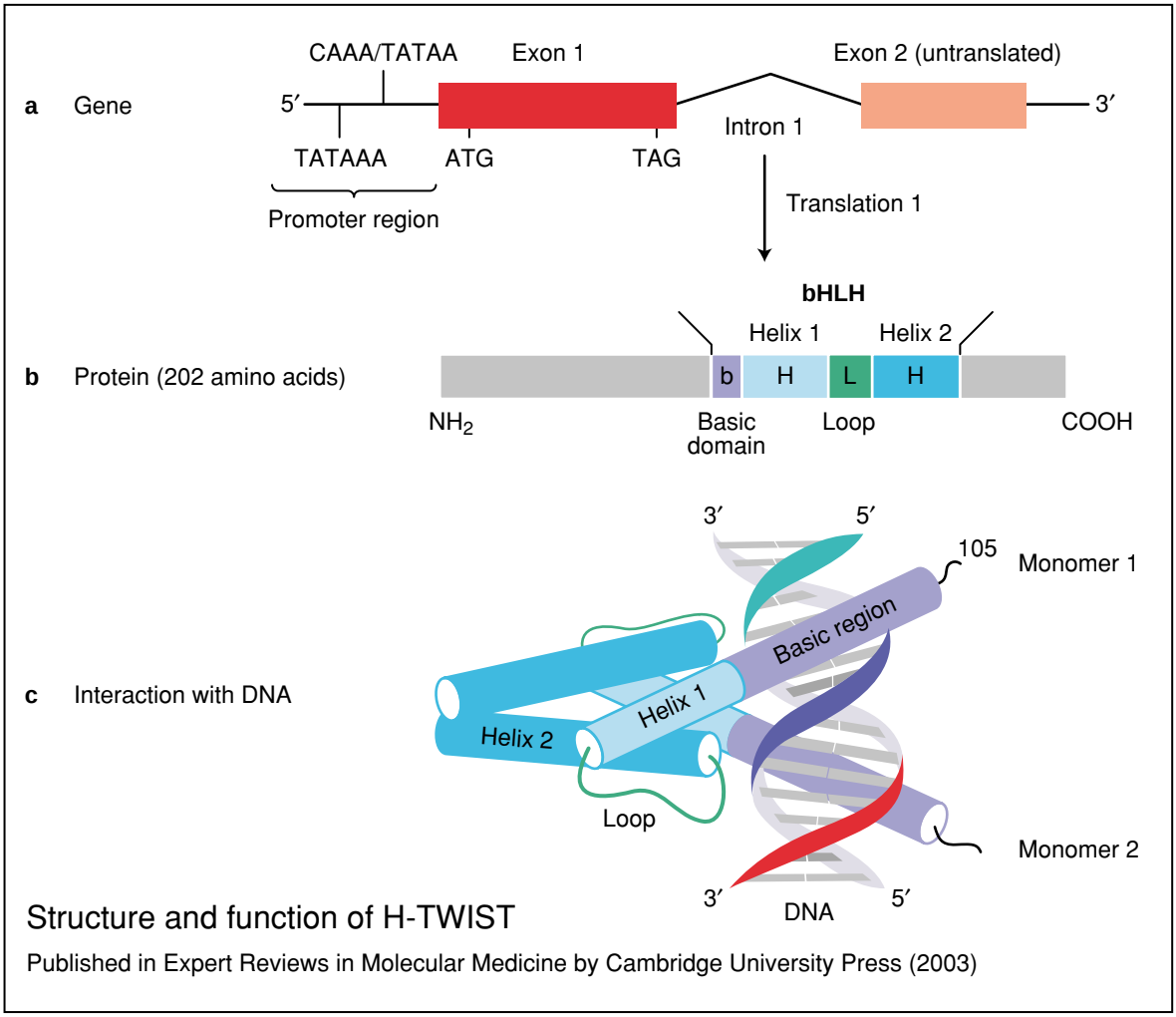

Figure 2. Structure and function of H-TWIST. (a) The gene encoding H-TWIST comprises two exons separated by a unique intron. (b) The H-TWIST protein is 202 amino acids with a basic helix–loop–helix (bHLH) domain. (c) TWIST acts as a transcription factor that dimerises to form a complex with DNA-binding activity. This three-dimensional model is adapted from Ref. 99 with permission from Elsevier Science (© 1994); in each bHLH monomer, helices are represented as cylinders and DNA sugar–phosphate backbones are represented by ribbons. Loss-of-function mutations causing Saethre–Chotzen syndrome affect the bHLH domain and abolish protein–DNA interactions. In vitro, TWIST is able to heterodimerise with E12, another bHLH transcription factor, to bind target gene promoters (not shown) **(fig002jbp)**.

