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REVIEW

Insights into Craniofacial Development and Anomalies: Exploring Fgf Signaling in Zebrafish Models

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Abstract

Purpose of Review To illustrate the value of using zebrafish to understand the role of the Fgf signaling pathway during craniofacial skeletal development under normal and pathological conditions.

Recent Findings Recent data obtained from studies on zebrafish have demonstrated the genetic redundancy of Fgf signaling pathway and have identified new molecular partners of this signaling during the early stages of craniofacial skeletal development.

Summary Studies on zebrafish models demonstrate the involvement of the Fgf signaling pathway at every stage of craniofacial development. They particularly emphasize the central role of Fgf signaling pathway during the early stages of the development, which significantly impacts the formation of the various structures making up the craniofacial skeleton. This partly explains the craniofacial abnormalities observed in disorders associated with FGF signaling. Future research efforts should focus on investigating zebrafish Fgf signaling during more advanced stages, notably by establishing zebrafish models expressing mutations responsible for diseases such as craniosynostoses.

Keywords FGF signaling pathway · Zebrafish · Craniofacial skeleton · Osteochondrodysplasia

Introduction

The fibroblast growth factor (FGF) signaling pathway is crucial in various biological processes during organ development and homeostasis, as well as at the cellular level, where it influences proliferation, migration, differentiation, and cell death [1–3]. In humans, FGF signaling includes 22 FGF ligands, 7 FGF receptors (FGFRs) with tyrosine kinase activity (resulting from alternative splicing of 4 genes: *FGFR1-4*), and numerous co-factors such as heparan sulfate and Klotho. The diverse functions of FGF signaling rely on precise regulation of expression and timing of FGFs, FGFRs, and co-factors [3]. Despite this tight regulation, multiple FGF signaling-related genetic disorders result in craniofacial anomalies (Tables 1 and 2) [51]. Gain-of-function (GOF) mutations in *FGFR1*, *2*, and *3* are involved in syndromic craniosynostoses, characterized by premature fusion of cranial sutures [52]. Common forms of syndromic craniosynostoses due to mutations on *FGFR2* include Crouzon, Apert, and Pfeiffer syndromes [32, 37, 38] with the latter resulting also from *FGFR1* GOF mutation [32]. *FGFR3* GOF mutations cause Muenke syndrome and Crouzon with acanthosis nigricans syndrome [47, 53]. *FGF9* mutations are also associated with craniosynostosis [16]. Additionally, cleft palate, skull base anomalies, and midface hypoplasia are observed in several of these syndromes [40, 54–56]. Craniofacial skeleton anomalies are also described in other FGFR3-related disorders such as achondroplasia (GOF mutation) where patients exhibit skull base, cranial vault, and mandibular defects in addition to rhizomelic dwarfism, and in CATSHL syndrome, (Loss-of-function mutation (LOF)) characterized by overgrowth associated with microcephaly and Wormian skull bones [48, 57–59]. All these disorders highlight the critical role of the FGF signaling pathway in craniofacial skeleton development.

Numerous mouse models have been developed to understand the role of the FGF signaling pathway during

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Table 1 Human FGFs and zebrafish orthologues: Expression patterns during craniofacial development and associated human diseases

Human gene	Zebrafish gene	% Homologies with Human Proteins	Early craniofacial skeleton development expression	Juvenile/adult craniofacial skeleton expression	Human pathologies affecting craniofacial skeleton
FGF1	*fgf1a*	52,4	No expression	No data	-
	fgf1b	35,5	No data	No data	
FGF2	*fgf2*	75,3	No expression	cranial vault osteoprogenitors [4••]	-
FGF3	*fgf3*	62,7	rhombomeres [5], pharyngeal pouches [6]	No data	Associated with craniosynostosis [7]—craniofacial microsomia [8]
FGF4	*fgf4*	62,3	Dental epithelium [9]	No data	Associated with craniosynostosis [7]—craniofacial microsomia [8]
FGF6	*fgf6a*	64,7	No expression	No data	-
	fgf6b	64,1	No data	No data	
FGF7	*fgf7*	48,8	No expression	cranial vault osteoprogenitors [4••]	-
FGF8	*fgf8a*	66,2	rhombomeres [5],paraxial mesoderm, pharyngeal mesoderm, mandibular archs [10–12]	chondocranium, kinetmoid bone, maxilla, cranial suture [13]	Kallman syndrome # [14, 15]
	fgf8b	61,3	No expression	No data	
FGF9	*fgf9*	32,8	No expression	No data	Craniosynostosis [16]
FGF10	*fgf10a*	57,7	pharyngeal archs, mandibular arch CNCCs [17, 18]	cranial vault osteoprogenitors and chondrocytes [4••]	Associated with cleft palate #[19, 20]
	fgf10b	52,9	No expression	No data	-
FGF16	*fgf16*	80,8	pharyngeal archs [21]	cranial vault osteoprogenitors [4••]	-
FGF17	*fgf17*	65,9	pharyngeal archs [19]	No data	Kallman syndrome [20]
FGF18	*fgf18a*	68,8	pharyngeal archs [19]	cranial vault osteoprogenitors [4••]	-
	fgf18b	63,6	pharyngeal archs [19]	cranial vault osteoprogenitors [4••]	-
	fgf24	61,3	pharyngeal archs, pharyngeal pouches [17, 19]	cranial vault osteoprogenitors [4••]	-
FGF19	*fgf19*	41,6	pharyngeal archs [22]	No data	craniofacial microsomia [8]
FGF20	*fgf20a*	72,5	No expression	inter-frontal joint osteoblasts, frontal bone osteoblasts [23]	-
	fgf20b	76,9	cranial neural crest, pharyngeal archs [24–26]	No data	-
FGF21	*fgf21*	31,7	No expression	No data	-
FGF22	*fgf22*	50,6	No expression	No data	-
FGF23	*fgf23*	32,2	No expression	No data	XLH (craniosynostosis) [27]

