

Gdf11/Smad signalling and Cdx proteins cooperate to activate the *Hoxc8* early enhancer in HepG2 cells

STEPHEN J. GAUNT*

Department of Zoology, University of Cambridge, Cambridge, U.K.

ABSTRACT Developing anatomy along the head-tail axis of bilaterian embryos is specified, to a large extent, by the overlapping patterns of expression of the *Hox* genes. *Hox* gene enhancers respond to a variety of signals in order to regulate these discreet domains of expression. For mouse *Hoxc8*, the 399bp “early enhancer” plays a major role. Activation of this enhancer is now examined using luciferase expression constructs transfected into HepG2 cells. Constructs are activated by the combined actions of Gdf11/Smad and Cdx protein signalling pathways, both of which are functional in early embryos. Each of these pathways alone has little stimulatory effect. Stimulation by the two pathways together exceeds the sum of the effects of each pathway alone, indicating synergistic activity. By mutation analysis, two Smad binding motifs are identified as mediators of the Gdf11 effect and two Cdx binding motifs mediate the Cdx effect. The two Smad motifs and one of the Cdx sites are conserved from fish to mammals. Gdf11 stimulation is partially inhibited by Specific Inhibitor of Smad3, suggesting that Smad3 plays a part in signal transduction. Fgf2 increases luciferase activation by the *Hoxc8* enhancer, but not, apparently, by specific interactions with either Gdf11 or Cdx effects.

KEY WORDS: *Gdf11*, *Smad*, *Hox*, embryo, cell culture

Enhancer elements regulate the position-specific expression of developmental genes, including *Hox* genes (Gaunt and Paul, 2012). Mouse *Hox* gene enhancers have typically been identified as short sequences of DNA that, when placed upstream of a minimal promoter and *lacZ* gene, can activate *lacZ* reporter transgene expression in mouse embryos in a *Hox*-like pattern. Examples include the ‘early enhancer’ of *Hoxc8* (Shashikant *et al.*, 2007, Shashikant and Ruddle, 1996, Wang *et al.*, 2004) and the region VIII enhancer of *Hoxd11* (Gaunt *et al.*, 2013, Gerard *et al.*, 1993). The location of chromosomal integration is apparently not critical for this particular pattern of transgene expression. Analysis of the enhancer sequence can lead to the identification of transcription factor binding sites and their activators, and thereby provide information about regulation of the gene.

The *Hoxc8* early enhancer (399bp fragment) activates *lacZ* expression in transgenic mouse embryos with anterior boundaries in mesoderm and neurectoderm that are similar to the expression of endogenous *Hoxc8* (Shashikant and Ruddle, 1996). The following studies indicate the importance of Cdx proteins in regulation of *Hoxc8*. 1) *Cdx1*^{-/-} mouse embryos show a one-segment posterior shift in endogenous *Hoxc8* expression within mesoderm (Subramanian *et al.*, 1995). 2) Mouse *Hoxc8* early enhancer/*lacZ* reporter

expression is activated by Cdx1 in *Xenopus* embryos (Schyr *et al.*, 2012). 3) *Hoxc8* early enhancer/*lacZ* transgene expression boundaries in mouse embryos are disrupted by mutations within two enhancer Cdx binding sites (Shashikant *et al.*, 2007, Shashikant and Ruddle, 1996). 4) EMSA studies reveal binding of Cdx2 to both of these Cdx binding sites (Taylor *et al.*, 1997). Cdx proteins bind to the motif [A/T] [T] [A/T] [A] [T] [A/G] (Margalit *et al.*, 1993).

Gain-of-function studies indicate Gdf11 protein as another activator of *Hoxc8*, although it is not established that this acts via the early enhancer. Thus, expressions of *Hoxc6* to *Hoxc10* genes, including *Hoxc8*, are shifted forward in chick neural tube following over-expression of Gdf11, with accompanying rostralized neural identity (Liu, 2006). Gdf11 is a member of the TGF- β family of growth factors (Feng and Derynck, 2005, Massague *et al.*, 2005). It binds predominantly to Acvr1IB and Alk5 surface receptors which, in turn, phosphorylate intracellular Smad2 and Smad3 proteins. These then bind to Smad4, permitting movement of the complex to the nucleus, and activation of Smad binding motifs within gene

Abbreviations used in this paper: FGF, fibroblast growth factor; GDF, growth differentiation factor.