loss-of-function mutations cause the Saethre–Chotzen syndrome phenotype and further emphasise the high sensitivity of the gene encoding Twist to dosage (Ref. 36). Support for this assertion is provided by the observation that trisomy 7p at the H-TWIST locus delays suture fusion (Ref. 37).

FGFR-associated craniosynostoses

The four fibroblast growth factor receptors (FGFR1–4) belong to the large family of tyrosine kinase (TK) receptors. They all present a common organisation, including an extracellular immunoglobulin (Ig)-like ligand-binding domain, a transmembrane (TM) domain and two intracellular TK subdomains (TK1 and TK2) (Fig. 3a). In FGFR1, -2 and -3, alternative splicing of two adjacent exons encoding the C-terminal half of the third Ig-like loop gives rise to two isoforms, IIIb and IIIc, with distinct properties and tissue-specific expression (Ref. 38). Binding of FGF to FGFR in association with heparan sulphate proteoglycan (HSPG) induces receptor dimerisation at the cell

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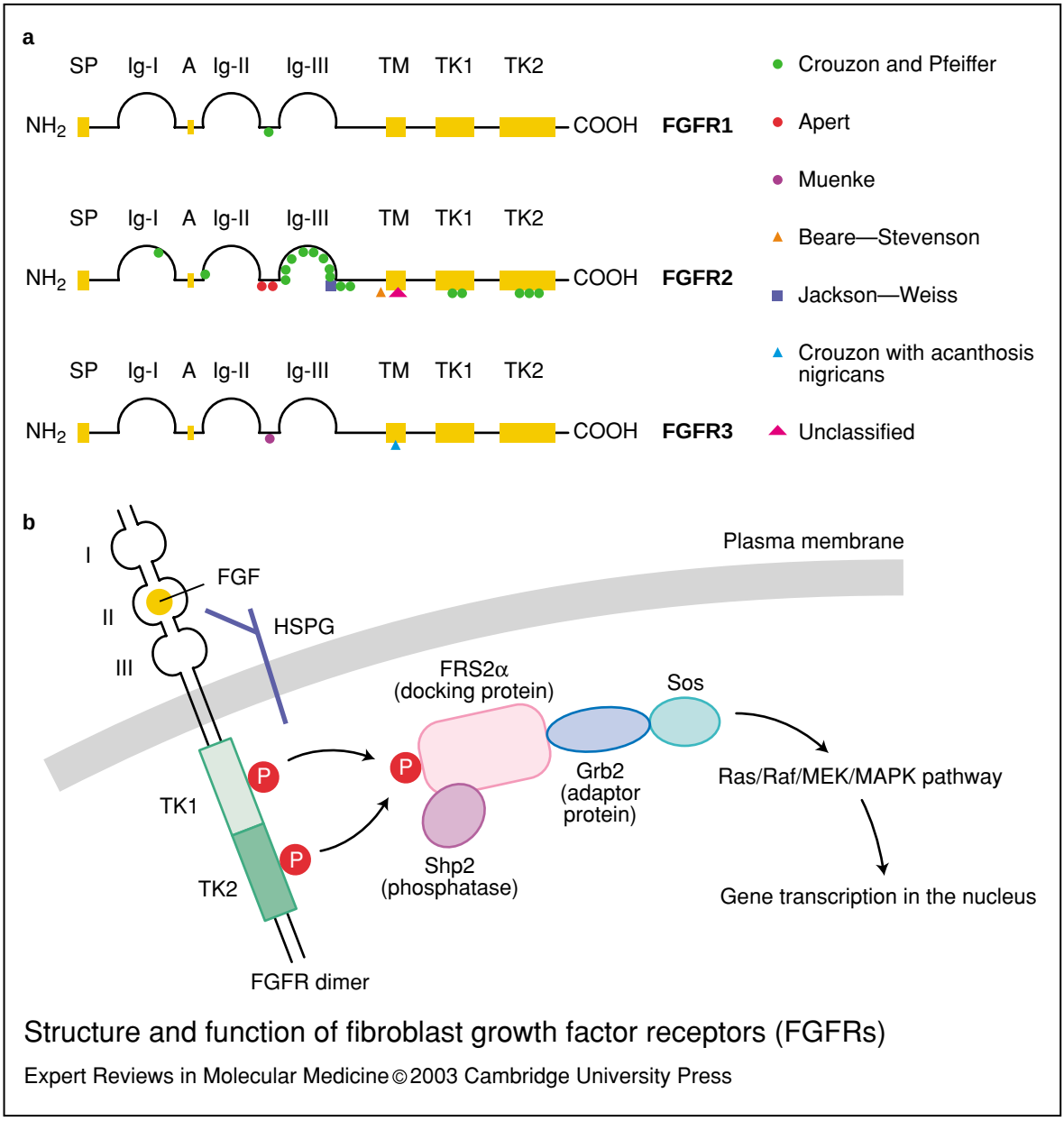

