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## Cell Mechanics of Craniosynostosis

**Zeinab Al-Rekabi**<sup>1,2</sup>, **Michael L. Cunningham**<sup>2,3</sup>, and **Nathan J. Sniadecki**<sup>1,4,\*</sup>

<sup>1</sup>Department of Mechanical Engineering, University of Washington, 3900 E Stevens Way NE, Seattle, WA, 98195, USA

<sup>2</sup>Seattle Children's Research Institute, Center for Developmental Biology and Regenerative Medicine, 1900 9<sup>th</sup> Ave, Seattle, WA, 98101, USA

<sup>3</sup>Department of Pediatrics, Division of Craniofacial Medicine and the, University of Washington, 1959 NE Pacific St., Seattle, WA, 98195, USA

<sup>4</sup>Department of Bioengineering, University of Washington, 3720 15<sup>th</sup> Ave NE, Seattle WA, 98105, USA

### Abstract

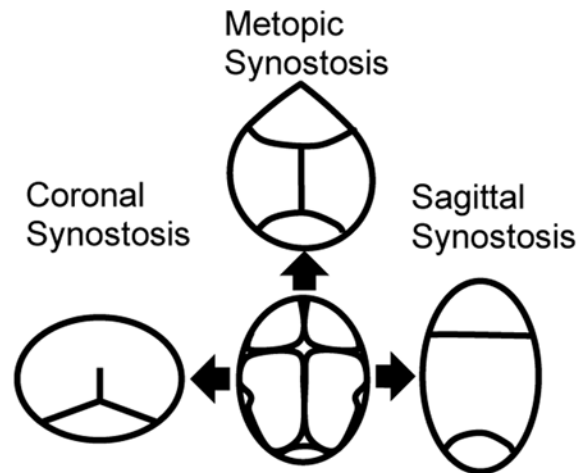
Craniosynostosis is the premature fusion of the calvarial sutures that is associated with a number of physical and intellectual disabilities spanning from pediatric to adult years. Over the past two decades, techniques in molecular genetics and more recently, advances in high-throughput DNA sequencing have been used to examine the underlying pathogenesis of this disease. To date, mutations in 57 genes have been identified as causing craniosynostosis and the number of newly discovered genes is growing rapidly as a result of the advances in genomic technologies. While contributions from both genetic and environmental factors in this disease are increasingly apparent, there remains a gap in knowledge that bridges the clinical characteristics and genetic markers of craniosynostosis with their signaling pathways and mechanotransduction processes. By linking genotype to phenotype, outlining the role of cell mechanics may further uncover the specific mechanotransduction pathways underlying craniosynostosis. Here, we present a brief overview of the recent findings in craniofacial genetics and cell mechanics, discussing how this information together with animal models is advancing our understanding of craniofacial development.

### For Table of Contents Use Only

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\*Corresponding nsniadec@uw.edu.

## Types of Craniosynostosis



### Keywords

Calvarial bone; Suture fusion; Molecular genetics; Development; Biomechanics

### Introduction

The human calvaria consists of five major bones: the paired frontal and parietal bones and the occipital bone at birth. These bones develop through intramembranous ossification, where the radial growth of each bone from a central locus of osteogenesis, approximates with an unossified mesenchyme to form a suture. The unossified mesenchyme is presumed to serve two major functions: it allows for both temporary deformation of the skull during birth and expansion of the cranial vault during brain growth. In normal development, the metopic suture, located between the paired frontal bones fuses at three to nine months of age,<sup>1</sup> while the other sutures fuse in the third decade of life.<sup>2</sup> Prior to these events, the balance of sutural elasticity, calvarial osteogenesis, and brain growth maintains healthy calvarial development.

Excessive bone growth at the osteogenic fronts or untimely reduction in brain growth can result in premature suture fusion. The four common types of synostosis are: metopic, coronal, sagittal and lambdoid synostosis (Fig. 1). Craniosynostosis divides into syndromic and non-syndromic forms with syndromic forms defined as those with recognizable patterns of craniofacial and non-craniofacial malformations. A number of mutations are associated with syndromic craniosynostosis.<sup>3-6</sup> Collectively, non-syndromic single-suture craniosynostosis (SSC) represents a common group of human malformations with a birth prevalence of 1 in 1,700-2,500 live births;<sup>7-8</sup> whereas syndromic forms have a prevalence of approximately 1 in 25,000.<sup>9-11</sup> Due both to its prevalence and the required medical and surgical management, craniosynostosis is one of the most clinically significant craniofacial disorders.

Premature suture fusion results in abnormalities in skull shape, usually becoming apparent between the last trimester of pregnancy and the first few months of life. Early suture fusion reduces further growth of the adjoining bones, in a direction orthogonal to the suture. Consequently, the normal expansion of the brain promotes compensatory overgrowth at other sutures, leading to progressive distortion in the skull shape. These changes in head shape can be associated with increased intracranial pressure that when untreated, may result in permanent brain injury.<sup>12-13</sup> In addition to these risks, craniosynostosis is also associated with alterations in craniofacial growth including mid-facial hypoplasia, abnormalities in dental alignment, orbital deformation, and other characteristics such as hearing loss or intellectual disability<sup>12-13</sup>. Generally, craniosynostosis is treated with cranioplasty in order to restore the normal shape of the head and relieve increased intracranial pressure. Due to its complexity, such procedures hold risk to significant morbidity.<sup>14-15</sup> To date, craniosynostosis remains a significant medical and dental health issue where there are no pharmacological treatments, nor earlier interventions to prevent suture fusion.

More recently, it has become evident that abnormal suture fusion may be caused by an interaction of a number of factors. One of the least understood factors that may be involved in this process is the role of mechanical forces in expansion of the calvaria, brain growth and its effect in maintaining suture patency, which is the focus of this review. The second factor is the intrinsic property of the suture, which has been reviewed elsewhere.<sup>16</sup> Finally, external forces acting on the calvaria, especially during fetal life, might also contribute to the onset of craniosynostosis, especially in non-syndromic cases of SSC.

