Endothelin signaling activates Mef2c expression in the neural crest through a MEF2C-dependent positive-feedback transcriptional pathway

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ABSTRACT
Endothelin signaling is essential for neural crest development, and dysregulated Endothelin signaling is associated with several neural crest-related disorders, including Waardenburg and other syndromes. However, despite the crucial roles of this pathway in neural crest development and disease, the transcriptional effectors directly activated by Endothelin signaling during neural crest development remain incompletely elucidated. Here, we establish that the MADS box transcription factor MEF2C is an immediate downstream transcriptional target and effector of Endothelin signaling in the neural crest. We show that Endothelin signaling activates Mef2c expression in the neural crest through a conserved enhancer in the Mef2c locus and that CRISPR-mediated deletion of this Mef2c neural crest enhancer from the mouse genome abolishes Endothelin induction of Mef2c expression. Moreover, we demonstrate that Endothelin signaling activates neural crest expression of Mef2c by de-repressing MEF2C activity through a Calmodulin-CamKII-histone deacetylase signaling cascade. Thus, these findings identify a MEF2C-dependent, positive-feedback mechanism for Endothelin signaling. Using genetic and pharmacological approaches, we further show that deletion of the enhancer from the genome of mice completely abolishes Endothelin signaling for proper craniofacial and melanocyte development and, based on the observation that MEF2C and Endothelin signaling regulate several common downstream targets (Agarwal et al., 2011; Ruest et al., 2004; Verzi et al., 2007), suggested that MEF2C functions in the Endothelin signaling pathway in the neural crest. Similarly, mutation of the Mef2c ortholog mef2ca in zebrafish results in craniofacial defects due to disrupted neural crest development (Miller et al., 2007). Moreover, mef2ca was shown to interact genetically with edn1 in zebrafish (Miller et al., 2007), suggesting a role for MEF2C as an effector of Endothelin signaling. However, how MEF2C responds to Endothelin signaling to control neural crest development and how its expression is regulated by Endothelin signaling has not previously been determined.

INTRODUCTION
The Endothelin signaling pathway triggers several intracellular signaling cascades coupled to a wide variety of cellular outputs (Barton and Yanagisawa, 2008; Kedzierski and Yanagisawa, 2001). There are three Endothelin ligands in mammals: Endothelin (ET)-1, ET-2 and ET-3 (encoded by the Edn1, Edn2 and Edn3 genes, respectively). Mature Endothelin peptides bind to and activate two seven-membrane-spanning G-protein-coupled receptors, referred to as ETα (encoded by the Ednra gene) and ETβ (encoded by the Ednrb gene) (Kedzierski and Yanagisawa, 2001). During embryogenesis, the primary role of Endothelin signaling is in the neural crest, a migratory and pluripotent cell population unique to vertebrates (Pla and Larue, 2003). Neural crest cells originate at the dorsal aspect of the nascent neural tube and then delaminate and migrate to many different locations in the embryo, where they differentiate into melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons, glia and other cell types (Knecht and Bronner-Fraser, 2002; Trainor, 2005).

Members of the Myocyte enhancer factor 2 (MEF2) family of MADS box proteins play crucial roles in development and postnatally by functioning as signal-responsive transcription factors (Black and Cripps, 2010; Potthoff and Olson, 2007). Studies performed in the mouse established a requirement for MEF2C for proper craniofacial and melanocyte development and, based on the observation that MEF2C and Endothelin signaling regulate several common downstream targets (Agarwal et al., 2011; Ruest et al., 2004; Verzi et al., 2007), suggested that MEF2C functions in the Endothelin signaling pathway in the neural crest. Similarly, mutation of the Mef2c ortholog mef2ca in zebrafish results in craniofacial defects due to disrupted neural crest development (Miller et al., 2007). Moreover, mef2ca was shown to interact genetically with edn1 in zebrafish (Miller et al., 2007), suggesting a role for MEF2C as an effector of Endothelin signaling.

