

The clinical manifestations, molecular mechanisms, and treatment of craniosynostosis

Eloise Stanton^{1,2}, Mark Urata³, Jian-Fu Chen¹, and Yang Chai¹

1. Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA 90033
2. Keck School of Medicine, University of Southern California, Los Angeles, CA 90033
3. Division of Plastic and Maxillofacial Surgery, Children's Hospital Los Angeles, Los Angeles, CA 90033

Correspondence author

Yang Chai, DDS, PhD
University Professor
George and MaryLou Boone Chair in Craniofacial Biology
University of Southern California
ychai@usc.edu

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Summary:

Craniosynostosis has broad clinical manifestations and a complex etiology. Using studies from humans and animal models, we discuss the developmental mechanisms of craniosynostosis and potential mesenchymal stem cell-based suture regeneration strategies to treat this disease.

Abstract

Craniosynostosis is a major congenital craniofacial disorder characterized by the premature fusion of cranial suture(s). Patients with severe craniosynostosis often have impairments in hearing, vision, intracranial pressure, and/or neurocognitive functions. Craniosynostosis can result from genetic mutations, chromosomal abnormalities, or adverse environmental effects, and can occur in isolation or in association with numerous syndromes. To date, surgical correction remains the primary treatment for craniosynostosis but it is associated with complications and with the potential for re-synostosis. There is therefore a strong unmet need for new therapies for patients with craniosynostosis. Here, we provide a comprehensive review of our current understanding of craniosynostosis, including typical craniosynostosis types, their clinical manifestations, cranial suture development, and genetic and environmental causes of craniosynostosis. Based on studies from animal models, we present a framework for understanding the pathogenesis of craniosynostosis, with an emphasis on the loss of postnatal suture mesenchymal stem cells (MSCs) as an emerging disease-driving mechanism. We evaluate emerging treatment options and highlight the potential of MSC-based suture regeneration as a therapeutic approach for craniosynostosis.

1. Introduction

Cranial sutures of the skull vault, including the metopic, coronal, lambdoid, and sagittal sutures, are fibrous joints that connect the skull bones, coordinate growth and development of the skull and the brain, enable minor movements, and serve as shock absorbers. Most calvarial sutures lie at neural crest-mesoderm boundaries, with the exception of the metopic suture (see Glossary, Box 1), which lies between the two neural crest-derived frontal bones, and the lambdoid suture, which lies between the two mesoderm-derived parietal and occipital bones (Balczerski et al., 2012; Di Ieva et al., 2013; Jiang et al., 2002; Lenton et al., 2005; Morriss-Kay and Wilkie, 2005). The metopic suture typically fuses by nine months of age in humans (Vu et al., 2001). Other sutures fuse in adulthood, from the sagittal around 22 years of age to the squamosal at around 60 years (Idriz et al., 2015). As the brain grows, the patent (i.e., non-ossified) calvarial sutures allow the skull to grow simultaneously (see Figure 1A). This process is essential for proper brain development (Morriss-Kay and Wilkie, 2005), as it accommodates postnatal brain enlargement (Nieman et al., 2012; Sharma, 2013).

The premature fusion of cranial suture(s) leads to craniosynostosis, a major congenital craniofacial disorder that affects between 1 in 2,100 and 1 in 2,500 live births in the United States. Craniosynostosis can occur either as part of a recognized genetic syndrome or as an isolated pathology (Sharma, 2013). Infants with syndromic craniosynostosis may present with other deformities or symptoms, including neurological and respiratory problems (Johnson and Wilkie, 2011). The four main sutures implicated in craniosynostosis are the sagittal, coronal, metopic, and lambdoid. Defects in each of these sutures present with distinct clinical manifestations (see Figure 1B) that often require surgical repair.

Currently, craniosynostosis treatment is almost entirely surgical, sometimes paired with postoperative helmet therapy for maintenance (Wolfswinkel et al., 2021) (see Box 3). Corrective procedures are complicated, long, and associated with the risk of numerous complications, including heavy blood loss and its sequelae (Erb and Meier, 2016; Wolfswinkel et al., 2017). In some practices, 94% of patients are reported to be admitted to the ICU after surgery (Wolfswinkel et al., 2017). Peri- and post-operatively, patients

with syndromic craniosynostosis are significantly more likely to experience complications and re-synostosis than non-syndromic patients (Lee et al., 2012). Although surgery may restore normal appearance, in some cases, patients experience persistent deficits in intellectual ability and cognitive function, the severity of which partially depends on the type and amount of suture fused (Maliapaard et al., 2014; Speltz et al., 2015; Zechi-Ceide et al., 2012). Overall, there is a strong unmet medical need for effective therapies to treat craniosynostosis and to prevent re-synostosis.

This review aims to summarize our current understanding of the clinical types and manifestations of craniosynostosis in humans and provide an overview of calvarial suture development, as well as genetic mutations and environmental risk factors implicated in the etiologies of craniosynostosis. We discuss the importance of animal models in pathophysiological studies of suture development and craniosynostosis. We highlight the complexity and shortcomings of current surgical treatment modality and ongoing efforts to investigate less invasive and/or biological strategies in animal models. We review and critique recent studies that have identified various populations of suture mesenchymal stem cells (MSCs) that could improve the treatment of craniosynostosis. This knowledge will allow the field to better understand the pathogenesis of craniosynostosis and to develop improved mechanism-based, biological therapies to treat patients with it.

2. The types and clinical manifestations of craniosynostosis

2.1: Craniosynostosis types and cranial phenotypes

Craniosynostosis is seldom diagnosed prenatally; it is most often identified through physical examination in the months after birth. However, some head shape and growth patterns can suggest syndromic craniosynostosis prenatally (Johnson and Wilkie, 2011). For example, severe forms of Pfeiffer syndrome (see Box 2; Table 1) can commonly be distinguished via ultrasonography due to its characteristic cloverleaf skull appearance. Apert syndrome (see Box 2) can also be indicated prenatally based on biparietal diameter and the presence of acrocephaly (see Glossary, Box 1) (Harada et al., 2019).

Craniosynostosis types and corresponding phenotypes are depicted in Figure 1B. The most common form of craniosynostosis is fusion of the sagittal suture, which is

responsible for 45% of cases in the United States (Kolar, 2011). The primary outcome of sagittal suture fusion is an elongated head shape (scaphocephaly) due to compensatory anterior-posterior lengthening (Dempsey et al., 2019). In around 20-25% of patients with craniosynostosis, the coronal suture is also affected, typically on only one side (called unilateral or unicoronal synostosis) (Cohen, 2000). Unicoronal synostosis is characterized by ipsilateral forehead flattening and a rotation ('twist' or 'yaw') of the midface. In bilateral coronal synostosis, anterior-posterior growth is restricted with compensatory temporal expansion, leading to brachycephaly (see Glossary, Box 1) (Dempsey et al., 2019). Most bicoronal cases can be attributed to a known genetic cause (Vinchon, 2019). The next most common form of craniosynostosis is metopic craniosynostosis, which is responsible for around 5-15% of cases (Cohen, 2000) and which leads to trigonocephaly (a triangular-shaped forehead) and to narrowing of the temporal region (Dempsey et al., 2019). Finally, lambdoid synostosis accounts for less than 5% of cases (Cohen, 2000; Hentz and Mathes, 2006). Unilateral lambdoid suture fusion typically presents with bulging of the mastoid process and lowering of the cranial base, as well as inferior-posterior ear shifting (Dempsey et al., 2019). Bilateral lambdoid craniosynostosis occurs less commonly and leads to a flat and widened occipital region with anterior-inferior ear displacement (Delashaw et al., 1991). Plagiocephaly (see Glossary, Box 1), which is seen in lambdoid synostosis, should be distinguished from deformational plagiocephaly. Deformational plagiocephaly is characterized by a flattened posterior skull due to positional molding and is a much more common cause of occipital asymmetry than lambdoid craniosynostosis (Rhodes et al., 2014).

2.2 Additional manifestations associated with craniosynostosis

Craniosynostosis can occur either as an isolated non-syndromic disorder or part of a recognized genetic syndrome (Sharma, 2013). Infants with craniosynostosis may present with non-suture deformities or symptoms (see Table 1; Box 2) (Johnson and Wilkie, 2011), such as hydrocephalus, hearing deficits, visual impairment, increased intracranial pressure (ICP), or neurocognitive dysfunction-related intellectual disabilities (ID). Hydrocephalus occurs in 12-15% of syndromic craniosynostosis patients (Cinalli et al., 1998; Di Rocco et al., 2011). This can be due to obstructed cerebrospinal fluid (CSF)

outflow from an abnormally small posterior skull with subsequent constriction of the fourth ventricle or to impaired CSF absorption (Collmann et al., 2005). Hearing deficits are frequently associated with craniosynostosis (Agochukwu et al., 2014; Biamino et al., 2016), and manifest as conductive hearing loss and otitis media with effusion (see Glossary, Box 1). Both result from the narrowing and abnormal angulation of the Eustachian tube due to the narrowing and contour of the skull base (Couloigner and Ayari Khalfallah, 2019). Visual pathologies are more common in syndromic (27%) relative to non-syndromic (17%) patients (Tunçbilek et al., 2010). The most common causes of vision loss in syndromic craniosynostosis are amblyopia and optic neuropathy; the latter results from increased ICP, which causes papilledema (see Glossary, Box 1) (Tay et al., 2006; Touzé et al., 2019). In general, these abnormalities are more prevalent or severe in syndromic relative to non-syndromic craniosynostosis.

Patients with syndromic craniosynostosis are significantly more likely to have ID, social problems, and attention deficits, and to show inhibited and withdrawn behaviors. Such neurocognitive deficits typically become apparent once a child begins schooling, at around 6-7 years of age (Da Costa et al., 2006; Maliepaard et al., 2014). While the prevalence and severity of ID vary across syndromes and patients, Apert syndrome has the highest rate of ID (Da Costa et al., 2006; Lajeunie et al., 1999; Lefebvre et al., 1986; Patton et al., 1988; Renier et al., 1996; Renier et al., 2000; Sarimski, 1997). Non-syndromic patients are significantly less likely to present with ID (Da Costa et al., 2006). The majority of studies have demonstrated that, in patients with non-syndromic craniosynostosis, intelligence typically remains within the normal range long-term (Arnaud et al., 1995; Magge et al., 2002; Shipster et al., 2003), although some patients may have ID (Sidoti et al., 1996; Speltz et al., 2015).

The premature fusion of cranial sutures restricts brain growth, leading to abnormal brain structure (i.e, microcephaly) and to abnormal cognitive functions in craniosynostosis. However, neurocognitive deficits have been largely neglected in basic research of craniosynostosis (Esparza and Hinojosa, 2008; Grova et al., 2012; Lee et al., 2012; Lee et al., 2019). The etiology of the anatomical and neurocognitive abnormalities seen in craniosynostosis patients largely remain unknown, raising questions as to how current clinical treatments and their timing can improve patients' neurological deficits. For

example, the role that different therapeutic interventions might play in attenuating the later-observed cognitive deficits, including behavioral, linguistic, or visuospatial difficulties, are unclear (Brooks et al., 2018; Esparza and Hinojosa, 2008).