Human pathology with existing zebrafish model.

craniofacial development [3, 60, 61]. Nevertheless, over the past quarter-century, zebrafish has emerged as a relevant model to study cellular and molecular mechanisms regulating craniofacial skeletal development and for the analysis of genetic variants underlying craniofacial defects [60, 62–64]. Approximately 70% of human genes have at least one homolog in zebrafish [65]. The presence of several homolog skeletal elements and the substantial conservation of the developmental mechanisms between zebrafish and mammals, makes the former an excellent model to study craniofacial skeleton formation. For example, the zebrafish ethmoidal plate of the anterior neurocranium is often described as analogous to the mammalian palate, the mammalian middle ear is analogous to the fish jaw. Cranial vault development and anatomy are also well conserved [31, 66]. From a technical point of view, the zebrafish model allows

Table 2 Human FGFRs and zebrafish orthologues: Expression patterns during craniofacial development and associated human diseases

Human gene	Zebrafish gene	% Homologies of the proteins of the human ortholog	Early craniofacial skeleton development expression in zebrafish	Juvenile/Adult craniofacial skeleton expression in zebrafish	Human pathologies affecting craniofacial skeleton
FGFR1	*fgfr1a*	FGFR1 IIIb vs Fgfr1a IIIb: 70,87 FGFR1 IIIb vs Fgfr1a IIIc: 68,99 FGFR1 IIIc vs Fgfr1a IIIb: 69,32 FGFR1 IIIc vs Fgfr1a IIIc:71,73	rhombomeres [28], mandibular archs, pharyngeal archs [22, 29, 30]	Osteoblasts [31], cranial vault osteoblasts [4••]	Pfeiffer syndrome [32], osteoglophonic dysplasia [33], Kallman syndrome [15]
	fgfr1b	FGFR1 IIIb vs Fgfr1b: 76,88 FGFR1 IIIc vs Fgfr1b: 80,3	No expression	inter-frontal joint osteoblasts, maxillary barbel [31, 34]	
FGFR2	*fgfr2*	FGFR2 IIIb vs Fgfr2 IIIb: 77,57 FGFR2 IIIb vs Fgfr2 IIIc: 76,02 FGFR2 IIIc vs Fgfr2 IIIb: 76,55 FGFR2 IIIc vs Fgfr2 IIIc: 78,78	rhombomeres [28], pharyngeal archs [35]	Cartilage, inter-frontal joint osteoblasts [31, 36••], cranial vault osteoprogenitors [4••]	Apert [37], Crouzon syndrome [38], Pfeiffer syndrome [39], Beare-Stevenson [40], cutis gyrata syndrome[41]
FGFR3	*fgfr3*	FGFR3 IIIb vs Fgfr3: 73,09 FGFR3 IIIc vs Fgfr3: 73,93	No expression	cartilage, osteoclasts, frontal bone osteoblasts [42], cranial vault osteoprogenitors and osteoblasts [4••]	Achondroplasia [43], hypochondroplasia [44], thanatophoric dysplasia [45], CAN syndrome [46], Muenke syndrome [47], CATSHL syndrome #[4••, 48, 49••]
FGFR4	*fgfr4*	66,22	No expression	maxillary barbel [34], cranial vault osteoblasts [4••]	-
FGFRL1	*fgfrl1a*	67,28	gill cartilage [50]	No data	-
	fgfrl1b	57,26	gill cartilage [50]	No data	

Human pathology with existing zebrafish model.

for high-resolution *in vivo* imaging during skull development thanks to the accessibility of the embryos and the relatively low cell count [67]. Finally, a wide array of genetic tools developed for this model, such as morpholino, Tol2 system, and CRISPR-Cas9, has facilitated the creation of transgenic lines and mutants, aiding in the study of craniofacial development under both normal and pathological conditions [68•, 69]. In this review, we present the literature data on Fgf signaling during zebrafish craniofacial development, providing insights into understanding FGF signaling-related anomalies in the craniofacial skeleton observed in human diseases, and offering promising avenues for future research in this field.

