Early Neural Ectodermal Genes Are Activated by Siamois and Twin during Blastula Stages

Steven L. Klein, **Sally A. Moody**[*]

Department of Anatomy and Regenerative Biology, George Washington University, School of Medicine and Health Sciences, 2300 I Street, Northwest, Washington, DC

Summary:

BMP signaling distinguishes between neural and non-neural fates by activating epidermis-specific transcription and repressing neural-specific transcription. The neural ectoderm forms after the Organizer secrets antagonists that prevent these BMP-mediated activities. However, it is not known whether neural genes also are transcriptionally activated. Therefore, we tested the ability of nine Organizer transcription factors to ectopically induce the expression of four neural ectodermal genes in epidermal precursors. We found evidence for two pathways: *Foxd4* and *Sox11* were only induced by Sia and Twn, whereas *Gmnn* and *Zic2* were induced by Sia, Twn, as well as seven other Organizer transcription factors. The induction of *Foxd4, Gmnn* and *Zic2* by Sia/Twn was both non-cell autonomous (requiring an intermediate protein) and cell autonomous (direct), whereas the induction of *Sox11* required Foxd4 activity. Because direct induction by Sia/Twn could occur endogenously in the dorsal-equatorial blastula cells that give rise to both the Organizer mesoderm and the neural ectoderm, we knocked down Sia/Twn in those cells. This prevented the blastula expression of *Foxd4* and *Sox11*, demonstrating that Sia/Twn directly activate some neural genes before the separation of the Organizer mesoderm and neural ectoderm lineages.

Keywords

Foxd5; Foxd4l1.1; Foxd4l1; Geminin; Sox11; Zic2; neural induction; Xenopus

INTRODUCTION

Establishment of the vertebrate neural ectoderm requires anti-BMP factors that are secreted by the Organizer mesoderm (reviewed in De Robertis and Kuroda, 2004; De Robertis, 2006; Itoh and Sokol, 2014; Khokha et al., 2005; Levine and Brivanlou, 2007; Wills et al., 2010). Repression of the BMP pathway enables the neural ectoderm to express numerous transcription factors that cooperatively solidify its neural fate (reviewed in Lee et al., 2014; Moody et al., 2013; Rogers et al., 2009; Sasai, 1998). The Organizer-derived diffusible factors, however, do not act directly on the neural genes to promote their transcription, but instead inhibit BMP receptor activation that leads to epidermis-specific transcription. For example, Vent transcription factors are activated by BMPs, up-regulate epidermal genes and

[*]Correspondence to: Sally A. Moody, Department of Anatomy and Regenerative Biology, George Washington University, School of Medicine and Health Sciences, 2300 I Street, Northwest, Washington, DC 20037, samoody@gwu.edu.

directly repress Organizer and neural genes (Ault et al., 1997; Hoppler and Moon, 1998; Henningfeld et al., 2000; Imai et al., 2000; Onichtchouk et al., 1996, 1998; Ramel and Lekven, 2004; Rogers et al., 2008; Taylor et al., 2006; Yoon et al., 2014). An important question yet to be resolved is: are early neural genes expressed simply due to lack of binding of repressive regulators like the Vents, or are they directly activated as well?

To address this question, we screened a large number of Organizer transcription factors for their ability to ectopically induce neural genes in an epidermal lineage. These included: (1) *Siamois* (*Sia*), *Twin* (*Twn*), *Goosecoid* (*Gsc*), and *Foxa4* (aka *Pintalavis*), which are expressed in both the dorsal-equatorial blastula cells that give rise to the Organizer and in the Organizer mesoderm itself; as well as (2) *Xanf* (aka *Hesx1), Lim1, Xnot, Otx2,* and *XlPOU2* (aka *Pou3f4*), which are only expressed in the Organizer mesoderm and not its blastula precursors (Blitz and Cho, 1995; Cho et al., 1991; Gont et al., 1996; Kuroda et al., 2004; Laurent et al., 1997; Lemaire et al., 1995; Reversade and De Robertis, 2005; Ruiz i Altaba and Jessell, 1992; Wessely et al., 2004; Witta and Sato, 1997; Taira et al., 1994; Zaraisky et al., 1995). We were particularly interested in those factors expressed in dorsal-equatorial blastula cells for several reasons. First, maternal β-Catenin translocates into the nucleus of these cells to activate a dorsalizing program (Schneider et al., 1996). Second, molecular interactions occurring in these cells are required to form the Organizer mesoderm (Bae et al., 2011; Suduo et al., 2012). Third, these cells are necessary to form the nervous system (Ishibashi et al., 2008; Kuroda et al., 2004). Fourth, these cells are the common precursors of both the Organizer mesoderm and of the neural ectoderm (Bauer et al., 1994; Vodicka and Gerhart, 1995). Thus, if Organizer transcription factors were to directly interact with neural genes it would be in a cell autonomous manner in cells that give rise to both tissues.

To test for direct activation of neural genes we focused on early-expressed neural ectodermal (NE) genes. Epistasis experiments in *Xenopus* show that at least 12 NE genes interact in a regulatory network that controls the early formation and differentiation of the neural plate (Lee et al., 2014; Moody et al., 2013; Yan et al., 2009). A key upstream factor in this network is Foxd4 (aka Foxd5, Foxd4l1.1, Foxd4l1), whose zygotic expression begins at blastula stages, is down-regulated as the neural folds close, and is required for the expression of the 11 other genes (Fetka et al., 2000; Sölter et al., 1999; Sullivan et al., 2001; Yan et al., 2009). It also directly up-regulates three of them: *Geminin* (*Gmnn*), *Zic2*, and *Sox11* (Yan et al., 2009). Together, these four transcription factors cooperatively maintain the NE in an immature, proliferative state, regulate its transition to neural plate stem cells, and delay neuronal differentiation (Sullivan et al., 2001; Yan et al., 2009).

In this study, we asked whether these four NE genes could be directly activated by Organizer transcription factors when ectopically expressed in epidermal precursors in the intact embryo. We found evidence for two pathways by which NE genes are activated: *Foxd4* and *Sox11* are ectopically induced only by Sia and Twn, whereas *Gmnn* and *Zic2* are ectopically induced by Sia, Twn, as well as seven other Organizer transcription factors. The ectopic inductions of *Sox11* by Sia and Twn were non-cell autonomous, indicating the necessity of an intermediate secreted factor. In contrast, the ectopic inductions of *Foxd4*, *Gmnn* and *Zic2* were both non-cell autonomous and cell autonomous, the latter

implicating direct transcriptional regulation. Direct transcriptional activation was confirmed by expressing hormone-inducible *Sia* and *Twn* constructs in the absence of protein synthesis. In these assays Sia directly activated *Foxd4*, both Sia and Twn directly activated *Gmnn* and *Zic2*, and neither directly activated *Sox11*. The cell autonomous inductions would require that the Organizer transcription factors activate the NE genes within the same cell before the separation of the Organizer mesoderm and NE lineages. Since fate map studies have identified such cells (Bauer et al., 1994; Vodicka and Gerhart, 1995), we knocked down Sia and Twn in the dorsal-equatorial region of the blastula and found that endogenous *Foxd4* and *Sox11* expression was eliminated. These results demonstrate that the blastula expression of Sia/Twn in cells that give rise to both the Organizer mesoderm and the NE can directly activate some NE genes before gastrulation.