RESULTS AND DISCUSSION
The Mef2c gene contains an Endothelin-dependent transcriptional enhancer
We previously identified an enhancer from the Mef2c locus with activity in multiple neural crest lineages (Agarwal et al., 2011). This enhancer, referred to as Mef2c-F1, contains a conserved MEF2 consensus site important for autoregulation of Mef2c expression (Fig. 1A; Agarwal et al., 2011), which we hypothesized might be a target of Endothelin signaling, given the possible links between
Endothelin signaling and MEF2C expression and function (Agarwal et al., 2011; Miller et al., 2007; Verzi et al., 2007; Wu et al., 2006). To determine whether the Mef2c-F1 enhancer was regulated by Endothelin, we crossed Mef2c-F1-lacZ transgenic mice to Ednra and Ednrb knockout mice (Fig. 1B-E). As previously described (Agarwal et al., 2011), the Mef2c-F1 enhancer was active in cranial and trunk neural crest populations at E9.5 (Fig. 1B). Interestingly, loss of either Endothelin receptor alone resulted in loss of enhancer activity in the corresponding neural crest population (Fig. 1C,D). Mef2c-F1-lacZ expression was abolished in cranial neural crest but remained present in trunk neural crest in the absence of Ednra (Fig. 1C). Conversely, deletion of Ednrb resulted in loss of Mef2c-F1-lacZ expression in trunk neural crest but did not affect expression in cranial neural crest (Fig. 1D). Importantly, simultaneous loss of both Endothelin receptors resulted in nearly complete loss of Mef2c-F1 enhancer activity (Fig. 1E). We also treated E9.5 Mef2c-F1-lacZ embryo explants with the dual ET_A/ET_B inhibitor bosentan (Clozel and Salloukh, 2005), which also strongly inhibited enhancer activity (Fig. 1F,G).

These data provide genetic and pharmacological evidence for the Endothelin-dependence of the Mef2c-F1 neural crest enhancer. To investigate further the Endothelin-responsiveness of the Mef2c-F1 enhancer, we treated E9.5 Mef2c-F1-lacZ embryo explants with ET-1 or PBS and examined β-galactosidase expression (Fig. 1H-L). A 300-bp minimal enhancer responds to ET-1 (dashed circles); two independent Mef2c-F1[3-3.3]-lacZ transgenic lines were examined, and both showed a similar ET-1 response. Note the absence of β-galactosidase expression in trunk neural crest cells in PBS-treated explants (H) and the complete overlap of β-galactosidase expression with Sox10^+ neural crest cells in ET-1-treated explants (I-K). BA, branchial arch; NT, neural tube. Scale bars: 100 µm.
The *Mef2c*-F1 neural crest enhancer contains a 300-bp minimal region that is sufficient to direct expression in vivo (Agrawal et al., 2011). This minimal enhancer also responded to ET-1 induction (Fig. 1L), establishing that the 300-bp core *Mef2c*-F1 enhancer contains cis-acting elements sufficient to respond to Endothelin signaling. Taken together, the gain- and loss-of-function of Endothelin signaling experiments shown in Fig. 1 establish that Endothelin signaling is required and sufficient for the activation of the *Mef2c*-F1 enhancer.

**Mef2c-F1 is a bona fide *Mef2c* enhancer required for Endothelin-responsiveness**

The location of the *Mef2c*-F1 enhancer in the third intron of the *Mef2c* gene and the similarity in the expression patterns of *Mef2c*-F1-lacZ and endogenous *Mef2c* in the neural crest strongly suggest that *Mef2c*-F1 is an enhancer of *Mef2c*. To test this idea explicitly, we used CRISPR/Cas9 technology to delete the *Mef2c*-F1 enhancer from the mouse genome (Fig. 2A). *Mef2c*Δ-F1 mice were crossed to *Mef2c*Δ/+ mice to generate *Mef2c*Δ-F1Δ transheterozygous mice (Fig. 2B). The *Mef2c*Δ allele contains a deletion in the coding region of *Mef2c*, and *Mef2c*ΔΔ (Mef2c-null) mice die at E10 from profound defects in cardiac morphogenesis (Lin et al., 1997). However, whereas the null allele ablates *Mef2c* protein production, it does not affect *Mef2c* transcription (Fig. 2A; Lin et al., 1997). From the cross depicted in Fig. 2B, wild-type, *Mef2c*Δ+/F1Δ and *Mef2c*Δ/+ mice were all viable and present in predicted Mendelian frequencies (Fig. 2B, and data not shown). By contrast, the majority of *Mef2c*Δ-F1Δ mice died at birth (Fig. 2B), with evidence of cleft palate and airway obstruction due to craniofacial defects (data not shown). This indicates that the heteroallelic combination caused lethality and provides strong genetic evidence that the *Mef2c*-F1 enhancer is a bona fide enhancer of *Mef2c*. The cleft palate phenotype and apparent craniofacial obstruction and neonatal lethality observed in *Mef2c*ΔΔ-F1Δ mice is similar to, but less severe than, the phenotype observed in *Mef2c* neural crest-specific conditional knockout mice (*Mef2c*ΔΔ/ΔΔ), which also die at birth due to cleft palate and craniofacial obstruction (Verzi et al., 2007). The less severe phenotype observed in *Mef2c*ΔΔ-F1Δ mice compared with *Mef2c*ΔΔ/ΔΔ mice is consistent with the fact that *Mef2c* has at least two neural crest enhancers, *Mef2c*-F1 and *Mef2c*-F10N (Aoto et al., 2015; De Val et al., 2008), and, as a result, deletion of *Mef2c*-F1 does not result in a complete loss of *Mef2c* expression in the neural crest.