The Fgf Pathway in Zebrafish

31 Fgfs, including 6 paralogs resulting from genome duplication during evolution after teleost radiation, are described in The Zebrafish Information Network database (Table 1) [70]. In mammals, FGFs are often classified based on their mode of action, which correlates with the coreceptors needed to stabilize the interaction between the ligand and the receptor. While most ligands are paracrine and associated with heparan sulfate coreceptors, exceptions include the endocrine FGF19 family linked to Klotho (α and βklotho) and the intracrine FGF11 family, which do not bind to a receptor [71]. To our knowledge, zebrafish *fgfs* have typically been categorized only by gene location and never by their modes of action [70]. However, some studies on zebrafish coreceptors offer insights into the conservation of Fgfs' mode of action. Expression of heparan sulphate proteoglycans is also described as regulating the majority of Fgf signalling [72–74]. *Klotho* and *klothob* are *αKLOTHO* and *βKLOTHO* orthologs, respectively [75]. In zebrafish, Fgf23 seems to interact with Klotho, and both *fgf23* and *klotho* mutants exhibit the same phenotype, suggesting conservation of the Klotho/Fgf23 system [76–78]. At last, while no evidence of intracrine action in zebrafish has been published for the Fgf11 family, the absence of the signal peptide domain in the C-terminus of Fgf11-14, akin to the human FGF11 family, suggests a conserved mode of action.

The zebrafish *fgfr*s include five genes encoding receptors with tyrosine kinase activity. Specifically, *fgfr1a* and *fgfr1b* are orthologs of *FGFR1*, and *fgfr2*, *fgfr3* and *fgfr4* are the orthologs of *FGFR2*, *FGFR3* and *FGFR4*, respectively (Table 2). In humans, FGFR1-3 receptors have isoforms resulting from alternative splicing of exon 8 or exon 9 corresponding respectively to the immunoglobulin domain IgIIIb or IgIIIc [79]. Similarly, Fgfr1a and Fgfr2 have isoforms due to an alternative splicing (exon 7 or exon 8). The IgIIIb and IgIIIc isoforms of Fgfr1a and Fgfr2 respectively align more closely with the corresponding human IgIIIb and c isoforms. Fgfr1b and Fgfr3 show greater homology with their corresponding human IgIIIc isoforms. Finally, similar to mammals, the Fgfr family in zebrafish includes a receptor lacking a tyrosine kinase domain; specifically, *FGFR5* has two orthologs in zebrafish, *fgfrl1a*, and *fgfrl1b*[50].

The Zebrafish Craniofacial Skeleton

The adult zebrafish skull consists of 73 bones (more than in mammals). There is a correlation between skull development and zebrafish size, therefore post-embryonic stages (beyond 5 days post-fertilization) are mostly determined by standard length (SL) rather than age [31]. The craniofacial skeleton is made up of neurocranium and viscerocranium. The viscerocranium is the most ventral part of the zebrafish skeleton and it is the first portion that develops starting from 48 h post fertilization (hpf). It is the feeding and respiratory apparatus and is composed of bones forming the jaw and five branchial arches: basibranchia, hypobranchials, ceratobranchials, epibranchials, and pharyngobranchials. The first four branchial arches support the gills, while the fifth carries the teeth. The neurocranium, supporting the brain and sensory systems, is comprised of four capsules: ethmoid, orbit, optic, and occipital, along with cranial vault bones [67, 80–82]. Cranial vault formation begins during the larval stage, around 7SL, approximately 1 month post-fertilization [31]. Similar to mammals, the zebrafish craniofacial skeleton is formed either through endochondral ossification or intramembranous ossification [83]. Zebrafish skull bones can be classified into four types: acellular and compact bones (e.g., frontal, parietal, occipital), cellular compact bones with osteocytes entrapped in the matrix (e.g., opercle, pterotic, sphenotic), tubular bones filled with adipose tissue (e.g., hyomandibula, basibranchial, ethmoid), and spongy bones filled with a trabecular network (e.g., quadrate, ceratohyal) [84].

Fgf Signaling and Early Craniofacial Development in Zebrafish

Despite its complexity, zebrafish craniofacial skeletal development closely resembles that of mammals. This model was widely used to study early step of craniofacial development including cellular dynamics and the involvement of signaling pathways like FGF signaling. The zebrafish skull bones derive from both the cranial neural crest cells (CNCCs) and the paraxial mesoderm [85, 86]. CNCCs originate from the junction between the neural tube and the ectoderm [87]. Around 12 hpf, coinciding with hindbrain segmentation into rhombomeres (R1-R7), CNCCs undergo epithelial-mesenchymal transition, delaminate, and migrate in three streams (mandibular, hyoid, and five branchial), populating

the seven pharyngeal arches (Fig. 1A) [88]. CNCCs that contribute to the formation of the mandibular arch delaminate adjacent to the posterior midbrain-R2 whereas CNCCs of the hyoid and branchial arches originate next to R4 and R6-R7, respectively. Each pharyngeal arch comprises of cylinders of CNCCs surrounding a core of mesoderm, bordered externally by ectoderm and separated from other arches by endodermal out pockets called pharyngeal pouches (Fig. 1A) [21, 24, 89, 90]. These arches serve as templates for craniofacial structure development in adulthood, with the first arch giving rise to the lower jaw and palate, the second arch to the ceratohyal and hyomandibular bones, and the third through seventh arches forming the ceratobranchials, epibranchials, and pharyngobranchials [63, 64, 67, 86, 91].

