Neurochirurgie 65 (2019) 202–209

Disponible en ligne sur ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com

Craniosynostosis: State of the Art 2019

Animal models of craniosynostosis

M. Cornille[a,1], E. Dambroise[a,1], D. Komla-Ebri[a,b,1], N. Kaci[a,c], M. Biosse-Duplan[a], F. Di Rocco[d,e,**], L. Legeai-Mallet[a,*]

[a] *Inserm U1163, Paris university, institut Imagine, 75015 Paris, France*
[b] *Molecular Endocrinology Laboratory, Department of Medicine, Imperial College London, W12 ONNLondon, United Kingdom*
[c] *Inovarion, 75013 Paris, France*
[d] *Centre de référence craniosténoses, université de Lyon, 69677 Bron France*
[e] *Service de neurochirurgie pédiatrique, université Lyon, hôpital Femme–Mère-Enfant, 69677, Bron, France*

ARTICLE INFO

Article history:
Received 11 July 2019
Received in revised form 22 July 2019
Accepted 16 September 2019
Available online 26 September 2019

Keywords:
Mouse model
Zebrafish
Syndromic craniosynostosis
Suture
Skull growth

ABSTRACT

Background. – Various animal models mimicking craniosynostosis have been developed, using mutant zebrafish and mouse. The aim of this paper is to review the different animal models for syndromic craniosynostosis and analyze what insights they have provided in our understanding of the pathophysiology of these conditions.
Material and methods. – The relevant literature for animal models of craniosynostosis was reviewed.
Results. – Although few studies on craniosynostosis using zebrafish were published, this model appears useful in studying the suture formation mechanisms conserved across vertebrates. Conversely, several mouse models have been generated for the most common syndromic craniosynostoses, associated with mutations in *FGFR1*, *FGFR2*, *FGFR3* and *TWIST* genes and also in *MSX2*, *EFFNA*, *GLI3*, *FREM1*, *FGF3/4* genes. The mouse models have also been used to test pharmacological treatments to restore craniofacial growth.
Conclusions. – Several zebrafish and mouse models have been developed in recent decades. These animal models have been helpful for our understanding of normal and pathological craniofacial growth. Mouse models mimicking craniosynostoses can be easily used for the screening of drugs as therapeutic candidates.

1. Introduction

The mouse and zebrafish genomes have been well known for two decades and demonstrate great similarity to the human genome. Various genetic tools allow manipulation of both animal genomes with high accuracy, to delete, add or replace specific sequences so as to produce several types of mutation and modulate gene expression and function. Despite differences in cell identity and timing during organogenesis, these two species possess a cranial vault the organization of which is analogous to humans. Skull development is a complex dynamic process involving multiple components: the various ossification centers of the head grow during embryonic life and form multiple membranous flat bones. The bones end up reaching each other and forming a connecting fibrous junction or suture that maintains a separation and allows smooth deformation during the growth of the brain and skull. Craniosynostosis occurs when maintenance of this borderline fails and a bony bridge forms between the two bones, causing premature suture fusion. Syndromic craniosynostosis is often associated with single-point mutation in specific genes. Among the most frequently mutated genes are the *FGFR1-3* and *TWIST* genes.

Mouse and zebrafish models have been helpful in understanding the complex pathophysiology of craniosynostosis. These animal models are useful not only for our knowledge of the condition but also to develop new therapeutic approaches. The current treatment of craniosynostosis is almost exclusively surgical and consists in reshaping the skull while removing the fused sutures by osteotomies. Because these surgeries are associated with morbidity and mortality, and successive craniofacial procedures are needed to reshape the face and skull of the child, especially in syndromic cases, pharmacological approaches are a promising therapeutic alternative.

* Corresponding author at: Inserm U1163, Paris university, institut Imagine, 24 boulevard du Montparanasse 75015 Paris, France.
** Co-corresponding author at: Service de neurochirurgie pédiatrique, université de Lyon, hopital Mère-Enfant, Lyon, France
E-mail addresses: federico.dirocco@chu-lyon.fr (F. Di Rocco), laurence.legeai-mallet@inserm.fr (L. Legeai-Mallet).
[1] Equal contributors.

https://doi.org/10.1016/j.neuchi.2019.09.010
0028-3770/

2. Material and methods

A literature search for relevant studies on animal models of craniosynostosis was performed, focusing on zebrafish and mouse models.

3. Results

Several zebrafish and mouse models have been reported. The most relevant mouse models, exhibiting FGFR-related craniosynostoses mimicking human pathologies, are summarized in Table 1.

4. Mouse models of craniosynostosis

4.1. Mouse model mimicking Pfeiffer syndrome (*Fgfr1*$^{P250R/+}$)

Zhou et al. generated a mouse model for Pfeiffer syndrome by inserting the P250R mutation in the murine *Fgfr1* gene using homologous recombination [1]. This mutation corresponds to the human Pro252Arg mutation causing the mild form of Pfeiffer syndrome. Heterozygous *Fgfr1*$^{P250R/+}$ animals presented normal skull structure at birth, but quickly (postnatal day 3) developed craniofacial anomalies and smaller size. At postnatal day 21 days, they showed fusion of the coronal, frontal and sagittal sutures. The skulls of these animals presented facial asymmetry, with a shortened and dome-shaped neurocranium. Hypertelorism and midface hypoplasia were also observed in several animals. However, *Fgfr1*$^{P250R/+}$ did not show skull base anomalies. Homozygous *Fgfr1*$^{P250R/P250R}$ animals displayed a similar phenotype to the heterozygous *Fgfr1*$^{P250R/+}$, but with smaller body size. Neither *Fgfr1*$^{P250R/+}$ or *Fgfr1*$^{P250R/P250R}$ animals exhibited anomalies of the growth plate in the long bones. Calvaria analysis of *Fgfr1*$^{P250R/+}$ and *Fgfr1*$^{P250R/P250R}$ mice revealed advanced bone mineralization at 7 days of age and both pre-osteoblast proliferation and osteoblast differentiation were increased in their cranial sutures. Differentiated osteoblasts showed increased expression of bone sialoprotein, osteocalcin and osteopontin. Mutant osteoblasts also showed higher expression of osteoblast differentiation activator core binding transcription factor α subunit 1 (CBFA1). *In vitro* studies then revealed that *Fgfr1*$^{P250R/+}$ induced increased expression of CBFA1 in murine fibroblasts in response to Fgf2 ligand.