*Address correspondence to: Stephen J. Gaunt, Department of Zoology, University of Cambridge, Downing Street, Cambridge, U.K., CB2 3EJ.
Tel: +44-1223-768917. Fax: +44-1223-336676. Email: sg397@cam.ac.uk  <http://orcid.org/0000-0001-6038-2272>

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enhancers. The Smad3 and Smad4 DNA binding motif contains a repeated AGAC sequence or its reverse complement GTCT (Denkler *et al.*, 1998). The optimal binding motif is the palindrome GTCTAGAC (Zawel *et al.*, 1998). Smad2 may bind to this motif via its complex with Smad4, but Smad2 does not itself have DNA binding activity (Feng and Derynck, 2005).

All three Cdx genes (Gaunt *et al.*, 2005) and *Gdf11* (Gamer *et al.*, 1999, McPherron *et al.*, 1999, Nakashima *et al.*, 1999) are expressed in the embryo tailbud, mesoderm and neural tissues at the time of *Hoxc8* activation. Both *Smad2* and *Smad3* are expressed together in most of the tissues of the embryo (Tremblay *et al.*, 2000).

Reporter transgenes expressed in mouse embryos provide a reductionist approach to the analysis of Hox gene expression. Any given enhancer is likely to be only one of multiple sites that affect expression of the endogenous Hox gene, even in controlling its expression up to its given anterior boundary. For example, deletions of the *Hoxc8* early enhancer (Juan and Ruddle, 2003) or the *Hoxd11* region VIII enhancer (Zakany *et al.*, 1997) each produce early posterior shifts in Hox expressions, but these later revert to normal. A further reductionist approach is now presented in the present paper where conditions are described for activation of mouse *Hoxc8* early enhancer/reporter constructs in cell culture. HepG2 cells are used since they respond to Gdf11 with activation of the Smad2/3 signalling pathway (Andersson *et al.*,

2006, Reissmann *et al.*, 2001). This is sufficient for activation of the *Hoxd11* region VIII enhancer (Gaunt *et al.*, 2013). The new findings for *Hoxc8* early enhancer show that Gdf11 and Cdx proteins each, alone, provide only weak activation in HepG2 cells. However, Gdf11 and Cdx proteins given together provide strong activation. The relevant binding motifs are identified in mutagenesis studies.

Results

Identification of candidate Smad and Cdx binding motifs in Hoxc8 early enhancer

Fig. 1 shows that the mouse *Hoxc8* early enhancer contains the motif GGCTAGACGTCTGGGC which is highly conserved from fish to mammals (blue box). This contains two putative variants of the optimal Smad binding motif with one inverted (on the opposite DNA strand) relative to the other. In comparison to the optimal motif, GTCTGGGC is 76.7% as effective in binding to Smad3, and GTCTAGCC is 79.6% as effective in binding to Smad4 (Zawel *et al.*, 1998).

One TTTATG (putative Cdx binding) motif conserved from fish to mammals is located downstream of the putative Smad motifs; and another TTTATG motif conserved in eutherian and marsupial mammals is located upstream (green boxes in Fig. 1). These are motifs that have already been shown to be essential for normal

expression of *Hoxc8/lacZ* reporter in the mouse embryo (Shashikant *et al.*, 2007, Shashikant and Ruddle, 1996), and to bind Cdx2 protein (Taylor *et al.*, 1997).

Gdf11 and Cdx proteins cooperate to activate Hoxc8 enhancer in HepG2 cells

In Fig. 2A, all cultures were transfected with *Hoxc8* reporter construct #1 (shown boxed), and all received 50ng/ml Gdf11. Cultures shown at bar on right were co-transfected with doxycycline-inducible *Cdx1* expression construct and show 10-fold stimulation in response to doxycycline. In control experiments: (i) cultures given no Cdx expression construct are not stimulated by doxycycline (left), and (ii) cultures co-transfected with a modified expression construct in which the *Cdx1* coding sequence truncates at the start of the homeobox show little or no stimulation by doxycycline (middle). In this latter case, the induced Cdx1 protein lacks the DNA binding homeodomain. Fig. 2B shows a Gdf11 dose response curve. All subsequent experiments were performed at 50ng/ml Gdf11.

