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ScienceDirect
Developmental Biology 308 (2007) 144–157
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

mef2ca is required in cranial neural crest to effect Endothelin1 signaling in zebrafish

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Received for publication 29 March 2007; revised 7 May 2007; accepted 16 May 2007
Available online 24 May 2007

Abstract

Mef2 genes encode highly conserved transcription factors involved in somitic and cardiac mesoderm development in diverse bilaterians. Vertebrates have multiple *mef2* genes. In mice, *mef2c* is required for heart and vascular development. We show that a zebrafish *mef2c* gene (*mef2ca*) is required in cranial neural crest (CNC) for proper head skeletal patterning. *mef2ca* mutants have head skeletal phenotypes resembling those seen upon partial loss-of-function of *endothelin1* (*edn1*). Furthermore, *mef2ca* interacts genetically with *edn1*, arguing that *mef2ca* functions within the *edn1* pathway. *mef2ca* is expressed in CNC and this expression does not require *edn1* signaling. Mosaic analyses reveal that *mef2ca* is required in CNC for pharyngeal skeletal morphogenesis. Proper expression of many *edn1*-dependent target genes including *hand2*, *bapx1*, and *gsc*, depends upon *mef2ca* function. *mef2ca* plays a critical role in establishing the proper nested expression patterns of *dlx* genes. *dlx5a* and *dlx6a*, known Edn1 targets, are downregulated in *mef2ca* mutant pharyngeal arch CNC. Surprisingly, *dlx4b* and *dlx3b* are oppositely affected in *mef2ca* mutants. *dlx4b* expression is abolished while the *edn1*-dependent *dlx3b* is ectopically expressed in more dorsal CNC. Together our results support a model in which CNC cells require *mef2ca* downstream of *edn1* signaling for proper craniofacial development.

Keywords: *mef2c*; *edn1*; Neural crest; Pharyngeal skeleton; Pharyngeal arch; *dlx*; *hand2*; Zebrafish

Introduction

Mef2 (*myocyte enhancing factor*) genes encode MADS domain-containing transcription factors that play critical roles in mesoderm development in invertebrates and vertebrates (reviewed in Black and Olson, 1998). Mammals have four *mef2* genes (*mef2a, mef2b, mef2c,* and *mef2d*). Mice lacking *mef2c* function die during early embryonic development due to severe heart and vascular defects (Bi et al., 1999; Lin et al., 1997, 1998). In addition to cardiac and vascular expression domains, *mef2c* is expressed in postmigratory cranial neural crest (CNC) within pharyngeal arch primordia (Dodou et al., 2004; Edmondson et al., 1994). The early embryonic lethality has, to date, precluded functional analysis in later *mef2c* expression domains such as CNC.

Most of the cartilages and bones of the vertebrate head are derived from CNC cells. In vertebrate embryos, three bilateral streams of CNC populate the first (mandibular), second (hyoid) and the set of more posterior branchial arches, respectively. Within each pharyngeal arch in gnathostome embryos, prominent dorsal and ventral cartilages form, separated by a dorsal–ventral joint. For example, in the first arch, CNC cells

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.05.018

form an upper (dorsal) and lower (ventral) jaw, articulating at the jaw joint. One potential function of the *mef2c* CNC expression domain could be to regulate aspects of pharyngeal skeletal development. A critical signaling pathway that patterns CNC at the stages that *mef2c* has been reported to be expressed is Endothelin1 (Edn1).

Development of the lower jaw in mice, chicks, and fish requires Endothelin signaling (Clouthier et al., 1998; Kempf et al., 1998; Kurihara et al., 1994; Miller et al., 2000; Nair et al., 2007). Edn1, a secreted signaling molecule, is expressed in pharyngeal arch epithelia and mesoderm, and signals to CNC cells, which express the G-protein-coupled transmembrane Endothelin receptor *EdnrA* (reviewed in Clouthier and Schilling, 2004). Genetic analyses in mice and zebrafish have revealed other molecules involved in regulating or transducing the Edn1 signal during craniofacial development. In mice, mutation of *Edn1, EdnrA*, or an endothelin converting enzyme (*ECE1*) gives similar lethal craniofacial phenotypes where the lower jaw is hypoplastic and malformed (Clouthier et al., 1998; Kurihara et al., 1994; Yanagisawa et al., 1998). The proprotein convertase Furin has been shown to biochemically cleave Edn1, and in zebrafish, reducing *furin* function causes phenotypes resembling *edn1* mutants (Denault et al., 1995; Walker et al., 2006). These studies suggest that Edn1 is cleaved twice, first by Furin and second by ECE1 and that these cleavages are required for Edn1 bioactivity. Further upstream of Edn1, the transcription factor Tbx1 is required in zebrafish for *edn1* pharyngeal arch expression (Piotrowski et al., 2003). In humans, mutation of *Tbx1* can cause one of the most common craniofacial defects, DiGeorge syndrome, highlighting the clinical importance of Edn1 signaling in craniofacial development (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001).