This model presents a strong craniofacial phenotype consistent with the human pathology. It is interesting to note that endochondral ossification does not seem to be affected by the mutation in either long bones or skull base. Membranous ossification is severely affected, with disruption of proliferation and differentiation of osteoblasts in the calvaria. The increased expression of CBFA1 detected in presence of the mutation suggests that it plays an essential mediating role in the pathophysiology of this syndrome.

4.2. Mouse models mimicking Apert syndrome (*Fgfr2*$^{S252W/+}$ and *Fgfr2*$^{P253R/+}$)

The mouse model for Apert syndrome was generated by ubiquitous expression of the Ser252Trp mutation in *Fgfr2* murine gene. Ser252Trp mutation is the most frequent mutation associated with Apert syndrome. It is located in the linker region of the protein, connecting the extracellular immunoglobulin II and immunoglobulin III domains for recognition of the ligand. It causes increased affinity for FGFR2 ligands and impaired binding specificity.

Fgfr2$^{S252W/+}$ mice present a phenotype of variable severity [2]. However, all animals showed a smaller size at birth and presented significantly shorter naso-anal length in later stages. No limb or paw abnormalities were found. However, they showed craniofacial abnormalities, with dome-shaped skulls, midface hypoplasia, malocclusion, and wide-spaced eyes. In the skull base, mutant newborns presented reduced presphenoid bone length. There were no strong anomalies in the growth plate of *Fgfr2*$^{S252W/+}$ long bones, suggesting that their reduced size may not be due to major disruption in endochondral ossification processes.

Craniosynostoses were noticeable mostly in coronal sutures, with significant overlapping between frontal and parietal bones and premature suture closure at embryonic stage E18.5. *Fgfr2*$^{S252W/+}$ mice calvaria also presented thinner bone. Closer examination of osteoblasts in newborn coronal sutures did not show alteration of proliferation and differentiation processes, as was observed in the *Fgfr1*$^{P250R/+}$ mouse model. However, increased levels of apoptosis were detected, providing a probable cause for the reduced bone thickness.

This model, while not mimicking the long bone phenotype observed in Apert syndrome, showed a strong craniofacial phenotype. The craniosynostosis observed in coronal sutures in presence of the equivalent human mutation makes it a good model for studying this syndrome.

Another model for Apert syndrome was created by inserting the Pro253Arg mutation into the *Fgfr2* murine gene [3]. This mutation is found in one-third of patients with Apert syndrome. *Fgfr2*$^{P253R/+}$ mice presented reduced size and brachycephaly. A third of the animals died before 20 days of age. Differently from *Fgfr2*$^{S252W/+}$ mice, syndactyly was present in several animals. Craniofacial phenotype featured a dome-shaped skull, wide-spaced eyes and midface hypoplasia with malocclusion of the teeth. A morphometric approach showed disproportion of the nasal and frontal bones and increased skull width. Fusion of coronal sutures was observed at 3 weeks of ages. Accelerated ossification and increased expression of osteoblast differentiation markers (CBFA1, osteopontin, osteocalcin) were observed in the calvaria of *Fgfr2*$^{P253R/+}$ animals. In accordance with the reduced body size of *Fgfr2*$^{P253R/+}$ animals, endochondral ossification was also affected by the mutation. The skull base was shortened in *Fgfr2*$^{P253R/+}$ mice, while the growth of secondary ossification and growth plate in long bones were delayed. The growth plate presented a decrease in proliferating cell number and in collagen X expression in hypertrophic chondrocytes. Bone-marrow cells from *Fgfr2*$^{P253R/+}$ mice presented overactivation of extracellular signal-regulated kinase 1/2 (Erk1/2). This kinase is part of the MAPK signaling pathway downstream of Fgfr2.

Yin L. et al. then performed *ex-vivo* cultures of calvaria and femurs of *Fgfr2*$^{P253R/+}$ embryos that they treated with the MEK1/2 inhibitor, PD98059. Reduced coronal suture fusion and increased femur length were observed after treatment with this inhibitor, showing the mediating role of the MAPK pathway in the mouse phenotype.

The *Fgfr2*$^{P253R/+}$ mouse model presents an interesting phenotype compared with the manifestations of the syndrome in humans. While presenting a severe craniofacial phenotype, it also presents syndactyly and shortening of long bones, as frequently observed in patients carrying the *Fgfr2* Pro253Arg mutation, showing the important role of *Fgfr2* in both endochondral and membranous ossification processes. The phenotypic rescue observed with the Erk1/2 inhibitor not only enables the molecular effect of the mutation on *Fgfr2* downstream signaling pathway to be deciphered, but also provides an interesting example of a drug-mediated strategy for treating syndromic craniosynostosis.

4.3. Mouse models mimicking Crouzon syndrome (*Fgfr2*$^{C342Y/+}$ and *Fgfr2*$^{W290R/+}$)

The first mouse model for Crouzon syndrome was generated by inserting the Cys342Tyr mutation, frequently found in human Crouzon and Pfeiffer syndromes, into the murine *Fgfr2* gene thanks

Table 1
Synopsis of the most common craniosynostosis mouse models.