As shown in Fig. 2C, Cdx1 (doxycycline) and Gdf11 alone each show little or no activation of *Hoxc8* reporter but in combination they produce about 8-fold stimulation. This suggests that Cdx1 and Gdf11 may exert a cooperative (synergistic) effect upon activation of *Hoxc8*. This is supported by plotting raw luminescence values (Fig. 2D), where activity in monolayers exposed to both Cdx1 (doxycycline) and Gdf11 is found greater than the sum of the values for cultures exposed



Fig. 1. Conservation between species in the *Hoxc8* early enhancer sequence. Alignments are shown only within the essential ca. 200bp enhancer region, as earlier identified in transgenic mouse embryos (Shashikant *et al.*, 2007, Wang *et al.*, 2004). Blue box, the pair of putative Smad binding elements. Green boxes, Cdx binding motifs. Elements A to E, shown red on the mouse sequence, are motifs that when mutated, alone or in combination, can disrupt *Hoxc8* reporter expression in mouse embryos (Shashikant *et al.*, 2007). The sequences, from Ensembl, are aligned by Clustal Omega.

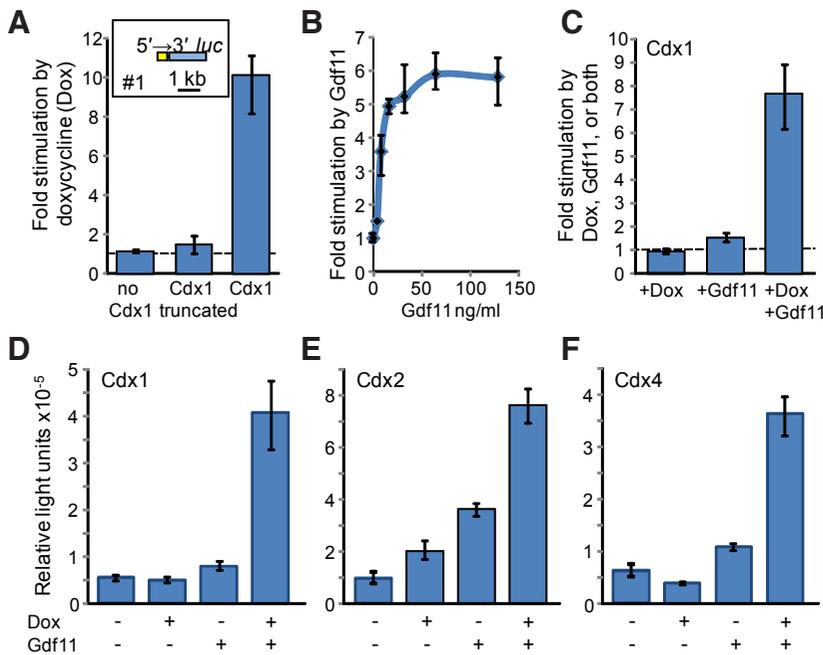


Fig. 2. Gdf11 and Cdx proteins activate *Hoxc8* synergistically. (A) All cultures were transfected with construct #1 (shown boxed: yellow bar is *Hoxc8* early enhancer; blue bar is SV40 minimal promoter/luciferase/SV40 polyA), and received Gdf11 at 50ng/ml. Cultures shown at right were co-transfected with doxycycline-inducible Cdx1 expression construct; cultures at middle were co-transfected with inducible Cdx1 construct lacking the homeobox; cultures at left were not co-transfected with Cdx construct. Fold stimulation shows effect of doxycycline relative to replicate cultures, similarly treated, but not given doxycycline (dotted baseline). (B) Gdf11 dose response curve. All cultures received *Hoxc8* construct #1, Cdx1 expression construct, and doxycycline. Data are plotted as fold stimulation relative to no Gdf11. (C-F) All cultures co-transfected with construct #1 and with a doxycycline inducible Cdx expression construct. In (C), data are plotted as fold stimulation relative to replicate cultures given no Gdf11 or doxycycline (dotted baseline). In (D-F) data are shown as raw luminometry readings. Throughout, each bar shows average values for three replicate cultures, and range bars are shown. luc, luciferase; Dox, doxycycline.