Recent epidemiological evidence consistent with contributions from fetal head constraint showed positive associations of craniosynostosis with twin pregnancies, multiple pregnancies, and high birth weight.<sup>17</sup> Previously, it was shown that compressive strain can increase osteogenesis at the suture.<sup>18</sup> Furthermore, *in vivo* mouse models of head constraint have been shown to induce craniosynostosis.<sup>19</sup> Recent work has demonstrated that the activity of an anabolic signaling factor as insulin growth factor 1 (IGF-1) affects human derived SSC osteoblast contractility and migration, providing valuable insight for phenotype-genotype correlation in SSC osteoblasts.<sup>20</sup> It is evident therefore, that there exists a complex interplay between suture patency, genetics, signaling pathways, and mechanotransduction processes which may be related to the pathogenesis of craniosynostosis. The purpose of this review is to provide an overview of the underlying developmental biomechanics of suture formation, followed by a discussion of the recent molecular genetics of craniosynostosis, supporting a role of cell mechanics in this disease; and finally, a consideration of future ideas and directions.

## **Developmental biomechanics of suture formation**

### **Calvarial bone formation and suture fusion**

The human calvaria is formed through intramembranous ossification which occurs within a condensed region of mesenchymal stem cells. Its formation is in contrast to the formation of endochondral bone such as long bones and the skull base, which advance initially through a stage of chondrogenesis before proceeding to osteogenesis.<sup>21</sup> The development of the human calvaria commences during the eighth week of gestation.<sup>9, 22-24</sup> At the initial site of

ossification (the ossification locus), mesenchymal osteoprogenitor cells differentiate into osteoblasts, secrete extracellular matrix (ECM) proteins, and initiate mineralization.<sup>9</sup> Osteogenesis in the human calvaria requires the differentiation of mature osteoblasts from undifferentiated proliferating mesenchymal osteoprogenitor cells. Growth of the calvaria is radially outward from the locus of osteogenesis, eventually approximating the bones to form the suture (Fig. 2).<sup>23</sup> The leading edges of these osteogenic fronts contain proliferative osteoprogenitor cells.<sup>25-26</sup>

Suture formation occurs through the progression of the two confronting osteogenic fronts. Therefore, a suture is a composite structure comprised of a region of unossified tissue between two calvaria bordered by osteogenic fronts and the overlying dura mater (the tough membrane that adheres to the inner surface of the cranial vault and separates it from the brain). Mature cranial sutures can withstand deformation in both tension and compression.<sup>27</sup> Their primary function is to enable the growth of the skull in coordination with the rapid expansion of the calvaria during brain growth.<sup>28</sup> Furthermore, the intracranial pressure of the brain growth produces tensile strains, which may either act directly on the suture or indirectly through mechanotransduction via the dura mater.<sup>18</sup> In addition, sutures allow deformation of the skull during birth, absorb cyclic mechanical loading during mastication and locomotion, and act as shock absorbers against external forces.<sup>29</sup>

Although cranial sutures start off as simple lines of separation between developing bones, they become increasingly interdigitated with age.<sup>30</sup> Mathematically, these meandering patterns have been previously described in terms of fractal geometry, with the fractal dimension increasing with age.<sup>30</sup> Furthermore, there have been analytical attempts to account for this behavior by employing reaction-diffusion models which incorporate diffusible factors, positive and negative feedback loops, mechanical strain, and time-dependent processes.<sup>27, 30</sup> Moreover, a recent study has suggested that the fractal nature of these meandering patterns may be due to the stochastic nature of craniosynostosis.<sup>31</sup> Therefore, these theoretical findings demonstrate that suture growth is likely to incorporate the interplay of cellular signaling pathways that are responsive to mechanical strain.

The major calvarial sutures fuse at different times during normal development. In humans, the metopic suture (between the frontal bones) fuses at three to nine months of age<sup>1</sup> while the others (coronal, sagittal and lambdoid) fuse in the third decade of life.<sup>2</sup> Although some investigation into the molecular processes of suture fusion has been conducted in humans, much of our understanding is drawn from animal models. Immunohistochemical studies of the coronal sutures in rats (between the frontal and parietal bones) reveal high concentrations of alkaline phosphatase at the osteogenic fronts on fetal day 19 (F19) prior to apposition of their osteogenic fronts.<sup>32</sup> At the time of apposition (F21), alkaline phosphatase activity decreases, demonstrating reduced bone formation, perhaps representing a mechanism serving to prevent synostosis. In contrast, *in vitro* studies of osteoblasts derived from prematurely fused human sutures demonstrate an increase in alkaline phosphatase production and osteocalcin expression, suggesting that osteogenic differentiation occurs in surplus of that present in normal sutures.<sup>33-34</sup> These studies suggest that regulation of bone differentiation and matrix production plays an important role in suture patency.

Apoptosis (programmed cell death) has also been widely explored during suture fusion in rodents. Through histologic evaluation of fetal and newborn mice, apoptotic bodies have been observed at the osteogenic front during bone apposition.<sup>35-38</sup> These findings suggest that the process of apoptosis may attenuate osteogenesis at the suture boundary, thereby preventing abnormal fusion. It appears therefore, that a harmonious balance of brain growth, <sup>2</sup> inhibited mineralization of the intrasutural mesenchyme,<sup>39</sup> growth of the calvarial bones at the osteogenic front,<sup>25-26</sup> and programmed cell death<sup>36</sup> maintains suture patency during skull growth. When the persistence of the unossified intrasutural mesenchyme of the calvaria is prematurely abolished or there exists an overgrowth of the osteogenic fronts, the neighboring calvaria begin to fuse, which then results into craniosynostosis. Craniosynostosis is therefore an etiologically heterogeneous condition with known genetic and presumed epigenetic causes.