Mouse model	Syndrome	Affected sutures	Cranial features	Facial features	Skull base features	Other features	References
Fgfr1$^{P250R/+}$	Pfeiffer	Coronal, frontal, sagittal	Short and dome-shaped skull	Snout deviation, hypertelorism, midface hypoplasia, malocclusion	–	Decreased body size	Zhou et al., 2000
Fgfr2$^{S252W/+}$	Apert	Coronal, delayed sagittal	Dome-shaped skull	Snout deviation, hypertelorism, midface hypoplasia, malocclusion	Short presphenoid bone	Decreased body size	Chen et al., 2003
Fgfr2$^{P253R/+}$	Apert	Coronal, delayed posterior frontal	Dome-shaped skull, brachycephaly	Snout deviation, hypertelorism, midface hypoplasia, malocclusion	Retarded growth	Decreased body size, retarded long-bone growth	Yin et al., 2007
Fgfr2$^{C342Y/+}$	Crouzon	Coronal, sagittal, lambdoid	Dome-shaped skull	Snout deviation, short face, hypertelorism, malocclusion	–	–	Eswarakumar et al., 2004
Fgfr2$^{W290R/+}$	Crouzon	Coronal, lambdoid	Dome-shaped skull	Snout deviation, midface hypoplasia, malocclusion	Presphenoid bone absent, basiphenoid dysmorphia	Decreased body size	Mai et al. 2010
Fgfr2$^{Y394C/+}$	Beare-Stevenson	Coronal, lambdoid, zygomaxillary, frontomaxillary, frontonasal, premaxillary-maxillary, intermaxillary	Dome-shaped skull, brachycephaly	Midface hypoplasia, malocclusion	Reduced sphenoid width	Decreased body size	Wang et al., 2012
Fgfr3$^{P244R/+}$	Muenke	Coronal, premaxillary-maxillary	Dome-shaped skull	Snout deviation, malocclusion	Premature fusion of intersphnoidal and sphenooccipital synchondrosis	Slightly decreased body size	Laurita et al., 2011, Twigg et al., 2009

to Cre recombinase under the control of PGK promoter [4]. The Cys342Tyr mutation is located in exon 9 of the gene and only affects the *Fgfr2IIIc* isoform. Heterozygous $Fgfr2^{C342Y/+}$ mice were viable and fertile while $Fgfr2^{C342Y/C342Y}$ animals died shortly after birth. $Fgfr2^{C342Y/+}$ mice presented shortened face and skewed snout, bulging eyes and dome-shaped skull. Frontal and parietal bone overlapped in E16.5 embryos, leading to coronal suture fusion in adult animals. Shortening of the sphenoid part of the skull base and reduction of maxillary bone with missing molar also accounted for this facial shortening.

Homozygous $Fgfr2^{C342Y/C342Y}$ mice presented a similar but more severe phenotype, with cleft palate closure failure and thickened skull base. Homozygous animals also presented an appendicular and axial phenotype, with shorter and thicker bones. Bony bridges were found in knee joints, with fusion of vertebrae. In addition, $Fgfr2^{C342Y/C342Y}$ mice presented defective development of tracheal cartilage and lungs, explaining the early lethality.

Eswarakumar et al. then looked at osteogenesis and chondrogenesis. Significantly increased expression of osteopontin and Cbfa1 was found in calvaria of E18.5 $Fgfr2^{C342Y/+}$ and $Fgfr2^{C342Y/C342Y}$ embryos. This correlated with increased proliferation of osteoprogenitors in the skull vault at early stages and in long bones. In contrast, expression of the genes related to chondrogenesis was not modified in the long bones of $Fgfr2^{C342Y/+}$ and $Fgfr2^{C342Y/C342Y}$ newborns.

These results suggest that endochondral and intramembranous ossification processes are affected differently by the mutation during embryonic development. $Fgfr2^{C342Y/+}$ mice, while mimicking all important features of Crouzon syndrome, highlighted an early role of *Fgfr2IIIc* signaling in osteoblast proliferation and a different role in chondrocyte and osteoblast lineages.

Another mouse model for Crouzon syndrome was generated by N-ethyl-N-nitrosourea (ENU) mutagenesis screen. Animals heterozygous for $Fgfr2^{W290R/+}$ mutation were obtained and bred to generate WT, $Fgfr2^{W290R/+}$ and $Fgfr2^{W290R/W290R}$ animals [5]. This mutation has been identified in many patients affected by Crouzon syndrome or Pfeiffer syndrome. Unlike the $Fgfr2^{C342Y}$ mutation, which affects only the IIIc isoform, this mutation is located in both IIIb and IIIc isoforms of *Fgfr2*. While *Fgfr2* expression was normal in heterozygous animals, increased expression was found in both isoforms in homozygous animals. Consistent with studies of molecular effects of the $Fgfr2^{W290R}$ mutation, increased activation of the Erk1/2 pathway was found in $Fgfr2^{W290R/W290R}$ animals.

$Fgfr2^{W290R/+}$ heterozygote animals presented craniofacial malformations aggravating with age, with short snout, dome-shaped skull and malocclusion of the incisors. Fusion of coronal and lambdoid sutures could be observed in young animals.

Homozygous $Fgfr2^{W290R/W290R}$ animals, however, died at postnatal day 1, showing severe craniofacial ossification retardation, and reduced cranial bones with reduced osteoblast differentiation. Additionally, $Fgfr2^{W290R/W290R}$ animals presented organ agenesis incompatible with life: no development of limbs or teeth, missing lacrimal and submandibular glands, and atrophied gastrointestinal tract and lungs.

Endochondral ossification was disrupted in both $Fgfr2^{W290R/+}$ and $Fgfr2^{W290R/W290R}$ animals, with dysmorphia of the skull base, thickened nasal septum cartilage and abnormal sternum cartilage. $Fgfr2^{W290R/W290R}$ animals also featured fused zygomatic arches and fusion of vertebrae.

The strong phenotype observed on homozygous animals can be explained by the effect of the mutation on both isoforms, while previous $Fgfr2^{C342Y/+}$ mice featured the modification of only one isoform. Interestingly, the phenotype observed in heterozygous animals can be associated with phenotypic traits found in Crouzon syndrome patients, while the homozygous phenotype is similar to observations in Pfeiffer syndrome: tracheal and visceral abnormalities, ankyloses of vertebral bodies. This finding highlights the strong clinical relevance of this model for understanding the role of *Fgfr2* in development and bone organogenesis.