RESULTS

Do Organizer Transcription Factors Ectopically Induce Early NE Genes?

In the blastula, the dorsal-equatorial cells that give rise to both the Organizer mesoderm and the NE (Bauer et al., 1994; Vodicka and Gerhart, 1995) are known to express four transcription factors (*Sia, Twn, Gsc, Foxa4*); in the gastrula, the Organizer mesoderm expresses these factors as well as *Xanf* (aka *Hesx1), Lim1, Xnot, Otx2,* and *XlPOU2* (aka *Pou3f4*) (Blitz and Cho, 1995; Cho et al., 1991; Gont et al., 1996; Kuroda et al., 2004; Ishibashi et al., 2008; Laurent et al., 1997; Lemaire et al., 1995; Reversade and De Robertis, 2005; Ruiz i Altaba and Jessell, 1992; Suduo et al., 2012; Taira et al., 1994; Wessely et al., 2004; Witta and Sato, 1997; Zaraisky et al., 1995). To determine if these factors might directly regulate NE genes we ectopically expressed each one by mRNA injection into the 16-cell blastomere precursor of the ventral epidermis (Moody, 1987) at doses that previous publications determined were optimal for inducing ectopic Organizer and/or dorsal axial tissue or for altering dorsal axis formation. This assay tests induction in a region of the embryo that is devoid of Organizer or neural gene expression. Because it is a region subjected to endogenous anti-neural signaling (via BMP and Wnt) and epidermal transcriptional activity (via Vents), it also tests whether the ectopically expressed transcriptional program can over-ride the epidermal program.

All four NE genes were ectopically induced at high frequencies by Sia and by Twn in gastrulae (stages 10.5-12.5; Fig. 1A). Some of the NE gene-expressing cells (blue arrows, Fig. 1B) were adjacent to Sia- and Twn-expressing cells that were marked by the nuclear βGal (nβGal) lineage tracer. This indicates non-cell autonomous induction via a diffusible factor, as would be expected for an Organizer transcription factor that acts indirectly on the ectoderm via secreted inhibitory factors. However, in addition, several *Foxd4-, Gmnn-* and *Zic2*-expressing cells were nβGal-positive, i.e., they were part of the Sia- or Twn-expressing clone of cells (red arrows, Fig. 1B). This suggests a cell autonomous induction within the same cell, indicating that Sia and Twn may also regulate *Foxd4, Gmnn* and *Zic2* directly. Since *Sox11* induction was only non-cell autonomous we conclude that its ectopic induction is only indirect via secreted factors.

Neither *Foxd4* nor *Sox11* were significantly induced by any other tested transcription factor; Gsc, which is a direct target of Sia (Bae et al., 2011; Kessler, 1997; Laurent et al., 1997;

Reid et al., 2012), ectopically induced *Foxd4* in only a few embryos. In contrast, *Gmnn* and *Zic2* were ectopically induced at high frequencies by Gsc and Foxa4 (Fig. 1A), which are expressed in both the dorsal-equatorial blastula cells and in the Organizer mesoderm. They also were induced at high frequencies by Lim1, Otx2 and XlPOU2 and at lower frequencies by Xanf and Xnot2 (Fig. 1A). In most cases the ectopic inductions of *Gmnn* and *Zic2* were both cell autonomous (in nβGal-positive cells; red arrows,) and non-cell autonomous (in adjacent nβGal-negative cells; blue arrows) (Fig. 1B). These results demonstrate that *Foxd4/ Sox11* and *Gmnn/Zic2* are differentially induced by the nine Organizer transcription factors. The non-cell autonomous inductions are most likely due to the Organizer gene activating expression of diffusible factors, whereas the cell autonomous inductions likely result from direct activation within the same cell.

Is the Ectopic Induction of NE Genes by Sia/Twn Direct?

To test whether the four NE genes could be direct targets of Sia or Twn, we expressed hormone-inducible *Sia* (*hGR-Sia*) or *Twn* (*hGR-Twn*) mRNAs in the cleavage stage blastomere precursor of the ventral epidermis. The hGR fusion proteins are synthesized shortly after injection of the mRNA, but the hGR domain forms a complex with endogenous HSP90 in the cytoplasm, preventing them from accessing the nucleus (Kolm and Sive, 1995; Mattioni et al., 1994). Embryos were treated with cyclohexamide (Chx) to block protein synthesis at blastula stages 8-8.5 (Cho et al., 1991), and treated 40 minutes later with the synthetic hormone dexamethasone (Dex) to uncouple the complex and allow nuclear translocation of the hGR-transcription factor. To ensure that the fusion protein was active, *hGR-Sia*-injected or *hGR-Twn*-injected embryos were treated only with Dex at either cleavage or blastula stages (Fig. 2A-C); for all four NE genes the ventral induction was as strong as that of the wild type constructs.

Both *Foxd4* and *Sox11* were ectopically induced after Dex treatment at high frequency by both constructs in early gastrulae (stages 10.5-11.5; Fig. 2A-C). In embryos incubated in Chx before Dex treatment, however, *Foxd4* was induced only by hGR-Sia; the frequency of induction was comparable to Dex treatment alone at either cleavage or blastula stages and significantly higher than in embryos not treated with Dex (Fig. 2A,D). In these cases only cells belonging to the Sia-expressing clone (nβGal-positive) expressed *Foxd4* (Fig. 2D), indicating only a cell autonomous induction. Chx treatment alone did not induce *Foxd4* (0/28 embryos), indicating that Chx was not indirectly preventing the activity of a repressor (Kurth et al., 2005). Interestingly, *Foxd4* was not induced by hGR-Twn in Chx+Dex-treated embryos. This indicates that Twn homodimers cannot directly activate *Foxd4*. In contrast, *Sox11* was not induced in Chx+Dex-treated embryos by either hGR-Sia or hGR-Twn (Fig. 2A,B,D). This is consistent with the lack of cell autonomous induction by the wild-type proteins (Fig. 1B), and indicates that the Sia-mediated induction of *Sox11* requires an intermediate protein.