Increased ICP on the cerebral cortex is thought to contribute to impaired neurocognition and intelligence (Morriss-Kay and Wilkie, 2005). This is supported by the fact that both syndromic and non-syndromic craniosynostosis patients display increased ICP (Erb and Meier, 2016; Tamburrini et al., 2005; Wolfswinkel et al., 2021); higher ICP statistically correlates with poorer performance on psychometric testing (Renier et al., 1982; Wolfswinkel et al., 2021). Given the crucial roles of ICP in impaired neurocognitive functions, as revealed by mouse models of craniosynostosis and as discussed later in this review, it is important to know the cause of elevated ICP in craniosynostosis. Historically, elevated ICP is mainly assumed to result from the premature fusion of the skull reducing the volume that the brain can occupy, leading to its compression (Johnson and Wilkie, 2011).

However, patients continue to have chronic elevated ICP even after surgical skull expansion, which suggests that there are other causes of ICP elevation. Accumulating evidence suggests that ICP elevation might partly result from venous malformations that lead to CSF accumulation and to elevated ICP (Cinalli et al., 1998; Di Rocco et al., 2011; Harada et al., 2019; Hayward, 2005; Stevens et al., 2007; Taylor et al., 2001). For example, in patients with complex craniosynostosis, venous malformations that particularly affect the sigmoid-jugular region appear to be the main contributors to increased ICP (Taylor et al., 2001). Such abnormalities and subsequent venous hypertension can, in turn, impair CSF absorption and flow (Hayward, 2005). Further evidence that CSF absorption contributes to increased ICP, independent of calvarial vault size, is provided by clinical reports that increased ICP is more common among patients with midline synostosis (i.e., sagittal) rather than coronal (Thompson et al., 1995a; Thompson et al., 1995b). It is postulated that the unusually high ICP in sagittal synostosis patients may arise from this suture's unique ability to compress the sagittal sinus and/or interfere with the arachnoid granulations' drainage into the sinus (Choi et al., 2016).

Indeed, the roles and mechanisms of cerebral vein malformations in ICP and in neurocognitive dysfunction warrant further investigation. Additional open questions in

craniosynostosis include whether diffuse malformation of the central nervous system, intrinsic brain dysfunction, brain metabolic defects, and/or hemodynamic instability might also contribute to the learning disabilities and cognitive deficits of craniosynostosis patients, independently of the physical compression of the brain imposed by the skull (Brooks et al., 2018).

3. Cranial suture development

Cranial suture development is a complex process that is coordinated with skull bone and brain development. A better understanding of the molecular and cellular regulatory mechanisms of cranial suture development is crucial for development of innovative treatments for craniosynostosis.

As calvarial bones develop, migratory mesenchymal cells contribute to suture formation. These mesenchymal cells originate from either head mesoderm or cranial neural crest (CNC) cells. Furthermore, cranial sutures are often positioned at the boundary of CNC- and mesoderm-derived bony elements (Ishii, et al., 2015; White et al., 2021).

Studies have shown that newborns with Apert syndrome have cranial suture developmental defects at 15 weeks of gestation, not long after the coronal sutures first become apparent (Mathijssen et al., 1999). This suggests that developmental defects in craniosynostosis occur at the early stages of coronal suture development. In 2012, Deckelbaum et al. used a mouse model to demonstrate the presence of coronal suture precursors in head mesoderm at E7.5-8.0 (Deckelbaum et al., 2012). These precursors ultimately migrate to the supraorbital ridge between E8.5-E9.0, followed by apical migration between E11.5-13.5. By E13.5, these cells give rise to the coronal suture and the overlapping parietal and frontal bones. The osteogenic fronts consist of fibroblast-like cells undergoing differentiation and are responsible for osteogenesis as the skull expands to accommodate the growing brain (Ishii et al., 2015). Overall, studies have shown that loss of boundary integrity between CNC- and mesoderm-derived cells, abnormal specification of suture cells during embryonic development and premature loss of mesenchymal stem cells in the suture at a later stage may constitute the cellular mechanisms of craniosynostosis (Ishii et al., 2021; White et al., 2021).

Regarding the molecular mechanisms of craniosynostosis, numerous genetic mutations can contribute to dysregulation of suture mesenchymal cells, and subsequent premature suture fusion. One such gene is the twist family bHLH transcription factor 1 (*TWIST1*), which encodes a basic helix-loop-helix transcription factor. Heterozygous loss-of-function *TWIST1* mutations lead to Saethre-Chotzen syndrome in humans (see Box 2; Table 1). Cre-based lineage tracing studies in mice identified that *Twist1* haploinsufficiency leads to a loss of the osteogenic-nonosteogenic boundary and subsequent ossification of the coronal suture, typically between P9-P13 (Behr et al., 2011; Merrill et al., 2006). *Twist1* mutants aberrantly express Notch2 in the suture mesenchyme; this early marker of osteogenesis is not normally expressed in this cell population (Ishii et al., 2015). Further, *Twist1* mutants have reduced ephrin A4 (*Efna4*) expression. Ephrin-Eph signaling has well-documented roles in boundary formation. Coupled with the finding that loss-of-function *EFNA4* mutations in humans lead to coronal synostosis, it is postulated to be a downstream effector of Twist1 in loss of boundary integrity (Merrill et al., 2006). Similarly, Ephrin B1 (*EFNB1*), a gene implicated in a craniosynostosis disorder known as craniofrontonasal syndrome, is predicted to be important in boundary maintenance. Gain-of-function mutation in *MSX2* causes Boston-type craniosynostosis. *Msx2* expression has been identified in the osteogenic front (Jabs et al., 1993), and is postulated to be involved in bone deposition and resorption.

Gain-of-function mutation in RUNX family transcription factor 2 (*RUNX2*) is also responsible for a variant of craniosynostosis. *RUNX2* is known to be inhibited by *TWIST1* (Kronenberg, 2004) and is involved in regulation of osteoblast differentiation (Komori, 2018), implicating it in the pathogenesis of premature suture fusion (See Figure 2). Reduced expression of ETS2 repressor factor (*ERF*) has been found to cause multi-suture craniosynostosis. Studies have identified Erf's ability to inhibit Runx2 activity, and thus subsequent osteogenesis and suture homeostasis (Twigg et al., 2013).

Heterozygous mutation of transcription factor 12 (*TCF12*), like that of *TWIST1*, can lead to the development of Saethre-Chotzen syndrome (Sharma et al., 2013). *Tcf12* forms a heterodimer with *Twist1* (Connerney et al., 2006), and compound heterozygosity has been found to generate a more severe craniosynostosis phenotype (Sharma, 2013). *Tcf12* has been found, like *Twist1*, to function in embryonic suture formation. In addition, loss

of *Tcf12* has been found to contribute to decreased asymmetry of the mesenchymal cells in the parietal and frontal bones of the coronal suture, suggesting that it may have an important role in maintaining the overlap of these two bones and thus preventing synostosis (Ting et al., 2022). Together, these findings provide evidence for a crucial protein-protein interaction between Twist1 and Tcf12 in regulating suture development.

In humans, loss-of-function mutation of RAB23, member RAS oncogene family (*RAB23*) leads to Carpenter syndrome with multi-suture synostosis, polysyndactyly, obesity, and cardiac defects (Jenkins et al., 2007). It has been suggested that RAB23's role in the pathogenesis of craniosynostosis involves negative regulation of both FGFR and hedgehog-Gli1 signaling, which are involved in osteoprogenitor cell recruitment and stem cell maintenance within the sutures (Hasan et al., 2020).

Interleukin-11 receptor alpha (*IL11RA*) missense mutations have been implicated in Crouzon-like craniosynostosis, characterized by multiple suture fusions and dental anomalies, such as delayed tooth eruption or supernumerary teeth. IL-11 is a cytokine involved in bone remodeling that functions by activating cells via the IL-11 receptor (Agthe et al., 2018).

More recently, mutations in Zic Family Member 1 (*ZIC1*); SMAD Family Member 6 (*SMAD6*); HECT, UBA, and WWE domain containing 1, E3 ubiquitin protein ligase (*HUWE1*); and Smoothed, Frizzled Class Receptor (*SMO*) have been identified as causal in the development of craniosynostosis, although their specific roles in suture development have not yet been investigated (Calpena et al., 2020; Moortgat et al., 2018; Twigg and Wilkie, 2015b; Wilkie et al., 2017).

Gain-of-function mutations in fibroblast growth factor receptor 1 (*FGFR1*), fibroblast growth factor receptor 2 (*FGFR2*), and fibroblast growth factor receptor 3 (*FGFR3*) are responsible for many craniosynostosis syndromes, including bent bone dysplasia and Crouzon, Apert, Pfeiffer, Beare-Stevenson, and Muenke syndromes, among others. The role of FGF/FGFR signaling pathways is well established in controlling differentiation and proliferation of mesenchymal and ectodermal cells and skeletal homeostasis (Moosa and Wollnik, 2016; Su et al., 2014).

The cranial suture harbors a niche for a population of mesenchymal stem cells (MSCs) that is crucial for craniofacial homeostasis and repair (Zhao et al., 2015). Zhao et

al. identified MSCs which express Gli1 in patent adult mouse sutures (Zhao et al., 2015). Lineage tracing showed that these cells give rise to osteogenic front, dura, periosteum, and the calvarial bones under normal and injury conditions. Two groups independently used mouse models to show that Axin2- and Prx1-expressing cells act like Gli1⁺ cells as postnatal suture MSCs and contribute to calvarial bone development and to regeneration (see Figure 4) (Maruyama et al., 2016; Wilk et al., 2017; Zhao et al., 2015). A separate study used the skeletal-targeting cre driver, cathepsin K-Cre (*Ctsk^{Cre}*), to label periosteal mesenchyme and identified a population of periosteal stem cells (PSCs) that are also present in the calvarium in mice; however, the functions of Cathepsin K-positive (Ctsk⁺) cells in the cranial suture have yet to be defined (Debnath et al., 2018). While the above studies used genetic lineage tracing to study suture MSCs *in vivo*, a recent study used a panel of surface markers (CD51⁺, CD200⁺, CD45⁻, Ter119⁻, Tie2⁻, Thy1.1⁻, Thy1.2⁻, 6C3⁻, CD105⁻) and identified a population of skeletal stem/progenitor cells (referred to as CD51⁺;CD200⁺ cells) in mouse cranial sutures (Menon et al., 2021) (see Figure 3).

In vitro and *in vivo* functional studies suggest that these different suture MSC lineages are heterogenous, can self-renew and undergo tri-lineage differentiation into bone, cartilage, and adipogenic stromal cells, and can participate in cranial suture development, homeostasis, and bone regeneration. Fundamental questions remain in the field regarding how these different populations of suture MSCs, including Gli1⁺, Axin2⁺, Prx1⁺, Ctsk⁺ CD51⁺;CD200⁺ cells, relate to each other, For example, do they act in a hierarchy of lineage restricted stem cells, as in the hematopoietic system, or do they localize in distinct spatial regions and act in parallel during suture development and homeostasis?

