Differentiation 86 (2013) 192–206

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

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

Arnaud Larbuisson, Julia Dalcq, Joseph A. Martial, Marc Muller*

Laboratory for Molecular Biology and Genetic Engineering, GIGA-R, Université de Liège, B34, Liège, Belgium

ARTICLE INFO

Article history:
Received 15 January 2013
Received in revised form
1 July 2013
Accepted 22 July 2013
Available online 29 October 2013

Keywords:
Zebrafish
Fgfr
Signaling
Cartilage
BMP

ABSTRACT

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 *fgr2* 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/Tgfß 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.

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 *tfap2a* characteristic to all cNCC cells (Barrallo-Gimeno et al., 2004), ectomesenchymal cartilage precursors are identified by early expression of *dlx2a* (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., 2012; 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 (Goldring 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., 2012).

* Corresponding author. Tel.: +32 4 366 4437; fax: +32 4 366 4198.
E-mail address: m.muller@ulg.ac.be (M. Muller).

0301-4681/$ - see front matter © 2013 International Society of Differentiation. Published by Elsevier B.V. All rights reserved.
Join the International Society for Differentiation (www.isdifferentiation.org)
http://dx.doi.org/10.1016/j.diff.2013.07.006

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; Walshe and Mason, 2003; Thisse and Thisse, 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, Fgf3 and Fgf8 are produced in pharyngeal endoderm and ectoderm and control segmentation of the pharyngeal endoderm and survival of cNCCs (Crump et al., 2004; David et al., 2002; Walshe and Mason, 2003). Five genes for Fgfrs have been identified: Fgfr1-4 (RTK) and Fgfr5/Fgfrl1. In Fgfrl1, the tyrosine kinase domain is replaced by a phosphatase domain: it thus acts as a negative regulator of Fgf signaling (Hall et al., 2006) and is also important for craniofacial cartilage formation (Hall et al., 2006; Trueb and Taeschler, 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 Fgfr 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; Sperber 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(hsp70l:dnfgfr1-EGFP)pd1* transgenic embryos to show a critical stage for Fgfr 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–Egr1–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(hsp70l:dnfgfr1-EGFP)pd1* (Lee et al., 2005) and *Tg(sox17-GFP)*^{s870} (Sakaguchi et al., 2006) were obtained from the ZIRC (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* (tMOFgfr1: 5′-GCAGCAGCGTGGTCTTCATTATCAT-3′ (Scholpp et al., 2004) or its 5′ UTR: MOFgfr1a: 5′-CAAAGATCCTCTACATCTGAACTCC-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′-ATTCAGTTGCATTCTCACCTGTAAC-3′ (Nakayama et al., 2008)) or *fgfr2* (4 ng; MOFgfr2: 5′-GCTCAAATGTCTTACCTTCAGGTGC-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′-CTGCTAATGACCCTGGCAAC-3′ and Fgfr2-MOtest-R; 5′-AGCTGTCTTTGGTCCAGACG-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 (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 unspecific 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 mMessage mMachine 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 *FGFR1* mRNA was injected alone or co-injected with morpholino at the one-cell stage. *fgfr2* mRNA was obtained by digestion of the pZL1-zfgfr2 (ZDB-GENE-030323-1) clone by BamHI and synthesized using mMessage mMachine Sp6 Kit. 100 pg/egg of this mRNA were injected alone or with morpholino directed against Fgf 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., 2012. Antisense probes were labeled with digoxigenin or DNP (2,4-dinitrophénol). Anti-digoxigenin-AP was used with NBT/BCIP for single *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) (Sperber and Dawid, 2008), *dlx2a*