4.4. Mouse model mimicking Beare-Stevenson syndrome ($Fgfr2^{Y394C/+}$)

A mouse model of Beare-Stevenson syndrome with cutis gyrata syndrome was generated to better understand the cellular and molecular pathogenesis of the syndrome by ubiquitous insertion of the Tyr394Cys mutation into the *Fgfr2* murine gene. $Fgfr2^{Y394C/+}$ animals displayed reduced growth and death by 2 weeks of age [6]. They presented premature fusion of coronal, zygomatic and squamosal sutures, midface hypoplasia and brachycephalic skull. Fusion of sternebral bones and enlarged umbilical stump were also observed. Coronal suture synostosis could be observed at E17.5, presenting an irregular osteogenic front, decreased cell proliferation and increased expression of osteoblast differentiation marker Runx2 and alkaline phosphatase between the osteogenic fronts. The skin phenotype of $Fgfr2^{Y394C/+}$ mice featured cutis gyrata, epidermal acanthosis and papillomatosis, with significantly thicker epidermis than in wild-type. Epidermal cell proliferation and epidermal differentiation markers were elevated. In contrast, apoptotic markers were decreased in $Fgfr2^{Y394C/+}$.

In vitro studies of $Fgfr2^{Y394C}$ then revealed constitutive ligand-independent overactivation of the receptor and of its downstream MAPK p38 pathway. Thus, *in utero* treatment of $Fgfr2^{Y394C/+}$ animals with p38 inhibitor SB203580 improved the skin phenotype, produced a minor effect on the craniofacial phenotype and did not improve the survival rate.

This mouse model presents a strong phenotype consistent with the craniofacial and skin phenotype observed in patients with Beare-Stevenson syndrome. Interestingly, the model displayed decreased osteoblast proliferation, while differentiation was increased. These results can be contrasted to previous *Fgfr* mouse models, where both mechanisms were increased, and suggest a complex involvement of both processes in suture fusion.

4.5. Mouse model mimicking Muenke syndrome ($Fgfr3^{P244R/+}$)

The mouse model for Muenke syndrome was generated using a Cre-LoxP system. Murine exon 7 of *Fgfr3* containing the Pro244Arg mutation, homologous to the human Pro250Arg mutation, was inserted into the gene with two LoxP sites by crossover [7,8]. The resulting mice were then crossed with mice expressing Cre recombinase under Gata1 promoter. The phenotype of $Fgfr3^{P244R/+}$ heterozygotes and $Fgfr3^{P244R/P244R}$ homozygous animals was variable, depending on genetic background and sex. While the first mutants were from a mixed background, the analyzed animals were generated by backcrossing into congenic lines. Only a minority of $Fgfr3^{P244R/+}$ animals presented craniofacial abnormalities, while penetrance was notably increased in homozygous mice. Interestingly, the sex bias observed in patients was reversed in $Fgfr3^{P244R/+}$ and $Fgfr3^{P244R/P244R}$ mice, with no heterozygous females affected, and 100% of homozygous males affected. The craniofacial phenotype was variable, with rounding of the skull, skewing of the snout and malocclusion of incisors and molars. Coronal synostosis was observed in only 2 animals, which do not allow it to be determined as the cause of the observed craniofacial variations. Fusion of zygomatic bone and synostosis of the ipsilateral premaxillary suture were, however, observed in mutant animals, associated with skewing of the snout. Additionally, analysis of the skull base of $Fgfr3^{P244R/+}$ mutant mice showed a premature closure of intersphenoidal and spheno-occipital synchondroses in a large number of animals at 3 weeks of age. This premature closure was correlated with malocclusion of incisors and molars.

Precise histological analysis of these synchondroses demonstrated abnormal endochondral ossification, with reduced chondrocyte differentiation and primary bone spongiosa, and accelerated secondary and perichondrial ossification, leading to the formation of bony bridges. This skull base phenotype can be related to the reduction in skull base length observed in patients. Furthermore, analysis of the temporomandibular joint of *Fgfr3*$^{P244R/+}$ mutant mice found delayed development of the condylar head. As in skull-base synchondroses, reduced chondrocyte proliferation and differentiation were observed after 3 weeks of age, with reduced primary bone spongiosa. Moreover, condylar cartilage presented arthritic features and all *Fgfr3*$^{P244R/+}$ mice presented low-frequency sensorineural hearing loss. Deep examination of the inner ear found an excess of pillar cells throughout the cochlear duct, suggesting an essential role of the *Fgfr3* signaling in inner-ear development. This phenotype is relevant, as hearing loss affects a large proportion of Muenke patients.

Unlike the craniofacial phenotype, no long-bone phenotype was observed in *Fgfr3*$^{P244R/+}$ mice, although heterozygous and homozygous animals presented reduction in femoral head and cortical head bone mineral density.

Interestingly, this model for Muenke syndrome showed a phenotype with low penetrance. Moreover, the sex bias was reversed compared to humans, with males more severely affected than females. The lower severity of the craniofacial phenotype of *Fgfr3*$^{P244R/+}$ mice in comparison to previously cited mouse models for *Fgfr1*- and *Fgfr2*-related craniosynostosis supports the idea that *Fgfr3* plays a later and more subtle role in membrane ossification and osteoblast maturation in mice.