Structure and function of fibroblast growth factor receptors (FGFRs)

Figure 3. Structure and function of fibroblast growth factor receptors (FGFRs). (a) Schematic representation of three of the four FGFRs and distribution of mutations causing craniosynostoses. The extracellular region of the FGFRs contains three immunoglobulin (Ig)-like loops (I, II and III), a signal peptide (SP) and an acidic box (A). The tyrosine kinase (TK) domain is split into two subdomains, TK1 and TK2. The receptor is anchored to the cell membrane through a transmembrane (TM) domain. Positions of mutations accounting for seven different craniosynostosis syndromes are shown. (b) Binding of FGF to FGFRs in association with heparan sulphate proteoglycan (HSPG) induces receptor dimerisation and autophosphorylation. This triggers phosphorylation (P) of downstream target proteins (Ref. 39), examples of which are shown here. FRS2α, Grb2, Shp2 and Sos are involved in the mitogen-activated protein kinase (MAPK) signalling pathway **(fig003jbp)**.

surface and autophosphorylation that triggers phosphorylation of downstream signalling proteins (Refs 39, 40; Fig. 3b). In FGFR4, the second half of the third Ig-like loop is encoded by a single exon, giving rise to only one transcript (Ref. 41).

In 1994, Crouzon syndrome was mapped to chromosome 10q25-26 and ascribed to mutations in FGFR2 (Refs 42, 43). Subsequently, more than

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30 different missense mutations affecting mostly the third Ig-like loop (Ig-III), but also the TK domains and Ig-I and Ig-II loops, have been reported (Ref. 44, 45). Allelic mutations of the gene encoding FGFR2 cause Apert, Pfeiffer, Jackson–Weiss and Beare–Stevenson syndromes (Refs 46, 47, 48, 49; Fig. 2a). Whereas Crouzon syndrome has been mostly ascribed to mutations that create or destroy cysteine residues, only two recurrent missense mutations (S252W and P253R) lying in the linker region between the Ig-II and Ig-III loops are implicated in more than 99% of Apert cases. However, in two cases, insertions of an Alu-element associated with ectopic expression of the FGFR2-IIIb isoform (KGFR) have been reported (Ref. 50). In Apert, Crouzon and Pfeiffer syndromes, 'de novo' FGFR2 mutations were demonstrated to arise exclusively on the paternal chromosome (Refs 51, 52).