There are several new directions in the study of molecular and cellular regulatory mechanisms of cranial suture formation and tissue homeostasis under normal and disease conditions that remain to be explored. First, how are sutures formed and how is their patency maintained? Whether and how do aforementioned key molecules affect the fate of MSCs in development and tissue homeostasis of cranial sutures? With cell lineage tracing and gene expression analysis at single-cell resolution, we are discovering a heterogenous MSC population that contributes to the formation and maintenance of cranial sutures (Holmes et al., 2021; Li et al., 2021). Second, mechanical transduction of either tensile or compressive loading can significantly impact suture morphology (Herring, 2008). As brain

growth exerts a force on the skull, how this force affects suture MSCs will be an important area of investigation. Third, it has been proposed that suture growth during brain development is regulated by various secreted factors (Twigg and Wilkie, 2015a). Dura-mediated signaling also influences suture development. The dynamic interaction between suture MSCs and the dura or periosteum will also be an important focus of our future study.

4. Etiology of craniosynostosis

4.1 Human genetics in craniosynostosis

The identification of pathogenic mutations in syndromic craniosynostosis patients has provided tremendous benefits in terms of genetic consulting, diagnostic testing, risk assessment, reproductive advice, prognostic guidance, treatment planning, and disease mechanism studies. Overall, the genetic basis for craniosynostosis can be identified in around only 20-30% of patients; 86% of these identifiable genetic causes consist of single-gene mutations and the remainder consist of chromosomal abnormalities (Johnson and Wilkie, 2011). The inheritance patterns of causative mutations are typically autosomal dominant. They are also less commonly X-linked (e.g. craniofrontonasal syndrome) or recessive (e.g. Baller-Gerold syndrome) (Lajeunie et al., 2005) (see Box 2; Table 1). The mechanism of these mutations in humans is seldom a complete loss-of-function, as total functional loss is likely to be lethal *in utero*, given that the mutated gene might be crucial for organogenesis early in embryonic development. By comparison, mammalian skull growth and cranial suture formation occur relatively late in embryogenesis. Thus, the genetic mechanisms of craniosynostosis instead often involve haploinsufficiency, dominant gain-of-function, recessive hypomorphic mutations, and are occasionally X-linked (see Glossary, Box 1) (Twigg and Wilkie, 2015a). As shown in Table 1, the common genes implicated in craniosynostosis include *TWIST1*, *FGFR1*, *FGFR2*, *FGFR3*, *EFNB1*, *ERF*, *ZIC1*, *SMAD6*, ~~*ERF*~~, *HUWE1*, *SMO*, *EFNA4*, *RAB23*, *RUNX2*, *IL11RA*, *MSX2*, *TCF12*, among others. Some mutations are associated with syndromic craniosynostosis (see Table 1), while others contribute to non-syndromic suture-fusion phenotypes (Calpena *et al.*, 2020; Cornille et al., 2019; Johnson and Wilkie, 2011; Twigg and Wilkie, 2015a; Wilkie *et al.*, 2017). Craniosynostosis is associated with over 180 syndromes (Durham et al., 2017), and this list is certainly non-exhaustive.

Mutations associated with familial craniosynostosis, including those in FGF/FGFR pathway genes, *TWIST1*, and *EFBN1*, among others, often occur in genes with critical functions in craniofacial and/or skeletal morphogenesis, and are thus highly penetrant or cause severe clinical abnormalities in patients. An alternative method to identify potential roles for common disease-modifying genes in craniosynostosis that might confer a relatively modest risk is the genome wide association study (GWAS). The first GWAS study on craniosynostosis identified susceptibility loci for non-syndromic sagittal craniosynostosis near bone morphogenetic protein-2 (*BMP2*) and within Bardet-Biedl Syndrome 9 (*BBS9*) (Justice et al., 2012). The same group recently performed an additional GWAS study, which implicated the *BMP7* locus as a risk factor for non-syndromic metopic craniosynostosis (Justice et al., 2020). Exome sequencing of parent-offspring trios identified *SMAD6* mutations with incomplete (<60%) penetrance causing non-syndromic midline (sagittal and metopic) craniosynostosis. Interestingly, all *SMAD6* mutations associated with aberrant phenotypes are associated with a *BMP2* mutant allele identified from previous GWAS studies (Timberlake et al., 2016). These findings suggest a two-locus inheritance mechanism for non-syndromic craniosynostosis that involves the inheritance of rare *SMAD6* and of more common *BMP2* alleles. We foresee that advanced human genetic and genomic approaches will uncover new variants that will advance our understanding of disease mechanisms and inform the development of new therapies.

4.2 Environmental risk factors in craniosynostosis

Genetic mutations are identified in a minority of craniosynostosis patients. The etiology of non-syndromic craniosynostosis in most infants is unknown and most cases occur in families with no history of the disease (Yilmaz et al., 2019). Overall, 70-80% of all craniosynostosis cases manifest with no described genetic cause, which indicates that new disease-causing mutations remain to be identified and/or potential environmental or gene-environment interactions (Durham et al., 2017; Hentz and Mathes, 2006; Johnson and Wilkie, 2011; Vinchon, 2019). The idea that environmental risk factors contribute to the etiology of craniosynostosis is also consistent with clinical variabilities, such as asymmetric suture fusions and variable phenotypic penetrance. Even in cases of syndromic craniosynostosis with defined genetic mutations, there is substantial variation in clinical

manifestations, suggesting the potential involvement of environmental risk factors. For example, patients that are haploinsufficient for *TWIST1* develop Saethre-Chotzen syndrome (see Box 2; Table 1). These patients display substantial phenotypic heterogeneity (Di Rocco et al., 2011; Lajeunie et al., 1995). This heterogeneity is also seen in *Twist1*^{+/-} mice, as discussed later in the review. Previous epidemiological studies have linked craniosynostosis to various environmental factors (Browne et al., 2011; Carmichael et al., 2008; Durham et al., 2017; Durham et al., 2019a; Grewal et al., 2008; Lajeunie et al., 2001; Rasmussen et al., 2007; Reefhuis et al., 2015; Reefhuis et al., 2011; Reefhuis et al., 2003; Sanchez-Lara et al., 2010; Sergesketter et al., 2019). These include maternal risk factors, such as diabetes (Durham et al., 2017; Sergesketter et al., 2019), smoking (Carmichael et al., 2008; Durham et al., 2017; Grewal et al., 2008; Hackshaw et al., 2011), high caffeine consumption (Browne et al., 2011), and thyroid disease (Rasmussen et al., 2007). The risk of craniosynostosis also increases in pregnant women taking certain medications, such as selective serotonin reuptake inhibitors (Durham et al., 2019a; Reefhuis et al., 2015) and clomiphene citrate, a medication used to treat infertility (Ardalan et al., 2012; Reefhuis et al., 2011; Reefhuis et al., 2003). Below we discuss some of these factors in more detail.

Diabetes

Mothers of infants with craniosynostosis have a significantly higher rate of gestational diabetes than mothers of non-affected infants (Sergesketter et al., 2019). Studies have demonstrated that 11.6-13.5% of mothers of children with craniosynostosis were diagnosed with diabetes mellitus during pregnancy (Ardalan et al., 2012; Sergesketter et al., 2019). This rate of gestational diabetes is significantly higher than that of mothers who have children without craniosynostosis, 2.9%. Further, a case-control study found diabetes to be a significant risk factor for having a craniosynostotic child (Ardalan et al., 2012).

Smoking

Maternal smoking appears to be the greatest risk (1.6 odds ratio) to the developing fetus after the first trimester, and the risk increases with the number of cigarettes smoked per day (Carmichael et al., 2008). A large retrospective study of 173,687 children born with malformations and 11.7 million controls found craniosynostosis to be 33% more likely to occur in children whose mothers smoked during pregnancy than in those whose mothers who did not (OR 1.33; CI 1.03-1.73) (Hackshaw et al., 2011). A case-control study found

maternal smokers to be 1.7 times (95% CI 1.2-2.7) more likely to have a child with craniosynostosis. In mothers who smoked more than one pack per day during pregnancy, the likelihood of having a child with craniosynostosis rose to 3.5 times that of non-smokers (Alderman et al., 1994). These studies provide evidence that smoking in pregnancy may be a risk factor for craniosynostosis.

Caffeine

A study by Browne et al. using data from the National Birth Defects Prevention Study demonstrated that mothers who consume high amounts of caffeine (≥ 300 mg per day) have significantly elevated relative odds (1.34) of having an infant with craniosynostosis (Browne et al., 2011). This study used data from 797 craniosynostosis cases and adjusted for covariates including maternal age, race/ethnicity, education, BMI, residence, fertility medication, and sickness during pregnancy. Thus far, this is the only study conducted on the relationship between caffeine and the development of craniosynostosis, so more research is indicated to elucidate caffeine's impact on cranial suture patency.

Thyroid Disease

For years, the relationship between thyroid disease and craniosynostosis had been primarily presented in small case studies (Cove and Johnston, 1985; Daneman and Howard, 1980; Johnsonbaugh et al., 1978; Nishihara et al., 2006; Penfold and Simpson, 1975). More recently, a study conducted by Rasmussen et al. using data from the National Birth Defects Prevention Study determined that mothers with thyroid disease had an odds ratio of 2.47 (95% CI 1.46-4.18), indicating heightened risk of giving birth to a child with craniosynostosis (Rasmussen et al., 2007). Associations with thyroid disease appeared to vary by suture. Sagittal (OR 3.32; CI 1.29-8.52) and coronal (OR 2.11; CI 0.99-4.48) suture synostosis had the highest odds ratios of single-suture synostoses. Overall, the association between maternal thyroid disease and craniosynostosis was strongest for multi-suture involvement (OR 8.73, CI 3.54-21.57) (Rasmussen et al., 2007). The results of this study suggest a relationship between thyroid disease and craniosynostosis; however, more investigation is necessary to gain a better understanding of this potential association.

Clomiphene Citrate

Clomiphene citrate, a fertility drug used in early pregnancy, has been found to be associated with craniosynostosis (Ardalan et al., 2012; Reefhuis et al., 2011; Reefhuis et

al., 2003). One case-control study on risk factors associated with both syndromic and non-syndromic craniosynostosis identified clomiphene citrate as one of the strongest independent risk factors (OR: 12.71; 95% CI 1.42-113.6) (Ardalan et al., 2012). A study using data from the National Birth Defects Prevention Study (1997-2005) found use of clomiphene citrate to increase odds of craniosynostosis development by 1.9 times (1.2-3.0 95% CI) after adjusting for potential confounds (Reefhuis et al., 2011).

Recent studies have also begun to investigate the impact of environmental factors on craniosynostosis in animal models (Durham et al., 2017; Durham et al., 2019a; Durham et al., 2019b). For example, although mice exposed to nicotine *in utero* do not develop craniosynostosis, murine coronal suture cells treated *in vitro* with nicotine show increased cell proliferation and altered calvarial growth (Durham et al., 2019b). *In utero* exposure to a serotonin selective reuptake inhibitor, citalopram, increased the incidence of craniosynostosis in mice and reduced the number of Gli1⁺MSCs in the sutures (Durham et al., 2019a). These Gli1⁺ cells are integral to suture maintenance and their loss precedes suture fusion in the *Twist1*^{+/-} mouse model of craniosynostosis (Zhao et al., 2015). Exposure *in utero* to excess thyroxine, a type of thyroid hormone involved in numerous metabolic functions, failed to alter the severity or frequency of the craniosynostosis phenotype seen in *Twist1*^{+/-} mice, although it slightly affected skull shape (Durham et al., 2017). These results underscore the difficulty of studying environmental factors and gene-environment interactions in craniosynostosis.