Both *Gmnn* and *Zic2* were ectopically induced after Dex treatment at high frequency by both constructs (Fig. 2A,B,D). In embryos incubated in Chx before Dex treatment, they both were induced by both hGR-Sia and hGR-Twn; the frequency of induction was comparable to Dex treatment alone at either cleavage or blastula stages and significantly higher than

in embryos not treated with Dex (Fig. 2A-D). In these cases, only cells belonging to the Sia-positive or Twn-positive clone (nβGal-positive) expressed *Gmnn* or *Zic2* (Fig. 2D). Neither *Gmnn* nor *Zic2* was induced by Chx treatment alone in either hGR-Sia (*Gmnn*, 0/28; *Zic2*, 0/24) or hGR-Twn (*Gmnn*, 0/15; *Zic2*, 0/16) injected embryos. These results confirm that *Gmnn* and *Zic2* are not indirectly induced by inhibiting the synthesis of repressors (Kurth et al., 2005), but instead are directly activated by both Sia and Twn.

Previously we reported that *Sox11*, *Gmnn* and *Zic2* expression in the neural ectoderm of the gastrula requires Foxd4, and that they each can be directly activated by Foxd4 (Yan et al., 2009). Since Sia and Twn also induce each of these NE genes, we tested whether Foxd4 activity is required. First, we injected *Foxd4* anti-sense morpholino oligonucleotides (FoxMOs), which previously were shown to effectively block *Foxd4* translation (Yan et al., 2009), into one 8-cell ventral animal blastomere. Next, one of the 16-cell daughter blastomeres was injected with wild-type *Sia* or wild-type *Twn* mRNA. The ectopic expression of *Sox11* by either Sia or Twn was nearly eliminated in these embryos (Fig. 3A,B), indicating that Foxd4 mediates the induction of *Sox11* by these two Organizer genes. Foxd4 knock-down also strongly reduced the Sia-mediated induction of *Gmnn* and *Zic2*, but the effects on Twn-mediated induction were much weaker, albeit statistically significant (Fig. 3A,B). Cells receiving only FoxMOs also did not ectopically express NE genes (0/74, *Sox11;* 0/73, *Gmnn;* 2/76, *Zic2*) These results indicate that Foxd4 activity is required for Sia induction of Gmnn and *Zic2*, but only moderately facilitates their Twn-mediated induction.

Is the Expression of NE Genes Initiated in the Blastula?

The ectopic induction assays indicate that Sia and Twn induce *Foxd4, Gmnn* and *Zic2* expression both indirectly, i.e., in adjacent cells, and directly, i.e., in the same cell. The indirect regulation likely occurs via the well characterized anti-BMP and anti-Wnt neural inductive signaling from the Organizer mesoderm to the adjacent ectoderm. However, since direct regulation must occur within the same cell we hypothesized that this interaction could occur within a dorsal-equatorial blastula precursor that gives rise to both the Organizer mesoderm and the NE. This seemed possible because these cells are required for both Organizer and neural development and they express both Organizer genes as well *Foxd4, Gmnn* and *Zic2* (Fig. 4). To test this, we used MOs to knock down both Sia and Twn in the dorsal-equatorial blastula cells on one side of the embryo; both proteins need to be knocked-down to observe a phenotype due to redundancy in DNA binding (Bae et al., 2011; Reid et al., 2012). The blastula expression of *Foxd4* and *Sox11* was eliminated in the MO-containing cells as early as stage 9, whereas *Gmnn* or *Zic2* expression was not detectably altered (Fig. 4). These results indicate that zygotic expression of *Foxd4* is initiated in the blastula by Sia/Twn, which in turn is required for *Sox11* expression before gastrulation. They also indicate that *Gmnn* and *Zic2* may not require Sia/Twn activation in the blastula.

DISCUSSION

Foxd4 is transiently expressed from blastula stages to the closure of the neural tube, and plays a key role in the early steps of NE development (Fetka et al., 2000; Sölter et al.,

1999; Sullivan et al., 2001; Yan et al., 2009). We previously showed that Foxd4 is a critical upstream regulator of a NE regulatory network (Lee et al., 2014; Moody et al., 2013; Yan et al., 2009). Decreasing the level of Foxd4 in the NE reduced the expression of 11 other early neural genes. Increasing the level of Foxd4 up-regulated *Gmnn* and *Zic2*, which promote a proliferative, immature neural ectoderm (Brewster et al., 1998; Kroll, 2007; Kroll et al., 1998; Seo et al., 2005; Seo and Kroll, 2006), and up-regulated *Sox11*, which transitions immature neural progenitors towards differentiation (Bergsland et al., 2006; Hyodo-Miura et al., 2002; Uwanogho et al., 1995). Furthermore, Foxd4 repressed BMP gene transcription and BMP signaling (Yan et al., 2009, 2010). Thus, Foxd4 plays a very early role in establishing the nascent NE and preventing its conversion to a non-neural fate. Its central and early role provided a unique opportunity to examine whether NE genes are activated directly.

The studies reported here provide the first evidence that the earliest-expressed NE genes are activated by the maternal β-Catenin targets, Sia and Twn, that their activation is direct, and that this activation begins in the blastula. Firstly, *Foxd4, Sox11, Gmnn*, and *Zic2* were ectopically induced by expressing Sia and Twn in ventral epidermal precursors. The evidence for direct activation is that *Foxd4, Gmnn* and *Zic2* are induced by Sia even when protein synthesis is inhibited, and that *Gmnn* and *Zic2* are induced by Twn following protein synthesis inhibition. Additionally, induction of *Foxd4, Gmnn* and *Zic2* was observed in both the cells expressing Sia/Twn and in neighboring cells following ventral injection of *Sia/Twn* mRNA, whereas this induction was observed only in the same cell when protein synthesis was blocked. Finally, the blastula expression of *Foxd4* and *Sox11* was prevented by knocking down of Sia/Twn in the dorsal-equatorial blastula cells that give rise to both the Organizer mesoderm and the NE. Taken together, these observations show that Sia/Twn can directly activate some NE genes in the blastula.