to either Cdx1 or Gdf11 alone. A similar result was obtained for Cdx4 plus Gdf11 (Fig. 2F). The results for Cdx2 plus Gdf11 are less indicative of a synergistic effect (Fig. 2E) although, for equal cell numbers, the added luminescence for Cdx alone and Gdf11 alone should ideally be compared with double the value shown for Cdx plus Gdf11 treated cultures.

The positive response to Gdf11 in cultures co-transfected with the un-induced *Cdx2* construct (Fig. 2E) are likely due to leakage in *Cdx2* transcription in absence of doxycycline, since no activation occurs when the *Hoxc8* enhancer includes mutations in its Cdx binding motifs (Fig. 3D). The reduced effects of un-induced *Cdx1* and *Cdx4* expression vectors upon response to Gdf11 (Fig.

2 D, F) may indicate either less transcriptional leakage from these constructs, or reduced sensitivity of the *Hoxc8* enhancer to Cdx1 and Cdx4 proteins relative to Cdx2.

Smad and Cdx response motifs identified in vitro by mutagenesis

Mutations were introduced into each or both of the putative Smad binding motifs (labelled SmadA and SmadB in Fig. 3A). Mutations within either of the two conserved Smad sites (constructs #2 and #3) result in a reduction of response to Gdf11, and response is reduced further by mutation of both sites (construct #4) (Fig. 3B). This result was observed irrespective of whether Cdx1 or Cdx4