Despite these tentative associations and experimental studies, the data associating environmental risk factors with the development of craniosynostosis remain inconclusive (Durham et al., 2017). We know little about which environmental risk factor(s) contribute to the phenotypic onset and progression of craniosynostosis nor to what extent, nor about how they differentially intersect with distinct genetic backgrounds to impact specific cell types and pathways that contribute to craniosynostosis. The effects of environmental factors might also vary depending on genotype, rendering some genetic variants important only in the presence of specific environmental factors. Thus, future efforts should seek to improve our understanding of the etiology and mechanisms of craniosynostosis that result from interactions among genetics and exposure to teratogens and other environmental factors.

5. Animal models and developmental mechanisms of craniosynostosis

5.1. Animal models of craniosynostosis

Multiple animal models are used to explore the mechanisms that underlie the pathophysiology of craniosynostosis. In particular, genetically modified mouse models have provided considerable insights into the molecular, cellular, and developmental mechanisms of craniosynostosis, and their skulls share developmental and anatomical similarities with those of humans. Researchers have also leveraged the rapid embryonic, external growth, and the large and transparent embryos of zebrafish, which enable development to be visualized *in vivo* into adulthood (Grova et al., 2012). However, this organism's interfrontal suture remains patent throughout life, unlike the corresponding metopic and interfrontal sutures of humans and mice, respectively (Quarto and Longaker, 2005). Due to its larger skull size, the rabbit is used to test therapeutic surgical manipulations and strategies (Grova et al., 2012). However, it is less genetically tractable than the mouse or zebrafish. Overall, the mouse serves as the most accurate animal model of craniosynostosis pathophysiology and is therefore the focus of this review.

Craniosynostosis has been studied extensively using mouse models generated to mimic human disease variants (see Figure 4; Table 2). Some of our mechanistic insights into the etiology of craniosynostosis are derived from *Twist1*^{+/-} and Fgf gain-of-function mouse models. For example, the same *TWIST1* haploinsufficiency patients display substantial phenotypic heterogeneity (Di Rocco et al., 2011; Lajeunie et al., 1995). *Twist1*^{+/-} mice are a well-established model of craniosynostosis in Saethre-Chotzen syndrome and exhibit unilateral or bilateral coronal suture fusion (See Figure 4c) with incomplete penetrance (see Table 2) (Behr et al., 2011; Bourgeois et al., 1998). This mouse model provides evidence for phenotypic heterogeneity, as only 40% develop bilateral coronal suture fusion. Recent studies have shown that *Twist1*^{+/-} mice with craniosynostosis also have elevated ICP and multiple neurocognitive behavioral abnormalities, including deficits in sociability, social memory, novel object recognition, and motor learning (Yu et al., 2021). These studies highlight that craniosynostosis is a complex congenital disorder of multiple tissues/organs. Future studies could determine to what extent genetically modified mice could model non-suture defects identified in craniosynostosis patients.

The contribution of gain-of-function mutations in the FGF signaling pathway to syndromic craniosynostosis has been well-documented. The variety of phenotypes is considered to be contingent upon the tissue affected as well as the specific mutation. *FGFR2* gain-of-function mutations contribute to Apert syndrome, Beare-Stevenson syndrome, Crouzon syndrome, Pfeiffer syndrome, and to bent bone dysplasia (see Box 2), each of which involves suture fusion (Jabs et al., 1994; Lajeunie et al., 1995; Merrill et al., 2012; Przylepa et al., 1996; Reardon et al., 1994; Rutland et al., 1995; Wilkie et al., 1995). Mutant mice carrying specific gain-of-function mutations in *Fgfr2* provide models of Apert syndrome. These mice have been engineered to carry targeted mutations, *Fgfr2*^{S252W/+} (Ser252Trp) (Chen et al., 2003) or *Fgfr2*^{253R/+} (Pro253Arg) (Yin et al., 2008), which together comprise 97% of Apert syndrome cases in humans. In mice, these mutations result in premature fusion of the coronal suture, which is a key clinical feature of Apert syndrome (Park et al., 1995b). Mice carrying two other gain-of-function *Fgfr2* mutations that can be found in humans with Crouzon syndrome - *Fgfr2*^{C342Y/+} (Cys342Tyr) (Eswarakumar et al., 2004) and *Fgfr2*^{W290R/+} - have phenotypes that recapitulate the clinical characteristics of this syndrome (Park et al., 1995a). *FGFR1* gain-of-function is primarily associated with Pfeiffer syndrome, which involves multi-suture synostosis (Muenke et al., 1994; Zhou et al., 2000). Mice carrying the P250R gain-of-function mutation in *Fgfr1* exhibit bicoronal synostosis, thus recapitulating the clinical features of Pfeiffer syndrome, as well as a dome-shaped skull and facial asymmetry (Cornille et al., 2019; Zhou et al., 2000). *FGFR3* gain-of-function mutation is responsible for both Muenke syndrome and Crouzon syndrome and is associated with multi-suture synostosis (Wilkie et al., 2017) (See Box 2; Table 1). *Fgfr3*^{P244R/P244R} (Pro244Arg) and *Fgfr3*^{P244R/+} gain-of-function mutations in mice have been generated to model Muenke syndrome and display variable phenotypes, with elevated penetrance of craniofacial abnormalities in homozygotes (Twiggs et al., 2009). However, in comparison to previously described *Fgfr1* and *Fgfr2* mouse models of craniosynostosis, this one falls short of recapitulating the human disease; it has a very low craniofacial phenotypic penetrance with a sex bias opposite to what is seen in humans (Cornille et al., 2019). In addition to suture and skull defects, other disease symptoms can be modeled in these mice. For example, patients with Apert syndrome have craniosynostosis of varying sutures, syndactyly, ocular anomalies, conductive hearing loss, cleft palate, and dental

anomalies (Wenger et al., 1993). It is important to investigate how these mouse models can recapitulate this wide spectrum of abnormalities in Apert syndrome patients. To date, most of these additional clinical features seen in various craniosynostosis syndromes have yet to be extensively investigated. An exception to this is Muenke syndrome, in which studies have been conducted in mice to evaluate the ability of the *Fgfr3*^{P244R/+} gain-of-function to produce sensorineural hearing loss like that seen in the human condition (Mansour et al., 2009).

A key limitation of these animal models is that a genetic mutation in humans might result in different phenotypes in animal models, making it difficult to clinically translate the findings or to investigate the mutation further. For example, some mouse models of disease mutations, such as those for Muenke syndrome, produce no phenotype or only a mild one (Holmes, 2012; Twigg and Wilkie, 2015a). These alterations are likely due to the species differences between humans and mice. Future *in vitro/ex vivo* human models or humanized chimeric mouse models of craniosynostosis should help with the translation of findings from animal models to humans.

5.2. Developmental mechanisms in craniosynostosis

A key strength of the mouse models of craniosynostosis is that they can be used to investigate early developmental abnormalities, even before any anatomical changes are evident. For example, in mouse models of *Twist1*^{+/-} craniosynostosis, the earliest detectable defect is the aberrant expression of *Notch2*, which is a marker of osteogenesis, and it is abnormally expressed in non-osteogenic suture mesenchymal regions in *Twist1*^{+/-} mouse embryos at E12.5 (Yen et al., 2010). This finding identified that the premature osteogenic differentiation of suture MSCs is a disease mechanism of craniosynostosis in *Twist1*^{+/-} mice. Studies of *Runx2* mutants have further supported this premature osteogenic differentiation of suture mesenchyme in craniosynostosis. *Runx2* is essential for osteoblastic differentiation and subsequent bone formation, and its duplication in humans leads to a craniosynostotic phenotype (Maeno et al., 2011), which is recapitulated in *Runx2* overexpression mouse models. These studies demonstrate that enhanced osteogenic differentiation, which occurs in *Twist1*^{+/-} suture MSCs, contributes to craniosynostosis. More importantly, double heterozygotes for *Twist1* and *Runx2* deletion have none of the

skull abnormalities observed in mutant mice (Bialek et al., 2004), which supports the notion that *Twist1*^{+/-} haploinsufficiency promotes Runx2 activity and leads to the premature osteogenic differentiation of suture MSCs, ultimately resulting in craniosynostosis.

The loss of the cellular boundary in suture development is another developmental mechanism that contributes to craniosynostosis discovered in *Twist1*^{+/-} mouse models. In *Twist1*^{+/-} mice with coronal synostosis, the frontal-parietal boundary is defective at E14.5 and neural crest cells invade the undifferentiated mesoderm-derived cells in the coronal suture. This neural crest cell invasion is coupled with an expansion in *Msx2* expression and reduction of *Efna4* (Merrill et al., 2006). *EFNA4* loss-of-function mutations in humans lead to craniofrontonasal syndrome with coronal synostosis (Ciurea and Toader, 2009; Schaefer et al., 1998). *EphA4*^{-/-} mutant mice exhibit defects in the coronal suture and neural crest-mesoderm boundary that phenocopy those in *Twist1*^{+/-} mice (Ting et al., 2009). Compound *Twist1*^{+/-};*EphA4*^{+/-} heterozygotes have a more severe craniosynostotic phenotype than do single-mutant heterozygous mice, as well as a higher penetrance of craniosynostosis. Labeling osteogenic precursor cells using DiI, a lipophilic membrane stain that diffuses to stain the entire cell, shows that *Twist1* and *EphA4* are required for the exclusion of such cells from the coronal suture. These results support the model in which loss of integrity of the osteogenic–non-osteogenic boundary leads to osteogenic cells crossing into the suture regions, resulting in suture MSCs taking on an osteogenic fate.

In addition to embryonic development deficits, postnatal suture MSCs have been recently identified for their role in calvarial injury repair. Further, ~~in calvarial homeostasis~~ emerging evidence suggests that loss of suture MSCs could also be a disease-driving mechanism for craniosynostosis. It has been reported that Gli1⁺ cells were significantly reduced in the *Twist1*^{+/-} craniosynostosis mouse model before the manifestation of suture closure defects; the ablation of this Gli1⁺ population resulted in craniosynostosis, indicating that the loss of Gli1⁺ cell is sufficient to cause craniosynostosis (Zhao *et al.*, 2015). CD51⁺;CD200⁺ cells are also significantly reduced in *Twist1*^{+/-} mice before suture fusion defects occur (Menon et al., 2021), although it remains unknown if the ablation of this cell population leads to craniosynostosis in mice. It also remains unknown if Axin2⁺ or Prx1⁺ cells are reduced in craniosynostosis mouse models. In contrast to the ablation of Gli1⁺

cells, that of Prx1⁺ cells does not result in a craniosynostosis phenotype (Wilk *et al.*, 2017), indicating that Prx1⁺ cells of the suture are dispensable for postnatal calvarial development. It is important to investigate how these suture MSCs behave and change their fates during the onset and progression of craniosynostosis. In addition, it remains to be investigated whether the lineage markers are mutually exclusive, or if cells can be positive for more than one marker. Such studies will provide insights into the regulation and functions of distinct suture MSCs under normal and disease conditions and will help guide stem cell-based therapeutic efforts to treat craniosynostosis and other craniofacial disorders.

