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Highlights

Fgf receptors Fgfr1a and Fgfr2 control the function of pharyngeal endoderm in late cranial cartilage development

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- The Fgf pathway is required around 30 hpf for head cartilage formation.
- Receptors Fgfr1a and Fgfr2 are expressed in pharyngeal endoderm beyond 26 hpf.
- Fgf signaling initiates a Runx3, Egr1, Sox9b regulatory cascade in pharyngeal endoderm.
- Fgfr1a and Fgfr2 are required for repression of the BMP inhibitor follistatin A.
Fgf receptors Fgfr1a and Fgfr2 control the function of pharyngeal endoderm in late cranial cartilage development

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A R T I C L E   I N F O

Article history:
Received 15 January 2013
Received in revised form 1 July 2013
Accepted 22 July 2013

Keywords:
Zebrafish
Fgf
Signaling
Cartilage
BMP

A B S T R A C T

Cranial cartilage derives mainly from cranial neural crest cells and its formation requires fibroblast growth factor (Fgf) signaling for early differentiation and survival of developing chondrocytes as well as patterning of the endodermal pouches.

Here, we investigate the role of Fgf receptors in chondrocyte maturation at later stages, beyond 24 hpf. Using inducible expression of a dominant-negative Fgf receptor, we show that Fgf signaling is required around 30 hpf for correct cartilage formation. The receptor genes fgfr1a and fgfr2 are expressed in pharyngeal endodermal pouches after 24 hpf or 26 hpf, respectively. Depletion of any of these two receptors by microinjection of antisense morpholinos results in severe defects in cartilage formation at 4 dpf and a decrease in expression of the late chondrocyte markers barx1 and runx2b. Although endodermal pouches are correctly formed and patterned, receptor knock down leads to decreased expression of runx3, egr1 and sox9b in this tissue, while expression of fsta, coding for a secreted BMP/Fgfr inhibitor, is clearly increased. Rescue experiments revealed that each Fgfr1a or Fgfr2 receptor is able to compensate for the loss of the other.

Thus, we show that minimal amounts of Fgfr1a or Fgfr2 are required to initiate a regulatory cascade in pharyngeal endoderm reducing expression of fsta, thereby allowing correct BMP signaling to the maturing chondrocytes of the head cartilage.

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1. Introduction

Craniofacial bone structures result from both membranous and endochondral or perichondral ossification, the latter requiring preliminary formation of a cartilaginous matrix. Pharyngeal cartilages derive from migration and differentiation of cranial neural crest cells (cNCC) within the pharyngeal arches. These cNCCs are formed at the neurulation stage and migrate in three streams into the seven pharyngeal pouches to form the different cartilage elements of the viscerocranium (mandible, hyoid, five ceratobranchial arches) (Schilling and Kimmel, 1994; Knight and Schilling, 2006). During and after this migration, the cNCCs undergo several differentiation steps to finally give rise to hypertrophic chondrocytes and osteogenic cells. After initial expression of fhp2u characteristic to all cNCC cells (Barrallo-Gimeno et al., 2004), ectomesenchymal cartilage precursors are identified by early expression of db2a (Sperber et al., 2008) while chondrogenic differentiation is characterized by the onset of sox9a expression required for production of the cartilage-specific collagen Col2a1 (Kluver et al., 2005; Yan et al., 2005). Finally, maturing hypertrophic chondrocytes express runx2b (Flores et al., 2008; Flores et al., 2004), a marker that is also present in bone-forming osteoblasts.

In the arches, the cNCCs are surrounded by and interact with different tissues such as pharyngeal endoderm and ectodermal epithelium. In casanova (cas) mutant zebrafish, endoderm is lacking (Alexander et al., 1999) and pharyngeal cartilages are not formed (David et al., 2002). Loss of function of several genes expressed in pharyngeal endoderm, such as egr1, runx3 or sox9b leads to severe reduction of head cartilage at 4 dpf (Dalcq et al., 2003; Kluver et al., 2005; Yan et al., 2005; Flores et al., 2006). Thus, interaction between endoderm and cNCCs is primordial for the correct formation of pharyngeal cartilage (Crump et al., 2004; Piotrowski and Nusslein-Volhard, 2000; Schilling et al., 1996) involving signaling pathways initiated by Bmp, Fgf or Hh ligands (Golding et al., 2006; Walshe and Mason, 2003). Recently, BMP signaling was shown to be required for early ventral arch development, upstream and simultaneously to endothelin1 (Edn1) (Alexander et al., 2011). An additional role for craniofacial patterning at later stages was also shown. We recently showed the existence of a regulatory cascade formed by the three transcription factors Runx3–Egr1–Sox9b, each being required for expression of the next, in pharyngeal endoderm at 30 hpf (Dalcq et al., in press).
This cascade controls late chondrogenesis by down-regulating expression of Follistatin A (fsta), a known antagonist of BMP signaling, thereby allowing correct activation of the BMP pathway required to activate runx2b expression in developing chondrocytes.

Fibroblast growth factor (Fgf) signaling is involved in proliferation, migration and specification of many cell types (Ornitz and Itoh, 2001; Walsh and Mason, 2003; Thissen and Thissen, 2005). It is highly conserved across different species (Itoh, 2007; Itoh and Ornitz, 2008) and is initiated by numerous Fgf ligands binding specific tyrosine kinase (RTK) receptors (Fgfrs). In mice, Fgf9 controls early hypertrophic chondrocyte differentiation (Hung et al., 2007) while in zebrafish, Fgf2a and Fgf8 are produced in pharyngeal endoderm and ectoderm and control segmentation of the pharyngeal endoderm and survival of CNCCs (Crump et al., 2004, 2005). The downstream target of Fgf signaling in the pharyngeal endoderm is Runx3 (Walshe and Mason, 2003, 2006). In humans, mutations in Fgfrs causing either increased or decreased Fgf signaling, generate craniofacial malformations resulting from deficient chondrogenesis (e.g. Apert syndrome, Crouzon syndrome, Pfeiffer syndrome, Kallmann syndrome 2, Jackson–Weiss syndrome) (Nie et al., 2006; Baldridge et al., 2010). In mice, fgfr1 controls endoderm patterning in the pharyngeal region and plays a crucial role in CNCC migration into the branchial arches (Trokovic et al., 2003). Different studies in zebrafish have shown that inhibition of Fgf signaling by SU5402 generates embryos lacking pharyngeal cartilage at 4 days post fertilization (dpf) and down-regulates expression of genes known to be crucial for chondrogenesis (Walshe and Mason, 2003; Spéder et al., 2008).

