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REVIEW

Clinical applications of molecular basis for Craniosynostosis. A narrative review.

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Abstract: Cranial sutures are specialized structures composed of the sutural mesenchyme, the overlying scalp, the dura and osteogenic fronts. Each one of these structures express important proteins for osteogenic maturation, membranous ossification of skull bones, and homeostasis of cranial sutures in a differential, spatial and temporal manner. These proteins include fibroblast growth factor (FGF) and its receptors (FGFR), the transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs), as well as transcription factors TWIST and MSX2, among others. The alteration in the expression of one or more of these proteins causes multiple pathological conditions; one of them is the premature closure of one or more cranial sutures, known as craniosynostosis. This malformation is commonly treated with surgery. However, advances in the fields of molecular and cellular biology have allowed to conduct research on some proteins involved in the development of craniosynostosis. The results of these studies can lead to future preventive therapeutic strategies that may be used as a complement to the surgical treatment of craniosynostosis. Possible strategies include the use of specific drugs that can regulate the expression and activation of FGF signaling pathways, TGF-β or BMPs, to prevent or avoid craniosynostosis or re-synostosis after a surgery.

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INTRODUCTION.

Craniosynostosis is a malformation characterized by the premature fusion of the cranial sutures[1]. The early closure of these sutures causes an abnormal shape of the skull due to a displacement of the brain as a compensatory mechanism and is frequently associated with an abnormal brain development.

The causes of this malformation are many, including: genetic, environmental, teratogenic, intrauterine factors, and hyperthyroidism, among others[1,2]; this has hindered the understanding of the development of this disease and limited its study. Much of the published research on craniosynostosis has been mainly focused on clinical descriptions.

Craniofacial malformations are some of the most common diseases in children, these may endanger the child's life or have unrecoverable consequences such as intellectual deficit, decreasing the quality of life of the patient due to the multiple and complex surgeries he/she has to undergo in order to get a more normal and functional facial appearance[3].

In an effort to bring together existing information from clinical studies and basic science research on the molecular, embryological, cellular, biochemical and genetic mechanisms that are associated with the development of craniosynostosis, we have done this narrative

review with a special interest in identifying therapeutic targets that may lead to the development of future research on novel and functional molecular treatments.

CLINICAL ASPECTS OF CRANIOSYNOSTOSIS.

Craniosynostosis is classified into two main groups: syndromic and non-syndromic. These names are assigned considering their characteristics in relation to the suture or sutures that have been affected. Knowledge of the characteristics of each group results in improved diagnosis and a better treatment of each specific type of craniosynostosis[3].

Globally, it is estimated that craniosynostosis occurs in one of every 2000 or 2500 live births, and no ethnic, racial or sex preferences have been reported. Approximately 85% of the reported cases are non-syndromic[4].

Clinical features

The total or partial premature fusion of one or more sutures, without the presence of malformations in other organs, is called simply craniosynostosis. Table 1 summarizes the types of simple craniosynostosis, the suture(s) affected and some of their clinical features.

Over 180 syndromes[5] have been associated with syndromic craniosynostosis. Some of the best-known syndromes are Apert, Carpenter, Pfeiffer and Saethre-Chotzen. They are also named as Acrocephalosyndactylia, as they produce alterations in skull, face, hands and feet[2,8].

These 180 syndromes have craniosynostosis as their main clinical feature, as well as other malformations, which distinguish them from each other. For example, Muenke syndrome is associated with hearing loss[8], Saethre-Chotzen with morphological alterations in the outer ear[2], *cutis verticis gyrata* and *acanthosis nigricans* as signs of Beare–Stevenson *cutis gyrate*[5] syndrome.

Anomalies of maxillofacial complex in syndromic craniosynostosis

Patients with complex craniosynostosis have both oral health problems and facial growth problems[9]. Because of this, children with craniosynostosis have a great need for dental care because of malformed teeth, missing teeth, delayed tooth eruption, dental crowding and susceptibility to dental caries and periodontal disease[9,10]. Malformations of the four most common types of syndromic craniosynostosis that affect the maxillofacial complex are summarized in Table 2.