6. Prospective treatments for craniosynostosis

Stem cell-based treatment: Given the shortcomings and complications of surgical intervention [as described above/in Box 3], pre-clinical studies have been conducted to develop alternative methods to address this unmet clinical need. One early study used autologous MSCs and the controlled release of transforming growth factor beta 3 (TGFβ3) during the operative correction of craniosynostosis (osteotomy) in rats (Moioli *et al.*, 2008). This procedure successfully mitigated ossification and secondary synostosis following suture regeneration (Moioli *et al.*, 2008). However, the independent replication of these findings has not been reported and subsequent progress has had a limited impact on current clinical practice on treating craniosynostosis.

The first animal model of neurological dysfunctions in craniosynostosis was recently established using *Twist1*^{+/-} mice (Jiang *et al.*, 2002; Yu *et al.*, 2021), a widely accepted model for Saethre-Chotzen syndromic craniosynostosis (Lee *et al.*, 2012; Maliepaard *et al.*, 2014; Speltz *et al.*, 2015). This study added significant clinical value to this animal model as we gained a more comprehensive understanding of adverse effects caused by craniosynostosis, which has promising potential for guiding the development of new therapies that could ameliorate these deficits in patients. Importantly, we have recently implanted suture MSCs on resorbable scaffolds and succeeded in regenerating a functional suture while mitigating skull dysmorphology and neurological defects in mice (Yu *et al.*, 2021) (see Figure 5). Specifically, removal of the fused suture followed by implantation of Gli1⁺ MSCs on a biodegradable scaffold could restore suture patency in *Twist1*^{+/-} craniosynostosis mice. Importantly, this intervention normalized the skull shape,

neurocognitive function, and normalized the ICP of *Twist1*^{+/-} mice. Such studies have been enabled by our previous identification of Gli1⁺ suture stem cells in the suture mesenchyme (Zhao et al., 2015), and by the finding that the premature loss of these cells might cause the premature fusion of the coronal suture in *Twist1*^{+/-} mice.

This stem cell-based treatment of craniosynostosis has been further supported by a recent study in *Twist1*^{+/-} mice in which CD51⁺;CD200⁺ suture MSCs were transplanted with Wnt3a following suturectomy and prevented re-synostosis (Menon et al., 2021). It is important to determine how implantation of CD51⁺;CD200⁺ and other suture stem cells (i.e. Axin2⁺, Prx1⁺, Ctsk⁺ cells) can regenerate cranial sutures and restore the skull and correct other associated anomalies in craniosynostosis.

These results highlight a potential paradigm shift in the future treatment of craniosynostosis, away from extensive surgery that incurs significant blood loss, to a less invasive stem cell-based biological solution. Most importantly, strategies that regenerate functional cranial sutures and that do not simply reshape the skull vault enable continued skull growth in coordination with brain development. They also normalize ICP, and thus likely reverse neurocognitive behavioral defects and eliminate the need for re-operation (Yu *et al.*, 2021).

Pharmacological treatment: The therapeutic potential of numerous bioactive molecules has been assessed in craniosynostosis animal models. In the *Twist1*^{+/-} mouse model of craniosynostosis, Pribadi et al. determined that *Kdm6a* and *Kdm6b*, two known promoters of osteogenesis, are upregulated in *Twist1*^{+/-} calvarial cells (Pribadi et al., 2020). Using this information, they applied GSK-J4, a pharmacological inhibitor of *Kdm6a* and *Kdm6b*, that led to decreased osteogenic differentiation in *Twist1*^{+/-} calvarial cells and reduced bone mineralization in *Twist1*^{+/-} calvarial organ cultures. *In vivo*, GSK-J4 treatment prevented fusion of coronal sutures in *Twist1*^{+/-} mice.

Recombinant periostin (an extracellular matrix protein) is also reported to mitigate coronal craniosynostosis in *Twist1*^{+/-} mice (Bai et al., 2018). Periostin is expressed in mesenchymal cells in the mouse skull and is repressed by Twist1. The results of this study demonstrated that periostin can reduce suture cell proliferation and differentiation through

upregulation of Wnt/ β -catenin signaling. This mitigated coronal craniosynostosis in *Twist1*^{+/-} mice.

Multiple studies have also targeted the role of FGFR in craniosynostosis. For example, intraperitoneal injection of the prolyl isomerase peptidyl-prolyl *cis*—*trans* isomerase interacting 1 (PIN1) inhibitor juglone into pregnant *Fgfr2*^{S252W/+} mice from E14.5-E18.5 prevents the development of Apert syndrome-like phenotypes in their offspring. PIN1 inhibition leads to attenuation of the elevated Runx2 levels seen in *Fgfr2*^{S252W/+} mice, and Runx2 regulates *Fgfr2* and *Fgfr3* expression to induce proliferation of osteoblast progenitor cells (Shin et al., 2018). Shukla et al. injected a MEK1/2 inhibitor intraperitoneally into pregnant mice from E13.0-18.0 and found that this inhibition of MEK-ERK signaling can also prevent *Fgfr2*^{S252W}-related craniosynostosis and rescue skeletal abnormalities in the offspring of these mice (Shukla et al., 2007).

In rat models of craniosynostosis, recombinant human (rh) noggin protein was shown to inhibit coronal suture fusion (Shen et al., 2009). The decision to investigate noggin was based on its known expression in patent suture mesenchyme and its absence from the mesenchyme of fused cranial sutures. Increased FGF2 signaling, as seen in various craniosynostosis syndromes, suppresses noggin expression in the suture. Noggin's inappropriate expression is also implicated in the pathogenesis of FGFR-related craniosynostosis; the implantation of human *FGFR2* mutant osteoblasts with rhNoggin in chimeric nude rats prevented suture fusion, while *FGFR2* mutant osteoblasts alone did not (Shen et al., 2009). The study provides evidence for noggin as an important mediator in the development of this disease and yet another possible future target for human therapies.

In another rat study, FGF activity was modulated *in utero* to assess the effects on the calvaria (Greenwald et al., 2001). The authors found that increased FGF2 activity resulted in the thickening of the parietal and frontal bones, as well as coronal suture fusion. They postulated that stimulation of FGF activity led to significant changes of cellular proliferation, TGF β 1 expression, and collagen I expression in the dura mater and suture; these molecular changes contributed to increased osteogenesis. Rabbit models have also been used to test bioactive molecules in craniosynostosis treatment owing to their larger skull size. One study of rabbits with familial delayed-onset craniosynostosis found that treatment of 25-day-old animals with TGF β 3 combined with slow-absorbing collagen

increased the width and area of the coronal suture (Chong et al., 2003). The exact mechanism behind these results has yet to be elucidated. However, there is evidence that isoforms of TGF β mediate suture cell homeostasis (Opperman et al., 2002; Opperman and Ogle, 2002).

Another study performed suturectomies on rabbits at 10 days of age, then treated them with anti-TGF β 2 antibodies and found that the treatment group developed a significantly greater suture area than controls. Blocking TGF β 2's function not only kept sutures patent, but also improved craniofacial growth. While the mechanism has not yet been fully investigated, the authors suggested based on previous *in vitro* and *in vivo* studies that TGF β 2 was able to prevent postoperative re-synostosis by controlling cell proliferation and apoptosis surrounding the osteogenic fronts (Mooney et al., 2007) (See Figure 5).

Overall, most of these pharmacological studies focused on one time point of morphological change in cranial sutures. The long-term beneficial effects and pharmacokinetic profiles of these bioactive molecules have yet to be carefully evaluated, and their efficacy and biosafety in treating craniosynostosis remain obscure. But, in time, these studies might help to identify potential pharmacological targets for the future prevention and/or treatment of craniosynostosis.

7. Conclusions and future directions

Despite research and technological advances improving clinical management of craniosynostosis, this congenital malformation remains challenging to manage and treat, both for patients and care providers. Given the complicated nature of the etiology of craniosynostosis, involving both genetic and environmental factors, more studies need to be conducted to elucidate this important relationship. Overall, the gene-environment crosstalk remains an understudied and important area, considering that only about a quarter ~~20-30%~~ of craniosynostosis cases have a currently identifiable genetic basis. Mutant animal models coupled with exposure to potential environmental insults will be invaluable for us to gain a deeper understanding of the mechanisms of craniosynostosis.

Although craniosynostosis mainly affects the shape and size of the skull, it needs to be investigated in the context of brain development and function because sutures function as sites of calvarial bone growth to accommodate the expansion of brain tissue in

postnatal development. As shown above, deficits in brain structure and function are detected in a population of craniosynostosis patients. However, these brain-related defects have not been fully explored in many of the mutant animal models with craniosynostosis and will require further investigation. From an evolutionary perspective, cranial sutures may act as targets for change in craniofacial morphology and have contributed to the diversity of skull shapes across mammals and other vertebrates (White et al., 2021). As expansion of brain structure and function is key to human evolution, we need to expand our efforts to gain a better understanding of the connection among the sutures, skull, and brain function.

Another important area of future study is the cranial suture niche environment. Mesenchymal cells within each cranial suture contain functionally indispensable MSCs and are highly heterogeneous (Farmer et al., 2021; Holmes et al., 2021; Li et al., 2021; Zhao et al., 2015). Furthermore, adjacent structures such as the dura mater may contribute to the homeostasis of suture MSCs during development and in suture regeneration (Yu et al., 2021). Further studies are necessary to investigate the role(s) of other structures, such as blood vessels and nerves, in regulating tissue homeostasis within the suture.

Finally, how can regenerative medicine be used to provide innovative treatment for patients with craniosynostosis? Suture MSC loss is a newly identified disease-driving mechanism of craniosynostosis. This discovery calls for a unique time window for therapeutic intervention. Proof-of-principle studies show that adding back Gli1⁺ suture MSCs after suture defects have occurred in mouse models can regenerate cranial suture and mitigate both skull and neurocognitive defects. It will be informative to determine the feasibility and efficacy of this heterogeneous suture MSC population in treating craniosynostosis, including Axin2⁺, Prx1⁺, Ctsk⁺, and CD51⁺;CD200⁺ cells. Combining bioactive small molecule-based pharmacological approaches with suture MSC implantation is also a promising area for future research. Overall, our pathophysiological studies will no doubt provide a better understanding of suture development, stem cell biology, tissue regeneration, and our disease mechanism-based therapeutic efforts will help bring improved treatment for patients with craniosynostosis.

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Declaration of conflict of interest

The authors declare that there is no conflict of interest.