While several signaling pathways, e.g. FGF and Wnt, also play important roles in the process of neural induction (Fletcher et al., 2006; Mulligan and Cheyette, 2012; Pera et al., 2014; Pinho et al., 2011; Rogers et al., 2011; Stern, 2006; Streit et al., 2000; Young et al., 2014), it is well established in several non-mammalian vertebrates that an essential step in inducing the neural ectoderm is the inhibition of BMP signaling by Organizer activity. Recent studies in invertebrates and in mouse ESCs and embryos confirm the critical role of inhibiting BMP signaling (Dang et al., 2012; Kozmikova et al., 2013; Li et al., 2013). Key transcriptional regulators of Organizer activity are Sia and Twn, two highly related proteins that directly activate the transcription of a number of genes required for initiating neural induction via secreted anti-BMP and anti-Wnt factors. However, there has not previously been evidence that Organizer genes directly up-regulate neural genes. Herein we demonstrate in an ectopic expression assay in the intact embryo that Sia and Twn can directly activate three genes that are important for establishing the NE, and that at least one of these, *Foxd4*, is activated in the blastula before the separation of the Organizer mesodermal and NE lineages. It is of interest that DUXO, a double-homeobox transcription factor that is unique to placental mammals and bears high homology to Sia and Twn in its homeodomains, also is required for Organizer gene expression in human embryonic stem cell cultures (Sharon et al., 2012), suggesting that there may be conservation of our findings with mammals.

From these observations we propose that two pathways regulate the expression of early NE genes (Fig. 5). First, within dorsal-equatorial blastula cells, Sia directly activates *Foxd4* expression, which in turn activates *Sox11*. Sia and Twn may directly activate *Gmnn* and *Zic2* as well (see below), but we were not able to demonstrate this due to the high levels of maternal transcripts. Then, at gastrulation, after dorsal-equatorial blastula lineages have segregated into Organizer mesoderm and NE, Sia and Twn activity in the Organizer up-regulates a number of other transcription factors, leading to the secretion of inhibitory factors. These in turn diffuse into the adjacent ectoderm to prevent BMP- and Wnt-mediated repression of NE genes, and thereby maintain the expression of NE genes.

Interestingly, Sia/Twn knock-down did not affect the blastula expression of *Gmnn* or *Zic2* even though they appear to be direct targets in the ectopic expression assay. There are two plausible explanations for this apparent discrepancy. First, Sia/Twn may not directly induce these two genes in the endogenous environment, whereas in the ectopic induction assay (Fig. 1) they may have an experimentally provided opportunity to bind to the enhancer/promoter regions of these NE genes. Alternatively, the bulk of the *Gmnn* and *Zic2* mRNAs detected at blastula stages is likely of maternal origin, which would not be affected by Sia/Twn MO knock-down in the blastula. We favor this interpretation because *Gmnn* and *Zic2* are reported to have strong maternal expression (Houston and Wiley, 2005; Kroll et al., 1998), and RNA-Seq data confirm that they have high levels of maternal expression from cleavage through blastula stages (Collart et al., 2014; Yanai et al., 2011). In contrast, *Foxd4* maternal mRNA is of low abundance and its zygotic mRNA levels significantly increase at blastula stages slightly after the zygotic expression of *Sia* (Collart et al., 2014; Yanai et al., 2011). By mining the data from a high resolution time-line of *Xenopus* transcription at blastula stages (Collart et al., 2014), we found that *Foxd4, Gmnn* and *Zic2* all show a transient uptick in mRNA levels just after the onset of *Sia* transcription (Yanai et al., 2011), and slightly in advance of a similar uptick in zygotic *Sox11* mRNA levels (Collart et al., 2014). These temporal data are consistent with a model in which Sia and Twn directly activate *Foxd4, Gmnn* and *Zic2* in the blastula, and Foxd4 directly activates *Sox11* (Fig. 5). However, using ChIP-Seq approaches will be needed to convincingly demonstrate this course of events.

Consistent with our previous epistasis studies of NE genes (Yan et al., 2009), *Sox11* induction in the blastula requires Foxd4 activity. In contrast, *Gmnn* and *Zic2* can be directly activated by both Sia and Twn, and only the Sia-induction is reduced in the absence of Foxd4. Since *Gmnn* and *Zic2* also can be ectopically induced by several Organizer-specific transcription factors, we conclude that they are regulated by multiple inputs. The Organizer transcription factors are not likely to act directly in the normal setting of the gastrulating embryo, but likely require intermediary secreted factors (Fig. 5). In fact, *Gmnn* and *Zic2* are known to be highly induced ectopically simply by expressing anti-BMP factors (Brewster et al. 1998; Kroll et al., 1998).

Sia and Twn have key regulatory roles in activating Organizer and neural genes. Several studies show that Sia is required during blastula stages for the formation of the Organizer and for the expression of several Organizer-specific genes (Darras et al., 1997; Fan and Sokol, 1997; Kessler, 1997; Kodjabachian and Lemaire, 2001). It can activate Chordin, Noggin and Cerberus independent of Nodal signaling and mesoderm formation (Agius et

al., 2000; Carnac et al., 1996; Darras et al., 1997; Ding et al., 1998; Kodjabachian and Lemaire, 2001; Medina et al., 1997), but Nodal signaling can enhance Sia induction of these genes (Crease et al., 1998; Darras et al., 1997; Engleka and Kessler, 2001) by enhancing its occupancy on their promoter/enhancer elements (Reid et al., 2012). Twn is highly related to Sia (Laurent et al., 1997; Nishita et al., 2000). Together with Sia it directly activates *Gsc, Chd*, and *Cerb* (Bae et al., 2011; Kessler, 1997; Laurent et al., 1997; Reid et al., 2012; Yamamoto et al., 2003). Several lines of evidence indicate that Twn and Sia redundantly activate Organizer genes. They bind to identical elements in the *Gsc* promoter, and they form homo- and heterodimers that bind equally well to this promoter (Bae et al., 2011). In certain assays there are no functional differences between the different types of dimers, and both proteins must be knocked down to completely eliminate *Gsc, Chd*, and *Zic1* expression (Bae et al., 2011). However, we detected differences in Sia versus Twn effects on the ectopic induction of the four NE genes. While both Sia and Twn could ectopically induce all four NE genes without protein synthesis, *Foxd4* was directly induced only by Sia, whereas *Gmnn* and *Zic2* were directly induced by both Sia and Twn. These differences suggest that there is some specificity in the regulation of the different NE genes that was not observed for *Gsc, Chd* and *Zic1*, which are likely context-dependent. Our evidence indicates that *Foxd4* can be induced by Sia homo-dimers but not by Twn homo-dimers, whereas *Gmnn* and *Zic2* can be induced by both. The causes of these differences need to be resolved by DNA-binding assays, and by considering the roles of other proteins, such as XIC (Snider and Tapscott, 2005), that modify Sia induction of Organizer genes.

