RESEARCH REPORT

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

Jianxin Hu1, Michael P. Verzi1, Ashley S. Robinson1, Paul Ling-Fung Tang1, Lisa L. Hua1, Shan-Mei Xu1, Pui-Yan Kwok1,2 and Brian L. Black1,3,†

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 induction and establish MEF2C as an immediate transcriptional effector and target of Endothelin signaling in the neural crest.

KEY WORDS: Endothelin, MEF2C, Craniofacial development, Melanocytes, Neural crest, Transcription, Mouse

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 ET\textsubscript{A} (encoded by the Ednra gene) and ET\textsubscript{B} (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. 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.

Here, we found that Endothelin signaling induces Mef2c expression through a conserved neural crest enhancer, and deletion of the enhancer from the genome of mice completely abolished the responsiveness of endogenous Mef2c to Endothelin signaling. Using genetic and pharmacological approaches, we found that the Mef2c-F1 enhancer requires Endothelin signaling for activity in vivo and that Endothelin signaling was sufficient to induce precocious activation of the enhancer. Intriguingly, Mef2c-F1 is activated in response to Endothelin signaling by MEF2C itself through a consensus MEF2 binding site. Finally, we demonstrate that Endothelin-dependent induction of Mef2c-F1 occurs through the de-repression of MEF2C by a Calmodulin-CamKII-histone deacetylase signaling cascade. Thus, these studies identify a MEF2C-dependent, positive-feedback mechanism for Endothelin signal transduction in the neural crest.

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-K). Compared with PBS, ET-1 induced precocious expression of β-galactosidase in the trunk neural crest in a pattern consistent with migrating neural crest cells (Fig. 1H). Co-staining of transverse sections with anti-β-galactosidase and anti-Sox10 antibodies confirmed that the induction was indeed in Sox10+ neural crest cells (Fig. 1I-K). Induction of Mef2c-F1-lacZ was not selective for ET-1, as the other two known Endothelin ligands, ET-2 and ET-3, also activated the enhancer in embryo explants in a pattern essentially identical to ET-1 (supplementary material Fig. S1). Interestingly, another neural crest-specific enhancer in the Mef2c locus, Mef2c-F10N (Aoto et al., 2015; De Val et al., 2008), did not respond in this ET-1 induction assay, suggesting that it is not similarly a target for Endothelin signaling (data not shown).
The Mef2c-F1 neural crest enhancer contains a 300-bp minimal region that is sufficient to direct expression in vivo (Aagarwal 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-F1/+ mice were crossed to Mef2c+/Δα mice to generate Mef2cF1+/Δα transheterozygous mice (Fig. 2B). The Mef2cΔα allele contains a deletion in the coding region of Mef2c, and Mef2c+/Δα (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+/F1Δ and Mef2c+/Δα mice were all viable and present in predicted Mendelian frequencies (Fig. 2B, and data not shown). By contrast, the majority of Mef2cF1+/Δα 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 Mef2cΔα/F1Δ mice is similar to, but less severe than, the phenotype observed in Mef2c neural crest-specific conditional knockout mice (Mef2cNCKO), which also die at birth due to cleft palate and craniofacial obstruction (Verzi et al., 2007). The less severe phenotype observed in Mef2cΔα/F1Δ 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 Mef2cF1Δ/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.
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 (ETα antagonist) and BQ-788 (ETβ 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 IP3 receptor (IP3R), 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 (Mef2cd Roosevelt; Wnt1a::Cre) [Mef2cNCKO] 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 Mef2cNCKO 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

Fig. 3. Endothelin activates Mef2c-F1 enhancer activity via a Calmodulin-HDAC-dependent pathway. (A-E) Inhibition of ETα and ETβ using BQ-123 and BQ-788 (B), IP3 receptor using 2-APB (C), Calmodulin (CaM) using W-7 (D) or CaMKII using KN-93 (E) was sufficient to block ET-1-induced activation of Mef2c-F1-lacZ in the developing neural crest (dashed circles), as in A. (F-I) Treatment of E9.5 transgenic embryo explants with trichostatin A (TSA), an HDAC inhibitor (H, I), activated Mef2c-F1-lacZ in an Endothelin-independent fashion compared with vehicle-treated explants (F,G) in trunk neural crest cells (black arrowheads and dashed circles). A minimum of four embryos was analyzed for each treatment. BA, branchial arch.
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 same connection between Endothelin signaling and MEF2 downstream of Endothelin signaling.

MEF2C, functions downstream of Endothelin signaling. Interestingly, MEF2C and Endothelin overlap significantly in tissues other than heart and neural crest derivatives, including in the vasculature (Firulli et al., 1996; Lin et al., 1998; Yanagisawa et al., 1988), suggesting that MEF2C might function as an effector of Endothelin in other contexts as well. It will be interesting to determine whether a feedback-loop similar to the one defined here functions in the heart or other tissues under normal or pathologic conditions.

MATERIALS AND METHODS

Transgenic and mutant mice

Rosa26<sup>tm2Egr</sup> (MGI:3716464), Mef2c<sup>loxP</sup> (MGI:3603182), Mef2c<sup>+/Δ</sup> (MGI:1857491), Mef2c-F1-lacZ (MGI:5508560), Mef2c-F1[3-3.3]-lacZ (MGI:5508561), Mef2c-F10N-lacZ (MGI:4357694) and Wnt1::Cre<sup>Tg</sup> (MGI:2386570) mice have been described (Agarwal et al., 2011; Danielian et al., 1997; De Val et al., 2008; Lin et al., 1997; Muzumdar et al., 2007; Vong et al., 2005). The Mef2c<sup>F1a</sup> allele was generated by CRISPR-mediated genome editing (Wang et al., 2013). 5′-ataactactgttagttgacgc-3′ and 5′-agtctcagcatggatt-3′ sgRNAs, which flank the F1 enhancer, were transcribed in vitro using the MEGAscript T7 kit (Life Technologies, AM1354) and were then purified using the MEGAgel 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<sup>F1a</sup>-ΔNCKO intercrosses and for crosses to Mef2c<sup>−/−</sup> mice. All animal experiments complied with federal and institutional guidelines and were reviewed and approved by the UCSC 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<sup>Tg</sup>; Rosa26<sup>tm2Egr</sup>) explants or three Mef2c<sup>−/−</sup> NCKO (Wnt1::Cre<sup>Tg</sup>; Mef2c<sup>−/−</sup>; Rosa26<sup>tm2Egr</sup>) 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...
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, Ab9361).

Acknowledgements
We thank D. McCulley, J. F. Martin and D. Clouthier for helpful discussions and M. Yanagisawa for providing mice.

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

Funding
J.H. was supported by an American Heart Association (AHA) postdoctoral fellowship [12POST11820060]. This work was supported by the National Institutes of Health [HL089707, HL064658 and DE019118 to B.L.B.]. Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.126391/-/DC1

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