Given that *Mef2c*-F1 is a bona fide enhancer of *Mef2c* and is Endothelin-responsive, we next tested the induction of endogenous MEF2C protein expression by Endothelin signaling in embryos with the *Mef2c*-F1 enhancer deleted. Embryos were explanted at E9.5 and were treated with ET-1 or PBS (Fig. 2C-F). In wild-type embryo explants, MEF2C protein expression was strongly induced in Sox10+ neural crest cells by ET-1 but not by PBS (Fig. 2C,D). Importantly, ET-1 did not induce detectable MEF2C protein expression in Sox10+ neural crest cells in *Mef2c*ΔΔ-F1Δ mutant embryos (Fig. 2F), further supporting the idea that the *Mef2c*-F1 enhancer is a bona fide enhancer of *Mef2c*. More importantly, these data indicate that Endothelin induction of endogenous *Mef2c* expression requires the *Mef2c*-F1 enhancer.

![Fig. 2. *Mef2c*-F1 is a bona fide Endothelin-responsive enhancer of *Mef2c*. (A) Schematics of the mouse *Mef2c* locus showing exons 4-6 (vertical black lines) and the *Mef2c*-F1 enhancer (green box). The *Mef2c*ΔΔ and *Mef2c*-null (*Mef2c*ΔΔ) knockout strategies are indicated. (B) Number of live and dead offspring of each indicated genotype from *Mef2c*ΔΔ-F1Δ/F1Δ intercrosses. Note that 23/23 wt and heterozygous (het) offspring survived, whereas only 2/7 *Mef2c*ΔΔ/ΔΔ survived (Fisher’s exact test, *P*=0.0001). (C-F) The *Mef2c*-F1 enhancer is required for ET-1 to induce endogenous MEF2C expression in trunk neural crest (NC) cells. ET-1 induced endogenous MEF2C protein expression in trunk neural crest cells (marked by Sox10 immunofluorescence) in ET-1-treated (D-D′) but not in PBS-treated (C-C′) explants. Note MEF2C expression in skeletal muscle (SkM) in both PBS- and ET-1-treated explants (C-D′). ET-1 treatment failed to induce endogenous MEF2C protein expression in trunk neural crest cells in *Mef2c*ΔΔ-F1ΔΔ explants (F). Note the absence of co-expression of MEF2C and Sox10 in *Mef2c*ΔΔ-F1ΔΔ explants treated with either PBS (E-E′) or ET-1 (F-F′). NT, neural tube. Scale bars: 100 µm.](Image:107x136 to 506x416)
**Endothelin induces the Mef2c-F1 neural crest enhancer through a MEF2C-dependent signaling cascade**

The potent induction of Mef2c-F1 by Endothelin suggested that it is a useful tool to define the signaling cascade and immediate transcriptional effectors of Endothelin signaling in the developing neural crest. Therefore, we next examined ET-1 induction of Mef2c-F1 in E9.5 transgenic embryo explants in the presence of pharmacological inhibitors that target potential downstream Endothelin signaling components (Fig. 3; supplementary material Table S1). Compared with ET-1 treatment only (Fig. 3A), inhibition of Endothelin receptors using BQ-123 (ET$_A$ antagonist) and BQ-788 (ET$_B$ antagonist) blocked ET-1 induction of Mef2c-F1-lacZ (Fig. 3B). Likewise, inhibitors that targeted any of the components of the Calmodulin/CamKII signaling pathway, including inhibitors of IP$_3$ receptor (IP$_3$R), Calmodulin (CaM) or CamKII, also blocked ET-1 induction of Mef2c-F1 (Fig. 3C-E; supplementary material Table S1). By contrast, inhibitors of MAPK signaling or PKC signaling components had no effect on ET-1 induction of Mef2c-F1 (supplementary material Table S1).