In zebrafish, the role of Fgf signaling in head cartilage formation was mainly studied by blocking the pathway at very early stages and thus possibly affecting multiple functions of this versatile signaling during early development. Here, we used heat-shock controlled expression of a dominant-negative Fgf receptor in Tg(hsp70:dnfgr1-EGFP)pd1 transgenic embryos to show a critical stage for Fgf activities in chondrogenesis around 30 hpf. We also show that fgfr1a and fgfr2 are both expressed in pharyngeal endoderm at this stage and we demonstrate that fgfr1a or fgfr2 depletion specifically causes severe cartilage defects at 4 dpf, which can be rescued by concomitant expression of exogenous zebrafish or human Fgf receptors. We further show that these two receptors are required for activation of the Runx3–Fgfr1a/Sox9b–Fsta cascade in the endoderm and for runx2b expression in developing chondrocytes. Finally, the defects in cartilage structure and gene expression observed in morphants for each of the receptors Fgfr1a or Fgfr2 can be rescued by ectopic expression of each of the two receptors, indicating that the exact identity of the receptor active in pharyngeal endoderm is not important, but rather the precise number of receptor molecules.

2. Materials and methods

2.1. Zebrafish maintenance and transgenic line

Adult zebrafish (Danio rerio) and embryos were raised as described (Westerfield, 2007). Embryos were kept in E3 medium at 28 °C and developed until the stages of interest according to Kimmel et al. (1995). The transgenic lines Tg(hsp70:dnfgr1-EGFP)pd1 (Lee et al., 2005) and Tg(sox17-GFP-yfp)Sakaguchi et al., 2006) were obtained from the ZRC (Eugene, Oregon, USA).

2.2. Ethics statement

All experiments and the entire study were evaluated by the Ethical Committee of the University of Liege, Belgium and accepted under the file numbers 377, 568 and 1074.

2.3. Knockdown of fgfr1a and fgfr2

One to two cell-stage embryos were injected with 4 ng of antisense morpholino oligonucleotides (MO, Gene Tools Inc.) complementary to the translational start site of fgfr1a (TMOFgfr1a: 5′-GACAGCGGTGTCCTCATATCAT-3′ (Scholpp et al., 2004) or its 5′ UTR: MOFgfr1a: 5′–CAAGATCTCTACTGATACCC-3′ (Thummel et al., 2006)). Splicing morpholinos targeting, respectively the second or first intron’s donor splice site in the coding region of fgfr1a (5 ng; sMOFgfr1a: 5′–ATTCAGTGTACCTACCTGAACC-3′ (Nakayama et al., 2008) or fgfr2 (4 ng; MO-Fgfr2: 5′–GCTAAATGCCTACCTAGATTCG-3′ were also used. Co-injection of tMOFgfr1a and MOFgfr2 was performed with 2 ng of each morpholino. Morpholinos were diluted in Danieau buffer and Tetramethylrhodamine dextran (Invitrogen, Belgium) was added at 0.5% to verify proper injection of the embryos by fluorescence stereomicroscopy. Standard control morpholino (MOCON) was injected at the same concentrations. The efficacy of the sMOFgfr1a splicing morpholino was tested previously by RT-PCR (Nakayama et al., 2008), while that of sMOFgfr2 was confirmed using the oligonucleotides Fgfr2-MOtest-F: 5′-CTGCTAATGACCCTGCACAG-3′ and Fgfr2-MOtest-R; 5′-AGGCTCTTGTTCCTAGACC-3′ targeting, respectively exon 2 and exon 3. Injection of sMOFgfr2 led to alternative splicing resulting in deletion of 22 nucleotides at the end of exon 2, thus coding for a truncated and inactive protein (Movie S1, Fig. S1H). Although no increase of cell death was observed in the Fgfr1a morphants, in absence or presence of co-injection of a morpholino directed against p53, this MOp53 was co-injected in all knockdown experiments to ensure inhibition of MO-induced unspecified cell death (Robu et al., 2007). The effects of morpholino injection were tested on at least 150 individuals, performed in at least three independent experiments.

2.4. Rescue experiments

Human FGFR1 mRNA was synthesized using mRNAExpress mRNA Machine Sp6 Kit (Ambion, TX, USA) from the IMAGE full length cDNA clone IRATp970D1237D (IMAGE ID: 3896359). The clone was digested using NotI. 80 pg of pZL1-zfgfr2 (ZDB-GENE-030323-1) digested using NotI. 80 pg of Human FGFR1 mRNA was synthesized using mRNAExpress mRNA Machine Sp6 Kit. 100 pg/egg of this mRNA were injected alone or with morpholino directed against Fgfr receptors.