Mutations in FGFR1 and FGFR3 can also cause craniosynostosis syndromes. Hence, a recurrent missense mutation (P252R) in the linker region between the Ig-II and Ig-III loops of FGFR1 was found in some cases of Pfeiffer syndrome (Refs 53, 54; Fig. 3a). FGFR2 mutations were frequently associated with more-pronounced ocular proptosis and broader thumbs than FGFR1 mutations. Although mutations in FGFR3 first appeared to be restricted to skeletal dysplasia affecting the growth of long bones (Ref. 55), additional studies have identified FGFR3 mutations in two autosomal dominant craniosynostoses (Fig. 3a). First, a recurrent mutation in the TM region of FGFR3 (A391E) was found to be specific for Crouzon syndrome with acanthosis nigricans (Ref. 56). Second, a common recurrent mutation (P250R) in the linker region between Ig-II and Ig-III loops was detected in patients with unilateral or bilateral fusion of coronal sutures (Muenke syndrome) (Refs 16, 57, 58). Phenotypic expressivity of this disease is highly variable: in several familial forms, members carrying the mutation had hearing impairment but no craniosynostosis.

In total, more than 50 different mutations (mostly missense) in one of FGFR1, -2 and -3 have been reported, further illustrating the genetic heterogeneity of Pfeiffer and Crouzon syndromes, which unexpectedly can also result from identical FGFR2 mutations affecting cysteines 278 and 342 (Ref. 47). The frequency of FGFR2 mutations causing five different craniosynostosis syndromes identifies this gene as a key regulator of skull development. It is also remarkable that the highly conserved proline residue at homologous positions (252 in FGFR1, 253 in FGFR2 and 250 in FGFR3) is prone to conversion into arginine, whereas no mutation in FGFR4 has been identified so far.

Functional studies have convincingly shown that most FGFR mutations induce gain-of-receptor function but different mechanisms could lead to the same end result. Several FGFR2 mutations either create or destroy cysteine residues in the extracellular domain to produce an unpaired cysteine that can form an intermolecular disulphide bond, resulting in constitutive activation of the receptor in a ligand-independent manner. Evidence for this mechanism has been provided by the demonstration of elevated TK activity in *Xenopus* oocytes injected with a mutant FGFR2 RNA (Ref. 59). Conformational changes of the Ig-III loop induced by mutations affecting residues other than cysteine also result in constitutive receptor activation (Ref. 60). Mutations causing Apert syndrome did not induce ligand independency but accentuated ligand binding in vitro (Ref. 61). They were also shown to induce loss of ligand specificity, allowing the FGFR2-IIIc isoform to be activated by ligands (FGF7 and -10) that are normally specific for the FGFR2-IIIb splice variant (Ref. 62). Therefore, aberrant signalling in mesenchymal cells (expressing the IIIc isoform) could explain the severity and unique clinical features of Apert syndrome. Alternatively, ectopic expression of the FGFR2-IIIb isoform in the mesenchymal cells of Apert patients could disrupt the mesenchymal–epithelial interactions, leading to visceral and limb abnormalities. Such a mechanism in Apert patients harbouring Alu-element insertions in FGFR2 (Ref. 50) was recently substantiated by the demonstration that mice lacking the FGFR2-IIIc isoform exhibited a phenotype mimicking some of the clinical features of Apert syndrome (Ref. 63).

Less is known regarding the mechanisms leading to the phenotype associated with the P252R FGFR1 and the P250R FGFR3 mutations. However, introduction of the P250R mutation into the *Fgfr1* murine gene (equivalent to the P252R FGFR1 Pfeiffer mutation) resulted in a shortened neurocranium with premature fusion of calvarial sutures in mutant animals (Ref. 64). Owing to the fact that the P250R FGFR3 and P253R

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FGFR2 substitutions are homologous, it is tempting to speculate that receptors carrying the P250R mutation would still be able to bind FGF molecules but that the ligand-binding specificity of the IIIc isoform, which is normally expressed at the osteogenic front (Ref. 65), could be affected or lost.