### Strain and suture patency

For over two decades, it has been suggested that *in utero* head constraint is associated with an increased incidence of premature calvarial suture fusion.<sup>2, 40-43</sup> Previous studies have shown that early descent into the pelvis, primiparity and other forms of fetal constraint have been implicated as causing both metopic and sagittal synostosis.<sup>17, 40, 44-45</sup> The proposed pathogenesis in these cases is that compression of the calvaria leads to reduced strain, at the osteogenic fronts and ultimately early suture fusion. These clinical examples are consistent with animal models of fetal constraint wherein cervical ligatures were used to prolong gestation resulting in craniosynostosis.<sup>46</sup> In addition to *in utero* constraint, reduced brain growth resulting in severe microcephaly is well known to be associated with premature fusion of the calvaria.<sup>2, 43</sup> Like *in utero* constraint, reduced brain growth has the effect of reducing quasi-static tensile strain across the calvarial sutures (Fig. 3). Similarly, treatment of hydrocephalus with ventriculoperitoneal shunting can lead to premature fusion of otherwise normal sutures.<sup>47</sup> Shunting decompresses the enlarged brain resulting in a reduction of the tensile strain experienced by the suture microenvironment. Although the exact pathogenesis of synostosis in these examples remains unclear, they serve to illustrate a possible relationship between quasi-static tensile strain and homeostasis of the suture microenvironment. These observations suggest that inhibition of normal suture strain associated with brain growth can result in premature suture fusion. These clinical and experimental models are in apparent disagreement with well-established animal data, which suggest that even in the absence of normal suture strain, the dura mater has an intrinsic ability to maintain suture patency.<sup>48-50</sup> Moreover, differential expression of transforming growth factors beta 1, beta 2, and beta 3 (TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3) and the type I TGF- $\beta$ 3 receptor in the suture microenvironment has been associated in the regulation of suture fusion through its control of proliferation and apoptosis.<sup>51-55</sup> This apparent inconsistency emphasizes the importance of improving our understanding of the role of strain in suture patency and calvarial development.

Much of our knowledge of suture biology comes from studies of facial sutures, rather than cranial sutures. For example, both oscillatory and continuous strains on the facial sutures are known to stimulate suture growth.<sup>56</sup> Tensile and compressive oscillatory strain of 1500 microstrain ( $\mu\epsilon$ ) have been demonstrated to increase suture growth, where enhanced

expression of ECM and mass of both osteoblast and fibroblast cells were observed<sup>56-57</sup> Fibroblast and osteoblast proliferation in response to mechanical strain is well recognized; however, there has been little work done in designing experimental models that mimic normal suture biology. As little as 500  $\mu\text{e}$  of oscillatory strain has been found to induce premaxillomaxillary suture osteogenesis.<sup>58</sup> Therefore, the oscillatory strain experienced in facial sutures induces suture growth with both compressive and tensile strain having an anabolic effect on the suture microenvironment.<sup>56</sup>

### Mechanical loading on sutures

The earliest studies of cranial suture biology sought to relate the morphology of a suture to its mechanical microenvironment.<sup>59-61</sup> More specifically, when sutures were transplanted into regions which did not experience mechanical loads, the new microenvironment was found to alter suture morphology.<sup>62</sup> Furthermore, previous studies investigating the relationship between mechanical loadings as a result of mastication observed an upregulation of sutural bone growth.<sup>63</sup> This study found that increased masticatory muscle mass and bite force would increase sagittal suture complexity in myostatin-knockout mice. Moreover, we have identified loss of nasofrontal suture complexity in the midface deficient *FGFR2* mutant model of Apert syndrome, where loss of normal incisor occlusion occurs (unpublished data: Fig. 4).<sup>64</sup> This suggests that the tissue surrounding the suture adapted to a particular mechanical loading regime achieved by differential bone growth at the suture<sup>63</sup>. In another study applying tensile testing on sagittal sutures from postnatal rats aged two to 60 days, increased sutural thickness and stiffness per length was also observed.<sup>65</sup> Interestingly, these aforementioned properties were found to be age dependent, suggesting that during development, the rat sagittal suture changes significantly after exposure to *in vivo* quasi-static tensile strain due to intracranial pressure.<sup>65</sup>

The craniofacial skeleton comprises intramembranous bones which exhibit growth following calvarial expansion<sup>66</sup> and mastication.<sup>67</sup> Change in masticatory forces has been shown to induce craniosynostosis, wherein osteopetrotic mice displayed premature fusion of the sagittal suture,<sup>68</sup> while rats on much softer diets exhibited internasal synostosis.<sup>69</sup> Expansion of the brain changes cranial growth, where absence of brain tensile forces observed in fetuses with microcephaly developed craniosynostosis.<sup>70-72</sup> Conversely, the presence of external pathological forces can also have a major impact on skull development. For example, primiparity,<sup>17</sup> multiple births,<sup>73</sup> low pelvic station,<sup>41</sup> and late-term pregnancies<sup>74</sup> have all been associated with the development of SSC.

### Modeling sutures and cranial growth

Novel developments in computer modeling are frequently employed when conducting detailed investigations into the effect of cranial vault loading. Analysis incorporating techniques in finite element analysis (FEA) and multibody dynamics analysis (MDA) have in the past, been used to examine such phenomena as musculoskeletal force generation, translations of bone plates and subsequent stress/strain distributions within the cranial vault. These techniques have previously been used to mechanically model biological systems, where understanding the force distribution of mastication in humans and other primates is under scrutiny.<sup>75-77</sup> It is essential however, that when developing a suitable computational

analogue, the specific material properties unique to the structure must be incorporated in the model to accurately predict the mechanical properties of the environment. This is more challenging in consideration of materials such as bone which are by their nature considered an anisotropic material where their properties vary not only between individuals but also throughout each specimen. Previously, one group performed a sensitivity study into the effects of using isotropic and anisotropic material properties in a *Macaca fascicularis* cranium.<sup>75</sup> The group observed that while more detailed models were more accurate when compared to their experimental strain counterparts, investigations defined by solely isotropic material produced comparable results. Congruently, one other study also validated these conclusions,<sup>77</sup> where they showed positive correlation to experimental data using isotropic material properties. These findings therefore indicate that investigations into complex three-dimensional structures applying isotropic materials yield highly successful results.<sup>78-80</sup>