2.5. Whole-mount in situ hybridization

Wild type and injected embryos were raised in presence of 0.003% of 1-phenyl-2-thiourea (PTU) until the desired stages, fixed for 2 h in 4% PFA and dehydrated in 100% methanol for storage at −20 °C. Embryos were rehydrated in PBS and whole mount in situ hybridization was performed as described and adapted from Dalcq et al. (2004). Antisense probes were labeled with digoxigenin or DNP (2,4-dinitrophénol). Anti-digoxigenin-AP was used with NBT/BCIP for single color in situ hybridization; anti-digoxigenin-HRP and anti-DNP-HRP were used with tyramide-Cy3 (Red) and tyramide-FITC (green) for the double fluorescent in situ hybridizations (Perkin-Elmer TSA Kit). The Fgfr1a (ZDB-GENE-980526-255) and Fgfr2 (ZDB-GENE-030323-1) riboprobes were prepared from cDNA clones with Sp6 and T7 RNA polymerase. Other probes used were barx1 (ZDB-GENE-050522-28) (Spéder and Dawid, 2008), dlx2a...
Fig. 1. fgfr1a is expressed in pharyngeal endoderm beyond 24 hpf. Sample in situ hybridization (ISH) using an fgfr1a probe of wt embryos at 24 (A), 48 (B) and 72 hpf (C); lateral views, anterior to the left. Single confocal sections of double fluorescent in situ hybridizations for expression of fgfr1a (red) and dlx2a (green) (D) or nkk2.3 (green) (E) at 24 hpf; sox9a (green) at 43 hpf (F) or nkk2.3 (green) at 43 hpf (G); (C) confocal projection for nkk2.3 (green) and fgfr1a (red) at 48 hpf in the domain delineated by the white rectangle in (G). Ventral views, anterior to the right (D–G). fgfr1a expression is detected in the pharyngeal region (pr), the midbrain/hindbrain boundary (MHB), the optic stalk (os) and the olfactory placode (op) at 24 hpf (A), fgfr1a mRNA is still observed in brain and in the pharyngeal endoderm (pe) at 48 (B) and 72 hpf (C). In the pharyngeal region at 24 hpf, fgfr1a (red) expression domains surround those of dlx2a (green) (D), while they overlap with those of nkk2.3 (green) cells in pharyngeal endoderm (E, yellow arrows). At 43 hpf, no colocalisation is observed between sox9a (green) and fgfr1a (red) (G); fgfr1a (red) continues to coexpress with nkk2.3 (green) in endodermal pouches (pe) at 48 hpf (G′; yellow arrows). Movies S1–S3: migration stream of nCC. Scale bar: 150 μm.

2.6. Alcian blue staining

To observe cartilage at 4 dpf, the embryos were stained with Alcian blue (Sigma, Bornem, Belgium) as described (Schilling et al., 1996).

2.7. Acridin orange staining

To detect unspecific cell death possibly due to morpholino injection, the treated larvae were exposed to a solution of acridin orange 5 μg/ml followed by 5 washing steps in E3 and finally observation of the fluorescent cells at 502 nm excitation.

2.8. Heatshock conditions

Tg(hsp70:dnfgr1-EGFP)pd1 embryos were heat shocked during 30 min at the desired stages by transferring into preheated E3 at 37 °C in a water bath. After that, the embryos were returned to 28 °C until the desired stages were reached. After 24 h, the embryos were screened for GFP fluorescence to sort transgenic individuals from their non-transgenic siblings, only strongly fluorescent individuals were used for the experiment.

2.9. Immunohistochemistry for MAPK phosphorylation

For immunohistochemistry, embryos were fixed in 2% PFA/Pipes 0.1 M/MgSO4 1 mM/EGTA 2 mM, pH=7 over night at 4 °C. After washing with PBT (PBS with Triton X100 0.3%), embryos were permeabilized with acetone at −20 °C. Then, samples were washed 4 times in PBT 0.3% and endogenous peroxidases were inhibited during 45 min with PBT 0.3%/H2O2 2% followed by 4 washes in PBT 0.3%. Finally, embryos were incubated in PBT 0.3%/4% BSA during 2 h at room temperature followed by incubation with P-MAPK antibodies 1/2000 in PBT 0.3%/4% BSA (Sigma M8159) overnight at 4 °C. Next, embryos were washed...
6 × 30 min in PBTr 0.3 and incubated 1 h in PBTr 0.3/4% BSA. The blocking solution was replaced by blocking solution supplemented with a 500-fold dilution of the secondary antibody from Vectastain ABC Elite Mouse kit (Vector Laboratories) and incubated overnight at 4 °C. Then, embryos were washed 6 times for 30 min in PBTr 0.3. Next, they were incubated 1 h in A+B solutions from Vectastain Kit (15 μL/mL). After 3 washes of 15 min in PBTr 0.3, embryos were incubated in amplification diluent from TSA Molecular Probe kit (Perkin Elmer) for 5 min. Colorimetric staining was performed with Tyramide-FITC (1/1000) or Tyramide-Cy3 (1/500) in amplification diluent during 45 min at room temperature in the dark and, finally, the embryos were washed 5 times in PBTr 0.3.

2.10. Imaging and analysis

Visible in situ hybridization and Alcian blue pictures were acquired on a Nikon Eclipse 90i microscope using NIS-Elements microscope imaging software. Fluorescent in situ images were captured on a Leica TCS SP2 confocal using LCS Leica SP2 software. Pictures and Z-stacks were analyzed with ImageJ; the same adjustments were used for all pictures from a same data group.

3. Results

3.1. fgfr1a and fgfr2 are expressed in pharyngeal endoderm.

Zebrafish fgfr1a expression was previously reported in brain, somites and in the pharyngeal region until 24 h post fertilization (Röhner et al., 2009; Schilling et al., 1996; Scholpp et al., 2004; Ota et al., 2010). We confirmed and analyzed more precisely fgfr1a expression in the pharyngeal region between 24 hpf and 72 hpf by simple ISH and by double fluorescent ISH with specific probes for fgfr1a, dlx2a, nklx2.3 and sox9a. At 24 hpf, fgfr1a expression is observed in the Midbrain/Hindbrain Boundary (MHB), optic stalk, olfactory placode and pharyngeal region (Fig. 1A), as expected. Moreover, fgfr1a mRNA co-localized with that for nklx2.3 in pharyngeal endoderm at 24 hpf (yellow arrows; Fig. 1E; Movie S1), but scarcely with dlx2a mRNA in cranial neural crest cells (cnCC) (Fig. 1D; Movie S2); areas of fgfr1a expression in the pharyngeal region mainly surround those of dlx2a. At 43 hpf, the sox9a expression domain in cnCC is mainly surrounded by fgfr1a expression domains in the endodermal pouches (Fig. 1F; Movie S3).

fgfr1a is still expressed at 48 hpf and 72 hpf in brain and in endodermal tissues (Fig. 1B, C, G, G′). Colocalisation of nklx2.3 and fgfr1a expression at 48 hpf is apparent in endodermal pouches in the pharyngeal region (yellow arrows; Fig. 1G, G′; Movies S4 and S5).