In summary, our results show that the Organizer transcription factors, Sia and Twn, can activate some NE genes at blastula stages. Sia directly activates *Foxd4*, which in turn activates *Sox11*. Once descendants of dorsal-equatorial blastula cells diverge into the Organizer mesoderm and NE lineages, Sia maintains NE genes indirectly via secreted factors. Although our experiments indicate that *Gmnn* and *Zic2* also can be directly activated by Sia/Twn in a cell autonomous fashion, which most likely occurs in the blastula, we could not directly confirm this by Sia/Twn knock-down because *Gmnn* and *Zic2* maternal mRNAs are very abundant. However, they do appear to be direct transcriptional targets of Twn, whereas their Sia induction is likely mediated by Foxd4 activity. In addition, our data are consistent with *Gmnn* and *Zic2* later being indirectly activated in the neural ectoderm downstream of several Organizer transcription factors via secreted factors. Thus, the experiments described herein demonstrate that Sia/Twn activity in the blastula cells that give rise to both the Organizer mesoderm and the NE directly activates some NE genes, initiating neural fate before gastrulation.

METHODS

mRNA Synthesis and Injection.

To generate a hormone-inducible *Twn* plasmid, the *Twn* ORF with a StuI site at its 5′ end and an XhoI site at its 3′ end was made using standard PCR methods and cloned into pCS2$^+$-hGR-MT using the StuI and XhoI sites in the polylinker. The pCS2$^+$-hGR-MT-Twn plasmid was confirmed by sequencing and used as template to generate mRNA (*hGR-MT-Twn*). mRNAs encoding *Foxa4* (500 pg/nl; Ruiz i Altaba and Jessell, 1992), *Foxd4* (100

pg/nl; Sullivan et al., 2001), *Goosecoid (Gsc;* 500 pg/nl; Cho et al., 1991), an activated form of *Lim1* (*3MLim1*, 750 pg/nl; Taira et al., 1994), *Xnot2* (100 pg/nl; Gont et al., 1996), *Otx2* (100 pg/nl; Blitz and Cho, 1995), *XlPOU2* (200 pg/nl; Witta and Sato, 1997), *Siamois* (*Sia*, 150 pg/nl; Lemaire et al., 1995), hormone-inducible *Siamois* (*hGR-Sia*; 150 pg/nl; Kodjabachian and Lemaire, 2001), *Twin* (*Twn*, 5-10 pg/nl; Laurent et al., 1997), hormone-inducible *Twin* (*hGR-Twn*; 200 pg/nl), and *Xanf* (50 pg/nl; Zaraisky et al., 1995) were synthesized *in vitro* (Ambion, mMessage mMachine kit). The dose of each mRNA used was determined from previous publications as being optimal for inducing a secondary axis or specific dorsal axial structure, indicating induction of ectopic Organizer tissue, or for altering dorsal axis formation. These mRNAs were mixed with nuclear-localized *βgal* mRNA (*nbgal*, 100 pg/nl) as a lineage tracer and microinjected at published optimal concentrations in embryos that were obtained, cultured and microinjected as previously described (Moody, 1999, 2000). One nl of each mRNA mixture was microinjected into one ventral animal blastomere of the 16-cell embryo that is a defined precursor of the epidermis (blastomere V1.1; Moody, 1987). The resulting embryos were examined by *in situ* hybridization (ISH) at stages when early NE genes are highly expressed (stages 11.0–12.5; Nieuwkoop and Faber, 1994) to observe the spatial relationship between the injected progeny and the cells expressing *Foxd4, Sox11, Gmnn* or *Zic2*. The uninjected side of the embryo was used as an internal control.

Morpholino Knock-Down of Foxd4 in Combination With *Sia/Twn* mRNA Injection.

To determine if Foxd4 is required for Sia- or Twn-mediated ectopic induction, we performed sequential injections of *Foxd4* antisense morpholino oligonucleotides (MOs) and then *Sia* or *Twn* mRNAs. A mixture of two *Foxd4* MOs (20 ng), which were lissamine-tagged to visualize the injected progeny and targets both alloalleles to effectively prevent mRNA translation (Yan et al., 2009), were injected into a single ventral animal blastomere of the 8-cell embryo. After the next cell division was completed, its midline daughter (blastomere V1.1) was injected with either *Sia* or *Twn* mRNA, mixed with *nβgal* mRNA as a lineage tracer. The resulting embryos were examined at stages when early NE genes are highly expressed (stages 11.0–12.5).

Morpholino knock-down of *Sia* and *Twn* expression.

The endogenous levels of Sia and Twn were knocked down by injecting previously characterized MOs (9 ng; Bae et al., 2011), labeled with lissamine, into the 16-cell common progenitor of the Organizer mesoderm and NE (blastomere D1.1; Bauer et al., 1994; Moody, 1987) on one side of the embryo. The resulting embryos were examined at late blastula (stage 9).

Hormone induced expression and cycloheximide treatment.

hGR-Sia or *hGR-MT-Twn* mRNA, mixed with *nbgal* mRNA as lineage tracer, was injected into one ventral animal blastomere of the 16-cell embryo. Injected embryos were incubated in synthetic hormone (10 μM dexamethasone, Dex) according to published protocols (Kolm and Sive, 1995). To ensure that the *hGR-Sia* and *hGR-MT-Twn* constructs functioned as expected, some injected embryos were treated with hormone immediately after mRNA injection or at blastula stage 9. These embryos phenocopied those injected with wild-type

mRNAs. Some embryos were injected with *hGR* mRNAs and raised in the absence of hormone; ectopic induction of target genes was observed in fewer than 10% of these embryos, indicating that the hormone-inducible constructs had little effect in the absence of hormone, in accord with published accounts (de Graaf et al., 1998; Hollenberg et al., 1993; Kolm and Sive, 1995; Mattioni et al., 1994). To block protein synthesis, embryos were injected with *hGR* mRNAs at the 16-cell stage and at stage 8–8.5 the vitelline membranes were manually removed and the embryos incubated in cycloheximide (Chx, 10 μg/ml; Kurth et al., 2005). Half of this dose of Chx is reported to block >95% of protein synthesis, as detected by either scintillation counting of [^{35}S]methionine incorporation or protein gel electrophoresis (Cho et al., 1991). After 40 minutes the Chx medium was supplemented with Dex (10 βM) and embryos allowed to develop until siblings reached stage 12.

Whole Embryo *In Situ* Hybridization.

Embryos were processed for ISH as previously described (Sive et al., 2000). Anti-sense Dig-labeled RNA probes were synthesized as previously described (Yan et al., 2009). The expression patterns of *Foxd4, Sox11, Gmnn*, and *Zic2* were compared on the experimental and control sides of embryos derived from at least three different clutches of eggs from different sets of adult parents. The frequencies at which embryos showed ectopic expression of early neural genes in the ventral epidermis were compared to controls using the Chi-squared test ($p < 0.05$).