Physiological studies

Role of Twist and MSX2 in skull vault formation

While MSX2 mutations in humans and Msx2 overexpression in mice induce premature suture fusion by increasing the number of osteogenic cells (Ref. 66), loss-of-function mutations in the gene encoding MSX2 cause an autosomal dominant disease characterised by deficient ossification of the parietal bone resulting in enlarged parietal foramina (holes in flat bones forming the skull vault) (Ref. 67). Together, these data indicate that MSX2 dosage is critical for human skull development. It appears that the normal function of MSX2 is to maintain the pre-osteoblastic cells of the osteogenic front in an undifferentiated stage and to stimulate their proliferation (Ref. 68). MSX2 is likely to be functionally linked to another homeodomain transcription factor, ALX4, because mutations in the gene encoding ALX4 also account for some forms of parietal foramina (Refs 69, 70).

Expression of Twist in the developing mouse calvaria is restricted both to the mesenchyme lying between calvarial bones and to the vicinity of the osteogenic fronts (Ref. 71, 72). Twist expression in bone cells strongly decreases after birth, suggesting that it could negatively regulate osteoblast differentiation. Support for this assertion has been provided by: (1) the demonstration that Twist overexpression resulted in osteoblast de-differentiation into an osteoprogenitor-like cell (Ref. 36), whereas Twist underexpression gives rise to a more-differentiated mature osteoblastic cell; and (2) the premature fusion of sutures in heterozygous Twist-null mice (Ref. 26). Likewise, the finding that Saethre–Chotzen syndrome is caused by loss-of-function mutations further emphasises the negative effect of Twist on osteoblast differentiation. Whether Twist interferes with an unknown bone-specific bHLH to inhibit osteoblast differentiation by titrating E12, similar to what occurs in muscle (Ref. 73), remains speculative and would not explain why missense mutations affecting the basic domain result in the same phenotype as nonsense mutations.

Roles of FGFRs in normal and pathological skull vault formation

Recent progress has been made in understanding how FGFR mutations affect the receptor function and its downstream signalling proteins, but less is known regarding the consequences of these mutations on the ossification process in the skull. Nevertheless, FGFR expression studies in different species have provided some phenotypic explanations. For instance, at six weeks of development in humans, FGFR1 and FGFR2 are expressed in the mesenchymal condensations pre-figuring long bones and in the mesenchyme of the skull vault, whereas FGFR3 is almost undetectable. At later stages, the three FGFRs are detectable in the pre-bone mesenchyme around the osteoid in pre-osteoblasts and in osteoblasts forming the mineralising tissue (Ref. 74).

Development of the cranial sutures appears to rely on a delicate balance between osteogenic cell proliferation and differentiation. In the coronal suture of the developing mouse embryo, Fgfr1 is expressed in cells close to or within the osteoid, while cells expressing Fgfr2 are more distant and correspond to proliferating osteogenic stem cells. Fgfr3 is detected at the periphery of the osteoid but at a lower level than Fgfr1 and 2 (Refs 75, 76). Fgfr1 expression seems to be associated with osteogenic differentiation, while signalling through Fgfr2 appears to regulate stem cell proliferation (Ref. 76). Fgfr3 could play a cooperative role during this process and is thought to exert a coronal-suture-specific function owing to the observation that the P250R FGFR3 mutation in humans is restricted to synostosis of coronal sutures without long bone defects (Refs 16, 17).