FEA is generally used when addressing questions concerning the impact of patent sutures on skull stresses/strains. To produce accurate measurements of strains experimentally, strain gauges are fixed to the surfaces of bones.<sup>81-83</sup> Generally, localized strains at these fixed locations are easily obtainable. However, to infer global strain measure over the entire cranial vault or the patent suture is a more challenging task. Therefore, FEA can be used to predict the stress/strain distributions for the entire structure.<sup>84-86</sup> A previous study assessing local and global strains carried out on a lizard skull revealed.<sup>87</sup> The first was strain modification was found to be greater in global patent sutures when compared to fused sutures. The second being that strain found to decrease in some areas of the skull was seen to increase in others.<sup>87</sup> In contrast, another study however suggested that patent sutures had little effect on skull strains in primates;<sup>88</sup> but appeared more important in animals with more patent sutures or a greater suture to bone volume as reptiles.<sup>87, 89</sup> These studies when combined with experimental data provide important information describing suture form and function.

In order to gain a wider insight in the impact of patent and fused sutures on load transfer within the cranial vault, more comprehensive analyses are needed. One way of doing this is to combine both MDA and FEA. These two techniques used in the reptile *Sphenodon* to predict separate biting loading regimen subsequently analyzed the structural performance of the skull under such regimens.<sup>90</sup> Subjecting the skull to many different loading regimens is important because cranial vault deformation varies greatly depending on the loading position and magnitude.<sup>90</sup> These findings demonstrated for the first time that patent sutures may in fact help in reducing the number of cranial areas with low-level strain throughout the reptile skull, leading to a more consistent method in predicting strain levels during mastication<sup>90</sup> Such findings are of clinical relevance due to their implications in respect to the remodeling and growth of bone in both juvenile and adult skulls, ensuring the normal trajectory of bone development.

## Molecular Genetics of Craniosynostosis

Our understanding of the genetic components of human craniosynostosis are modest at best. Presently, there are 57 genes known to be causally related to craniosynostosis, which have been reviewed in great detail elsewhere.<sup>16</sup> Herein, this review will briefly describe the

genetic pathophysiology linked to some of the more common forms of craniosynostosis. In humans, syndromic synostosis (hereditary) is caused by mutations in the genes for fibroblast growth factor receptors (*FGFR*) and twist-related protein 1 (*TWIST1*). The following syndromes - Apert, Crouzon, Pfeiffer, and Jackson Weiss - are all due to specific gain of function mutations of *FGFR2* in either the second inter-loop domain (Apert) or third immunoglobulin-like domain (Crouzon, Pfeiffer, Jackson Weiss).<sup>91-96</sup> This is similar to the gain of function mutations in the second inter-loop domains of *FGFR1* and *FGFR3*, which result in Pfeiffer and “Muenke Type” craniosynostosis, respectively.<sup>97-100</sup> Other less common mutations of *FGFR2* and *FGFR3* have been associated with syndromic craniosynostosis.<sup>101-102</sup> A single point mutation in *MSX2* is believed to increase transcriptional activity, resulting in “Boston-type” craniosynostosis.<sup>103</sup> To date, the only other transcription factor found to be associated with craniosynostosis is the basic helix-loop-helix (bHLH) protein TWIST1. Several loss of function mutations in DNA binding and loop domains of TWIST1 have been found to be responsible for Saethre-Chotzen syndrome.<sup>104-109</sup>

### Saethre-Chotzen syndrome

Saethre-Chotzen syndrome (SCS, acrocephalo-syndactyly type III) is one of the more common forms of syndromic craniosynostosis.<sup>109-110</sup> Patients with SCS typically present premature fusion of one or more sutures of the calvaria, brachycephaly, facial asymmetry, a low frontal hairline, ptosis, maxillary hypoplasia, and small ears with a prominent superior crus.<sup>111</sup> Although any sutures in the calvaria can undergo premature fusion in SCS, coronal sutures is the most common. Associated limb anomalies may include brachydactyly or cutaneous syndactyly of the second and third digits of the upper extremities. As SCS is an autosomal dominant trait, it is accepted that the SCS phenotype is caused by a functional haploinsufficiency of *TWIST1*.<sup>112</sup> This is further supported by animal models such as the heterozygous *TWIST1* mutant mouse (*TWIST1<sup>tm1Bhr</sup>*) that reveals premature coronal suture fusion mimicking that of the human SCS phenotype.<sup>105, 113-114</sup>

### *TWIST1*<sup>+/-</sup> mutant and haploinsufficiency

High-throughput sequence analysis has identified many intragenic *TWIST1* mutations in patients with SCS.<sup>109, 112</sup> Nonsense mutations inhibiting translation of the DNA and the HLH domains have been identified from the 5' end of the coding sequence to the end of the HLH motif. Though missense mutations cluster within the functional domains, specific mutational loci have yet to be identified. In recent studies, the functional effects of *TWIST1* mutations have also been examined. In these studies, nonsense mutations were found to increase the synthesis of truncated proteins that rapidly degraded, leading to functional haploinsufficiency.<sup>115-116</sup> Missense mutations involving helical domains were found in contrast, to result in a loss of heterodimer formation, which subsequently altered nuclear translocation.<sup>115-116</sup> Moreover, in-frame insertion or missense mutations within the loop domain were found to alter dimer formation while these mutations in the basic domain altered DNA binding. Taken together, these findings suggest that both protein degradation and altered subcellular localization, may in part, account for the loss of functional *TWIST1* protein (functional haploinsufficiency) in SCS patients.