Expression of fgfr2 is detected in wild type 24 hpf embryos in hindbrain rhombomeres (R1–4), the tectum, the optic stalk and olfactory placodes and weakly in the pharyngeal region (Fig. 2A). Expression increases in the pharyngeal region at 26 hpf in the most posterior arch and was present until 72 hpf (Fig. 2B–D), while its expression in the brain was maintained during these developmental stages. At 38 hpf, fgfr2 mRNA was observed in the same cell type than nklx2.3 i.e. in endodermal pouches of the pharyngeal region (yellow arrows; Fig. 2E; Movie S6). No coexpression of fgfr2 mRNAs was observed with those of sox9a in cnCC of the pharyngeal region at 72 hpf (Fig. 2G; Movie S8); the fgfr2 expression domains surround those for sox9a and correspond to pharyngeal endoderm. Double fluorescent ISH using probes for fgfr1a and fgfr2 confirms co-expression of these two genes in endodermal pouches and in otic vesicles at 48 hpf (yellow; Fig. 2F; Movie S7).

In conclusion, both fgfr1a and fgfr2 are predominantly expressed in cells of the pharyngeal endoderm.

3.2. Viscerocranium cartilage formation requires Fgf signaling beyond 24 hpf

To determine the importance of Fgf signaling specifically at stages beyond 24 hpf, we used Fgfr1 translation initiation codon (Nakayama et al., 2008) caused an absence of ceratobranchial arches, a strong reduction of mandible and hyoid (types B and C) through increasingly affected viscerocranium with a complete absence of ceratobranchials, a reduction of Meckel’s cartilage and palatoquadrate, axis modification or absence of ceratohyoid (types D and E) to finally only remnants of the neurocranium (type F). Embryos that underwent heat shock at 26 or 30 hpf presented the highest proportion (Fig. 3G) of strongly affected cartilage structures. Heat shock treatments at earlier (before 25 hpf) or later stages (beyond 40 hpf) led to a lower proportion of highly affected larvae. The extent of MAPK phosphorylation was drastically reduced 15 h after heat shock treatment both at 24 and at 28 hpf (Movie S1, Fig. S1A–D), showing that Fgf signaling was similarly reduced in both cases. Siblings, identified as non-transgenic by the absence of GFP fluorescence formed normal head cartilage after any of the heat shock treatments, proving that the observed defects are indeed due to impairment of Fgf signaling by the transgene.

These observations indicate that Fgfr signaling plays a crucial role for pharyngeal cartilage formation between stages 26 and 40 hpf.

3.3. Both Fgfr1a and Fgfr2 receptors are required for cranial cartilage formation

To determine the effect of fgfr1a and fgfr2 loss of function, we assessed cartilage formation in morpholino-injected larvae at 4 dpf by alcian blue staining. Injection of tMOFgfr1a, directed against the fgfr1 translation initiation codon (Nakayama et al., 2008) caused an absence of ceratobranchial arches, a strong reduction of mandible and hyoid and the observed defects were not caused by unspecific apoptosis (not shown). In addition, no increase of apoptotic or necrotic cells was observed in the morphants by acridine orange staining, with or without co-injected MOP53 (not shown). Nevertheless, we performed all subsequent knock-down experiments by co-injecting MOP53. To further confirm the specificity of the observed effects for Fgfr1a depletion, we co-injected tMOFgfr1a and mRNA coding for human FGF1R1 into one-cell stage eggs. At 4 dpf, the proportion of strongly affected embryos had considerably decreased, whereas 48% (n = 146/305) of the embryos presented only slight modifications (Fig. 4C).

For further experiments, the alcian blue stained larvae were classified according to their cranial cartilage pattern, from wt (Type A, Fig. 5A), mildly affected (Fig. 5B), strongly affected head cartilage but with ceratobranchial arches still present (Fig. 5C), strongly affected and lacking ceratobranchial arches (Fig. 5D) to only remnants of the neurocranium (type E, Fig. 5E). Finally, type F
corresponds to a slightly more intensely stained cartilage and an abnormal position of the ceratohyals (Fig. 5F). The proportions of each observed phenotype at the indicated conditions are summarized in the form of a table (Fig. 5G).

Two additional morpholinos targeting \textit{fgfr1a} mRNA were tested, one directed against the 5’ UTR region of \textit{fgfr1a} (5’ UTR MOFgfr1a) and one splicing morpholino (sMOFgfr1a). Microinjection resulted in 42% and 47% of type D cartilage, respectively for sMOFgfr1a and 5’ UTR MOFgfr1a (Fig. 5G). Microinjection of a splicing morpholino directed against \textit{fgfr2} mRNA (MOFgfr2) resulted in less severe cartilaginous defects than those injected with MOFgfr1a. Only 16% of Fgfr2 morphants are strongly affected (type D), while 65%...
presented a type C head cartilage (Fig. 5G). Evaluation of MAPK phosphorylation revealed that both tMOFgfr1a and MOFgfr2 injection lead to a significant decrease of Fgf signaling at 30 hpf (Movie S1, Fig. S1E–G). Co-injection of mRNA coding for zebrafish Fgfr2 together with MOFgfr2 resulted in rescue of the severe defects, with larvae presenting normal (type A) or mildly affected (type B) head cartilage. Ectopic expression of either human FGFR1 or zebrafish Fgfr2 alone generated larvae with normal or mildly affected head cartilage (type A, B; Fig. 5) or with slightly hypertrophic cartilage elements (14% and 25%, respectively) (Fig. 5F, G). Co-injection of tMOFgfr1a and MOFgfr2 generated respectively 52% of type C, 38% of type D and 12% of type E cartilage. Efficacy of the splicing morpholinos was verified by RT-PCR on mRNA from 48 hpf morphants (Movie S1, Fig. S1H). Taken together, these results clearly suggest that both Fgfr1a and Fgfr2 are crucial for viscerocranium formation during chondrogenesis.