In zebrafish, early craniofacial development is characterized by redundant use of Fgf signaling components.

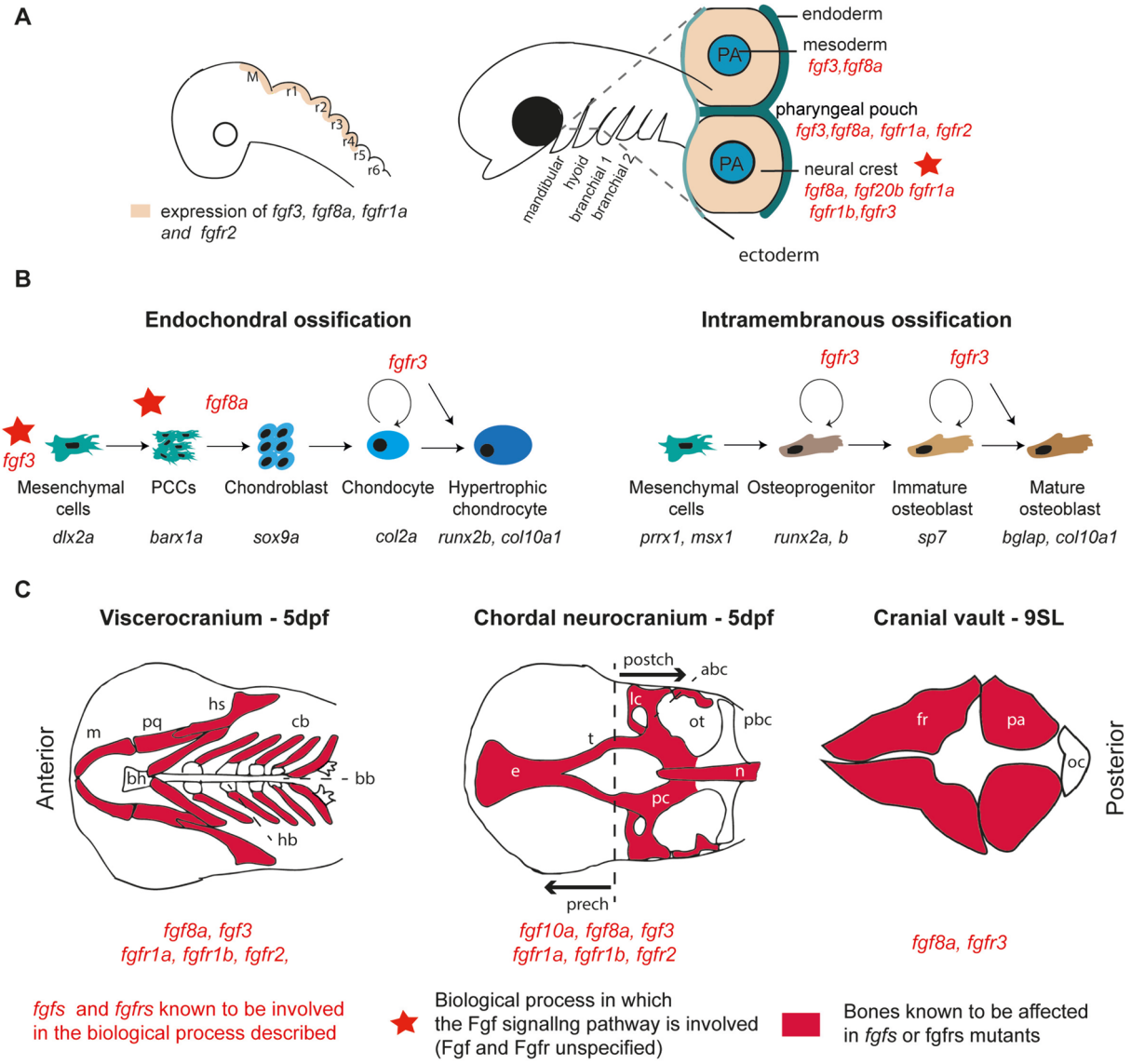

Fig. 1 Involvement of the Fgf signaling pathway during craniofacial skeleton development in zebrafish. **A** Diagram of the first steps of craniofacial development from the formation of the rhombomeres to the formation of the pharyngeal arches and pharyngeal pouches m: midbrain, r: rhombomere, PA: pharyngeal arch. **B** Endochondral and intramembranous ossification. The most important genes expressed during these processes are in italic. PCC: precartilaginous condensations. **C** Diagram showing all bones affected in the different *fgf* and *fgfr* zebrafish models published, in the viscerocranium and chordal neurocranium at 5dpf and in the cranial vault at 9SL. abc: anterior basicranial commissure, ac: auditory capsule, bb: basibranchial, bh: basihyal, cb: ceratobranchial, e: ethmoid plate, hb: hypobranchial, hs: hyosymplectic, lc: lateral commissure, m: Meckel's cartilage, n, notochord, ot: otic capsule, pc: parachordal, pq: palatoquadrate, pbc: posterior basicapsular commissure, postch: postchordal neurocranium, prech: prechordal neurocranium, t: trabeculae, fr: frontal bone, pa: parietal bone, oc: occipital bone