4.6. Mouse model mimicking Saethre-Chotzen syndrome (*Twist*$^{-/-}$)

Because *TWIST* haploinsufficiency leads to Saethre-Chotzen syndrome, the mouse model proposed for this syndrome is a Twist$^{+/-}$ heterozygous mouse [9]. Several studies have evaluated the Twist$^{+/-}$ Saethre-Chotzen mouse model at various ages and focusing on different parameters [10–12]. Homozygous Twist-null mice present a lethal embryonic phenotype. Embryo and new-born studies reported extra toes on one or both hindfeet in Twist$^{+/-}$ heterozygotes and minor defects in several bones of the calvaria (squamosal, interparietal, and supraoccipital). From P9 to P13, these mice showed an abnormal coronal suture closure process that has been shown to be governed by endochondral ossification. Coronal suture fusion is not the only defect in the skull of *Twist*$^{+/-}$ mice, which also mice present a limited regenerative capacity of the cranial vault (analyzed on a resynostostosis model) and anomalies in the development of the skull base. Accelerated spheno-occipital synchondrosis tether formation has been reported in *Twist*$^{+/-}$ mice. However, this anomaly occurs at P25-P30, well after normal coronal suture fusion time. For this reason, it is unlikely that the observed skull base defects are responsible for the coronal fusion. The Saethre-Chotzen syndrome mouse model presents incomplete penetrance and variable expressivity, and noteworthy that this has also been reported in human patients.

Another mouse model exists using *Twist 1*, based on the finding, in a cohort of patients presenting isolated sagittal and coronal craniosynostosis, of 1 case with an isolated sagittal synostosis due to a novel mutation in *Twist1* box: Ser188Leu. [13]. The homologous Serine in Twist1 is mutated in a mouse model called Charlie Chaplin (CC). CC mice were generated through ENU mutagenesis. *CC*$^{/+}$ mice harbor the heterozygous single amino acid substitution mutation Ser192Pro in the Twist box of Twist1. The phenotype of this model, however, includes single sagittal stenosis. The mice present hindlimb polydactyly and anomalies of sagittal, coronal and lambdoidal sutures. However, the CC mouse phenotype is variable, with 50% of the animals presenting polydactyly in both hindlimbs. The large majority of the analyzed *CC*$^{/+}$ mice (94%) showed coronal synostosis at P10.

The model was presented in a study highlighting the inhibitory role of Twist proteins toward Runx2 and their necessary inhibition to determine osteoblast differentiation [14]. However, since the mouse model does not present an isolated single-suture fusion, but sagittal, coronal and lambdoidal suture irregularities at the same time, the CC model is far from ideal to study isolated sagittal synostosis.

4.7. Mouse model mimicking Boston-type craniosynostosis (*Msx2* transgenic)

Two mouse lineages express the *Msx2* mutation Pro7His, also observed in Boston-type craniosynostosis patients, under two different promoters: TIMP1 and CMV (ubiquitous promoters) [15]. The third model overexpresses the wild-type form of *Msx* (CMV promoter). All three models present premature fusion of sagittal, coronal and lambdoidal sutures from P1 with variable expressivity. With these results, Liu et al. concluded that the Msx2 mutations responsible for the craniosynostosis are gain-of-function mutations. Nonetheless, it is also interesting to note that these models have incomplete penetrance: 30% of mutant Msx2-expressing mice and 70% of WT overexpressing mice show a craniosynostosis phenotype.

4.8. Mouse model mimicking trigonocephaly (*Frem1*^{ba}, *Frem1*^{Qbrick})

Frem1 mice were developed following the identification of 5 patients with de novo CNVs involving Frem1 gene and 3 patients presenting missense mutation in the same gene [16]. Firstly, Vissers et al. used *in situ* hybridization to investigate the pattern of expression of *Frem1*. They observed *Frem1* presence in the skull area where the posterior-frontal suture would arise in E14.5-E16.5 mice. Secondly, immunostaining for Frem1 in P0 mice revealed consistent expression of the protein in pericranium and dura mater surrounding the frontal bones. *Frem1* expression was also observed in osteoprogenitors localized in between the frontal bones, suggesting a role in the developing frontal and nasal sutures.

To validate their findings, they compared the pathological human phenotype with two *Frem1* mouse models. The first, named *Frem1*^{bat}, results from a single nucleotide substitution that affects the normal mRNA splicing of *Frem1*. The substitution leads to frameshift and premature truncation of the Frem1 protein, and for this reason *Frem1*^{bat} is thought to be a hypomorphic allele. The second mouse model is *Frem1*^{Qbrick}, which presents a null allele. In both models, homozygote mice present peculiar dysmorphologies compared to control mice. Morphometric analyses showed deformation of frontal and nasal bones in mutant mice. As previously shown in other craniosynostosis mouse models, incomplete penetrance was observed: only 45% of the homozygote Frem1 mutant mice presented defects in the frontal region of the skull, in the form of pronounced deviation of the midface and a shortened snout. Moreover, both *Frem1*^{bat} and *Frem1*^{Qbrick} P28 mice displayed advanced posterior frontal suture fusion compared to wild-type control mice. Furthermore, heterozygous mutant mice partly exhibit these anomalies.

4.9. Mouse model mimicking EFNA4 non-syndromic coronal synostosis (*Epha4*$^{-/-}$)

Loss-of-function heterozygous mutations in EFNA have been observed in patients with non-syndromic coronal synostosis [17].

To validate the link between the phenotype and these genetic findings, Ting et al. carried out analyses with an EphA4 mutant mouse presenting coronal synostosis [18]. The phenotype of *Epha4*$^{-/-}$ mice includes uni/bi-lateral coronal synostosis, a phenotype that was previously observed in *Twist1*$^{+/-}$ mice (and presented in the Saethre-Chotzen syndrome section above). This observation, paired with the fact that analyses of *Epha4* performed by *in situ* hybridization showed decreased expression of this protein in *Twist1*$^{+/-}$ mice, suggests a role of *Epha4* as an effector of *Twist1*. At the same time, *Twist1* expression in *Epha4*$^{-/-}$ mice was unchanged, suggesting that Twist1 acts through Eph-ephrin signaling, regulating coronal suture development.