Diagnosis

Diagnosis can be divided into prenatal and preoperative; the first is based on the exploration of the limbs and craniofacial deformities by means of ultrasound[1] or by means of genetic testing through amniocentesis, with the aim of finding common deletions[16,17] or *Fgfr* mutations, or other

Table 1. Suture(s) affected in simple craniosynostosis and clinical characteristics.

Name	Affected suture	Clinical Characteristics
Scaphocephaly	Sagittal[5]	Impossibility of transverse cranial growth. The result is an elongated anteroposterior head reminiscent of an overturned boat.
Brachycephaly	Both coronal sutures[5,6]	There is severe growth restriction of the sphenoid bone, resulting in a shortening of the skull in anteroposterior direction and a large, flat forehead.
Trigonocephaly	Metopic[3]	The development of the head is triangle form, the forehead is narrow and prominent in its midline showing hypotelorism.
Anterior plagiocephaly	Coronal[7]	Asymmetrical skull with oblique aspect. Major unilateral anteroposterior growth.
Posterior plagiocephaly	Lambdoidal[7]	Ipsilateral cranial flattening of the occipital region with a compensatory lump on the mastoid bone.
Oxycephaly Turricephaly	Coronal and sagittal sutures[7] Multiple sutures[7]	Pointy skull at the level of the great fontanelle. Cloverleaf skull.

Table 2. Complex maxillofacial abnormalities occurring in patients with syndromic craniosynostosis.

Syndrome	Manifestations
Apert syndrome	Underdevelopment of midface in an anteroposterior manner, anterior open bite, retained deciduous teeth, late eruption of permanent teeth, ectopic dental eruption, impacted teeth, supernumerary teeth, congenitally missing teeth, posterior lingual cross-bite by inadequate mediolateral growth of maxilla, narrow arched palate, decreased volume of the nasopharynx that predisposes to chronic oral breathing, gingival hypertrophy[11,12].
Saethre-Chotzen syndrome	Midface hypoplasia, anterior open bite, posterior lingual cross-bite, pseudomandibular prognathism, wide mesiodistal dimension of crown, thin teeth roots[13].
Crouzon syndrome	Poor growth of the maxilla with normal jaw; pseudomandibular prognathism, anterior cross-bite, class III molar, dental ectopic eruption producing dental crowding, tooth rotation, hypodontia, delayed eruption and unerupted teeth in permanent and deciduous dentition[14].
Pfeiffer syndrome	Dental crowding, natal teeth, supernumerary teeth. The midface is hypoplastic with frontal bossing, and anterior open bite[15].

genes associated with complex craniosynostosis[17-19].

On the other hand, surgeons perform a preoperative diagnosis that helps them plan surgical treatment in a better way. MRIs, CT scans, 3D-CT angiography, cephalometric exams and even models of dental studies[9,20-22] are commonly used.

MOLECULES INVOLVED IN SUTURAL MORPHOGENESIS AND THEIR IMPLICATIONS FOR THE DEVELOPMENT OF CRANIOSYNOSTOSIS.

During the formation, development and maturation of sutures and skull bones, including cells (mesenchymal cells, osteoblasts and osteocytes), a series of molecular factors are involved, which have been described as key proteins (Figure 1). The absence or failure of one of them leads to the development of pathological conditions such as parietal foramina, alteration in membranous ossification, sutural defects or craniosynostosis[2]. The most studied molecular factors and their role in sutural morphogenesis are described below.

Fibroblast growth factor (FGF) and its receptor (FGFR)

Eighteen types of fibroblast growth factors (FGFs) in mammals (FGF1-FGF10 and FGF16-FGF23) have been identified. They are involved in proliferation, migration, cell differentiation, mitogenesis, angiogenesis, embryogenesis and tissue repair[23]. The effects of FGF in these physiological processes are mediated by the activation of several signal transduction pathways through binding to its receptor (FGFR).