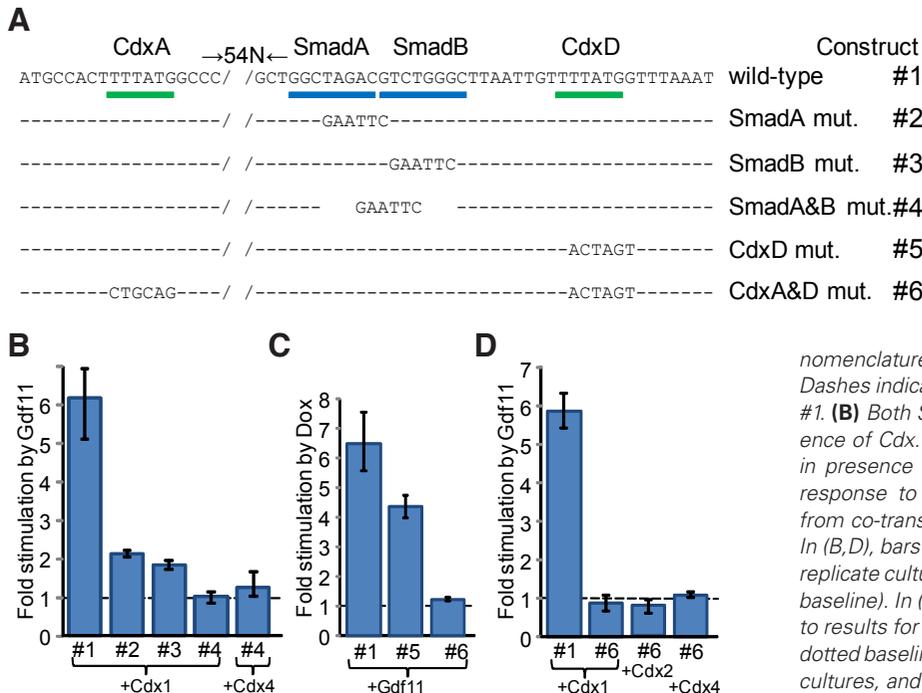


Fig. 3. Gdf11 and Cdx stimulatory effects inhibited by mutations in the putative Smad and Cdx binding motifs. (A) Mutations introduced (constructs #2 to #6) to putative Smad motifs (blue underline) and Cdx motifs (green underline). Cdx sites are designated A and D in accordance with the nomenclature of other researchers (Fig. 1) (Shashikant et al., 2007). Dashes indicate identity with the wild-type sequence of construct #1. (B) Both Smad motifs contribute to Gdf11 stimulation in presence of Cdx. (C) Both Cdx motifs contribute to Cdx1 stimulation in presence of Gdf11. (D) Mutations in the Cdx motifs inhibits response to Gdf11. Cdx proteins were provided, as indicated, from co-transfected doxycycline-inducible expression constructs. In (B, D), bars show fold stimulation by Gdf11 relative to results for replicate cultures given doxycycline but no Gdf11 (shown as dotted baseline). In (C), bars show fold stimulation by doxycycline relative to results for replicates given Gdf11 but no doxycycline (shown as dotted baseline). Each bar shows average values for three replicate cultures, and range bars are shown. Dox, doxycycline.

was provided as co-activator.

As shown in Fig. 3C, mutation of the downstream CdxD binding motif (construct #5) results in reduced response to Cdx1 when given in presence of Gdf11, and this is reduced further by the additional mutation of the upstream CdxA binding motif (construct #6). The double Cdx mutant (construct #6) is also severely impaired in its response to Gdf11 when given in presence of either Cdx1, Cdx2 or Cdx4 (Fig. 3D). This provides further evidence that response to Gdf11 depends upon synergistic action of Cdx protein binding, and both Cdx binding sites appear to contribute to this function.

Effect of SIS3, an inhibitor of Smad3

SIS3, Specific Inhibitor of Smad3, is a dose-dependent inhibitor of Smad3 phosphorylation, Smad3-DNA binding, and interaction of Smad3 with Smad4. SIS3 does not affect phosphorylation of Smad2 or the expression of Smad4 (Jinnin *et al.*, 2006). In HepG2 cells, Gdf11 plus Cdx1 activation of *Hoxc8* reporter construct #1 is inhibited by SIS3 in a dose-dependent manner (Fig. 4A). This suggests that Smad3 mediates, at least in part, the effect of Gdf11 upon *Hoxc8*.

Effect of Fgf2

The effect of Fgf2 was examined since this is proposed to act cooperatively with Cdx2 and/or Gdf11 in activation of the endogenous *Hoxc8* gene (Bel-Vialar *et al.*, 2002, Liu, 2006, Mazzoni *et al.*, 2013). Fgf2 was given at 100ng/ml because this is the concentration used in the earlier, *in vitro* study (Mazzoni *et al.*, 2013). In the HepG2 assay Fgf2 increased luciferase activation by the *Hoxc8* enhancer under all conditions tested, including in cells grown without Gdf11 or Cdx proteins (Fig. 4 B,C). There is no clear evidence that Fgf2 specifically operates synergistically with either Gdf11 or Cdx signalling. Notably, synergy between Gdf11 and Cdx on the *Hoxc8* early enhancer is clearly greater than is any possible synergy between either Fgf2 and Cdx, or Fgf2 and Gdf11 (Fig. 4 B,C).

Discussion

Identification of factors that activate an enhancer element *in vitro* does not prove that the same mechanism operates in the early embryo. The chances of this being so are, however, increased if, as here, the enhancer investigated is known to regulate a Hox-like reporter expression pattern in transgenic embryos, and the activating transcription factors identified are known to be functional in the embryo. Indeed, an *in vitro* system offers certain advantages over *in vivo* studies since large numbers of assays can readily be conducted, allowing activators, inhibitors and co-factors to be more easily, and quantitatively, tested.