## **TWIST1<sup>+/-</sup> mutant and cell specifications**

*TWIST1* and other bHLH transcription factors play an important role in specifying and maintaining cell identity.<sup>117-118</sup> *TWIST1* was initially characterized in *Drosophila* as being necessary during gastrulation in the establishment of the mesodermal germ layer and embryos with *TWIST1* mutations failing to develop mesoderm.<sup>119</sup> In *Drosophila*, *TWIST1* expression persists at high levels in the mesoderm until its differentiation into the somatopleura and splanchnopleura when its expression diminishes.<sup>120</sup> During mouse development, *TWIST1* is expressed in the neural crest cells that populate the cephalic region and branchial arches, which differentiate into connective tissue, muscle, cartilage, and bone.<sup>121</sup> Migratory populations of cephalic neural crest cells are the origin of the membranous bones of the skull and its intervening sutures, overlying dermis, and underlying dura mater,<sup>122-125</sup> which infers a crucial role in early calvarial development. *TWIST1* has also been shown previously to inhibit differentiation of multiple cell lineages, including muscle<sup>126-129</sup> and bone.<sup>130-131</sup> Taken together, these findings propose that *TWIST1* may function to maintain cells in a less differentiated state during craniofacial development. In support of this hypothesis, recent studies suggests that *TWIST1* is indeed necessary for normal osteocalcin expression in human osteoblasts,<sup>132</sup> perhaps acting through a RUNX2 dependent pathway.<sup>133</sup> While the precise function of osteocalcin still remains unclear, its secretion by osteoblasts during differentiation suggests a likely role in matrix mineralization. Furthermore, an additional investigation into the role of *TWIST1* in osteoblast biology observed its binding to the promoter of periostin (*OSF2*) by which upregulating its expression.<sup>134</sup> As a secreted ligand of  $\alpha_5\beta_3$  and  $\alpha_5\beta_5$  integrins, periostin is therefore believed to play a role in cellular adhesion. Together, these recent discoveries suggest that *TWIST1* might serve to regulate both matrix mineralization and cellular adhesion. While information from disease-specific mutations and their genetic/biochemical characteristics provide a clear benefit to understanding craniosynostosis, it is when we view genetics in relation to cell mechanics (i.e. signaling pathways and mechanotransduction processes) that we gain a more detailed understanding of the external and internal factors influencing this disease.

## **Role of Cell Mechanics**

### **Mechanical properties of sutures**

Characterizing the mechanical properties (elastic modulus) of bone is an important step in the understanding of craniofacial development. By invoking tools in tensile testing or three-point bending, the elastic modulus of calvarial bones and sutures in normal skulls has been able to be quantified.<sup>135-138</sup> More recently however, nanoindentation has been used as an alternative method in examining tissue samples less than 0.1 mm in size, making it an ideal method for measuring the properties of cranial bone, and even sutures, in rodents and other small animals.<sup>139</sup> When using a Crouzon mouse model (*Fgfr2<sup>C342Y/+</sup>*), a difference in the elastic modulus of the frontal bones between wild type and *Fgfr2<sup>C342Y/+</sup>* mutant mice was observed at the early stages of post-natal development.<sup>139</sup> In contrast however, this study also demonstrated that the elastic modulus of the parietal bones and their sutures were comparatively more similar between these two groups.<sup>139</sup> It is therefore likely that such

variations in the mechanical properties of the calvaria may result from different patterns of strain as a consequence of suture fusion.

### Traction forces of sutures

*In vivo*, both mechanical forces and properties of the ECM influence cellular physiology. The translation of physical information into a cellular response is now believed to be a critical component in many biological pathways.<sup>140-142</sup> Extracellular nanoscale forces have been shown to influence numerous signaling pathways both *in vivo* and *in vitro*.<sup>143-147</sup> Such nanoscale forces can arise through stretch or compression of the microenvironment, fluid shear stress or localized forces occurring at focal adhesion sites, which have been shown to result in cytoskeletal remodeling, changes in cellular orientation and alignment, alterations in gene regulation, and the determination of cell fate.<sup>143-147</sup> Understanding the role of mechanotransduction is therefore quintessential in expanding our understanding of how physical forces are generated and transmitted through living cells.

Mechanical forces imposed on osteoblasts are a well-known inductor of osteogenic markers. In particular, cyclic strain has been shown to induce the production of these osteogenic markers, including osteocalcin, osteopontin, alkaline phosphatase, and type I collagen.<sup>148</sup> Osteoblasts differentiated from mesenchymal stem cells, have been shown to increase in response to mechanical factors like cell shape;<sup>149-150</sup> substrate stiffness,<sup>151</sup> and applied strain.<sup>152</sup> Moreover, when a strain regimen was applied *in vivo* to the tibia of transgenic mice selectively overexpressing IGF-1, a five-fold increase in bone formation as compared to wild-type mice was observed.<sup>153</sup> This suggests therefore that traction forces are an essential factor for the mechanotransduction of cell shape, substrate stiffness, and applied strain shape.<sup>140, 154-156</sup>

The causative factors leading to craniosynostosis is of great interest due to relatively high frequency of SSC when compared to other birth defects, and its far-reaching clinical burden. Previously, the family of TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 were found to play an important role in suture morphogenesis by regulating and maintaining suture patency and calvarial bone growth.<sup>55</sup> Furthermore, cyclical loading on murine calvaria was also found to induce suture fusion and show upregulation of alkaline phosphatase, a non-specific bone marker of osteoblastic activity.<sup>157</sup> Recently, IGF-1 expression has been correlated to SSC osteoblast contractility and migration, where increased expression levels led to larger traction forces and reduced migration speeds in diseased osteoblasts.<sup>20</sup> Furthermore, in our previous study we identified a number of genes (*FGFR3*, *TGFBR1*, *TGFB3*, *WNT3*, *WNT5B*, *WNT16*, *CTBP2*, *DTX4*, *DVL2* and *ITGB1*) whose expression was correlated with contractility and/or migration in SSC osteoblasts, all of which have been previously implicated in bone development.<sup>158-161</sup> These findings suggest that there exists interplay between the IGF-1 pathway and the regulation of the aforementioned genes, which may act in an integrative manner leading to the development of SSC.