Receptors of fibroblast growth factors (FGFR) are 4 (FGFR1-FGFR4). They are expressed in osteoprogenitor cells, osteogenic fronts and the dura differentially, both spatially and temporally[23,24] as seen in Figure 1.

FGF-FGFR signaling plays an important role in the normal craniofacial development and morphogenesis during embryogenesis and postnatal growth[23]. For example, they induce the formation of cranial neural crest[25]. FGF is expressed in osteoblasts and mesenchymal cells of mouse calvaria[24].

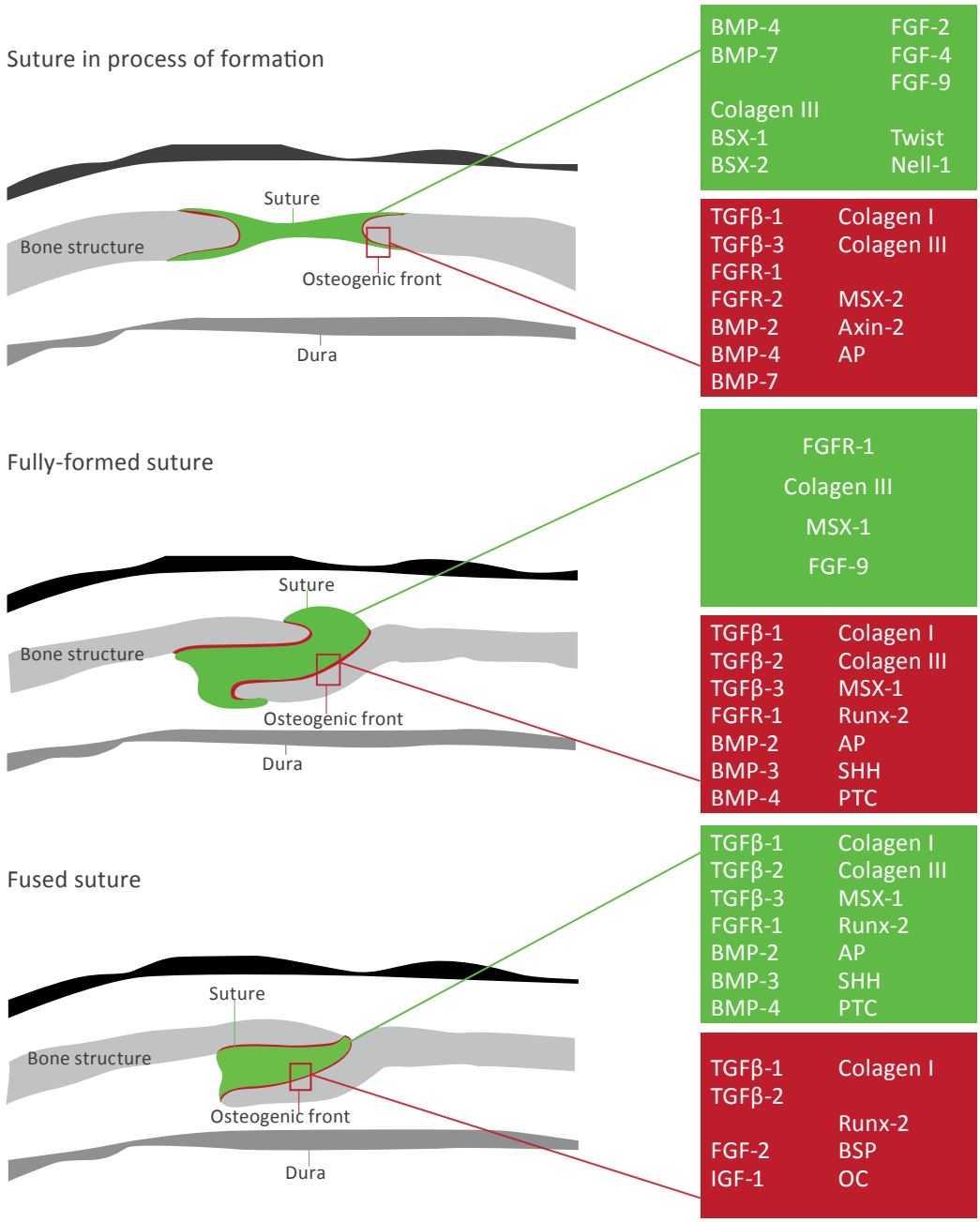
Morris-Kay *et al*. showed that low levels of FGF are required for the development of normal coronal suture and for maintaining the proliferation of osteogenic cells in bone margins, where the suture is formed. However, high levels of FGF have been associated with osteogenic differentiation[26].