Animals.

Fertilized eggs were obtained from adult frogs under in accordance with national and NIH guidelines and via protocols approved by the GWU IACUC.

ACKNOWLEDGMENTS

Abbreviations:

ISH	in situ hybridization
NE	neural ectodermal

LITERATURE CITED

Agius E, Oelgeschlager M, Wessely O, Kemp C, De Robertis EM. 2000. Endodermal Nodal-related signals and mesoderm induction in *Xenopus*. Development 127:1173–1183. [PubMed: 10683171]

Ault KT, Xu RH, Kung HF, Jamrich M. 1997. The homeobox gene PV.1 mediates specification of the prospective neural ectoderm in *Xenopus* embryos. Dev Biol 192:162–171. [PubMed: 9405105]

Bae S, Reid CD, Kessler DS. 2011. Siamois and twin are redundant and essential in formation of the Spemann organizer. Dev Biol 352:367–381. [PubMed: 21295564]

Bauer DV, Huang S, Moody SA. 1994. The cleavage stage origin of Spemann's organizer: Analysis of the movements of blastomere clones before and during gastrulation in *Xenopus*. Development 120:1179–1189. [PubMed: 8026328]

Bergsland M, Werme M, Malewicz M, Perlmann T, Muhr J. 2006. The establishment of neuronal properties is controlled by *sox4* and *sox11*. Genes Dev 20:3475–3486. [PubMed: 17182872]

Blitz IL, Cho KW 1995. Anterior neurectoderm is progressively induced during gastrulation: The role of the *Xenopus* homeobox gene *orthodenticle*. Development 121:993–1004. [PubMed: 7743941]

Brewster R, Lee J, Ruiz i Altaba A. 1998. Gli/zic factors pattern the neural plate by defining domains of cell differentiation. Nature 393:579–583. [PubMed: 9634234]

Carnac G, Kodjabachian L, Gurdon JB, Lemaire P. 1996. The homeobox gene *siamois* is a target of the wnt dorsalization pathway and triggers organizer activity in the absence of mesoderm. Development 122:3055–3065. [PubMed: 8898219]

Cho KW, Blumberg B, Steinbeisser H, De Robertis EM. 1991. Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene *goosecoid*. Cell 67:1111–1120. [PubMed: 1684739]

Collart C, Owens NDL, Bhaw-Rosun L, Cooper B, De Domenico E, Patrushev I, Sesay AK, Smith JN, Smith JC, Gilchrist MJ. 2014. High-resolution analysis of gene activity during the *Xenopus* mid-blastula transition. Development 141:1927–1939. [PubMed: 24757007]

Crease DJ, Dyson S, Gurdon JB. 1998. Cooperation between the activin and wnt pathways in the spatial control of organizer gene expression. PNAS USA 95: 4398–4403. [PubMed: 9539748]

Dang LTH, Wong L, Tropepe V 2012. Zfhx1b induces a definitive neural stem cell fate in mouse embryonic stem cells. Stem Cells Development 21:2838–2851. [PubMed: 22594450]

Darras S, Marikawa Y, Elinson RP, Lemaire P 1997. Animal and vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: Implications for the patterning of the organizer. Development 124:4275–4286. [PubMed: 9334276]

de Graaf M, Zivkovic D, Joore J. 1998. Hormone-inducible expression of secreted factors in zebrafish embryos. Dev Growth Differ 40:577–582. [PubMed: 9865967]

De Robertis EM. 2006. Spemann's organizer and self-regulation in amphibian embryos. Nat Rev 7:296–302.

De Robertis EM, Kuroda H. 2004. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annu Rev Cell Dev Biol 20:285–308. [PubMed: 15473842]

Ding X, Hausen P, Steinbeisser H. 1998. Pre-MBT patterning of early gene regulation in *Xenopus*: The role of the cortical rotation and mesoderm induction. Mech Dev 70:15–24. [PubMed: 9510021]

Engleka MJ, Kessler DS. 2001. Siamois cooperates with TGFβ signals to induce the complete function of the Spemann-Mangold organizer. Int J Dev Biol 45:241–250. [PubMed: 11291853]

Fan MJ, Sokol SY. 1997. A role for siamois in Spemann organizer formation. Development 124:2581–2589. [PubMed: 9217000]

Fetka I, Doederlein G, Bouwmeester T. 2000. Neuroectodermal specification and regionalization of the Spemann organizer in *Xenopus*. Mech Dev 93:49–58. [PubMed: 10781939]

Fletcher RB, Baker JC, Harland RM. 2006. fgf8 splice-forms mediate early mesoderm and posterior neural tissue formation in *Xenopus*. Development 133: 1703–1714. [PubMed: 16554360]

Gont LK, Fainsod A, Kim SH, De Robertis EM. 1996. Overexpression of the homeobox gene *Xnot-2* leads to notochord formation in *Xenopus*. Dev Biol 174: 174–178. [PubMed: 8626017]

Henningfeld KA, Rastegar S, Adler G, Knochel W. 2000. smad1 and smad4 are components of the bone morphogenetic protein-4 (BMP-4)-induced transcription complex of the *Xvent*-2B promoter. J Biol Chem 275:21827–21835. [PubMed: 10791953]

Hollenberg SM, Cheng PF, Weintraub H. 1993. Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. PNAS USA 90:8028–8032. [PubMed: 8396258]

Hoppler S, Moon RT. 1998. BMP-2/-4 and Wnt-8 cooperatively pattern the *Xenopus* mesoderm. Mech Dev 71:119–129. [PubMed: 9507084]

Houston DW, Wiley C. 2005. Maternal *Xenopus zic2* negatively regulates *Nodal-related* gene expression during antero-posteror patterning. Development 132:4845–4855. [PubMed: 16207750]

Hyodo-Miura J, Urushiyama S, Nagai S, Nishita M, Ueno N, Shibuya H. 2002. Involvement of NLK and *sox11* in neural induction in *Xenopus* development. Genes Cells 7:487–496. [PubMed: 12047350]

Imai Y, Gates MA, Melby AE, Kimelman D, Schier AF, Talbot WS. 2001. The homeobox genes *vox* and *vent* are redundant repressors of dorsal fates in zebrafish. Development 128:2407–2420. [PubMed: 11493559]

Ishibashi H, Matsumura N, Hanafusa H, Matsumoto K, De Robertis EM, Kuroda H. 2008. Expression of *siamois* and *twin* in the blastula chordin/noggin signaling center is required for brain formation in *Xenopus laevis* embryos. Mech Dev 125:58–66. [PubMed: 18036787]

Itoh K, Sokol SY 2014. Early Development of Epidermis and Neural Tissue. In: Moody SA, editor. Principles of Developmental Genetics", 2nd ed. NY: Elsevier. pp 189–201.