In other tissues, Calmodulin-CamK signaling stimulates the nuclear export of class II histone deacetylases (HDACs) (McKinsey et al., 2000). Therefore, we predicted that inhibition of class II HDACs might be sufficient to activate Mef2c-F1. Indeed, treatment of Mef2c-F1 transgenic embryo explants with the HDAC inhibitor trichostatin A (Marks et al., 2001), strongly induced enhancer activity compared with treatment with vehicle alone, even in the absence of ET-1 (Fig. 3F-I).

**MEF2C is required for Mef2c-F1 enhancer activation and Endothelin-responsiveness**

A major function of class II HDACs is to repress MEF2 transcription factor activity, and the nuclear export of HDACs in response to Calmodulin/CamKII activity results in de-repression of MEF2 activity (McKinsey et al., 2000, 2002). Notably, the core Endothelin-responsive element in the Mef2c-F1 enhancer contains a conserved, perfect consensus MEF2 site (Fig. 1A), suggesting that the Mef2c-F1 enhancer might require MEF2C itself for activation and for Endothelin responsiveness. Consistent with this idea, Mef2c-F1 enhancer activity was completely abolished in the neural crest lineage in Mef2c knockout embryos (Fig. 4A). Likewise, the MEF2 site in the Mef2c-F1 enhancer was required for Endothelin responsiveness. ET-1 treatment of transgenic embryo explants strongly induced expression of the wild-type Mef2c-F1-lacZ transgene in neural crest cells but did not similarly induce expression of a version of the transgene with a mutated MEF2 site (Fig. 4B). Importantly, MEF2C was also required for the precocious activation of endogenous Mef2c in the neural crest by ET-1. In the results shown in Fig. 4C, wild-type and neural crest-specific Mef2c knockout (Mef2c$^{dW/w};$ Wnt1a::Cre) [Mef2c$^{NCKO}$] embryos were explanted and treated with ET-1 or PBS. Neural crest cells were then sorted, and Mef2c expression was determined by qPCR using primers recognizing both wild-type and mutant transcripts. Importantly, ET-1-induced Mef2c expression more than fourfold in wild-type neural crest cells but did not induce any Mef2c expression in Mef2c$^{NCKO}$ neural crest cells (Fig. 4C).

Based on the work presented here, we propose that MEF2C functions in the Endothelin pathway as an immediate downstream transcriptional effector to activate the transcription of Mef2c and other neural crest genes (Fig. 4D). In this model, early migrating neural crest cells express Endothelin receptors and are competent to receive the Endothelin signal, which in turn, activates gene expression by alleviating HDAC inhibition of an initial pool of MEF2C. The Endothelin signal is then amplified in a positive transcriptional feedback-loop via MEF2C-dependent activation of its own expression through the Endothelin-responsive Mef2c-F1 enhancer element (Fig. 4D). Importantly, this model requires an initial pool of MEF2C expression that is independent of Endothelin signaling. Indeed, Mef2c-F1 contains SOX binding sites that are...
bound by Sox10 and are essential for the initial activation of the enhancer in vivo (Agarwal et al., 2011). Additionally, Mef2c expression could be activated initially through other neural crest enhancers, including F10N (Aoto et al., 2015; De Val et al., 2008) or other unidentified enhancers.

Previously, an Endothelin-MEF2 signaling cascade was shown to function in cardiomyocytes (Wu et al., 2006). Here, we identified the same connection between Endothelin signaling and MEF2 activity via the CamKII-HDAC pathway in the developing neural crest. Importantly, we extend those earlier studies by defining the MEF2C-dependent activation of Endothelin-induced gene expression during neural crest development.