FGF is highly expressed in the celular differentation region, while, it is poorly express in the suture. This means that the expression of FGF in the normal sutural development is different in time and site specific.

TGF-β or transforming growth factor beta

Transforming growth factor beta (TGF-β) include 33 polypeptides. The most important for craniofacial bone

Figure 1. Proteins involved in different stages of maturation and fusion of sutures.



BMP: Bone morphogenetic protein; **FGF:** fibroblast growth factor; **TGF-β:** transforming growth factor β; **AP:** alkaline phosphatase; **BSP:** bone sialoprotein; **OC:** osteocalcin; **IGF:** insulin growth factor.

growth are TGF-β1, TGF-β2 and TGF-β3[27].

These growth factors are involved in several events of bone development and growth in embryogenesis, differentiation of osteoblasts, their organization and death. They also have been implicated in the repair of fractures and osteogenesis[28].

Shakir *et al*. showed that stimulation with TGF-β in rabbits with suturectomy improved bone formation, and that the bone formed in the sutures was morphologically similar to the native bone. This suggests that TGF-β is involved in the correct formation of the skull and suture[18].

The expression of TGFβ-3 is important in stages where

sutures are required to be open, while TGFβ-2 is over-expressed in the premature fusion of the sutures, which means that it is involved in the fusion of these structures[29]. Thus it has been observed that TGFβ-2 and TGFβ-3 are involved in sutural homeostasis, the antagonistic action of both molecules regulates the precise closure of cranial sutures (Figure 1).

The proposed molecular mechanism for its antagonistic action is by its competition for the same receptor binding site that triggers in opposing signalling pathways. That is, TGF-β2 induces phosphorylation of Erk1/2 and inhibits expression of Smad2/3, which likely induces the production of bone matrix and the premature closure of the sutures. Furthermore, the binding of TGFβ-3 to its receptor activates Smad2/3 phosphorylation and inhibits Erk1/2 pathway, therefore decreasing osteogenesis and increasing the stages in which cranial sutures are kept open[30].

This molecular characterization has been experimentally confirmed with possible clinical applications, because the administration of inhibitor Erk1/2 (PD980509) is able to prevent premature suture fusion and maintain Smad2/3 expression[19,30].

BMPs (bone morphogenetic proteins)

Bone morphogenetic proteins (BMPs) are secreted proteins that belong to the superfamily TGF-β. They are found in the extracellular matrix. Twenty-two BMPs have been identified[31]. They are divided into 4 subfamilies according to their homologous amino acid sequence, their structure and function[32].

BMPs are involved in the formation of bone and cartilage of mesenchymal cells, in the induction of the development of mesoderm, in the formation of cranial bone (Figure 1), in the induction of apoptosis in osteoblasts, in the homeostatic maintenance of joints, in initiating the repair of fractures and in vascular remodeling[33].

BMP2 induces differentiation of mesenchymal cells in osteoblast precursors and promotes their maturation by increasing Runx2 expression and other genes specific to osteoid phenotype[34]. BMP2 and 4 through BMP7 and 9 have been shown to induce endochondral and membranous bone formation.