Kessler DS. 1997. Siamois is required for formation of Spemann's organizer. PNAS USA 94:13017–13022. [PubMed: 9371792]

Khokha MK, Yeh J, Grammer TC, Harland RM. 2005. Depletion of three BMP antagonists from Spemann's organizer leads to a catastrophic loss of dorsal structures. Dev Cell 8:401–411. [PubMed: 15737935]

Kodjabachian L, Lemaire P. 2001. Siamois functions in the early blastula to induce Spemann's organizer. Mech Dev 108:71–79. [PubMed: 11578862]

Kolm PJ, Sive HL. 1995. Efficient hormone-inducible protein function in *Xenopus laevis*. Dev Biol 171:267–272. [PubMed: 7556904]

Kozmikova I, Candiani S, Fabian P, Gurska D, Kozmik Z. 2013. Essential role of bmp signaling and its positive feedback loop in the early cell fate evolution of chordates. Dev Biol 382:538–554. [PubMed: 23933491]

Kroll KL, Salic AN, Evans LM, Kirschner MW. 1998. Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. Development 125:3247–3258. [PubMed: 9671596]

Kroll KL. 2007. Geminin in embryonic development: Coordinating transcription and the cell cycle during differentiation. Front Biosci 12:1395–1409. [PubMed: 17127390]

Kuroda H, Wessely O, Robertis DEM. 2004. Neural induction in *Xenopus*: Requirement for ectodermal and endomesodermal signals via chordin, noggin, beta-catenin, and cerberus. PLoS Biol 2:0623–0634.

Kurth T, Meissner S, Schackel S, Steinbeisser H. 2005. Establishment of mesodermal gene expression patterns in early *Xenopus* embryos: The role of repression. Dev Dyn 233:418–429. [PubMed: 15779047]

Laurent MN, Blitz IL, Hashimoto C, Rothbacher U, Cho KW 1997. The *Xenopus* homeobox gene *twin* mediates wnt induction of *goosecoid* in establishment of Spemann's organizer. Development 124:4905–4916. [PubMed: 9428427]

Lee HK, Lee HS, Moody SA. 2014. Neural transcription factors: From embryos to neural stem cells. Mol Cells 37:705–712. [PubMed: 25234468]

Lemaire P, Garrett N, Gurdon JB. 1995. Expression cloning of *siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. Cell 885–894. [PubMed: 7553849]

Levine AJ, Brivanlou AH. 2007. Proposal of a model of mammalian neural induction. Dev Biol 308:247–256. [PubMed: 17585896]

Li L, Liu C, Beichele S, Zhu Q, Song L, Lanner F, Jing N, Rossant J. 2013. Location of transient ectodermal progenitor potential in mouse development. Development 140:4533–4543. [PubMed: 24131634]

Mattioni T, Louvion JF, Picard D. 1994. Regulation of protein activities by fusion to steroid binding domains. Meth Cell Biol 43:335–352.

Medina A, Wendler SR, Steinbesser H. 1997. Cortical rotation is required for the correct spatial expression of *nr3, sia* and *gsc* in *Xenopus* embryos. Int J Dev Biol 41:741–745. [PubMed: 9415495]

Moody SA. 1987. Fates of the blastomeres of the 16-cell stage *Xenopus* embryo. Dev Biol 119:560–578. [PubMed: 3803718]

Moody SA. 1999. Cell lineage analysis in Xenopus embryos. In: Tuan RS, Lo CW, editors. Methods in Molecular Biology, Vol. 135. Developmental Biology Protocols. Humana Press Inc.: Totowa, NJ.

Moody SA. 2000. Testing the cell fate commitment of single blastomeres in *Xenopus laevis*. In: Richter J, editor. Advances in Molecular Biology, Oxford University Press. pp. 355–381.

Moody SA, Klein SL, Karpinski BA, Maynard TM, LaMantia AS. 2013. On becoming neural: What the embryo can tell us about differentiating neural stem cells. Amer J Stem Cells 2:74–94. [PubMed: 23862097]

Mulligan KA, Cheyette BN. 2012. Wnt signaling in vertebrate neural development and function. J Neuroimmune Pharmacol 7:774–787. [PubMed: 23015196]

Nieuwkoop PD, Faber J. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland, Amsterdam.

Nishita M, Hashimoto MK, Ogata S, Laurent MN, Ueno N, Shibuya H, Cho KWY. 2000. Interaction between wnt and TGF-β signaling pathways during formation of Spemann's organizer. Nature 403:781–785. [PubMed: 10693808]

Onichtchouk D, Gawantka V, Dosch R, Delius H, Hirschfeld K, Blumenstock C, Niehrs C. 1996. The *Xvent-2* homeobox gene is part of the BMP-4 signalling pathway controlling dorsoventral patterning of *Xenopus* mesoderm. Development 122:3045–3053. [PubMed: 8898218]

Onichtchoul D, Glinka A, Niehrs C. 1998. Requirement for *Xvent-1* and *Xvent-2* gene function in dorsoventral patterning of *Xenopus* mesoderm. Development 125:1447–1456. [PubMed: 9502725]

Pera EM, Acosta H, Gouignard N, Climent M, Arregi I. 2014. Active signals, gradient formation and regional specificity in neural induction. Exp Cell Res 321:25–31. [PubMed: 24315941]

Pinho S, Simonsson PR, Trevers KE, Stower MJ, Sherlock WT, Khan M, Streit A, Sheng G, Stern CD. 2011. Distinct steps of neural induction revealed by *asterix, obelix* and *TrkC*, genes induced by different signals from the organizer. PLoS One 6:e19157 [PubMed: 21559472]

Ramel MC, Lekven AC. 2004. Repression of the vertebrate organizer by wnt8 is mediated by vent and vox. Development 131:3991–4000. [PubMed: 15269175]

Reid CD, Zhang Y, Sheets MD, Kessler DS. 2012. Transcriptional integration of wnt and nodal pathways in establishment of the Spemann organizer. Dev Biol 368:231–241. [PubMed: 22627292]

Reversade B, De Robertis EM. 2005. Regulation of ADMP and bmp2/4/7 at opposite embryonic poles generates a self-regulating morphogenetic field. Cell 123:1147–1160. [PubMed: 16360041]