This is evidenced by the absence of distinguishable phenotypes in single mutants of the receptors *fgfr1a*, *fgfr1b*, *fgfr2*, and *fgfr3* [92, 93••]. This differs from mice, where ubiquitous knock-out of *fgfr1* or *fgfr2* receptors are embryonic lethal [94, 95]. Nevertheless, the zebrafish studies contributed widely to reveal that *fgf3* and *fgf8* are part of a regulatory network controlling pharyngeal pouches and CNCCs homeostasis (Fig. 1A). *Fgf8* is initially expressed in the lateral mesoderm, in the midbrain-hindbrain boundary (MHB) and in R2 and R4 domains. Its expression overlaps *fgf3's* in neural MHB and R4 domains [5, 96, 97]. Between 18 and 28hpf, *fgf3* and *fgf8a* are expressed in pharyngeal pouches adjacent to *dlx2a*-expressing CNCCs. Their expression in the mesoderm close to the endoderm is crucial for proper endodermal cell migration, segmentation of the pharyngeal endoderm into pouches, and CNCCs proliferation [98]. A link was reported between Fgf signaling and Tbx1, which deletion in human is associated to DiGeorge syndrome and developmental defects of the pharyngeal arches and pouches [99]. Research conducted in zebrafish brought two significant findings: firstly, Tbx1 triggers directional pocket growth through Fgf8a [10]; secondly, in pharyngeal pouches regulated by Pax1a and Pax1b, Tbx1 along with Fgf3 influences the expression of *dlx2a* in nearby CNCCs located in pharyngeal arches 3 to 6. Dlx2a is essential in guiding the differentiation of CNCCs into ectomesenchymal cells and chondrocytes [100•]. To complete this network, Fgf signaling functions downstream of Twist1 to suppress s*ox10* expression in the CNCCs while simultaneously activating *dlx2a* expression [101]. Additionally, it was recently demonstrated that Fgf8 is also involved in CNCCs differentiation through the negative regulation of Nkx2.3 [102].

Further, Fgf20b interaction with Fgfr1 is required for the ectomesenchyme formation [25]. *fgf24* is expressed in pharyngeal pouches, and *fgf8b, fgf17, fgf16, fgf18a, fgf18b* expression was observed in pharyngeal arches but no data have been reported about their role during first step of pharyngeal arches morphogenesis [21, 24, 89]. By 24hpf, *fgfr1a* and *fgfr2* are expressed in the MHB, hindbrain rhombomeres (R1-4), and pharyngeal pouches, with *fgfr1a* showing earlier expression [28]. Research using morpholino injection or CRISPR Cas9 mutagenesis revealed that while *fgfr1a, fgfr1b,* and *fgfr2* are unnecessary for CNCCs migration into the pharyngeal pouches, they are vital for CNCCs maintenance [28, 92]. *fgfr3* is also expressed in pharyngeal pouches [49••]. The role of Fgf signaling during pharyngeal arches morphogenesis, and CNCCs homeostasis is critical as it influences the later patterning of the viscerocranium and the neurocranium either via endochondral or intramembranous ossification [93••, 98].

FGF Signaling and the Viscerocranium Development: Focus on Endochondral Ossification

Zebrafish viscerocranium is exclusively derived from CNCCs and consists of bones formed via both endochondral and intramembranous ossification [83, 86, 103•, 104••]. In this paragraph, we focus on endochondral ossification. As in mammals, itoccurs principally during growth and is characterized by a cartilaginous intermediate matrix formed by chondrocytes and invaded by blood vessels and osteoblasts that eventually convert the cartilage template into bone at larval stages. Initially, CNCCs of the pharyngeal arches differentiate into ectomesenchymal cartilage precursors expressing *dlx2a* and aggregate from 48hpf into precartilaginous condensations (PCCs) expressing *barx1* [105, 106]. These PCCs dictate the morphology of the facial cartilage [36••]. At 60 hpf, cartilage precursors start expressing *sox9a* (necessary for producing the cartilage-specific collagen Col2a*)* and initiate chondrocyte differentiation. Subsequently, between 72 and 84 hpf, chondrocytes stack to form the pharyngeal cartilage [107, 108]. Chondrocytes differentiation is then completed by their maturation into *runx2b* and *col10a1*-expressing enlarged hypertrophic cells (Fig. 1B). As in mammals, zebrafish chondrocytes contribute to osteoblasts, adipocytes, and mesenchymal cells within the adult bones [109]. However, hypertrophic cells in zebrafish minimally contribute to bone growth and appear to be transient, as they are no longer present in later stages of development [103•]. The spatial organization of epiphyseal growth zones in zebrafish resembles mammalian long bone growth plates. The ceratohyal exhibits a similar organization to the long bones of mammalian limbs, featuring two prominent growth zones at each end and a marrow cavity. However, it is important to note the absence of secondary ossification in zebrafish [104••]. Each growth zone consists of a resting zone (Col2a1a+) followed by the proliferative zone (Pcna+) and a hypertrophic zone (Col10a1+). Pharyngeal bones are separated by synchondroses, as in mammals, that produces a bidirectional growth formed by a resting zone flanked by two proliferative and hypertrophic zones [103•, 104••].