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Box 1. Glossary

Acrocephaly: a congenital abnormality of the skull in which the top of the skull presents with a conical shape

Amblyopia: colloquially known as “lazy eye,” and is characterized by poor vision in one eye as a result of abnormal vision development

Biparietal diameter: the maximum diameter of the fetal skull at the eminences of the parietal bones; this is one of the basic parameters used to assess fetal skull size

Brachycephaly: a shortened skull shape with a shorter than normal length to width ratio

Crouzonoid faces: common features present in patients with Crouzon syndrome, including midface hypoplasia, a convex nose, a prognathic mandible, exophthalmos, and posteriorly angled ears

Deformational plagiocephaly: when an infant develops a flattened posterior of the skull or spot on the side of the head due to prolonged positioning/pressure on that area

Gain-of-function mutation: a mutation that leads to increased/new activity of a protein

Haploinsufficiency: when one copy of a gene is inactivated/deleted, and the remaining copy is unable to appropriately compensate to maintain normal function

Hypomorphic mutation: a mutation that confers a gene product with a decreased activity level

Loss-of-function mutation: a mutation that leads to decreased/ablated activity of a protein

Metopic suture: fibrous joint that connects the two frontal bones

Otitis media with effusion: a collection of fluid in the middle ear in absence of an ear infection

Papilledema: swelling of the optic disc as a result of increased intracranial pressure

Plagiocephaly: flattening of the posterior skull caused by lambdoid synostosis

Scaphocephaly: long and narrow “boat-shaped” head caused by sagittal suture fusion

Trigonocephaly: triangularly shaped forehead caused by metopic suture fusion

X-linked mutation: a mutation that occurs on the X chromosome; for this reason, males are more likely to be affected by these mutations as they only carry one X chromosome

Box 2. Craniosynostosis associated disorders. See Table 1 for genetic abnormalities and modes of inheritance associated with each disorder.

Apert syndrome: a rare genetic condition characterized by craniosynostosis and distinctive malformations of the skull, hands, feet, and face. Affected children often also have intellectual disability.

Baller-Gerold syndrome: a rare condition characterized by craniosynostosis (most commonly coronal), bulging eyes, a prominent forehead, and bone abnormalities.

Beare-Stevenson syndrome: a genetic disorder characterized by craniosynostosis, furrowed and wrinkled skin (cutis gyrate), bulging eyes, and an underdeveloped maxilla.

Bent bone dysplasia: an often-lethal skeletal disorder characterized by craniosynostosis, osteopenia, hypoplastic pubic bone and clavicles, bent long bones, and poor skull mineralization.

Craniofrontonasal syndrome: a rare condition characterized by coronal craniosynostosis, wide-set eyes, a cleft nose tip, a wide nasal bridge, and often cleft lip and/or palate.

Crouzon syndrome: a rare genetic disorder characterized by craniosynostosis, bulging eyes, a prominent forehead, midface hypoplasia, and a short upper lip.

Muenke syndrome: a rare genetic disorder characterized by craniosynostosis, wide-set eyes, and flattened cheekbones.

Pfeiffer syndrome: a rare genetic disorder characterized by craniosynostosis, broad and medially deviated thumbs and big toes.

Saethre-Chotzen syndrome: a rare genetic condition characterized by coronal craniosynostosis, ptosis, a broad nasal bridge, and widely spaced eyes. Affected children can also have small and rounded ears.

Box 3. Surgical treatment of craniosynostosis

- The current treatment for craniosynostosis is surgical
- Various surgical treatments offered include distraction osteogenesis springs, endoscopically assisted strip craniectomy, and open calvarial vault remodeling
- Postoperative helmet therapy to help with skull reshaping can be implemented following surgery
- More recently, technological advancements including machine learning, intraoperative near-infrared spectroscopy, and improved 3D imaging have improved the accuracy of diagnostics and surgical planning
- Commonly reported surgical complications include postoperative bleeding, puncture/lacerations, pneumonia, seizures, cardiorespiratory shock, hypovolemic shock, CSF fistula, and hematoma
- Sutures can also re-fuse postoperatively, requiring surgical revision
- The appropriate timing of surgical intervention is not well established, is case-specific, and involves weighing the benefits and risks of early versus later intervention
- Early intervention has been shown to improve neurological and developmental outcomes, yet it carries the risk of heavy blood loss and complications of general anesthesia, including possible neurodevelopmental defects
- Many patients require reoperation driven by poor cosmetic outcomes, the desire for improved cranial morphology, re-synostosis, and/or persistent increased intracranial pressure

Table 1. Gene mutations, craniosynostosis and other defects in humans

Human Gene	Syndrome	Inheritance Pattern	Penetrance	Suture(s) Affected	Major Clinical Characteristics	Reference
<i>EFNA4</i>	Craniofrontonasal syndrome	XLD		Coronal	Frontonasal dysplasia, ocular hypertelorism, broad nasal bridge, frontal bossing, sloping shoulders and dysplastic clavicles, syndactyly, nail splitting, more severe in females	Schaefer <i>et al.</i> (1998)
<i>EFNB1</i>	Craniofrontonasal syndrome	XLD		Uni-or-bi- coronal	Frontonasal dysplasia; ocular hypertelorism, bifid nasal tip, nail splitting	Ciurea and Toader (2009)
<i>ERF</i>	ERF-related craniosynostosis	AD		Multi-suture	Facial dysmorphism, Chiari type I malformation, postnatal onset of craniosynostosis	Glass <i>et al.</i> (2019)
<i>FGFR1</i>	Pfeiffer	AD	100%	Coronal	Craniofacial deformations, syndactyly, digit/limb abnormalities	Muenke <i>et al.</i> (1994)
<i>FGFR2</i>	Apert	AD	100%	Bi-coronal, multi-suture	Dysmorphic facies, ocular anomalies, conductive hearing loss, cleft palate, syndactyly	Wenger <i>et al.</i> (1993)
	Pfeiffer	AD	100%	Multi-suture	Crouzonoid facies, Broad thumbs, sometimes cutaneous syndactyly, high forehead, maxillary hypoplasia	Johnson and Wilkie (2011)
	Crouzon	AD	100%	Commonly coronal, but can have late onset pansynostosis	Crouzonoid facies, absence of major abnormalities of hands/feet, shallow orbits, normal limbs	Johnson and Wilkie (2011)
<i>FGFR3</i>	Muenke	AD	87% in females; 76% in males	Uni-or- bi-coronal	Low-frequency sensorineural hearing loss, normal to dysmorphic face	Doherty <i>et al.</i> (2007); Twigg <i>et al.</i> (2009)

	Crouzon with acanthosis nigricans			Multi-suture	Crouzonoid facies, choanal stenosis, hydrocephalus, acanthosis nigricans	Arnaud-López et al. (2007)
HUWE1		XLD		Multi-suture or metopic	Learning difficulties	Moortgat <i>et al.</i> (2018)
IL11RA	Craniosynostosis and dental anomalies (CRSDA); also known as Crouzon-like craniosynostosis disorder	AR		Multi-suture	Craniosynostosis; delayed tooth eruption; some have supernumerary teeth and conductive hearing loss	Nieminen et al. (2011)
MSX2	Boston-type craniosynostosis	AD	100%	Multi-suture	Highly variable craniosynostosis ranging from fronto-orbital recession to clover-leaf skull deformity	Jabs <i>et al.</i> (1993)
RAB23	Carpenter syndrome	AD		Multi-suture	Polysyndactyly, obesity, cardiac defects	Jenkins <i>et al.</i> (2007)
RUNX2	Cleidocranial dysplasia	AD	Near 100%		Delayed closure of fontanelles, dental abnormalities, hypoplastic clavicles	Jaruga et al. (2016)
TCF12		AD	53%	Coronal	May have Saethre-Chotzen syndrome appearance; alternatively, can have isolated uni- or bi-coronal synostosis	di Rocco et al. (2014)
SMAD6				Multi-suture variable	Cardiac defects, brain anomalies	Calpena <i>et al.</i> (2020)
SMO	Curry-Jones syndrome			Multi-suture variable	Cerebral malformations, iris colobomas, polysyndactyly, patchy skin lesions	Wilkie <i>et al.</i> (2017)

<i>TWIST1</i>	Saethre-Chotzen	AD	100%	Uni-or-bi-coronal	Abnormal extremities (variable broad digits, syndactyly, and/or brachydactyly). Facial asymmetry	Johnson and Wilkie (2011)
<i>ZIC1</i>		AD		Coronal	Severe learning disability	Twigg et al. (2015)

AD: autosomal dominant
AR: autosomal recessive
XLD: X-linked dominant

Table 2. Animal models of craniosynostosis: affected sutures and other phenotypic characteristics.

Gene	Type of Mutation	Animal	Model	Penetrance	Suture(s) Affected	Stage of phenotypic appearance	Major Phenotypic Characteristics	Reference
<i>Efna4</i>	LOF	Mouse	<i>EphA4</i> ^{-/-}	40%	Coronal	By P21	Coronal suture defects; defective formation of neural crest-mesoderm boundary at coronal suture	Ting <i>et al.</i> (2009)
		Rabbit					*	Taylor <i>et al.</i> (2018)
<i>Efnb1</i>	LOF	Mouse	<i>EphrinB1</i> ^{Lox/+} , <i>EphrinB1</i> ^{Lox/Lox} , <i>EphrinB1</i> ^{Lox/**}			By E18.5	Complete ablation: perinatal lethality with varying defects in neural crest cell-derived tissues, incomplete closure of body wall and abnormal skeleton; shortened skulls, some cleft palate, more severe in females	Compagni <i>et al.</i> (2003); Davy <i>et al.</i> (2004)
		Rabbit					*	Taylor <i>et al.</i> (2018)
<i>Erf</i>	Hypomorph	Mouse	<i>Erf</i> ^{fllox} , <i>Erf</i> ^{fllox/-**}		Coronal, sagittal, lambdoid	P3-6w	Postnatal multi-suture craniosynostosis	Twigg <i>et al.</i> (2013)
		Rabbit					Excluded as causing craniosynostosis phenotype in rabbits	Gilbert <i>et al.</i> (2019)
<i>Fgfr1</i>	GOF	Mouse	<i>Fgfr1</i> ^{P250R/+}	100%	Coronal, interfrontal, sagittal	P6-21	Facial asymmetry, shortened, dome-shaped skull	Zhou <i>et al.</i> (2000)

		Rabbit					Excluded as causing craniosynostosis phenotype in rabbits	Gallo et al. (2013)
<i>Fgfr2</i>	GOF	Mouse	<i>Fgfr2</i> ^{S252W/+}	100%	Coronal, sagittal, lambdoid	By P20	Shortened midface, dome-shaped skull. None exhibit limb phenotype	Chen et al. (2003)
			<i>Fgfr2</i> ^{P253R/+}	100%	Coronal	E16.5-P21	Dome-shaped skull, brachycephaly, shortened cranial base and growth of long bones	Yin et al. (2008)
			<i>Fgfr2</i> ^{C342Y/+}	100%	Coronal	E16.5-P21	Coronal, sagittal and lambdoid synostosis, dome-shaped skull, midface hypoplasia	Eswarakumar et al. (2004)
			<i>Fgfr2</i> ^{W290R/+}	100%	Coronal	P3-P6w	Coronal and lambdoid synostosis, dome-shaped skull, short snout, midface hypoplasia	Mai et al. (2010)
		Rabbit					*	Gallo et al. (2013)
<i>Fgfr3</i>	GOF	Mouse	<i>Fgfr3</i> ^{P244R/+} <i>Fgfr3</i> ^{P244R/P244R}	Variable (0-100%) based on sex and whether homozygous vs. heterozygous	Coronal	P7 onward	Incomplete penetrance, minority of heterozygotes and many homozygotes show round skull, short snout, malocclusion	Ting et al. (2009)
		Rabbit					*	Gallo et al. (2013)
<i>Il1ra</i>	LOF	Mouse	<i>Il1ra</i> ^{-/-}	50%	Synostosis of facial sutures	By adulthood	Twisted snouts, shortened skulls	Agthe et al. (2018)
<i>Msx2</i>		Mouse	<i>Timp1</i> - <i>Msx2</i> ^{P7H} , <i>CMV</i> - <i>Msx2</i> ^{P7H} , <i>CMV</i> - <i>Msx2</i>	30-71%	Coronal, sagittal, lambdoid	By P14	Multi-suture synostosis; incomplete penetrance	Liu et al. (1995)