Please cite this article as: Larbuisson, A., et al., Fgf receptors Fgfr1a and Fgfr2 control the function of pharyngeal endoderm in late cranial cartilage development. Differentiation (2013), http://dx.doi.org/10.1016/j.diff.2013.07.006

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**Fig. 3.** Inhibition of Fgf signaling beyond 24 hpf perturbs craniofacial cartilage formation. Alcian blue staining at 4 dpf of transgenic Tg(hsp70:dnFgfr1-eGFP)ped1 embryos after a heat shock at 37 °C during 30 min starting at the indicated times after fertilization. (A–F) Ventral views, anterior to the left. Six different phenotypes are observed, ranging from (A) wild type like, all cartilaginous structures are present through (B) all structures are present but reduced in size, (C) all structures are present but a strong modification of the ceratohyoid axis and a reduction of the mandible size is observed, (D) strong modifications of the ceratohyoid and the mandible and absence of ceratobranchial arches, (E) remnants of the viscerocranium and defects in the neurocranium to (F) no viscerocranium and strongly affected neurocranium. m: Meckel’s cartilage; pq: palatoquadrate; ch: ceratohyoid; hs: hyosymplectic; n: neurocranium; cb1-5: ceratobranchial 1–5. (G) The proportion of strongly affected cartilage (D–F) dramatically increases when heat shock is performed between 26 hpf and 30 hpf, while treatment after 40 hpf causes only weak cartilage defects (B, C).

**Fig. 4.** Knockdown of fgfr1a expression generates cartilage defects at 4 dpf. Ventral view, anterior to the left. Wild type and control MO injected embryos display a normal growth of the different pieces of pharyngeal cartilage (A). At this stage, Meckel’s cartilage (m), palatoquadrate (pq), ceratohyoid (ch), hyosymplectic (hs) and ceratobranchials (cb1-5) are formed. In tMOFgfr1a morpholino injected embryos, we predominantly observe an absence of the ceratobranchial cartilage, a dorsal modification of the ceratohyoid axis and a reduction of the mandible (B) (47%). When mRNA for human FGFR1 is coinjected with tMOFgfr1a, all pharyngeal cartilage pieces are restored (C; 38%).
Fig. 5. Cranial cartilage structures observed in 4 dpf larvae previously injected with fgfr1a and fgfr2 morpholinos and/or mRNA. At this stage, Meckel's cartilage (m), palatoquadrate (pq), ceratohyoid (ch), hyosymplectic (hs) and ceratobranchials (cb1-5) are formed. Panels A–F illustrate the different cartilage types that were observed in these experiments, lateral and ventral views, anterior to the left. (A) Wild type and control Mo injected embryos display a normal growth of the different pieces of pharyngeal cartilage. (B) Mildly affected cartilage. (C) Strongly affected head cartilage with ceratobranchial arches still present, (D) Strongly affected cartilage also lacking ceratobranchial arches. (E) Only remnants of the neurocranium are observed. (F) Embryos displaying a stronger staining of cartilage and an abnormal position of the ceratohyal. (G) Table summarizing the proportions of each type of head cartilage pattern observed in the indicated experiments. Column “n” indicates the number of observed individuals for that particular experiment. Bold numbers indicate the highest proportion of cartilage phenotype in each experiment and red numbers concern the increased cartilage phenotype.
3.4. Early neural crest cell differentiation does not require Fgfr1a and Fgfr2

To test whether one receptor can rescue depletion of the other, we co-injected fgfr2 mRNA into tMOFgfr1a morphants, leading to rescue of the observed defects with a decrease in the proportion of strongly affected larvae (type D, from 49% to 8%; Fig. 5G) and an increase of embryos with only slight modifications of cartilage structure (type B, from 18% to 66%; Fig. 5G).

To investigate the function of fgfr1a and fgfr2 genes in early differentiation of neural crest cells, we performed ISH on morphants using probes for dlx2a, a marker of migrating and post migratory NCs (Sperber et al., 2008; Akimenko et al., 1994) and for sox9a, a transcription factor involved in chondrocyte maturation (Yan et al., 2005). In all experiments, the expression patterns of dlx2a (Fig. 6A–F; Movie S3, Fig. S3) and sox9a (Fig. 6G–L; Movie S2, Fig. S2; Movie S3, Fig. S3) remained unaffected both in Fgfr1a and Fgfr2 morphants at 24 and 48 hpf, although a slight decrease of dlx2a expression was observed at 48 hpf in branchial arches 1 and 2 in both fgfr1a and fgfr2 morpholino-injected embryos (Fig. 6D–F). Double fluorescent in situ hybridization in control embryos at 24, 30 and 48 hpf revealed that dlx2a-expressing cells are intermingled with those expressing sox9a in the pharyngeal arches, with patches of cells expressing only one of these factors neighboring domains of cells expressing both mRNAs (Movie S3, Fig. S3 A, D, G). Moreover, the expression and colocalisation domains of sox9a and dlx2a mRNAs are not affected in fgfr1a or fgfr2 morphants (Movie S3, Fig. S3 A–L) although a slight reduction of their expression domains is observed at 48 hpf. Similarly, expression of GFP in flt-GFP transgenic embryos was not affected at 48 hpf after injection of tMOFgfr1a or MOFgfr2, although Fgfr1a knockdown led to perturbations in the branchial arches (Movies S4, Fig. S4). The absence of substantial modifications in dlx2a, sox9a and flt1 expression in morphants suggests that migration of neural crest cells and their first steps of differentiation within the endodermal pouches are normal.
3.5. *Fgfr1a* and *Fgfr2* are required for condensation and late differentiation of chondrocytes

Barx1 is a transcription factor involved in condensation of cranial neural crest cells whose expression was shown to be controlled by Fgf signaling (Sperber and Dawid, 2008). Moreover, *barx1* expression is essential for *runx3* expression, a transcription factor absolutely required for chondrocyte maturation (Flores et al., 2006; Flores et al., 2004) in the pharyngeal region. At 48 hpf, *barx1* mRNA is detected in chondrocytes of the ceratobranchial arches, the hyosymplectic and the ceratohyal in control embryos (Fig. 7A) (Sperber and Dawid, 2008). At this stage, *fgfr1a* or *fgfr2* knock-down caused a reduction of *barx1* expression, to different extents (Fig. 7B, E).