Implantation of beads soaked with FGF2 onto foetal mouse suture or loaded with a neutralising antibody to FGF2 into chick cranial tissue, respectively promoted (Refs 75, 76) or blocked (Ref. 77) osteogenic differentiation. However, the action of FGF2 is concentration dependent and varies with the stage of cell differentiation. Hence, low levels of FGF2 induced proliferation of cranial neural crest cells, whereas high FGF2 levels mediated skeletogenic differentiation of these cells into osteoblasts (Ref. 78). Unexpectedly, FGF2-null mutant mice showed no embryonic abnormalities of skull formation (Ref. 79), suggesting that

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compensation by another member of the FGF family could explain this result. Although FGF9, which is expressed in osteogenic mesenchymal cells and binds to the IIIc isoforms of FGFR2 and -3 has been evoked (Ref. 71), FGF18 has recently emerged as a more crucial player in the process of osteogenesis (Refs 80, 81). Fgf18-null mouse embryos exhibit considerable delay of suture closure owing to decreased proliferation of osteogenic mesenchymal cells and delayed differentiation of osteoblasts (Ref. 81). FGF18 might be one of the physiological ligands of the FGFR3-IIIc isoform but binds with a lesser affinity to FGFR2-IIIc than FGF2. This indicates that at least three different ligands acting on three receptors are likely to regulate skull development.

Ectopic expression of FGF2 in the normal mouse coronal suture seems to mimic, in the short term, some of the effects of activating mutations in the FGFRs that cause craniosynostosis (Ref. 71). This increase in the amount of FGF2 is accompanied by downregulation of FGFR2 expression (Ref. 76). Similarly, in Apert syndrome, FGFR2 mutations were shown to induce selective downregulation of FGFR2 expression in pre-osteoblasts and osteoblasts (Ref. 82), a process that could contribute to premature osteoblast differentiation at the suture level (Ref. 18). However, it is not yet firmly established whether FGFR mutations or FGF supplementation induce skeletogenic differentiation with low-proliferative activity being a consequence of accelerated differentiation, or whether they inhibit proliferation resulting in premature differentiation.

Possible relationships between FGFRs and transcription factors in bone growth

The finding that mutations in both the FGFRs and Twist produce the same end result (i.e. premature fusion of sutures) through two different mechanisms (respectively, constitutive activation and haploinsufficiency), raises the question of whether these genes lie in the same or in parallel signalling pathways during skull bone ossification. Experiments in invertebrates support the idea that they share the same pathway: (1) a physical interaction between Ce-Twist and the promoter of egl-15 (a *C. elegans* homologue of Fgfr) has been described (Ref. 83); and (2) appropriate expression of *Drosophila melanogaster* DFR1/htl (heartless), one of two *Drosophila* Fgfr genes, has been shown to require D-Twist (Ref. 84).

On the basis of these data, a first model in which transcription factors NF-κB, Id (an HLH factor with no basic domain) and Twist interact to control FGFR expression in vertebrates was proposed over a decade ago (Ref. 85; Fig. 4). This model was supported by the demonstration of an altered expression of Fgfr2 in the calvaria of heterozygous Twist-null mice (Refs 71, 86), suggesting that Twist could negatively regulate Fgfr2 during skull development. A more recent model is based on the demonstration that Twist can also directly interact, through its amino-terminal domain, with histone acetyl transferase p300/CBP (Ref. 87); in this model, negative regulation of Fgfr3 expression by Twist was assumed to require formation of a multi-component complex comprising E12, p300/CBP and Twist (Ref. 88).

The question remains as to whether Twist acts directly on the FGFR promoters as found in *C. elegans* (Ref. 83). The failure to demonstrate a direct binding of the Twist protein to FGFR2 and FGFR3 promoters suggests that regulation of FGFRs by Twist could be only indirect in vertebrates (Ref. 86; Fig. 4). Finally, it is noteworthy that genes encoding Twist and MSX disclose overlapping expression patterns and are associated with calvarial bone disorders. Whether they are involved in the same or different signalling pathways remains to be elucidated but both have been postulated to regulate expression of the *Cbfa1* gene, which is a crucial regulator of cell differentiation specifically expressed in the osteochondrogenic lineage (Ref. 89; Fig. 4).