### Migration of osteoblasts derived from sutures

Previous studies have implicated IGF-1 signaling in mediating focal adhesion formation and cell migration.<sup>162-163</sup> Indeed recent transcriptomic studies reveal an upregulation in IGF-1

expression in calvarial osteoblasts derived from patients with SSC, which was accompanied by a further positive correlation to an increase of ECM-mediated focal adhesion proteins.<sup>164</sup> This anabolic signaling factor appears to promote the association of the IGF-1 receptor to focal adhesion proteins, leading to increased cellular migration and invasion.<sup>162</sup> In our previous study, we found that not only did IGF-1 expression correlate to cell contractility, but also to cell migration.<sup>20</sup> Furthermore, a number of factors that have been found to influence skeletal development have been correlated to migration in osteoblasts derived from SSC patients.<sup>20</sup> Previous work has identified *RUNX2* as an osteogenic marker that induces osteoblast and chondrocyte differentiation by enhancing their migration through coupling with PI3K-Akt signaling.<sup>165</sup> Furthermore, Akt signaling is activated by IGF-1 through the PI3K pathway and therefore, IGF-1 plays an important role in *RUNX2*-dependent osteoblastic differentiation in MC3T3-E1 cells.<sup>165</sup>

Healthy patterned growth of the calvarium is dependent on a tightly regulated program of cell proliferation, differentiation, and migration. Investigating the contributions of these processes is crucial in understanding how the calvarial pattern is established in cranial growth and how developmental pathologies like craniosynostosis arise. Osteoblast migration has previously been demonstrated to be an important factor in the patterned growth of calvarial bones, where its impairment was found to lead to craniosynostosis in *TWIST1* and *EphA4* mutant mice.<sup>166</sup> These findings were consistent with previous work,<sup>167</sup> supporting the notion that cell migration is a significant morphogenetic force in the patterned growth of the skull vault. Therefore, it appears that the migration of osteoprogenitor cells from osteogenic front may contribute to the apical expansion of calvarial bones.

More precise techniques in identifying the progenitor cell populations which comprise the suture, as well as understanding the mechanotransduction processes that guide their migration and differentiation, will help further advance our understating of the mechanisms that underlie the patterned growth of the skull as well as the pathophysiology of craniosynostosis.

## Future Ideas and Directions

One of the most exciting areas of craniofacial research is investigating the integrative role of mechanical forces, signal transduction, and gene regulation in the onset of craniosynostosis. By employing mutant mouse models, we can identify candidate genes affected as result of changes in mechanical strain mimicking that of an expanding brain. Furthermore, developing an *in vitro* model that allows us to study the transduction of mechanical signal into biochemical changes will advance our understanding of the role of strain induced by brain growth and other mechanical forces (mastication or pulsatile blood flow) in normal calvarial development and suture development. Given the importance of environmental factors in craniosynostosis, including frequent asymmetry in suture fusion, the contribution of genetic and epigenetic influences are all crucial areas of interest that should be explored further in hope to yield diagnostic treatments on a case-by-case basis.

While the precise mechanisms preceding craniosynostosis are complicated and unclear at present, current advances in the field suggest it is a bridge between suture biology and cell

mechanics which may affect the normal onset of suture fusion. Further investigations which raise disease-specific cell mechanics to their genetic counterparts are therefore necessary in order to provide deeper insights into the mechanisms regulating the development of craniosynostosis and other developmental disorders.