Downstream of EdnrA, G-proteins transduce the Edn1 signal, as mice doubly mutant for *GalphaQ* and *Galpha11* have *Edn1*-like craniofacial defects (Ivey et al., 2003). G-protein activation by EdnrA likely leads to activation of Phospholipase C (Plc), because in zebrafish, mutation of *plcβ3* causes phenotypes similar to *edn1* mutants and *plcβ3* and *edn1* interact genetically (Walker et al., 2007). The Edn1 signal eventually activates a transcriptional hierarchy in CNC involving *Dlx5*, *Dlx6*, and *Hand2*(*dHAND*), and nearly all CNC expression of these three genes requires Edn1 signaling (Miller et al., 2003; Ruest et al., 2004; Walker et al., 2006). *Dlx5* and *Dlx6* regulate the CNC expression of *Hand2 (dHAND),* an effector of *edn1* signaling in mice and zebrafish (Charite et al., 2001; Depew et al., 2002; Miller et al., 2003; Ruest et al., 2004; Thomas et al., 1998). A *Hand2* pharyngeal arch enhancer requires *Edn1* signaling, as enhancer expression is absent in the arches of *EdnrA* mutant mice (Charite et al., 2001). Targeted deletion of this *Edn1*-responsive *Hand2* enhancer reveals it to be required for craniofacial development (Yanagisawa et al., 2003). Biochemical analyses of this *Hand2* pharyngeal arch enhancer found that this enhancer binds Dlx6 (but not Dlx2, Dlx3, or Dlx5) (Charite et al., 2001). Thus in mice, Dlx6 appears to participate in transducing the Edn1 signal to *Hand2*.

Dlx genes have nested expression patterns within each pharyngeal arch in mice, many aspects of which are conserved in zebrafish (Depew et al., 2002; Walker et al., 2006). *Dlx1* and *Dlx2* are expressed broadly throughout seemingly all postmigratory pharyngeal arch CNC. *Dlx5* and *Dlx6* are more ventrally restricted within the pharyngeal arch primordia. *Dlx3* and *Dlx4* are even more ventrally restricted. Genetic experiments in the mouse have revealed this *Dlx* code to be of paramount importance in establishing regional identity within each pharyngeal arch (Depew et al., 1999, 2002, 2005; Qiu et al., 1995). *Dlx6*, together with *Dlx5,* specifies ventral identity within the pharyngeal arches. Mice doubly mutant for *Dlx5* and *Dlx6* have a fantastic homeotic phenotype in which the lower jaw is transformed into an upper jaw (Depew et al., 2002).

A similar ventral-to-dorsal transformation is seen in *Edn1* and *EdnrA* mutants (Ozeki et al., 2004; Ruest et al., 2004). In zebrafish mutant for Edn1 pathway genes or with reduced levels of Edn1, a ventral hyoid bone can be homeotically transformed into a more dorsal bone (Kimmel et al., 2003). Gene expression studies show that the dorsally restricted *eng2* expression ectopically expands ventrally in *edn1* mutants (Miller et al., 2003). Furthermore, overexpression of Edn1 protein can cause the reciprocal transformation, where dorsal cartilages appear transformed into ventral cartilages (Kimmel et al., in press). Thus, *Edn1* is a master regulator of lower jaw and other ventral pharyngeal arch fates and in its absence the ventral arch assumes dorsal fates. *Edn1* seems to exert this regulation primarily through *Dlx* genes. Despite this fundamental importance of the *Dlx* genes, little is known about what establishes their nested expression patterns.

Large scale genetic screens in the zebrafish have identified over 100 loci required for head skeletal patterning (Neuhauss et al., 1996; Nissen et al., 2003; Piotrowski et al., 1996; Schilling et al., 1996). One class of mutations was put into an “anterior arch” class for phenotypically similar defects in the anterior pharyngeal arch skeleton, particularly the first and second arches. This anterior arch class contains four loci, *sucker*, *schmerle*, *sturgeon*, and *hoover* (Piotrowski et al., 1996). We have previously shown these first three loci to encode Endothelin1, FurinA, and Plcβ3, respectively (Miller et al., 2000; Walker et al., 2006, 2007).

Within this “anterior arch” class, both strong and weak phenotypes are seen. Strong phenotypes, represented by typical *edn1* and *plcβ3* mutants, include highly penetrant severe reduction of lower jaw and other ventral pharyngeal cartilages, absence of the jaw joint and other dorsal–ventral pharyngeal joints, and loss of ventral pharyngeal bones (Kimmel et al., 1998, 2003; Miller et al., 2000; Walker et al., 2007). Weak phenotypes, represented by typical *furinA* and *hoover* mutants, or partial reduction of Edn1 signaling by morpholino knockdown, include only mild, if any, reductions in ventral cartilage, and incompletely penetrant absence of joints and expansion of hyoid pharyngeal bones (Kimmel et al., 1998, 2003; Miller and Kimmel, 2001; Walker et al., 2006).

Here we present fine mapping, sequencing, and morpholino phenocopy data showing that *hoover* is the zebrafish *mef2ca*