Role of apoptosis in normal and pathological suture formation

Apoptosis (programmed cell death) is part of cellular differentiation and developmental processes controlling tissue homeostasis. Apoptosis of osteoblastic cells forming calvaria and coronal sutures occurs at the same time and location as suture initiation (Ref. 90), a sign of its involvement in suture development. In Apert syndrome, osteoblasts and osteocytes expressing the mutant FGFR2 display a higher propensity to undergo programmed cell death than do control cells (Refs 91, 92), suggesting that apoptosis also participates in premature suture fusion by more-rapidly clearing cells that must die in order for mineralisation to occur. Consistent with the massive wave of apoptosis in cranial ganglia

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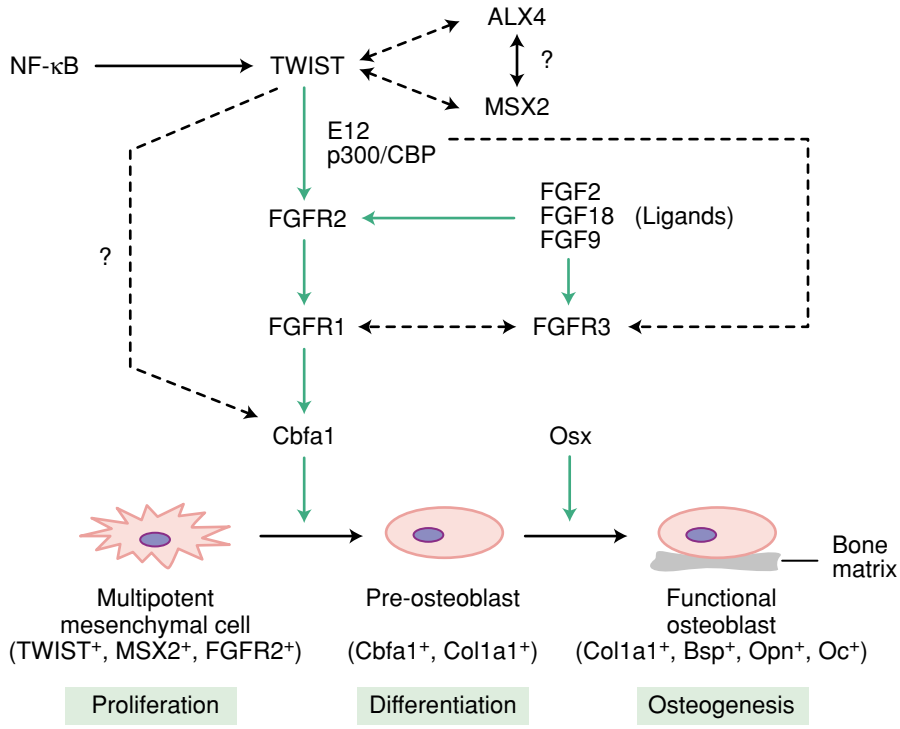

Proposed interactions between signalling molecules and transcription factors during skull formation

Figure 4. Proposed interactions between signalling molecules and transcription factors during skull formation. This model suggests how the fibroblast growth factors (FGFs) and their receptors (FGFRs) might interact with the transcription factors TWIST, MSX2, ALX4, Cbfa1 and Osx. Where evidence is available for protein–protein binding or interactions between proteins and gene promoters, solid arrows are used. By contrast, dashed arrows are used to depict interactions between transcription factors for which conclusive additional data are missing. Hypothetical interactions are indicated by question marks. At the suture level, proliferation of mesenchymal cells is followed by differentiation into pre-osteoblasts that further differentiate into bone-matrix-forming mature osteoblasts. These cells secrete collagen type I (Col1a1), bone sialoprotein (Bsp), osteopontin (Opn) and osteocalcin (Oc), which are deposited at the edges of the osteogenic fronts. FGF molecules (FGF2, -9, -18) are expressed by mesenchymal cells of the suture separating two osteogenic fronts **(fig004jbp)**.

of homozygous Twist-null mice (Ref. 93), TdT-mediated dUTP nick end-labelling (TUNEL) analysis of coronal sutures from Saethre–Chotzen syndrome patients also showed increased osteoblast and osteocyte apoptosis (Ref. 94), confirming the previously reported anti-apoptotic capacities of Twist (Ref. 95). Increased apoptosis in craniosynostoses caused by either FGFR2 or H-TWIST mutations further emphasises the possible relationship between the two proteins. FGFR2 overexpression could be a result of H-TWIST haploinsufficiency, leading to a local upregulation of osteoblast apoptosis in the sutures of Saethre–Chotzen patients.