A large number of BMPs are ligand of type I receptor of bone morphogenetic proteins (BMPRI). This complex (BMP-BMPRI) binds in turn to the BMP type II receptor (BMPRII), which phosphorylates serine-glycine of type I receptor. Activation of BMRI phosphorylates Smad proteins, which together can induce translocation to the nucleus of transcription factors such as Runx2, which transcribes genes that encode proteins involved in the maturation and activation of osteoblasts[35].

Insulin growth factor (IGF)

This growth factor is a mitogenic peptide hormone. Two isoforms have been identified in bone matrix, IGF-I and IGF-II, having 40-50% homology between them. Both have mitogenic function for osteoblasts, and stimulate the production of type I collagen fiber, osteocalcin and alkaline phosphatase through binding to the IGFR receptor[36].

The absence or increase in IGF-I and II expression has been associated with diseases involved in the development of craniofacial pathologies such as Graves' disease, problems in the mandibular condyle, among others[37,38].

NELL-1 (binding protein to C kinase protein)

It is a polypeptide of 810 amino acids, which has an important role in inducing bone regeneration in calvarial defects, and in promoting osteoblast differentiation and mineralization[39]. In humans, NELL-1 is expressed in mesenchymal cells, osteoblasts of the osteogenic fronts, and along the parasutural bone margins[40] (Figure 1).

Nell-1 gene is expressed in calvarial osteoprogenitor cells of a mouse. Mutation of this gene in mice has resulted in simple and complex phenotypes of craniosynostosis[41].

AXIN-2 (negative regulatory protein of Wnt/B-catenin signaling pathway)

AXIN-2 promotes *in vivo* and *in vitro* osteoblast proliferation and differentiation, participating in membranous ossification. It is expressed in cells of the neural crest and has an important role in skeletal morphogenesis and sutural development (Figure 1). AXIN-2 deficiency in mice causes craniosynostosis; it is expressed in osteogenic fronts and periosteum of developing sutures in mice[42].

Msx-2 (homeobox gene protein)

It is involved in disorders affecting craniofacial organogenesis. For example, *msx-2* gene deficiency (in mice) creates defects in the ossification of the cranial vault and calvarial foramina. This phenotype is caused by a defect in proliferation of osteoprogenitor cells in osteogenic fronts during craneosutural development and MSX2 haploinsufficiency, which is associated with parietal foramina[43,44].

TWIST (Twist-related protein)

It is a transcription factor that belongs to the family of proteins with basic helix-loop-helix structure. *Twist-1* gene is expressed in the osteoprogenitor cells of the mesenchymal of the sagittal and coronal sutures of mice in the embryonic period and its expression decreases postnatally[40] (Figure 1).

It has roles in the development of neural tube; in inhibiting the synthesis of Twist in calvarial organs during the natal period of mice; it causes fusion of the coronal suture because it promotes osteoblast differentiation and prevents the suture from being established and maintained[35].

CLINICAL TREATMENT.

Craniosynostosis requires surgical treatment. It is intended to correct esthetic and physiological alterations. In simple craniosynostosis, surgery aims to correct the intracranial hypertension and abnormal appearance of the skull. In complex craniosynostosis, in addition to the above, treatment aims to correct abnormal facial appearance and abnormal upper limbs[45,46].

There are several types of surgeries, ranging from the removal of all the abnormal areas of the cranial skeleton and endoscopic surgery, to osteogenic distraction. The type of surgery depends on the characteristics of the malformation and the patient's history[1,7,20,47].

Because of the complexity of the functional and structural alterations, the care of patients with craniosynostosis should be provided by a multidisciplinary team. The American Cleft Palate-Craniofacial Association defines the interdisciplinary team as consisting of the following disciplines: maxillofacial surgery, anesthesiology, ophthalmology, craniofacial surgery, nursing, genetics, hand surgery, intensive care, otolaryngology, social work, language and speech pathology, radiology, psychology, neurosurgery, prosthodontics, orthodontics and pediatric dentistry[48,49]. The role of dental specialties is relevant for restoring facial aesthetics and maxillofacial function[50].