Both of the Cdx binding motifs identified have already been shown to be essential for the mouse embryo expression patterns of *Hoxc8* early enhancer transgenes (Shashikant *et al.*, 2007, Shashikant and Ruddle, 1996). The principal new finding now made in HepG2 cells is that effective Cdx activation requires cooperation by

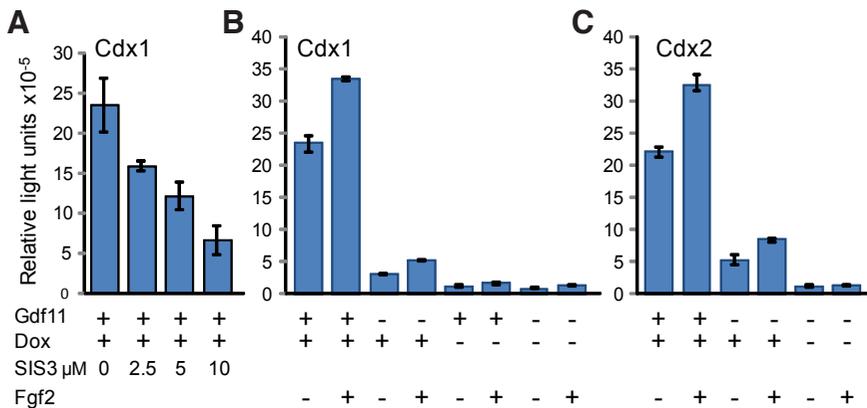


Fig. 4. SIS3 and Fgf2 effects upon Gdf11 and Cdx activation of mouse *Hoxc8* reporter. (A) SIS3, an inhibitor of Smad3 activities. (B,C) Fgf2. *Hoxc8* reporter construct #1 was co-transfected with Cdx1 (A,B) or Cdx2 (C) doxycycline-inducible expression construct. Each graph bar shows average values for three replicate cultures, and range bars are shown. Each culture in (A) was exposed to 1% DMSO solvent. Dox, doxycycline.

Gdf11/Smad signalling. Two putative Smad binding motifs located in the enhancer near to the Cdx motifs are shown by mutagenesis to be essential for this synergistic effect. This is in-keeping with a common finding that enhancer activation by Smad2/3 requires the nearby binding of a major transcription factor (Mullen *et al.*, 2011). *Hoxc8* early enhancer regulates mid to posterior thoracic vertebral patterning and neural control in limbs (Juan and Ruddle, 2003). Evolutionary change in either of these functions could, in an early mammalian ancestor, potentially have been facilitated by acquisition of the additional Cdx binding site.

The questions now arise as to whether Smad signalling is also required during embryonic activation of the endogenous *Hoxc8* gene and, if so, whether this pathway is activated by Gdf11. Overexpression of Gdf11 in the chick neural tube induces increase in phosphorylated Smad2/3 proteins and accompanying forward shifts in the expression boundary of *Hoxc8* (Liu, 2006). Conversely, neural overexpression of follistatin, an inhibitor of endogenous Gdf11, produces posterior shift in *Hoxc8* expression (Liu, 2006). These results indicate that the Smad2/3 signalling pathway activates *Hoxc8* *in vivo*. However, they do not prove that Gdf11 is the primary signal since other TGF β ligands, including Gdf8, activin and nodal, also bind to the Acvr11B receptor to activate Smad2/3, and are also inhibited by follistatin. *Acvr11B*^{-/-} embryos show posterior shifts in the paraxial mesoderm expressions of a variety of more posteriorly-active Hox genes including *Hoxc8* (Oh and Li, 1997) but, again, this does not specifically identify Gdf11 as the primary ligand.

In spite of the positive evidence that Gdf11 may be a physiological activator of *Hoxc8* in the embryo (Liu, 2006) there are apparently contradictory findings. *Gdf11*^{-/-} mouse embryos at 9.5 to 12.5 days show normal anterior boundaries of *Hoxc8* expression in mesoderm and neural tissues, but with caudally-extended posterior boundaries (Jurberg *et al.*, 2013, Liu, 2006, McPherron *et al.*, 1999). This may indicate that Gdf11/Smad signalling represses, rather than activates, embryo expression of *Hoxc8*. More posterior genes *Hoxc10* and *Hoxc11* show caudal shifts of their entire expression domains (Jurberg *et al.*, 2013, McPherron *et al.*, 1999). The role of Gdf11/Smad signalling in embryonic *Hoxc8* expression is, therefore, far from clear but the following possibilities are suggested. 1) The