The zebrafish studies highlighted that Fgf signaling plays a key role during the first steps of chondrogenesis, including the regulation of *dlx2a* expression and the differentiation of CNCCs into ectomesenchyme *(see previous section)*. Recently, Paudel et al. demonstrated that Fgf signaling participates also to PCC formation as it regulates *barx1* expression directly and indirectly by inhibiting *jag1,* whose expression is inversely proportional to *barx1*. [36••]. With these insights, it becomes evident

that disruptions in the Fgf pathway during early chondrogenesis have significant repercussions on the formation of cartilage, particularly within the viscerocranium. This assertion finds support in several studies. Firstly, the inhibition of Fgf signaling in zebrafish embryos between 24 and 36hpf, a crucial period for CNCCs differentiation into chondrocytes, using the pan-FGFR kinase inhibitor BGJ-398, resulted in smaller viscerocranium bones and mineralization defects at later stages (5dpf) [93••]. Secondly, the absence of both Fgf8 and Fgf3 has been shown to hinder posterior viscerocranium formation and significantly impact the development of the anterior viscerocranium [14, 98, 110]. Thirdly, *fgfr1a; fgfr1b* double mutants or *fgfr1a; fgfr1b; fgfr2* triple mutants exhibit significant defects in the viscerocranium, including anomalies in the ceratobranchials, hyosymplectic, palatoquadrate, and Meckel's cartilage (Fig. 1B and C). The triple mutant shows even more severe viscerocranium defects, with the involvement of the ceratohyal as well [92]. Finally, it was discovered that Fgf signaling is regulated partially by the von Willebrand factor A domain (VWA1), during CNCCs aggregation and differentiation, and its absence leads to chondrocytes disarrangement and deformities of craniofacial cartilage in zebrafish and to Hemifacial microsomia in Human [111].

To date, limited data are available regarding the molecular partners and roles of Fgf3, Fgf8, Fgfr1a, Fgfr1b, and Fgfr2 in the zebrafish endochondral ossification. However, studies have shown that the absence of Fgf8 or inhibition of FGF signaling results in the impairment of key genes involved in bone formation, such as *runx2a, sp7, col1a1 and col9* [14, 93••]. A recent elegant study, revealed that, the stabilization of *fgf8* mRNA by the rRNA-processing protein Nucleolin is essential for the proper formation of the viscerocranium in osteochondroprogenitors [112••]. In contrast, Fgfr3 plays a distinct role. It is highly expressed during viscerocranium development: observed at 60 hpf in the mandibular and hyoid arches cartilage, followed by expression at 72hpf in chondrocytes of branchial arches 1–5. Although its expression diminishes by 4 dpf, Fgfr3 persists in the head cartilage until adulthood [42, 49••]. Similar to its function in mammals, Fgfr3 serves as a crucial regulator of the endochondral ossification process in zebrafish as it was demonstrated by the analysis of *fgfr3* LOF zebrafish, established using CRISPR-Cas9 technology, mimicking CATSHL syndrome with cranial vault and hyoid anomalies, along with midface hypoplasia [4••, 49••] *fgfr3* LOF zebrafish model, Sun et al. described that the function of Fgfr3 is conserved between tetrapod and teleost during endochondral ossification. Fgfr3 serves as a negative regulator of chondrocyte proliferation and is also involved in the differentiation of chondrocytes into hypertrophic cells (Fig. 1B) [49••, 113]. They demonstrated that this regulation occurs in part via the activation of the canonic Wnt/β-catenin and Ihh pathways. During the endochondral process, Fgfr3 regulates not only chondrogenesis but also osteogenesis. This is evidenced by the delayed ossification of pharyngeal bones and the reduced number of osteoblasts observed in *fgfr3* LOF fish. Finally, Fgfrl1a and Fgfrl1b also appear to play a role in cartilage formation, especially in the development of the gills. This is intriguing considering that in mammals, FGFR5 interacts with other FGFRs to modulate Fgf signaling in cartilage [50, 114].