MOLECULAR TREATMENTS OF CRANIOSYNOSTOSIS.

As mentioned above, current treatments for craniosynostosis are limited to correct the anomalies, and improve the appearance and functionality of patient's stomatological system. However, these treatments do not solve the underlying problem. This is because the causes of this disease or the factors involved in its development remain unknown.

In the last two decades disciplines such as genomics and proteomics have developed considerably. They have helped to clarify the molecular causes of craniosynostosis. This has encouraged the study of each of these molecules as therapeutic targets for prevention, to stop the progression of the disease or limit its consequences and complications.

Several mutations of FGF and their receptors, or their overexpression, have been identified to favor the development of craniosynostosis. In most patients with Apert syndrome, mutation in FGFR2 (S252T and P253R) has been identified[51], while multiple substitution of different cysteines in this receptor are associated with Crouzon syndrome[52]. The mutation in FGFR1 (P252R) is associated with Pfeiffer syndrome and mutation in FGFR3 (P250R) was found in Muenke syndrome[53].

Several animal models have been developed to study the effect of various mutations in FGFR. *Fgfr1* and *Fgfr2* deletion is lethal in mice[54,55]. The single mutation of FGFR2 showed the development of a Crouzon-like syndrome. Samples of bone marrow of these mice showed greater rate of proliferation in osteoprogenitor cells, indicating the importance of FGFR2 in the early stages of sutural formation[56].

Various efforts have been made to inhibit FGF signaling pathway on order to maintain patency of cranial

sutures. Yokota *et al*. inhibited the activity of FGF2 impregnating a nanogel with soluble FGFR2 and placing it on the coronal suture in an Apert syndrome model, this prevented fusion of the coronal suture[51]. In another approach for the treatment of craniosynostosis, researchers used pathway inhibitors of MAP kinases involved in FGF signaling pathway such as PD98059 and U0126, which inhibit ERK1/2, resulting in the inhibition of the occurrence of craniosynostosis[5,45].

Transcription factor TWIST1 has also been studied as a therapeutic target, because it has been found that in most patients with Seathre Chotzen syndrome and in some patients with non-syndromic craniosynostosis, it has mutated to a degree that it has completely lost its function[57]. Then, the partial loss of TWIST1 expression causes bone pathological conditions that are related to osteoblast differentiation, in part, because it interferes partially the Runx2 pathway[40].

TGF-βs are involved, as mentioned above, in sutural biology. TGF-β2 seems to promote fusion of sutures and TGF-β3 prevents melting and maintains patency. This knowledge has been of great help in the search of molecular therapies. The administration of anti-TGF-β3 antibodies inhibits the normal fusion of sutures[35,58].

Justice *et al*. conducted a study on 130 children with non-syndromic sagittal synostosis analyzing their genomic profile in comparison with a large group of children with normal cranial suture development. They identified a polymorphism of a single nucleotide (SNP) located in the downstream region of *Bmp2*, possibly a regulatory protein of this gene[59].

Mutations in other genes and proteins that can trigger the development of craniosynostosis, although less common, such as *Msx2* and *Erf* have also been described. The mutation of a single base to replace a proline by a histidine at position 7 of MSX2 homeodomain increases affinity for DNA binding and accelerates the formation of the sutures, causing Boston-type craneosinosotosis. The *Erf* gene, whose protein (EFNB) is a transcription factor that inhibits ER1/2 pathway, has also been associated with the development of craneosinosotosis. Multiple mutations in the *Efr* gene in a small group of patients with multisutural or unisutural synostosis have been demonstrated[60].

Table 3 summarizes and shows the application of mo-

Table 3. Molecular therapies for craniosynostosis.