	GOF/overexpression	Rabbit					*	Gilbert <i>et al.</i> (2019)
Rab23	LOF	Mouse	<i>Rab23</i> ^{-/-}	100%	Coronal, parietal-temporal, fronto-nasal, lambdoid	By E18.5	Skeletal patterning defects in limb; embryonically lethal	Hasan <i>et al.</i> (2020)
Runx2	Overexpression	Rabbit	<i>Prx1-Runx2</i>	100%	Pan-synostosis	E13-18.5	Multi-suture synostosis, ectopic bone formation, limb defects	Maeno <i>et al.</i> (2011)
Tcf12	LOF	Mouse	<i>Ella-Cre;Tcf12</i> ^{lox/+} ; <i>Twist1</i> ^{+/-}	100%	Coronal	By P21	Tcf12/Twist1 together synergistically cause craniosynostosis. Tcf12 not yet studied on its own	Sharma <i>et al.</i> (2013)
		Rabbit					*	Gilbert <i>et al.</i> (2019)
		Zebrafish	<i>tcf12;twist1b</i>		Coronal		Combined loss of twist1 and tcf12 causes coronal synostosis	Teng <i>et al.</i> (2018)
Twist1		Mouse	<i>Twist1</i> ^{+/-}	71%	Coronal	P9-14	Coronal suture fusion, skull base anomalies, extra toes	(Behr <i>et al.</i> 2011; Cornille <i>et al.</i> (2019)
		Rabbit					*	Gallo <i>et al.</i> (2013)
		Zebrafish	<i>tcf12;twist1b</i>		Coronal		Combined loss of twist1 and tcf12 causes coronal synostosis	Teng <i>et al.</i> (2018)
Zic1		Mouse	<i>Zic4</i> ^{-/-} ; <i>Zic1</i> ^{-/-} ; <i>Zic1</i> ^{-/-} ; <i>Zic4</i> ^{-/-}	100%		By P5	Cerebellar hypoplasia and vertebral defects	Blank <i>et al.</i> (2011)

**Conditional allele

*Excluded as causing craniosynostosis phenotype in rabbits

GOF = gain-of-function

LOF= loss-of-function

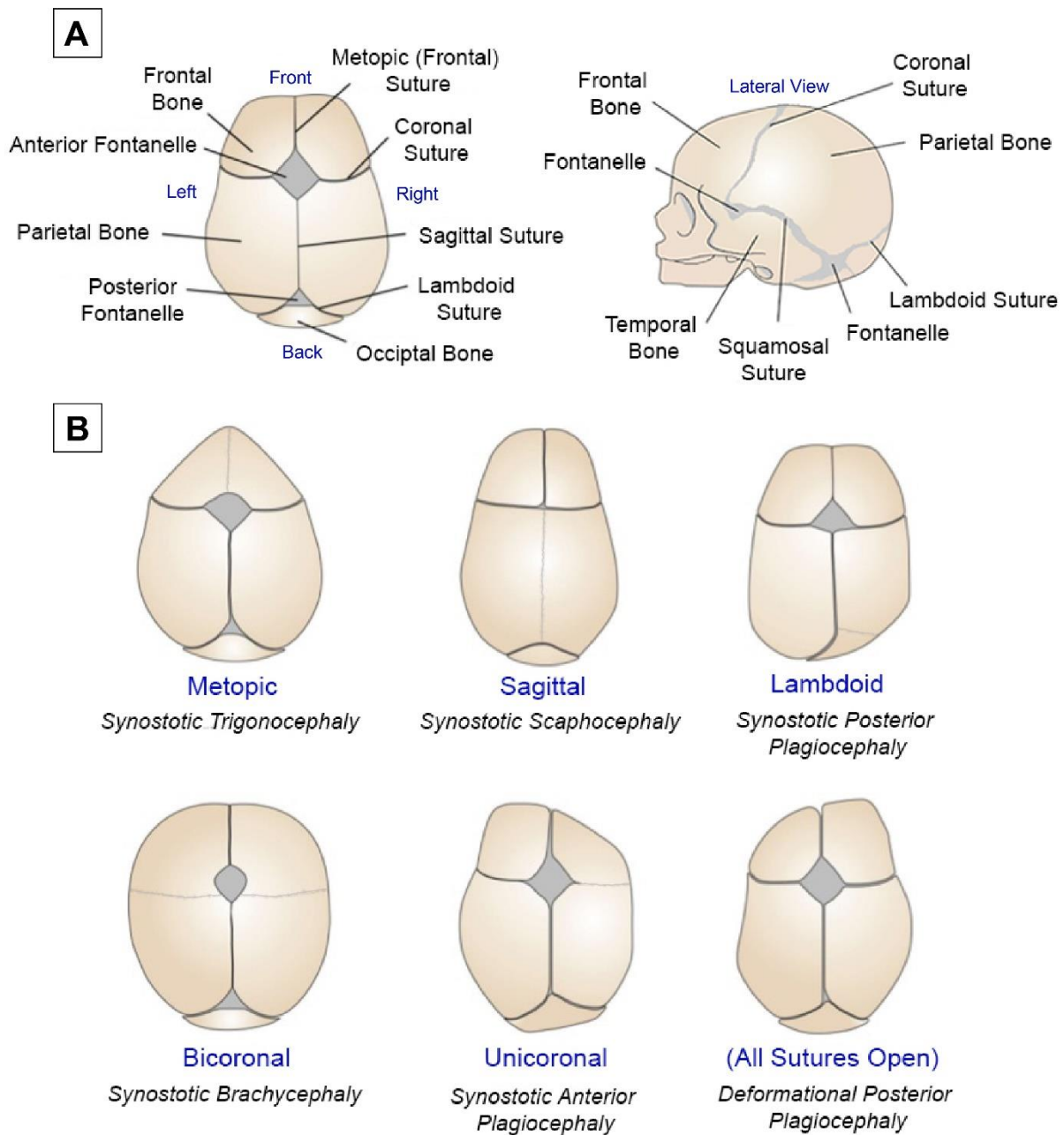


Fig. 1: Cranial sutures and craniosynostosis in humans. (A) A normal adult human skull labeled with different bones and connecting cranial sutures, shown from above. (B) Different skull deformities caused by different forms of craniosynostosis. (a) Metopic suture fusion leads to synostotic trigonocephaly. (b) Sagittal suture fusion leads to synostotic scaphocephaly. (c) Lambdoid suture fusion leads to synostotic posterior plagiocephaly. (d) Bilateral coronal suture fusion leads to synostotic brachycephaly. (e) Unilateral coronal suture fusion leads to synostotic anterior plagiocephaly. (f) Deformational posterior plagiocephaly (in which all sutures are open and suture patency is normal) is not caused by craniosynostosis but is shown as a comparison to synostotic anterior plagiocephaly, which is caused by unilateral coronal suture fusion. See Glossary, Box 1 for description of medical terms. Figure adapted from Buchanan et al. (2017) under the terms of Creative Commons CC BY-NC 3.0 license.