4.10. Mouse model mimicking Greig cephalopolysyndactyly (Gli3$^{Xt-J/Xt-J}$*)*

Greig cephalopolysyndactyly syndrome is caused by missense mutations in GLI3 and characterized by craniofacial abnormalities including macrocephaly and scaphocephaly (long and narrow cranial vault, respectively). Craniosynostosis has also been reported in patients with Greig cephalopolysyndactyly, although it is not always present. One of the peculiar features observed in these patients is the presence of polydactyly in both hands and feet.

In wild-type mouse embryos, Gli3 transcripts were highly and strictly expressed on the interparietal side of the lambdoid and interfrontal sutures, suggesting a role of the protein in their development. A mutant mouse model called Extra-toes (Gli3$^{Xt-J/Xt-J}$), presenting a deletion of Gli3, is considered a valid model for Greig cephalopolysyndactyly syndrome [19]. Extra-toes is a spontaneous semi-dominant mouse mutation, and the affected mice exhibit polydactyly as well as defects in the craniofacial region. In E18.5 Gli3$^{Xt-J/Xt-J}$ mice, a dome-shaped skull was observed, with lambdoid suture fusion and cranial base anomalies. Rice and colleagues showed, through in situ hybridization experiments, that Gli3$^{Xt-J/Xt-J}$ mice show abnormal expression of Ptch1 in the area where the lambdoid suture subsequently develops, while at the same time Twist1 expression is downregulated [20]. Lack of Twist1 expression in the suture stops the inhibition of Runx2, allowing osteoblast differentiation, bone formation and, consequently, craniosynostosis. Interestingly, the same group demonstrated an increase in cell proliferation in E16.5 Gli3$^{Xt-J/Xt-J}$ mice at suture level and were able to rescue the craniosynostosis phenotype using FGF2 beads.

5. Zebrafish models of craniosynostoses

Zebrafish has been used to study craniofacial development. As in mammals, the zebrafish skeleton is formed via both endochondral and membranous ossification processes. A majority of studies focused on the early stages of craniofacial skeleton development, including the formation of the neurocranium and viscerocranium. The former provides a support for the brain and sensory systems while the latter corresponds to the mandibular elements and respiratory apparatus. Both of these structures are mostly formed of cartilage at 5 days after fecundation and are later replaced by bone. At this stage, the zebrafish presents a simplified skeleton with a cartilage consisting of a few cell layers that are easy to study using Alcian blue/Alizarin red staining or live imaging. The formation of these structures early during zebrafish development has been extensively studied due to structure homology between fish and mammals [21]. For example, the bones forming the jaw and connecting the viscerocranium to the ear in fish are evolutionarily speaking the precursor of the mammalian middle ear. Likewise, the anterior neurocranium is an excellent model for the hard palate. Both of these structures derive from cranial neural crest cells and their formation seems to be regulated by a conserved genetic network across vertebrate species. Several studies demonstrated that knockdown of genes involved in human cleft palate formation, such as irF6, faf 1 or crispld2, induces a similar phenotype in zebrafish [22–24].

Contrary to the neurocranium and the viscerocranium, zebrafish cranial vault development occurs at 3–6 weeks post-fertilization, at the juvenile stage. In zebrafish as in mammals, the calvaria is formed by thin membranous bones separated by sagittal and coronal sutures, separating frontal and parietal bones. One specificity of the zebrafish calvaria is the persistence of the sutures following skull extension and growth throughout life [25]. The same characteristic is observed in rodents. Moreover, zebrafish bones flanking the coronal sutures derive from mesoderm, whereas in mammals coronal sutures mark the boundary between anterior neural crest bones and the posterior mesoderm bones. Despite these differences, the conservation of the anatomical calvaria structures and the accessibility of the skull make zebrafish a good model to study suture formation.

Unlike in mouse models, only a few studies of skull formation and craniosynostosis were performed in zebrafish, due to a lack of adult zebrafish mutants. Most craniofacial mutants are obtained by large-scale forward-genetic chemical ENU treatment or insertional mutagenesis screening focused on severe craniofacial defects mainly affecting neurocranium and viscerocranium. Also, mutants were screened at early embryonic stages (5–6 days post-fecundation), which limits the screening to the identification of genes that are important in early development, while genes controlling later stages were not studied.

6. Discussion

Animal models have proved useful for understanding craniofacial growth.

In particular, because of analogous craniofacial development between mice and humans, several models mimicking human pathology have been generated.

The mouse interfrontal suture, localized between frontal bones and analogous to the human metopic suture, undergoes fusion between postnatal days 7 and 12. The other sutures (sagittal, coronal, lambdoid) remain patent throughout the life of the mouse. For many years, various research groups have studied the role of key genes and their signaling in craniosynostoses processes, using various mouse models. These models mimicked the human pathology, with varying exactness, and allowed pharmacological treatment of the phenotype to be tested. These studies constitute a first step and promise progress in treatment design for the pathology.

Zebrafish is also a valuable model for studying craniofacial skeletal development and cranial vault formation. The use of this teleost fish as a predictive model for human development and diseases has been increasing for 30 years, for multiple reasons. Firstly, as a vertebrate, zebrafish share a high degree of sequence homology with the human genome; 70% of human genes have a zebrafish orthologue, and the genome of zebrafish was sequenced in the early 2000s. Secondly, the rapidity of embryogenesis, the external development and the optical transparency of the embryo allow live imaging experiments and developmental studies. The short generation time and the huge number of descendants (about 100 per clutch) make zebrafish a powerful model for genetic screening and therapeutic trials.

Studies on skull formation using zebrafish demonstrated the relevance of the model, with suture formation mechanisms conserved across vertebrate species. Topcweska et al. demonstrated, by *in situ* hybridization, that most of the genes involved in suture development in mammals are conserved in zebrafish:

col1a1a, *col2a1a*, *col10a1*, *bglap/osteocalcin*, *fgfrs*, *twist2*, *twist3*, *runx2a*, *runx2b*, *sp7*, and *osteopontin* [25]. Moreover, study of the expression of IL11RA, which is involved in premature fusion of cranial sutures in patients with Crouzon-like syndromes and pansynostosis, revealed its presence in the coronal sutures of zebrafish and mouse. These results confirm the role of IL11ra in frontal and parietal bone separation [26].