**Materials and Methods**

**Transgenic and mutant mice**

Rosa26^mTmG^+/+ (MGI:3716464), Mef2c^lox^ (MGI:3603182), Mef2c^+/^ (MGI:1857491), Mef2c-F1-lacZ (MGI:5508560), Mef2c-F1[3-3.3]-lacZ (MGI:5508561), Mef2c-F10N-lacZ (MGI:4357694) and Wnt1::Cre^Te^ (MGI:2386570) mice have been described (Agarwal et al., 2011; Danieian et al., 1997; De Val et al., 2008; Lin et al., 1997; Muzumdar et al., 2007; Vong et al., 2005). The Mef2c^F1A^ allele was generated by CRISPR-mediated genome editing (Wang et al., 2013). 5'-ataactcagttgtgcgc-3' and 5'-agctcagctcagttgcgc-3' sgRNAs, which flank the F1 enhancer, were transcribed *in vitro* using the MEGASHortscript T7 kit (Life Technologies, AM1354) and were then purified using the MEGAclean kit (Life Technologies, AM1908). Purified sgRNAs and *in vitro*-transcribed Cas9 mRNA were co-injected into the cytoplasm of fertilized mouse oocytes using standard transgenic technology as described previously (De Val et al., 2004). Two independent F0 transgenic founders were each outcrossed to wild-type mice, and F1 offspring were used for subsequent Mef2c^F1A^ intercrosses and for crosses to Mef2c^−/−^-mice. All animal experiments complied with federal and institutional guidelines and were reviewed and approved by the UCSF IACUC.

**Embryo explant culture**

Embryos were explanted at E9.5 and cultured as described (Rojas et al., 2005) for 1 h prior to 16 h treatment with 10 µM ET ligand and/or pharmacological inhibitors. The concentration of pharmacological compounds used is indicated in supplementary material Table S1. For qPCR detection of Mef2c expression, three control (Wnt1::Cre^Te^, Rosa26^mTmG^+/+) explants or three Mef2c^NCKO^ (Wnt1::Cre^Te^, Mef2c^−/−^-Rosa26^mTmG^+/+) explants were pooled for each sample following ET-1 or PBS treatment and were digested in 300 µl 0.25% trypsin, 0.7 mM EDTA in PBS for 30 min at 37°C. A single-cell suspension was formed and FACs-sorted, and RNA was prepared. cDNA was amplified from 2 ng of

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**Fig. 4. MEF2C is required for Mef2c-F1 enhancer activity.** (A) E9.0 Mef2c-F1-lacZ (F1-lacZ) transgenic embryos on a wild-type/Mef2c^+/^ (A) or Mef2c^−/−^- (A) background were stained with X-gal. Loss of MEF2C resulted in a nearly complete loss of enhancer activity in cranial (black arrows) and trunk (dashed circulars) neural crest, BA, branchial arch. (B) Wild-type Mef2c-F1-lacZ [F1-lacZ(wt)] transgenic embryos and embryos with a Mef2c-F1-lacZ transgene with a mutated MEF2 site [F1-lacZ (mMEF2)] were explanted and treated with PBS or ET-1 and then assayed for β-galactosidase activity via quantitative luminescent assay. Mutation of the MEF2F site in the Mef2c-F1 enhancer significantly reduced enhancer activity induced by ET-1. (C) Mef2c neural crest conditional knockout (Mef2c^NCKO^) and control embryo explants were treated with ET-1 or PBS, and neural crest cells from the caudal region of the explant were sorted and assessed for endogenous Mef2c expression by qPCR. Note that ET-1 failed to induce endogenous Mef2c expression in the absence of MEF2C function in the neural crest. (D) A positive-feedback model for MEF2C-dependent activation of Endothelin-induced gene expression during neural crest development. Error bars in B,C represent s.d.
total RNA using the Ovation RNA-seq System V2 (Nugen). qPCR was performed using the SYBR Green system (Applied Biosystems).

**X-gal staining, luminescent β-galactosidase assay, immunofluorescence and in situ hybridization**

X-gal staining, quantitative luminescent β-galactosidase assay, in situ hybridization with digoxigenin-labeled antisense probes, cryosectioning and immunofluorescence were performed as described (Agarwal et al., 2011; Anderson et al., 2004; Rojas et al., 2005). Immunolabeling was performed using the following primary antibodies at 1:100 dilutions in PBS with 3% BSA and 0.1% Triton X-100: anti-SOX10 (R&D, AF2864); anti-MEF2C (D80C1, Cell Signaling, #5030); anti-β-galactosidase (Abcam, ab3961).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

J.H. performed experiments, analyzed data and helped to write the manuscript; M.P.V., A.S.R., L.L.H., P.-M.F.T., S.-M.X. and P.-Y.K. performed experiments and analyzed data; B.B.B. conceived and directed the project, analyzed data and wrote the paper. All authors commented on and approved the written manuscript.

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**Supplementary material**

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