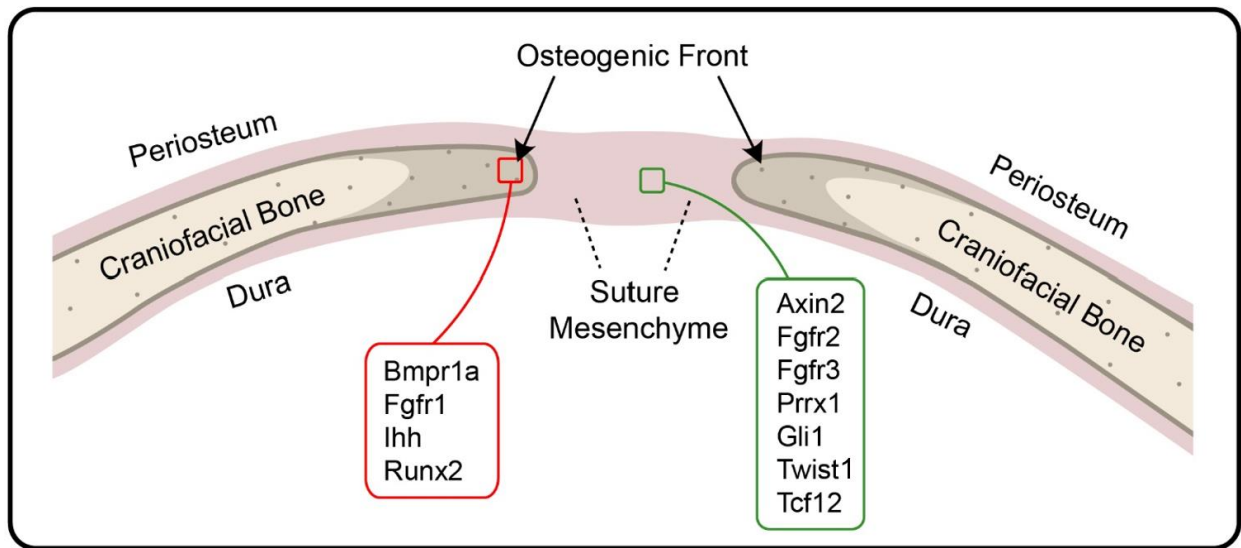
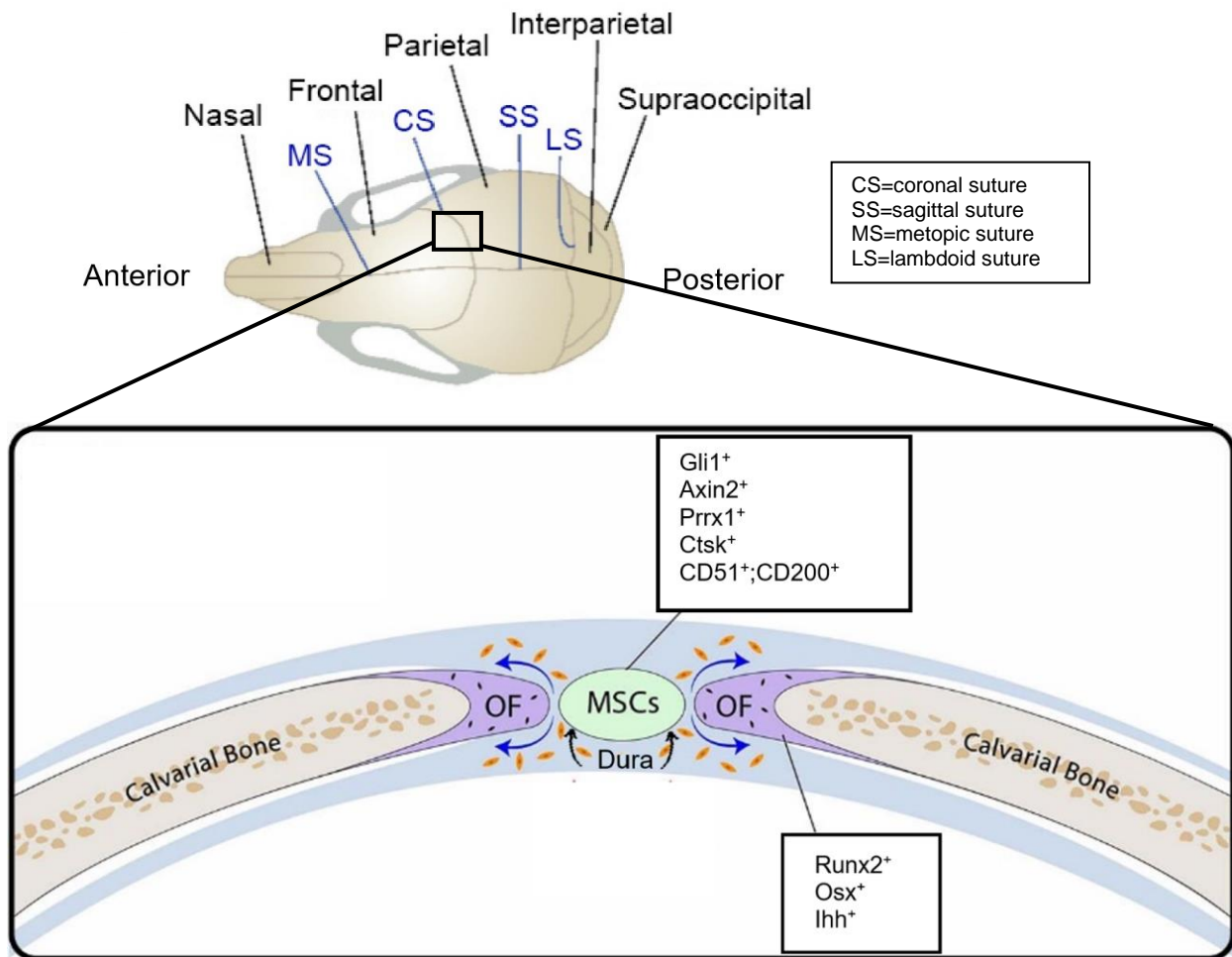
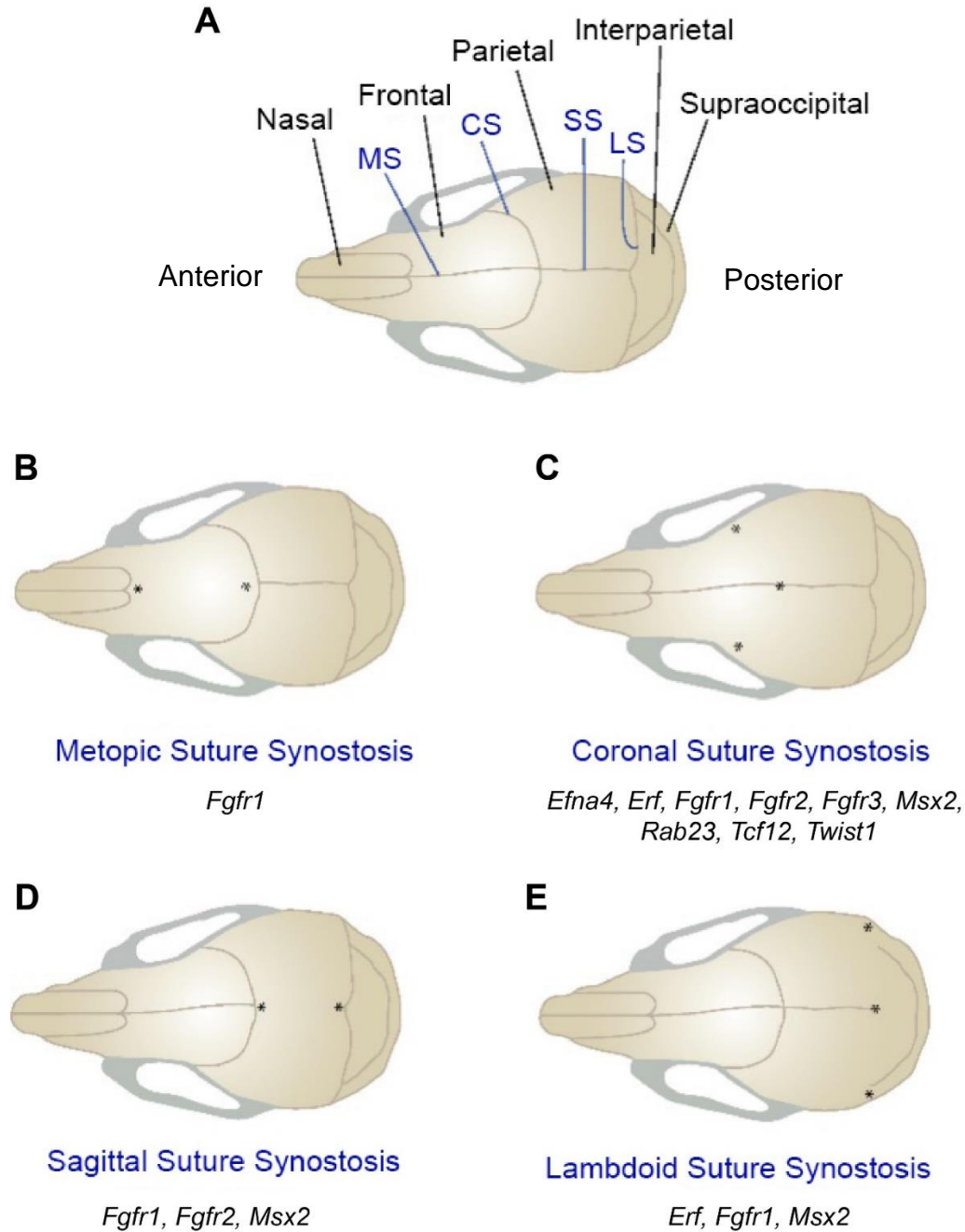


Figure 2. Suture development at postnatal stage. This schematic drawing highlights some genes that are expressed in the suture mesenchyme versus the ones that are expressed at the osteogenic front. Specifically, Axin2, Fgfr2, Fgfr3, Twist1 and Tcf12 are expressed in the suture mesenchyme. At the osteogenic front, Bmpr1a, Fgfr1, Ihh, and Runx2 are present. These gene expression patterns are dynamic and may change throughout postnatal suture development.



OF=osteogenic front
 MSC=mesenchymal stem cells

Fig. 3: Suture MSCs and their loss in craniosynostosis animal models. Here depicts a cross-section of the coronal suture of a mouse, with orange dots representing cells in the suture mesenchyme and black dots representing cells in the osteogenic front (OF). Genetic lineage tracing and surface marker purification has identified multiple populations of suture mesenchymal stem cells (MSCs) in mouse calvarial sutures, including Gli1⁺, Axin2⁺, Prx1⁺, Ctsk⁺, CD51⁺;CD200⁺ cells. Loss of Gli1⁺ and CD51⁺;CD200⁺ suture MSCs occurs during postnatal stages of mouse development and suture MSCs undergo premature differentiation into osteogenic cells in *Twist1*^{+/-} craniosynostosis mouse embryos.



CS=coronal suture
 SS=sagittal suture
 MS=metopic suture
 LS=lambdoid suture

Fig. 4: The genetic mutations associated with each type of suture fusion in an adult mouse are shown. Asterisks indicate fused sutures. A) Normal adult mouse skull. B) Metopic (interfrontal) suture synostosis; can be caused by *Fgfr1* mutation. C) Coronal suture synostosis; can be caused by mutation in *Efn4, Erf, Fgfr1, Fgfr2, Fgfr3, Msx2, Rab23, Tcf12, Twist1*. D) Sagittal suture synostosis; can be caused by mutation in *Fgfr1, Fgfr2, Msx2*. E) Lambdoid suture synostosis; can be caused by mutation in *Erf, Fgfr1, Msx2*.

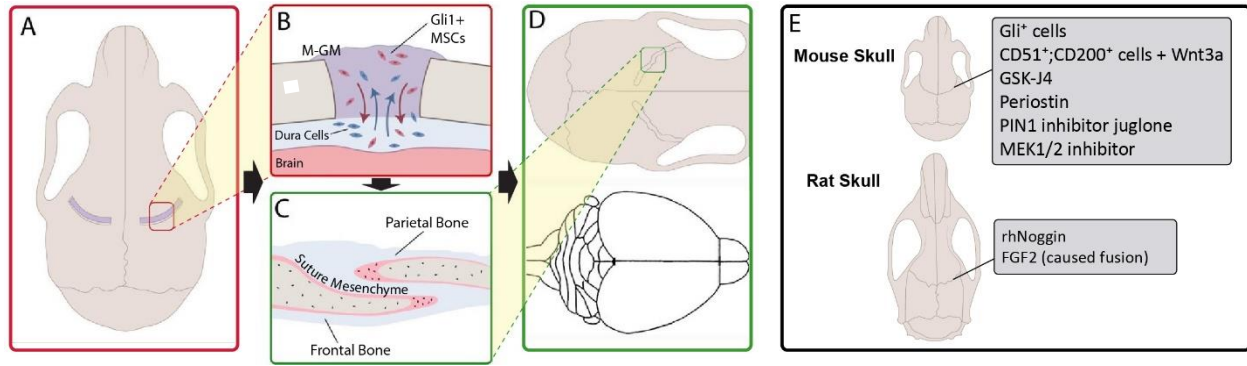


Fig. 5: Gli1⁺ MSC-mediated suture regeneration mitigates skull and neurocognitive defects in craniosynostosis.

(A) A rectangular defect 0.3-0.4 mm wide was made over each of the fused coronal sutures in *Twist1*^{+/-} mice. (B) Gli1⁺ cells were mixed with GelMA (methacrylated gelatin) modified with Matrigel and collagen I (which we refer to here as modified M-GM) and implanted into the empty space generated by surgical removal of the fused suture in *Twist1*^{+/-} mice. (C) Regenerated sutures exhibited normal morphology and act as natural sutures in terms of their gene expression and functions. (D) Regenerated sutures rescued skull deformity (top) and defective brain structure (microcephaly) (bottom figure represents restored brain) and functions in *Twist1*^{+/-} mice with craniosynostosis. (E) Various therapeutic approaches have been shown to regenerate cranial suture or prevent suture fusion. Specifically, Gli1⁺ MSCs or CD51⁺;CD200⁺ stem cells plus Wnt3a can support cranial suture regeneration in *Twist1*^{+/-} mice. Pharmacological agent GSK-J4, recombinant Periostin, PIN1 inhibitor juglone, and MEK1/2 inhibitor can prevent cranial suture fusion in mice. In the rat model, rhNoggin can prevent coronal suture fusion. Increased FGF2 activity can lead to suture fusion.