Other studies highlighted mechanisms involved in calvaria and suture formation and in premature fusion of the sutures. Due to skull accessibility and existence of transgenic lines with promoters driving fluorescent reporter expression in the developing craniofacial skeleton (e.g. runx2, sp7, type I collagen), zebrafish is a suitable model for live imaging. Kague et al. used this strategy to study the dynamic process involved in calvaria formation and demonstrated the importance of *Sp7*, a gene required for osteoblast differentiation, in this process [27]. *Sp7* mutant fish present abnormal calvaria formation with no normal patterned sutures and skull bones. Using a transgenic line expressing the green fluorescent protein (GFP) under the control of Runx2, expressed in early osteoblasts, they demonstrated that signaling from differentiated osteoblasts is critical to limiting overproliferation of immature osteoblasts, leading to abnormal bone overlap and suture formation. Their study thus highlighted a new mechanism involved in skull patterning and showed that signals from the bone itself are required for orderly recruitment of precursor cells and growth along the edge.

It is well known that retinoic acid (RA) influences suture formation. In humans, partial loss of CYP26B1, an enzyme able to inactivate RA, induces severe defects in the calvarial plates of the skull roof, including premature fusion of the coronal sutures between the frontal and parietal plates. The phenotype of CYP26B1-null humans is more severe, with smaller and fragmented calvaria. Laue et al., using a hypomorphic CYP26B1 zebrafish model, demonstrated that premature fusion is due to premature transition of osteoblasts to preosteocystes within the coronal sutures [28]. This premature accumulation of preosteocystes within the suture of the hypomorphs leads to ectopic mineralization of the sutural matrix, whence the apparent hyperossification. A more recent study completed previous results and explained the different phenotypes observed between hypomorph and amorph patients. Treating juvenile zebrafish with a high level of exogenous RA or with CYp26 inhibitors, Jeradi et al. obtained a phenotype similar to amorph patients, with thinner and fragmented calvaria [29]. They demonstrated that the fragmented calvaria is due not only to premature differentiation of osteoblasts into preosteocytes but also to activation of osteoclasts through the resultant preosteocytes.

In the last decade, new tools were developed to perform mutagenesis of candidate genes, such as CrispR/Cas9 technology, which targets the Cas 9 endonuclease at specific sites in the genome using specific guide RNAs. This technology is fast improving and it is now possible to generate both targeted knockout and knock-in mutants in zebrafish. The development of this technology will be useful for testing putative disease alleles and studying the impact of mutations on skull formation from a dynamic cellular and molecular point of view.

7. Conclusion

Several zebrafish and mouse models mimicking craniosynostoses have been developed in recent decades. These animal models have been helpful for our understanding of normal and pathological craniofacial growth. They can be easily used for the screening of drugs as therapeutic candidates.

Disclosure of interest

The authors declare that they have no competing interest.