At 48 hpf, *runx2b* mRNAs are observed in the mandible, the hyoid, the ceratobranchial arches, the ethmoid plate and in the cleithrum (Fig. 8A). In *Fgfr1a* or *Fgfr2* morphants, all *runx2b* expression domains are absent in the pharyngeal region except 1

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Please cite this article as: Larbuisson, A., et al., Fgf receptors *Fgfr1a* and *Fgfr2* control the function of pharyngeal endoderm in late cranial cartilage development. Differentiation (2013), http://dx.doi.org/10.1016/j.diff.2013.07.006
for its expression in the cleithrum (Fig. 8B–D). Rescue experiments reveal that co-injection of tMOFgfr1a with human FGFR1 mRNA or zebrafish fgfr2 restores wild type runx2b expression in 61% (n=149/245) and 67% (n=131/196) of the larvae, respectively (Fig. 8E, F). Similarly, MOFgfr2-injected embryos recover expression of runx2b in the pharyngeal region when FGFR1 mRNA or mRNA fgfr2 is co-injected, respectively in 61% (n=134/220) and 65% (n=172/264) of injected embryos (Fig. 8G, H). These data suggest that the two receptors are involved in condensation and late maturation of chondrocytes.

3.6. Fgfr1a and Fgfr2 are not essential for formation of the endodermal pouches

To determine the function of the two receptors in endodermal pouch formation, we performed in situ hybridization with the endodermal marker nksx2.3 (Lee et al., 1996) on both morphants. At 24 and 48 hpf and in comparison with control embryos, loss of function of fgfr1a or fgfr2 does not affect nksx2.3 expression, only a modification of the endodermal pouch patterning was observed in the ceratobranchial 5 (cb5) region (arrows; Fig. 9E, F) at 48 hpf. We also observed that Fgfr1a depletion does not affect fgfr2 expression and conversely Fgfr2 depletion does not affect fgfr1a expression in the pharyngeal endoderm at 48 hpf (Fig. 10). Similarly, injection of both morpholinos into sox17-GFP (Sakaguchi et al., 2006) transgenic embryos revealed that expression of this endodermal marker is maintained, albeit again displaying an aberrant patterning of the cb5 pouches at 48 hpf (Movie S5, Fig. S5 B, C).

These results suggest that both Fgfr1a and Fgfr2 are not required for formation of the pharyngeal endoderm, but contribute to its correct shaping at 48 hpf.

3.7. Fgfr1a and Fgfr2 activate the Runx3–Egr1–Sox9b–fsta cascade in pharyngeal endoderm

Recently, a regulatory cascade was described in zebrafish pharyngeal endoderm involving the transcription factors Runx3–Egr1–Sox9b (Dalcq et al., in press) that inhibits expression of Follistatin A, a BMP inhibitor. This down-regulation allows the full activity of BMP signaling required for expression of runx2b in cartilage mesenchyme. We investigated the effect of Fgfr1a or Fgfr2 depletion on this cascade.

In control embryos, runx3 expression was observed at 48 hpf in pharyngeal endoderm, the cleithrum and the trigeminal ganglion (Fig. 11A, C), as expected (Flores et al., 2006). Knockdown of each receptor decreases runx3 expression in the pharyngeal endoderm (Fig. 11B, D), while its expression in the cleithrum and in the trigeminal ganglia is still present. This effect is specific, as co-injection of MOFGfr2 with fgfr2 or human FGFR1 mRNA caused respectively 87% (n=47/54) or 85% (n=59/70) of larvae to recover runx3 expression in the pharyngeal endoderm.

At 72 hpf, egr1 expression can be detected in oral epithelium, in the brain and in the pharyngeal endoderm of control embryos (Fig. 11G, I). Fgfr1a and Fgfr2 morphants showed an absence of egr1 expression in the pharyngeal endoderm whereas the other expression domains remain unchanged (Fig. 11H, J).

At 48 hpf, sox9b mRNA is localized in pharyngeal endoderm, in the hindbrain, in the tectum and in the pectoral fin bud in control embryos (Fig. 11K, M) (Yan et al., 2005). In situ hybridizations indicate that Fgfr1a or Fgfr2 knock down leads to an absence of sox9b expression in the endodermal pouches (Fig. 11L, N) and a decrease in the mandibular and hyoid region, whereas expression in the hindbrain is maintained.

Sox9b was shown to repress expression of the secreted BMP inhibitor follistatin A in pharyngeal endoderm, thereby allowing correct BMP signaling to the developing chondrocytes (Dalcq et al., in press). In control embryos, we observed weak fsta expression in the pharyngeal region at 48 hpf (Fig. 11O, Q), while fsta transcription was strongly upregulated when tMOFGfr1a or MOFGfr2 were injected into one-cell stage embryos (Fig. 11P, R).