observations so far made upon *Hoxc8* expression in *Gdf11*^{-/-} embryos are likely, at 9.5 to 12.5 days, to be too late to detect a caudal shift in anterior expression limits. Mouse embryos deleted for the *Hoxc8* early enhancer show posterior shift in *Hoxc8* expression at 8 days, but not at 8.5 days or later (Juan and Ruddle, 2003). 2) *Gdf11* may be partially redundant with other TGF β proteins in its role as a Hox gene activator. If *Hoxc8* is more sensitive to Smad signalling than *Hoxc10* and *Hoxc11*, as proposed in a morphogen gradient hypothesis (Liu, 2006), then this might explain why caudal shifts in anterior expression boundaries are more readily detected for the more posterior genes. Notably, *Gdf8* function and expression in embryonic mesoderm is known to overlap with that of *Gdf11* (Amthor *et al.*, 2002, Lee *et al.*, 2005, McPherron *et al.*, 2009). 3) Separate regulatory elements, lying outside the early enhancer, may be inhibited by *Gdf11*/Smad signalling to explain the extended posterior boundary of endogenous *Hoxc8* expression in *Gdf11*^{-/-} embryos.

In the *Hoxc8* reporter assays of the present paper *Fgf2* provided a general stimulatory effect upon luciferase activity but this was not apparently due to specific synergistic actions with either *Cdx* or *Gdf11*/Smad signalling. This conclusion is, however, limited to activity within the *Hoxc8* early enhancer. An earlier report that *Fgf2* acts synergistically with *Cdx2* to activate endogenous Hox genes, including *Hoxc8*, did not determine whether *Fgf2* acts directly upon the *Hoxc8* early enhancer (Mazzoni *et al.*, 2013).

Materials and Methods

Expression constructs

The mouse *Hoxc8* early enhancer was cloned as a 399bp DNA fragment. This is the same fragment that, in *lacZ* transgenes, has been shown to give a *Hoxc8*-like pattern of expression in both embryonic neuroectoderm and mesoderm (Shashikant and Ruddle, 1996). The regulatory elements in the enhancer are thought to be located within 200bp located at the 3' end of the 399bp fragment (Fig. 1) (Shashikant *et al.*, 2007, Wang *et al.*, 2004). For use in luciferase reporter assays, the 399bp fragment was inserted, in 5' to 3' orientation, upstream of the minimal promoter in *pGL3-promoter* (Promega) (construct #1; Fig. 2A). Various mutations were introduced by PCR into the putative Smad and *Cdx* binding motifs of the *Hoxc8* enhancer (constructs #2 to #6), as shown in Fig. 3A.

Cdx expression constructs were prepared by cloning full-length coding sequences of mouse *Cdx1*, *Cdx2* or *Cdx4*, with Kozak motif upstream of ATG, into the *pTRE3G-IRES* vector (Clontech). A *Cdx1*-minus-homeobox/*pTRE3G-IRES* plasmid was also prepared for use as a control.

Cell culture and luminometry

Cells were the HepG2 Tet-On Advanced transgenic cell line (Clontech, cat. 631150) designed for use with the doxycycline-inducible *pTRE3G-IRES* plasmids. Cell culture in gelatin-coated 24-well plates, transfections using Lipofectamine 2000 (Invitrogen), and luciferase assays (Promega, cat. E1500) were carried out in accordance with manufacturers' instructions and as described earlier (Gaunt and Paul, 2011), but with use of a Biotech Synergy HT luminometer. Cultures were maintained in DMEM supplemented with tetracycline-free foetal bovine serum (Clontech).

All data shown within any one bar chart were obtained in the same experiment. Each bar on each chart shows the mean value obtained from three replicate cultures (n=3). Range bars show the values obtained from the highest and lowest of these three biological replicates. Range bars are preferred to statistical error bars where n is small, including n=3 (Krzywinski and Altman, 2013).

Gdf11 and *Fgf2* were from R&D systems; *SIS3* from Calbiochem; and doxycycline hyclate from Sigma. Unless otherwise indicated *Gdf11* was given at 50ng/ml; *Fgf2* at 100ng/ml; and doxycycline at 10 μ M. Exposure

of transfected cultures to these reagents was for 18 hours, prior to lysis for luciferase assay.

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