References

[1] Zhou YX, Xu X, Chen L, Li C, Brodie SG, Deng CX. A Pro250Arg substitution in mouse Fgfr1 causes increased expression of Cbfa1 and premature fusion of calvarial sutures. Hum Mol Genet 2000;9:2001–8.
[2] Chen L, Li D, Li C, Engel A, Deng C-X. A Ser252Trp [corrected] substitution in mouse fibroblast growth factor receptor 2 (Fgfr2) results in craniosynostosis. Bone 2003;33:169–78.
[3] Yin L, Du X, Li C, Xu X, Chen Z, Su N, et al. A Pro253Arg mutation in fibroblast growth factor receptor 2 (Fgfr2) causes skeleton malformation mimicking human Apert syndrome by affecting both chondrogenesis and osteogenesis. Bone 2008;42:631–43, http://dx.doi.org/10.1016/j.bone.2007.11.019.
[4] Eswarakumar VP, Horowitz MC, Locklin R, Morriss-Kay GM, Lonai P. A gain-of-function mutation of Fgfr2c demonstrates the roles of this receptor variant in osteogenesis. Proc Natl Acad Sci USA 2004;101:12555–60, http://dx.doi.org/10.1073/pnas.0405031101.
[5] Mai S, Wei K, Flenniken A, Adamson SL, Rossant J, Aubin JE, et al. The missense mutation W290R in Fgfr2 causes developmental defects from aberrant IIIb and IIIc signaling. Dev Dyn 2010;239:1888–900, http://dx.doi.org/10.1002/dvdy.22314.
[6] Wang Y, Zhou X, Oberoi K, Phelps R, Couwenhoven R, Sun M, et al. p38 Inhibition ameliorates skin and skull abnormalities in Fgfr2 Beare-Stevenson mice. J Clin Invest 2012;122:2153–64, http://dx.doi.org/10.1172/JCI62644.
[7] Laurita J, Koyama E, Chin B, Taylor JA, Lakin GE, Hankenson KD, et al. The Muenke syndrome mutation (FgfR3P244R) causes cranial base shortening associated with growth plate dysfunction and premature perichondrial ossification in murine basicranial synchondroses. Dev Dyn 2011;240:2584–96, http://dx.doi.org/10.1002/dvdy.22752.
[8] Twigg SRF, Healy C, Babbs C, Sharpe JA, Wood WG, Sharpe PT, et al. Skeletal analysis of the Fgfr3(P244R) mouse, a genetic model for the Muenke craniosynostosis syndrome. Dev Dyn 2009;238:331–42, http://dx.doi.org/10.1002/dvdy.21790.
[9] Bourgeois P, Bolcato-Bellemin AL, Danse JM, Bloch-Zupan A, Yoshiba K, Stoetzel C, et al. The variable expressivity and incomplete penetrance of the twist-null heterozygous mouse phenotype resemble those of human Saethre-Chotzen syndrome. Hum Mol Genet 1998;7:945–57.
[10] Behr B, Longaker MT, Quarto N. Craniosynostosis of coronal suture in twist1 mice occurs through endochondral ossification recapitulating the physiological closure of posterior frontal suture. Front Physiol 2011;2:37, http://dx.doi.org/10.3389/fphys.2011.00037.
[11] Carver EA, Oram KF, Gridley T. Craniosynostosis in Twist heterozygous mice: a model for Saethre-Chotzen syndrome. Anat Rec 2002;268:90–2, http://dx.doi.org/10.1002/ar.10124.
[12] Hermann CD, Lee CSD, Gadepalli S, Lawrence KA, Richards MA, Olivares-Navarrete R, et al. Interrelationship of cranial suture fusion, basicranial development, and resynostosis following suturectomy in twist1(+/-) mice, a murine model of Saethre-Chotzen syndrome. Calcif Tissue Int 2012;91:255–66, http://dx.doi.org/10.1007/s00223-012-9632-3.
[13] Seto ML, Hing AV, Chang J, Hu M, Kapp-Simon KA, Patel PK, et al. Isolated sagittal and coronal craniosynostosis associated with TWIST box mutations. Am J Med Genet A 2007;143A:678–86, http://dx.doi.org/10.1002/ajmg.a.31630.
[14] Bialek P, Kern B, Yang X, Schrock M, Sosic D, Hong N, et al. A twist code determines the onset of osteoblast differentiation. Dev Cell 2004;6:423–35.
[15] Liu YH, Kundu R, Wu L, Luo W, Ignelzi MA, Snead ML, et al. Premature suture closure and ectopic cranial bone in mice expressing Msx2 transgenes in the developing skull. Proc Natl Acad Sci USA 1995;92:6137–41.
[16] Vissers LELM, Cox TC, Maga AM, Short KM, Wiradjaja F, Janssen IM, et al. Heterozygous mutations of FREM1 are associated with an increased risk of isolated metopic craniosynostosis in humans and mice. PLoS Genet 2011;7:e1002278, http://dx.doi.org/10.1371/journal.pgen.1002278.
[17] Merrill AE, Bochukova EG, Brugger SM, Ishii M, Pilz DT, Wall SA, et al. Cell mixing at a neural crest-mesoderm boundary and deficient ephrin-Eph signaling in the pathogenesis of craniosynostosis. Hum Mol Genet 2006;15:1319–28, http://dx.doi.org/10.1093/hmg/ddl052.
[18] Ting M-C, Wu NL, Roybal PG, Sun J, Liu L, Yen Y, et al. EphA4 as an effector of Twist1 in the guidance of osteogenic precursor cells during calvarial bone growth and in craniosynostosis. Dev Camb Engl 2009;136:855–64, http://dx.doi.org/10.1242/dev.028605.
[19] Hui CC, Joyner AL. A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the *Gli3* gene. Nat Genet 1993;3:241–6, http://dx.doi.org/10.1038/ng0393-241.
[20] Rice DPC, Connor EC, Veltmaat JM, Lana-Elola E, Veistinen L, Tanimoto Y, et al. Gli3Xt-J/Xt-J mice exhibit lambdoid suture craniosynostosis which results from altered osteoprogenitor proliferation and differentiation. Hum Mol Genet 2010;19:3457–67, http://dx.doi.org/10.1093/hmg/ddq258.
[21] Mork L, Crump G. Zebrafish Craniofacial Development: A Window into Early Patterning. Curr Top Dev Biol 2015;115:235–69, http://dx.doi.org/10.1016/bs.ctdb.2015.07.001.

[22] Dougherty M, Kamel G, Grimaldi M, Gfrerer L, Shubinets V, Ethier R, et al. Distinct requirements for wnt9a and irf6 in extension and integration mechanisms during zebrafish palate morphogenesis. Dev Camb Engl 2013;140:76–81, http://dx.doi.org/10.1242/dev.080473.
[23] Ghassibe-Sabbagh M, Desmyter L, Langenberg T, Claes F, Boute O, Bayet B, et al. FAF1, a gene that is disrupted in cleft palate and has conserved function in zebrafish. Am J Hum Genet 2011;88:150–61, http://dx.doi.org/10.1016/j.ajhg.2011.01.003.
[24] Yuan Q, Chiquet BT, Devault L, Warman ML, Nakamura Y, Swindell EC, et al. Craniofacial abnormalities result from knock down of nonsyndromic clefting gene, crispld2, in zebrafish. Genes NYN 2000 2012;50:871–81, http://dx.doi.org/10.1002/dvg.22051.
[25] Topczewska JM, Shoela RA, Tomaszewski JP, Mirmira RB, Gosain AK. The Morphogenesis of Cranial Sutures in Zebrafish. PloS One 2016;11:e0165775, http://dx.doi.org/10.1371/journal.pone.0165775.
[26] Keupp K, Li Y, Vargel I, Hoischen A, Richardson R, Neveling K, et al. Mutations in the interleukin receptor IL11RA cause autosomal recessive Crouzon-like craniosynostosis. Mol Genet Genomic Med 2013;1:223–37, http://dx.doi.org/10.1002/mgg3.28.
[27] Kague E, Roy P, Asselin G, Hu G, Simonet J, Stanley A, et al. Osterix/Sp7 limits cranial bone initiation sites and is required for formation of sutures. Dev Biol 2016;413:160–72, http://dx.doi.org/10.1016/j.ydbio.2016.03.011.
[28] Laue K, Pogoda H-M, Daniel PB, van Haeringen A, Alanay Y, von Ameln S, et al. Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid. Am J Hum Genet 2011;89:595–606, http://dx.doi.org/10.1016/j.ajhg.2011.09.015.
[29] Jeradi S, Hammerschmidt M. Retinoic acid-induced premature osteoblast-to-preosteocyte transitioning has multiple effects on calvarial development. Dev Camb Engl 2016;143:1205–16, http://dx.doi.org/10.1242/dev.129189.