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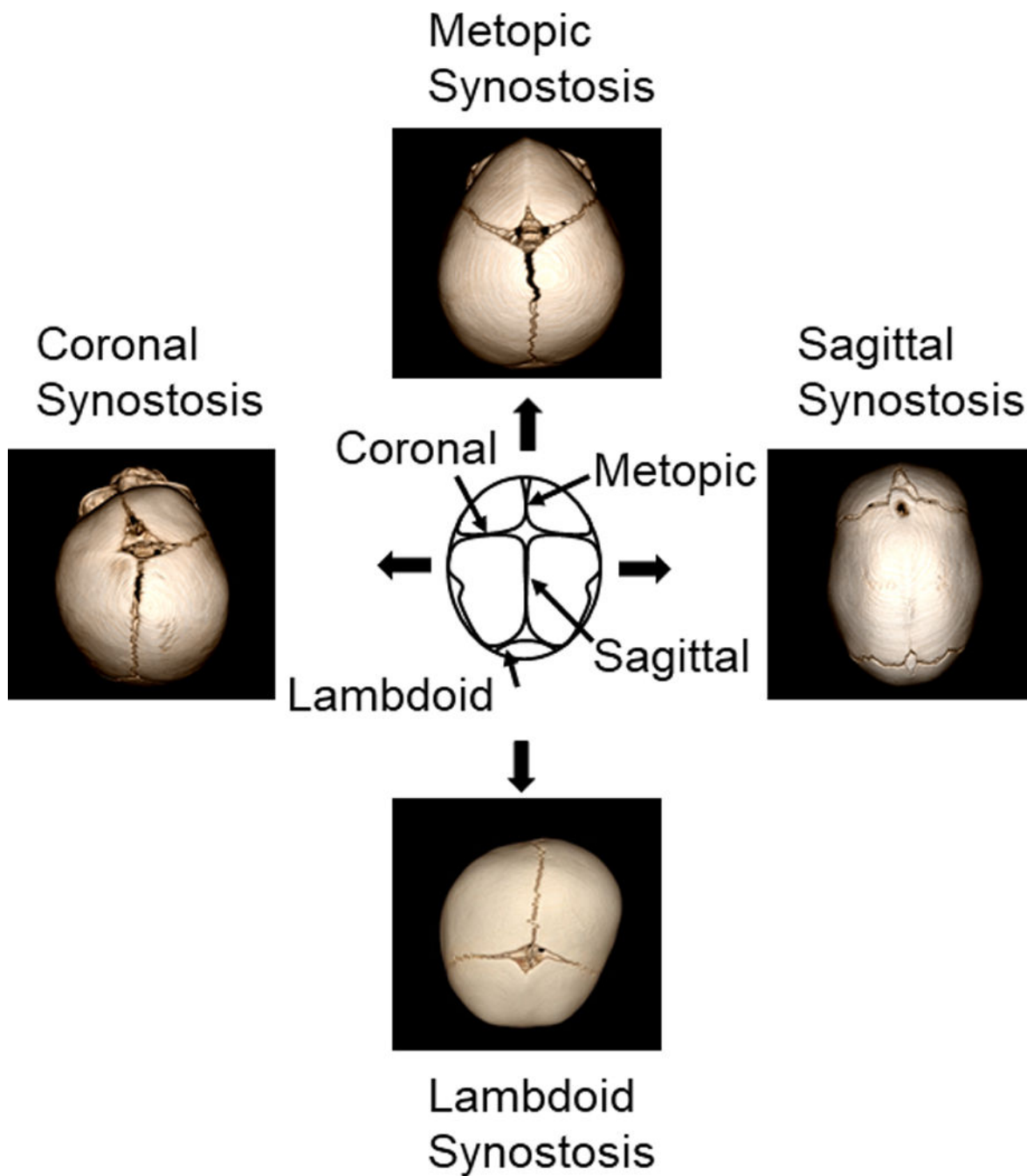
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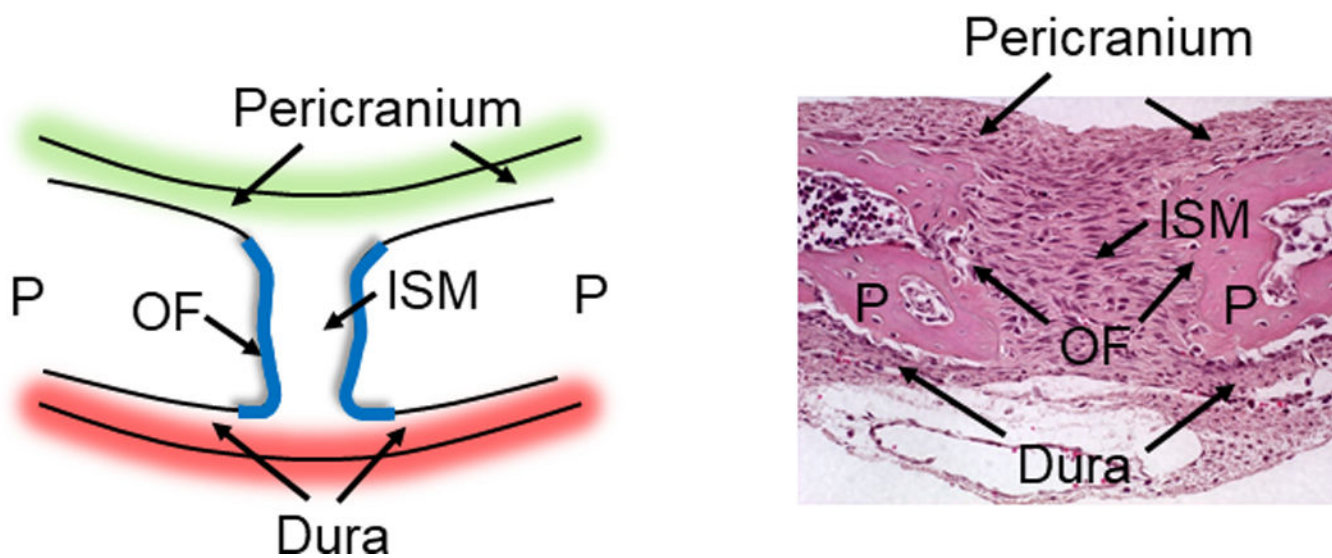
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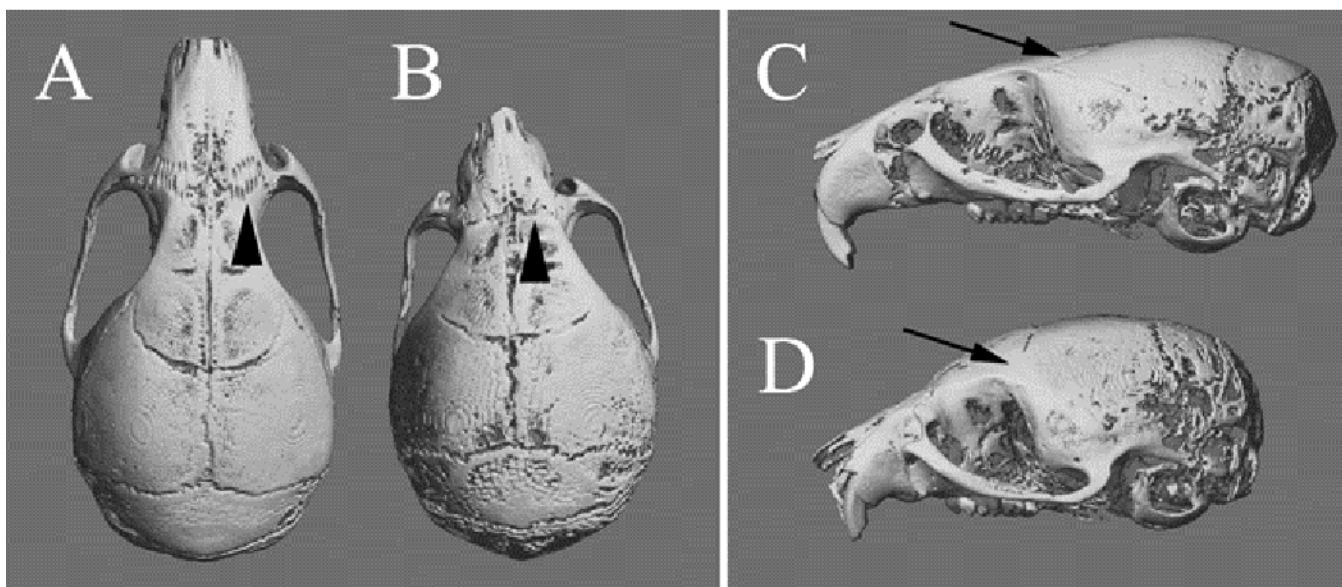
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**Figure 1.** Types of craniosynostosis. Center: schematic representation of the top view of a normal cranium with all identified sutures (metopic, coronal, sagittal and lambdoid). To either side of the normal presentation of the skull, CT scans showing skull shapes with coronal (left) and sagittal (right) synostosis. Finally, metopic synostosis is shown at the top, while lambdoid synostosis is shown at the bottom.