Fgf Signaling and the Zebrafish Cranial Vault Development: Focus on Intramembranous Ossification

The zebrafish cranial vault is composed mainly by pairs of frontal and parietal bones formed via intramembranous ossification and the supraoccipital bone formed via endochondral process. These bones originate from both CNCCs and mesoderm, but contrary to mammals, where the boundary between CNCCs and mesoderm-derived cells lies between the frontal and parietal bones, in zebrafish, this boundary is situated within the frontal bones, with the anterior and posterior parts respectively derived from CNCCs and mesoderm [86]. The cranial vault bones, are the largest ones formed via the intramembranous process, and offer an ideal opportunity to analyse the cellular mechanisms involved in this process due to their prominent location, and their late development [115].

Intramembranous ossification begins with the differentiation of CNCCs and mesodermal cells to mesenchymal cells, expressing *paired related homeobox 1a* (encoded by *prrx1a*), *muscle segment homeobox msx1 and 3*, which initially aggregate to form the ossification center. Subsequently, these cells differentiate into osteoblasts through highly conserved mechanisms. The growth of cranial vault bones occurs at the periphery of the newly formed bone through the continuous differentiation of mesenchymal cells into osteoprogenitors expressing *twist*1a, *2* and *3* and *runx2a and b* genes. Osteoblast differentiation progresses with the expression of *osterix (sp7)*, followed by the sequential expression of genes encoding bone matrix proteins such as *osteopontin (spp1)*, *collagen type 1 (Col1)*, *col10a1, and osteocalcin (bglap)* (Fig. 1B) [4••, 31, 116]. The expression of *col10a1* in osteoblasts is noteworthy, as it is typically restricted to hypertrophic chondrocytes in mammals [117]. Furthermore, zebrafish cranial vault bones are acellular, as the maturation of osteoblasts in these bones does not lead to their embedding into the bone matrix and their transformation into osteocyte [84].

At the end of the cranial vault development, the bones come together and overlap, with a thin layer of suture

mesenchymal stem cells (SuSCs) and fibrous tissue forming the cranial suture. Specific sutures delineate the boundaries between bones: the metopic suture (frontal-frontal), two coronal sutures (frontal-parietal), sagittal suture (parietal-parietal), and lambdoid suture (parietal-supraoccipital). Interestingly, all cranial sutures in zebrafish exhibit overlapping bones similar to the coronal and lambdoid sutures in mammals, and none feature bones facing each other, as seen in the metopic and sagittal sutures of mammals [118, 119]. The sutures impart flexibility to the cranial vault, supporting its growth until brain development concluded. It is worth noting that, unlike in mammals, where cranial sutures fuse by adulthood, the zebrafish cranial sutures remain an area of slow intramembranous ossification throughout the animal's lifespan due to continuous growth [31]. At the cellular level, mammals exhibit four main clusters of SuSCs*: gli1+, axin2+, prrx1+, and Ctsk+* SuSCs. In zebrafish, however, only *gli1+, prrx1+,* and *grem1a+* SuSCs have been identified thus far, necessitating further investigation [120–124]. Despite these differences, the limited studies on cranial vault formation in zebrafish emphasize the highly conserved nature of this process, which relies on well-orchestrated cellular and molecular mechanisms [31, 125–127].

Single cell RNAseq performed during zebrafish cranial vault development highlighted that some *fgfs* are expressed in the osteoprogenitors *fgf2, fgf10a, fgf16, fgf18b*, *fgf2*, *fgf7, fgf24* and finally *fgf18a*, which has the highest expression. *fgfr2* is mainly expressed in osteoprogenitors, *fgfr1a,* and *fgfr4* in osteoblasts and *fgfr1b* in chondrocytes. Interestingly and contrary to what has been described in mice, *fgfr3* is the most strongly expressed during cranial vault development and can be detected in late osteoprogenitors and osteoblasts [4••]. At adult stages, *fgfr1a*, *fgfr1b, fgfr2, fgfr3* are still expressed in the cranial suture. Expression of *fgf8a* was also detected [13, 31, 42, 49••]. Despite the important role of FGF signalling in cranial vault formation, as evidenced by how *FGFR1, FGFR2* and *FGFR3* are all involved in craniosynostosis, limited studies have investigated Fgf signaling and cranial vault development in zebrafish. The *fgfr3* LOF zebrafish model mentioned earlier, presenting cranial bone growth delay, wormian bones and cranial sutures anomalies, has provided us with an invaluable tool to study the role of Fgfr3 during cranial vault development. This model is the only *fgfr3* LOF animal model with cranial vault anomalies, and has enabled us for the first time to highlight that Fgfr3 is an activator of osteoblasts expansion and differentiation during cranial vault development (Fig. 1B) [4••]. Further studies on the involvement of Fgfr3 during cranial suture formation are ongoing. *Fgf8a* plays also a role in cranial formation and *fgf8a* haploinsufficiency leads to adult skeletal defects including irregular patterns of cranial suturing, and ectopic bone formation (Fig. 1C) [13].