To further support the involvement of Fgfr1a and Fgfr2 receptors in activation of the endodermal regulatory cascade, we tested whether ectopic expression of Egr1 or Runx3 would be able to rescue the cartilage defects in Fgfr morphants. Injection of either egr1 or runx3 mRNA together with tMOFGfr1a or MOFGfr2 resulted in a drastic decrease of the severe phenotypes observed in Fgfr morphants (table in Fig. 5G). In 48 hpf embryos, co-injection of runx3 mRNA with tMOFGfr1a resulted in partial (43%, n=57/133) or near complete (52%, n=69/133) rescue of runx2b expression (Fig. 12A–D). Ectopic Runx3 expression partially (33%, n=38/115)
or completely (62%, n=71/115) rescued Fgfr2 knockdown, while injection of runx3 mRNA alone had no effect (Fig. 12E–H). Similarly, injection of runx3 mRNA rescued the loss of barx1 expression caused by tMOFgfr1a (100% rescue, n=48/48) or MOFgfr2 (100% rescue, n=64/64)(Fig. 7C, F).

These results show that in pharyngeal endoderm, both Fgfr1a and Fgfr2 control the endodermal cascade involved in chondrogenesis.

4. Discussion

4.1. The receptors Fgfr1a and Fgfr2 are required for late differentiation of pharyngeal chondrocytes

The development of head cartilage, deriving from cranial neural crest cells and forming the matrix for subsequent endochondral bone formation, depends on a complex and precisely controlled interplay between different extracellular signaling pathways. Among these, Fgf signaling has been extensively studied in zebrafish. Fgf3 and Fgf8 ligands produced in the hindbrain and lateral mesoderm are required during early segmentation stages for correct organization of the endodermal pouches (Crump et al., 2004). Later, Fgf signaling is required in cNCCs for their conversion into the eotomesenchymal lineage, characterized by dbx2a expression, after migration into the endodermal pouches (Blentic et al., 2008). Endodermal expression of fgf3 is required for dbx2a expression in post migratory cNCCs and their survival in the posterior arches (David et al., 2002; Nissen et al., 2003), while loss of function of both Fgf3 and Fgf8 affect differentiation and survival of cNCCs as well as dbx2a expression in all the arches (Crump et al., 2004; Walshe and Mason, 2003). Thus, Fgf signaling acts during segmentation stages before 24 hpf on pharyngeal cartilage formation through cell-autonomous mechanisms within cNCC-derived chondrocyte precursors and indirectly through patterning of the pharyngeal endoderm.

In this study, we examined the role of Fgf signaling during craniofacial cartilage formation using the transgenic line Tg (hsp70l:dnfgfr1-EGFP)pd1 which allows controlled expression of a dominant negative Fgf receptor mutant (Ota et al., 2010). We show an additional function for Fgf signaling at later stages in chondrogenesis, which is most effective after 30 hpf compared to earlier (24–26 hpf) or later (beyond 40 hpf) stages. At this stage, conversion of cNCCs to eotomesenchyme is finished and the first steps of chondrocyte differentiation, such as sox9a expression, are initiated.

As the dominant negative receptor blocks signaling by interacting with endogenous receptors in the cells where it is expressed, we decided to study the role of Fgfr1a and Fgfr2 receptors by antisense morpholino injection, as both were previously shown to be expressed in the pharyngeal region at 24 hpf (Tonou-Fujimori et al., 2002; Ota et al., 2010). Depletion of each Fgfr1a or Fgfr2 leads to severe defects in cranial cartilage formation, similar to those observed upon Fgf inhibition after 24 hpf. Efficacy of the splicing morpholinos was verified by RT-PCR, while unspecific effects due to morpholino injection were excluded by co-injecting a morpholino against p53 to block unspecific apoptosis (Robu et al., 2007) in all experiments. Results obtained in the presence of MOp53 were similar to those obtained without co-injection (data not shown). In addition, we tested three different morpholinos directed against fgfr1a with similar results and we show that the defects caused by depletion of each receptor are rescued by ectopic expression of exogenous receptor. Furthermore,
ectopic expression of the receptors alone led to either no defect or an increase in cartilage formation.

When we investigated the effect of receptor depletion on chondrocyte differentiation, we observed that expression of *dbx2a* and of *sox9a* is maintained at 24 and 48 hpf. By double fluorescent *in situ* hybridization at 24, 30 and 48 hpf, we observed three types of domains within the ectomesenchymal condensations: one domain mostly consisting of cells expressing both *dbx2a* and *sox9a*, the other two formed of cells expressing either *dbx2a* or *sox9a*. One possible explanation for this observation would be that cells expressing first *dbx2a* only subsequently differentiate into cells expressing both factors and finally into *sox9a*-only expressing cells. Importantly, we did not observe a substantial modification of this combined expression pattern in Fgf receptor morphants, suggesting an absence of effect on developmental timing.

In contrast, expression of the late chondrocyte marker *runx2b* was completely abolished in the pharyngeal arches, indicating that CnCC formation, migration and early differentiation are not affected, while late maturation is absent.

In addition, we observe a decreased expression of *barx1* in the pharyngeal arches of both Fgfr1a and Fgfr2 morphants. In mouse, *Barx1* is expressed in cranio-facial ectomesenchyme and the stomach (Tissier-Seta et al., 1995), it is required for differentiation of the stomach epithelium by controlling expression of Wnt signaling inhibitors and for spleen morphogenesis (Woo et al., 2011). In zebrafish, *barx1* expression was observed in a subset of CnC at 19 hpf, in the three streams of cranial neural crest at 24 hpf and in developing ectomesenchyme at later stages (Sperber and Dawid, 2008). Its expression was abolished in the presence of the Fgf inhibitor SU5402. Morphants for Barx1 displayed a
Fig. 12. Co-injection of runx3 mRNA rescues the defects in tMOFgr1a and MOFgr2 morphants. Lateral views of in situ hybridizations, anterior to the left. At 48 hpf, runx2b expression is detected in the mandible (ma), in the ceratohyoid (ch), in the hyosympletic (hs), in ceratobranchials (cb1-5) and in the ethmoid plate (ep) (A, F) in control embryos, while it is only observed in the cleithrum (cl) in Fgr1a or Fgr2 morphants (B, E). Ectopic expression of runx3 mRNA in Fgr1a morphants restores expression of runx2b in the pharyngeal cartilages partially in 43% (n=57/133) of embryos (C) and completely in 52% (n=69/133) of injected embryos (D). Co-injection of runx3 mRNA and MOFgr2 restores wild type runx2b expression in 62% (n=71/115) of embryos (G), while 33% (n=38/115) of embryos present a decreased expression of runx2b (F). Injection of runx3 mRNA alone does not affect runx2b expression in injected embryos (H). Scale bar: 100 μm.