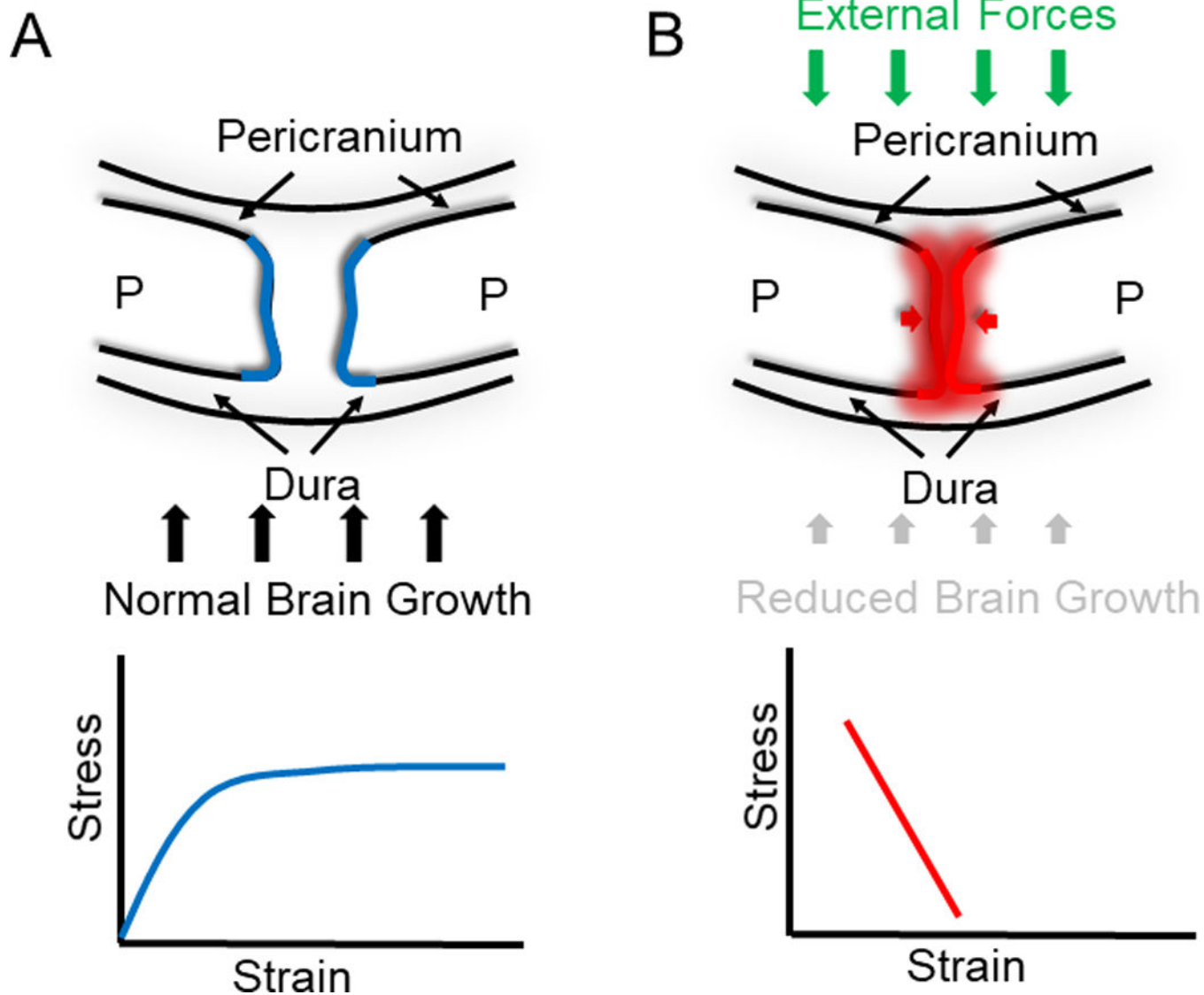
**Figure 2.** Schematic and histological presentation of the sagittal suture. (A) A schematic and (B) histological appearance of the sagittal suture showing the paired parietal bones (P) and the relative positions of the osteogenic fronts (OF), intrasutural mesenchyme (ISM), the pericranium and the dura mater. The leading edges of these osteogenic fronts contain proliferative osteoprogenitor cells and the sagittal suture is a composite structure that consists of the osteogenic fronts and the intrasutural mesenchyme.



**Figure 3.**

Strains and suture patency. (A) Cross-sectional depiction of the sagittal suture depicting the paired parietal bones (P). The dura mater is the tough membrane that adheres to the inner surface of the cranial vault, which separates it from the brain. The pericranium is located apically. The growth of the cranial vault is regulated by a harmonious balance of proliferating and differentiating cells occurring within the suture (blue). This growth takes place in synchrony with an expanding brain (black arrows). Therefore, we can describe this behavior by plotting the effect of normal expanding brain and its effect on the suture as a stress-strain curve. (B) Conversely, in craniosynostosis, this balance is disturbed by external forces as *in utero* constraints during pregnancy (green arrows), poor brain expansion (vertical black arrows) and/or abnormal signal transduction within the suture (red shade). Generally, reduced brain growth has the effect of reducing quasi-static tensile strain across the calvarial suture as shown in the stress-strain graph.





**Figure 4.** MicroCT images were obtained of the nasofrontal suture of both control and Apert mice carrying the *Fgfr2*(S252W/+) mutation. (A) The control mouse is showing the interdigitated suture (black arrow). (B) The Apert mouse does not have normal occlusion or maxillary and mandibular incisors; therefore, the nasofrontal suture is not strained and loses normal interdigitation (black arrow).