Study model	Molecule used	Treatment	Effect	References
In vivo Mouse model with suturectomy.	Gremlin	Injectable hydrogel *in situ.*	Delays postoperative re-synostosis.	Hermann *et al.*, 2014[61]
In vivo Mouse model with postoperative re-synostosis treated with suturectomy.	Noggin	Cells transfected.	Inhibits bone formation with Noggin.	Cooper *et al.*, 2009[62]
In vivo Rabbit models with coronal craniosynostosis and surectomy.	TGF-β3	Neutralization with the use of TGF-β3 antibody craniofacial development.	Delays postoperative re-ossification and improves.	Gilbert *et al.*, 2016[30]
In vitro Culture of calvarial organ of mouse with Crouzon syndrome, FGFR2, C342Y/+	FGFR2	PLX052 inhibitor of FGFR tyrosine kinase.	Prevents the fusion of the coronal suture.	Perlyn *et al.*, 2006[63]
In vivo Mouse with Apert syndrome, FGFR2, S252W/+	ERK1/2	Intraperitoneal injection of the U0126 inhibitor of MEK1/2.	Represses the phenotype of craniosynostosis in Apert syndrome.	Eswarankumar *et al.*, 2006[64]

lecular therapies implemented in animal models for the treatment of craniosynostosis, ranging from the prevention of surgical complications, inhibition of ERK1/2 activation, prevention of tyrosine kinase activity of FGF-FGFR pathway, and the use of specific inhibitors of BMPs or TGF-β antibodies, among others[5,45,61].

CONCLUSION.

The treatment of choice for patients with craniosynostosis remains surgical treatment. However, thanks to scientific advances on the molecular causes of this disease, new frontiers for implementing innovative molecular therapies have opened. According to the results of recent studies, mostly in animal models, manipulation of FGF-FGFR pathways of TGF-β are the most promising therapeutic targets.

Therapies based on the use of proteins for bone regeneration are promising, but they are not yet perfect. Finding new powerful, but specific, osteo-inductive molecules is one the biggest obstacles, as well as the implementation of effective release systems that can maintain the desired concentrations of the molecule localized, and at the same time control the timing of their release, all this to minimize adverse effects. The above certainly involves the recent use of nanotechnology in drug release.

It is therefore necessary to make further investigations on the effect of inhibition or induction of FGF expression or its receptors, of TGF or BMPs, or TWIST and Runx2 transcription factors in animal models by controlling the time and manner of administration, so that they can be used as viable treatments in human patients.

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Aplicaciones clínicas de las bases moleculares de la craneosinostosis. Revisión narrativa.

Resumen: Las suturas craneales son estructuras especializadas compuestas por el mesénquima sutural, el pericráneo suprayacente, la duramadre y los frentes osteogénicos. Cada una de estas estructuras expresan de forma diferencial, espacial y temporalmente, proteínas importantes para la maduración osteogénica, la osificación membranosa de los huesos calvarios y la homeostasis de las suturas craneales. Estas proteínas incluyen el factor de crecimiento fibroblástico (FGF) y sus receptores (FGFR), el factor de crecimiento transformante beta (TGF-β), las proteínas morfogenéticas óseas (BMPs), así como factores de transcripción TWIST y MSX2, entre otros. La alteración en la expresión de una o varias de estas proteínas provoca múltiples condiciones patológicas, una de ellas es el cierre prematuro de una o varias suturas craneales, conocido como craneosinostosis. Esta malformación es comúnmente tratada con cirugía. Sin embargo, los avances en los campos de la biología molecular y celular han permitido investigar algunas proteínas que participan en el desarrollo de la craneosinostosis. Los resultados de estos estudios pueden generar futuras estrategias terapéuticas preventivas o que complementen los tratamientos quirúrgicos de la craneosinostosis. Algunas estrategias posibles son el uso de fármacos específicos que puedan regular la expresión y activación de las vías de señalización del FGF, el TGFβ o de las BMPs, para prevenir la craneosinostosis o evitar la resinostosis tras una cirugía.

Palabras clave: *Craneosinostosis, Suturas, Terapia molecular.*

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