Conclusion and future prospects

Despite significant advances in the surgical management of patients, whereby the deformed areas of the skull and face are remodelled by specialised teams of neurosurgeons, craniosynostoses still represent a considerable medical challenge, particularly as rapid re-ossification occurs after separation of fused sutures, prompting the need for repeated surgery in some cases. During past years, many efforts have been devoted to elucidating the molecular bases of these disorders, but in spite of technical improvements in the mutation-detection methods (Ref. 45), few mutations have been identified

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in nonsyndromic coronal craniosynostoses, suggesting that additional genes might be involved. It is now clearly established that mutations in TK receptors (FGFRs) and transcription factors (MSX and Twist) account for most well-delineated syndromic craniosynostoses. These genes might also cause milder phenotypes that are difficult to diagnose. Identifying mutations in these cases could facilitate diagnosis and patient care. It is also unclear why FGFR genes are so prone to highly recurrent mutations that arise exclusively on the paternal allele and why the same FGFR2 mutation can produce mostly craniofacial defects (Crouzon syndrome) or both skull and limb anomalies (Pfeiffer syndrome). Studies involving in vitro and in vivo animal models have disclosed sophisticated cellular mechanisms controlling skull ossification but results are still controversial (Refs 63, 96) since only subtle differences such as the levels of FGF signalling may profoundly affect the phenotype. Additional complexity is provided by the ability of FGFs to bind to different FGFR isoforms with variable affinities, as well as by the diversity and spatiotemporal expression of FGF ligands in the developing skull. Although three FGFs, namely FGF2, -9 and -18, have been conclusively shown to play a role in this process, additional members of this expanding family, including FGF20, might be involved (Ref. 97). Identifying these ligands and their receptor specificities, as well as understanding how ligands regulate the tight balance between osteogenic cell proliferation and differentiation, will be the goals of future studies. These studies should also aim to decipher the precise role of FGFRs and transcription factors (Twist, MSX and ALX) in the development and growth of intramembranous bones. Finally, determining the possible interactions between these proteins, extracellular bone matrix proteins (e.g. collagen I, bone-sialoprotein, osteopontin, osteocalcin) and bone-specific transcription factors, including the runt-related transcription factor 2 (Cbfa1) and the recently identified zinc-finger factor Osterix (Osx) (Ref. 98), will be a challenging task that should be facilitated by newly designed powerful methods for genetic manipulations in mice and other species.

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Approaches to diagnosis and surgery for craniosynostosis are described on the websites of the Texas Pediatric Surgical Associates, from the Division of Pediatric Surgery at the University of Texas Medical School (Houston, TX):
http://www.pedisurg.com/PtEduc/Craniosynostosis.htm

and the website for Plastic & Craniofacial Surgery for Children (Dallas, TX):
http://www.kidsplastsurg.com/craniosynostosis.html

The National Institute of Neurological Disorders and Stroke (NINDS) supports biomedical research on disorders of the brain and nervous system, and features an entry for FAQs concerning craniosynostosis:
http://www.ninds.nih.gov/health_and_medical/disorders/craniosytosis_doc.htm

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Features associated with this article

Figures

Figure 1. Structure of the human skull at birth and histological characteristics of normal and prematurely fused sutures (fig001jbp)
Figure 2. Structure and function of H-TWIST (fig002jbp)
Figure 3. Structure and function of fibroblast growth factor receptors (FGFRs) (fig003jbp)
Figure 4. Proposed interactions between signalling molecules and transcription factors during skull formation (fig004jbp)

Table

Table 1. Main syndromic craniosynostoses and their frequency (tab001jbp)

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