4.2. Fgfr1a and Fgfr2 control the function of pharyngeal endoderm in head cartilage formation

At this point, it was important to determine the precise tissues that express these Fgf receptors at the stages beyond 24 hpf. We show that both Fgfr1a and Fgfr2 receptors are expressed in pharyngeal endoderm by double fluorescent in situ hybridization revealing their coexpression with the endodermal marker nkx2.3, while their expression domains are clearly distinct from those of cNCC markers such as dll2a or sox9a. We further show that fgr1a and fgr2 expression largely overlaps in pharyngeal endoderm at 48 hpf. This is consistent with the previously described expression for fgr1a in the pharyngeal arches (Ota et al., 2010) and fgr2 in axial mesoderm and endoderm at 24 hpf (Tonou-Fujimori et al., 2002). In our experiments, fgr2 mRNA was weakly detected in the pharyngeal region at 24 hpf, but clearly observed at 26 hpf, suggesting that its endodermal expression starts around this stage. With the observed timing of the requirement for Fgf signaling around 30 hpf, this observation could explain the somewhat weaker effects observed upon Fgfr2 depletion. Earlier requirement for Fgf signaling in cNCCs during segmentation (Blenitc et al., 2008) suggests the presence of an Fgf receptor at this stage in cNCCs, however the exact nature of this receptor was not determined.

Our loss of function studies reveal that both receptors act on the function of pharyngeal endoderm during chondrogenesis. Formation and initial patterning of pharyngeal pouches is not affected, as shown by the intact expression patterns of nkx2.3 and sox17. Only a slight defect is observed at 48 hpf in segmentation of the most posterior pouch, which might arise as a secondary effect due to the defects in cNCC differentiation. In contrast, expression of later markers of endodermal pouches, such as runx3, egr1 and sox9b is severely reduced, indicating that the function of the mature endodermal pouches is abolished. Expression of the three transcription factors runx3, egr1 and sox9b was recently shown to be required in pharyngeal endoderm to reduce expression of fsta, coding for the BMP inhibitor follistatin (Dalcaq et al., 2006). Consistent with this model, we observed a clear increase of fsta expression in Fgfr1a and Fgfr2 morphants. Furthermore, the defects observed in our receptor morphants could be rescued by expression of exogenous Runx3 or Egr1, indicating that the loss of this endodermal regulatory cascade is the major cause for cartilage defects in Fgfr1a or Fgfr2 morphants. Taken together, these observations suggest that the two Fgf receptors are required to initiate the regulatory cascade in pharyngeal endoderm that controls expression of fsta, and thus allows correct BMP signaling to the cNCC.

The various Fgf ligands bind to the different receptors with specific affinities and further variation is brought about by the presence of different splicing isoforms for Fgfr1, 2 and 3 (Zhang et al., 2006; Ornitz and Itoh, 1996). Using the human FGFR isoforms, it appears that the two major Fgfs involved in cartilage formation, Fgfs3 and Fgfs8 are able to bind to at least one isoform of each receptor 1 or 2. The fact that loss of function of each Fgfr1a or Fgfr2 results in cartilage defects points at a non-redundant, specific function for each receptor, which could be brought about by specificity for a particular ligand or specificity in downstream signaling for each receptor. Our results showing that the defects caused by lack of one receptor can be rescued by expression of the other argue against such specificity. We also show that knockdown of one receptor does not affect expression of the other. Although we cannot completely rule out rescue of specific functions of one receptor by exogenous over expression of the other, we favor the explanation of a requirement for a precise amount of receptors to ensure activation of the regulatory cascade. Further support for this interpretation comes from the similarity of the defects observed upon knockdown of each receptor.

Considering the importance of Fgf signaling for the entire development (Itoh and Ornitz, 2011), specifically during segmentation, for...
formation of the pharyngeal arches (Blentic et al., 2008; Crump et al., 2004), it seems surprising that the effects of fgfr1a or fgfr2 knockdown are not more severe. Indeed, the general morphology of the embryos was not much affected, brain segmentation was normal, and the pattern of the pharyngeal endoderm was close to normal and the first markers for chondrocyte differentiation are present. This is in sharp contrast to the defects observed upon complete Fgf inhibition during segmentation (Crump et al. 2004). Assuming that knockdown of the receptors is probably not complete in our experiments, these observations indicate that the requirement for Fgfr1a and Fgfr2 during these early stages is less stringent and/or that their absence is more efficiently compensated by other members of the Fgfr family, such as Fgfr3.

In conclusion (Fig. 13), we show that Fgfr receptors Fgfr1a and Fgfr2 are expressed in zebrafish endodermal pouches beyond 24 hpf, where they are required to ensure activation of a regulatory cascade in pharyngeal endoderm. This cascade reduces expression of the BMP antagonist fsta, thereby allowing full activity of BMP signaling in the pharyngeal region to induce chondrocyte maturation in head cartilage.

Acknowledgments

This work was supported by the “Fonds de la Recherche Fondamentale Collective” projects 2.4555.99/2.4542.00/2.4561.10, the “Pôle d’Attraction Interuniversitaire (PAI): P5/35, the University of Liège, GAME project; the European Space Agency (ESA) and the Belgian Space Agency Proxem. A.L. was supported by the “Fonds National de la Recherche Scientifique” and the “Fonds Léon Frédéricq”; M.M. is a “Chercheur Qualifié du Fonds National de la Recherche Scientifique”. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We also want to thank the sequencing and imaging platforms as well as the zebrafish facility of the GIGA-R center.

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