Many questions persist regarding FGF signaling in cranial vault and suture development. Studying LOF mutants of other *fgfs* and *fgfrs* in later stages could elucidate their roles. Establishing models with GOF mutations is essential for understanding the pathophysiological mechanisms of craniosynostosis related to FGF signaling. The zebrafish model's relevance for these diseases has already been demonstrated as for example the Saethre-Chotzen syndrome and craniosynostoses linked to *Cyp26b1* [60, 124, 128, 129].

Fgf Signaling and Pre and Post Chordal Neurocranium Development

Finally, FGF signaling is also associated with other craniofacial anomalies such as cleft palate (observed in multiple synostoses syndrome type 3 related to FGF9), in Kallman, Apert, Beare-Stevenson and Crouzon syndrome, and with skull base defects as in Achondroplasia, Apert and Crouzon syndromes [15, 40, 54–57, 130]. In zebrafish the palate and the skull base are interconnected, delineating the prechordal (anterior) and postchordal (posterior) regions of the neurocranium. The first is made up of CNCC-derived cells and the second mostly of mesoderm-derived cells [63, 67, 86, 110]. The zebrafish palate consists of the ethmoidal plate, trabeculae and parasphenoid bones. Clefts, truncations, hypoplasia, or absence of these structures indicate orofacial clefts. The postchordal neurocranium includes the parachordal cartilages, anterior and posterior basicapsular commissures around the developing ear, lateral commissures, and occipital arches. Few zebrafish studies have explored FGf signaling and its role in these structures' development (Fig. 1C). Notably, one study identified *fgf10a* expression in both CNCCs and oral ectoderm, suggesting its necessary in palatogenesis. Morpholino-induced *fgf10a* knockdown results in shortened trabeculae and parasphenoid bones. Fgf10a likely regulates *shh* expression, guiding CNCCs migration towards the midline, triggering chondrogenesis, and facilitating trabeculae formation. A deeper analysis of Fgf10a's role during this process would be relevant, as FGF10 has been associated with pathologies resulting in dental anomalies and cleft palate (homozygous or heterozygous knock-out mice are either non-viable or exhibit only a very slight phenotype) [131, 132]. Interestingly, fish carrying the $fgf8^{ti282}$ LOF mutation display ethmoidal plate defects similar to the ones described in humans with Kallman syndrome (characterised by cleft lip and palate) and due to mutations in *FGF8* [14]. Further, Fgf8 and Fgf3 appear to be key regulators in postchordal neurocranium development by stimulating specification of mesoderm-derived progenitors [110]. *fgfr3* expression is observed in ethmoid plates. Further analysis of pre- and postchordal neurocranium development in *fgfr3* LOF mutants would be intriguing given their severe

craniofacial phenotype [4••, 49••]. Anomalies in the post-neurocranium were observed in the triple mutant (*fgfr1a, fgfr1b, and fgfr2*), supporting their involvement in cranial base formation. However, their redundant activity during early craniofacial development impedes the determination of their respective roles. Thus, the development of a zebrafish line expressing *fgfr* GOF mutations could provide insight into their specific roles.

Conclusions

In conclusion, we have underscored the strengths of the zebrafish model, highlighting its close resemblance to mammals in craniofacial skeleton formation. This model proves invaluable tool for elucidating the role of the Fgf signaling pathway in the cellular mechanisms driving developmentand complement mammalian models, as seen in cases like mutant mice showing early lethality while zebrafish models display milder phenotypes (e.g., *fgfr1, fgfr2*, and *fgf10*). Conversely, there are instances where mice show no phenotype, yet the zebrafish model exhibits one, such as craniofacial anomalies in CATSHL syndrome linked to FGFR3. Studies using zebrafish, consistently demonstrate Fgf signaling's involvement at every stage of craniofacial development, from CNCCs to the formation of numerous craniofacial bones. From early investigations to recent ones, conducted during the early stages of zebrafish cranial development, they all emphasize the pivotal role of the Fgf signaling pathway during this stage that profoundly influences the development of various structures constituting the craniofacial skeleton. These data partially account for craniofacial anomalies observed in FGF signaling-related disorders, including cleft palate, cranial base defect or midface hypoplasia. Future research efforts should aim to elucidate the precise roles of specific Fgf ligands and receptors at later stages of craniofacial skeletal formation. Notably, studies utilizing *fgfr3* LOF zebrafish at later stages align with this direction and demonstrate the zebrafish's potential as a valuable tool in understanding FGF signaling-related craniosynostosis. Advancements in genome editing techniques in zebrafish, including base or prime editing, present opportunities to introduce point mutations associated with human pathologies. This will accelerate our comprehension of FGF-related craniofacial skeletal disorders and allow to establish zebrafish model for exploring new therapeutic strategies for these diseases.

Author contributions R.P. and E.D. wrote the main manuscript and prepared figure and tables. All authors reviewed the manuscript

Declarations

Competing interests The authors declare that they have no competing interests.

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- • Of importance
- •• Of major importance

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