Rogers C, Archer TC, Cunningham DD, Grammer TC, Casey SEM. 2008. *sox3* expression is maintained by FGF signaling and restricted to the neural plate by vent proteins in the *Xenopus* embryo. Dev Biol 313:307–319. [PubMed: 18031719]

Rogers CD, Ferzli GS, Casey ES. 2011. The response of early neural genes to FGF signaling and inhibition of BMP indicate the absence of a conserved neural induction module. BMC Dev Biol 11:74– doi: 10.1186/1471-213X-11-74. [PubMed: 22172147]

Rogers CD, Moody SA, Casey ES. 2009. Neural induction and factors that stabilize a neural fate. Birth Defects Res C: Embryo Today 87:249–262. [PubMed: 19750523]

Ruiz i Altaba A, Jessell TM. 1992. *Pintallavis*, a gene expressed in the organizer and midline cells of frog embryos: Involvement in the development of the neural axis. Development 116:81–93. [PubMed: 1483397]

Sasai Y 1998. Identifying the missing links: Genes that connect neural induction and primary neurogenesis in vertebrate embryos. Neuron 21:455–458. [PubMed: 9768831]

Schneider S, Steinbeisser H, Warga RM, Hausen P. 1996. Beta-catenin translocation into nuclei demarcates the dorsalizing center in frog and fish embryos. Mech Dev 57:191–198. [PubMed: 8843396]

Seo S, Herr A, Lim JW Richardson GA, Kroll KL. 2005. Geminin regulates neuronal differentiation by antagonizing brg1 activity. Genes Dev 19: 1723–1734. [PubMed: 16024661]

Seo S, Kroll KL. 2006. Geminin's double life: Chromatin connections that regulate transcription at the transition from proliferation to differentiation. Cell Cycle 5:374–379. [PubMed: 16479171]

Sharon N, Mor I, Zahavi E, Benvenisty N. 2012. DUXO, a novel double homeobox transcription factor, is a regulator of the gastrula organizer in human embryonic stem cells. Stem Cell Res 9:261–269. [PubMed: 23010573]

Sive HL, Grainger RM, Harland RM. 2000. Early Development of *Xenopus laevis*. A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Snider L, Tapscott SJ. 2005. XIC is required for siamois activity and dorsoanterior development. Mol Cell Biol 25:5061–5072. [PubMed: 15923623]

Sölter M, Köster M, Holleman T, Brey A, Pieler T, Knöchel W 1999. Characterization of a subfamily of related winged helix genes, *XFD-12/12′/12•* (*XFLIP*), during *Xenopus* embryogenesis. Mech Dev 89:161–165. [PubMed: 10559492]

Stern CD. 2006. Neural induction: 10 years on since the 'default model'. Curr Opin Cell Biol 18:692–697. [PubMed: 17045790]

Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD. 2000. Initiation of neural induction by FGF signaling before gastrulation. Nature 406:74–78. [PubMed: 10894544]

Suduo N, Yamamoto S, Ogino H, Taira M. 2012. Dynamic in vivo binding of transcription factors to cis-regulatory modules of *cer* and *gsc* in the step-wise formation of the Spemann-Mangold organizer. Development 139:1651–1661. [PubMed: 22492356]

Sullivan SA, Akers L, Moody SA. 2001. *foxD5a*, a *Xenopus* winged helix gene, maintains an immature neural ectoderm via transcriptional repression that is dependent on the C-terminal domain. Dev Biol 232: 439–457. [PubMed: 11401404]

Taira M, Jamrich M, Good PJ, Dawid IB. 1994. The LIM domain-containing homeobox gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. Genes Dev 6:356–366.

Taylor JJ, Wang T, Kroll KL. 2006. Tcf- and Vent-binding sites regulate neural-specific *geminin* expression in the gastrula embryo. Dev Biol 289:494–506. [PubMed: 16337935]

Uwanogho D, Rex M, Cartwright EJ, Pearl G, Healy C, Scotting PJ, Sharpe PT. 1995. Embryonic expression of the chicken *sox2*, *sox3* and *sox11* genes suggests an interactive role in neuronal development. Mech Dev 49:23–36. [PubMed: 7748786]

Vodicka MA, Gerhart JC. 1995. Blastomere derivation and domains of gene expression in the Spemann organizer of *Xenopus laevis*. Development 121:3505–3518. [PubMed: 8582265]

Wessely O, Kim JI, Geissert D, Tran U, De Robertis EM. 2004. Analysis of Spemann organizer formation in *Xenopus* embryos by cDNA microarrays. Dev Biol 269:552–566. [PubMed: 15110719]

Wills AE, Choi VM, Bennett MJ, Khokha MK, Harland RM. 2010. BMP antagonists and FGF signaling contribute to different domains of the neural plate in *Xenopus*. Dev Biol 337:335–350. [PubMed: 19913009]

Witta SE, Sato SM. 1997. 2 is a potential regulator of Spemann's organizer. Development 124:1179–1189. XlPOU [PubMed: 9102305]

Yamamoto S, Hikasa H, Ono H, Taira M. 2003. Molecular link in the sequential induction of the Spemann organizer: Direct activation of the *cerberus* gene by Xlim-1, xotx2, mix.1 and siamois, immediately downstream from nodal and wnt signaling. Dev Biol 257:190–204. [PubMed: 12710967]

Yan B, Neilson KM, Moody SA. 2009. foxd5 plays a critical upstream role in regulating neural fate and onset of differentiation. Dev Biol 329:80–95. [PubMed: 19250931]

Yan B, Neilson KM, Moody SA. 2010. Microarray identification of novel downstream targets of *FoxD5*, a critical component of the neural ectodermal transcriptional network. Dev Dyn 239:3467–3480. [PubMed: 21069826]

Yanai I, Peshkin L, Jorgensen P, Kirschner MW. 2011. Mapping gene expression in two *Xenopus* species: Evolutionary constraints and developmental flexibility. Dev Cell 20:483–496. [PubMed: 21497761]

Yoon J, Kim JH, Kim SC, Park JB, Lee JY, Kim J. 2014. PV.1 suppresses the expression of *FoxD5b* during neural induction in *Xenopus* embryos. Mol Cells 37:220–225. [PubMed: 24608799]

Young JJ, KJolby RA, Kong NR, Monica SD, Harland RM. 2014. Spalt-like 4 promotes posterior neural fates via repression of *pou5f3* family members in *Xenopus*. Development 141:1683–1693. [PubMed: 24715458]

Zaraisky AG, Ecochard V Kazanskaya OV Lukyanov SA, Fesenko IV Duprat AM. 1995. The homeobox-containing gene *XANF-1* may control development of the Spemann organizer. Development 121:3839–3847. [PubMed: 8582293]
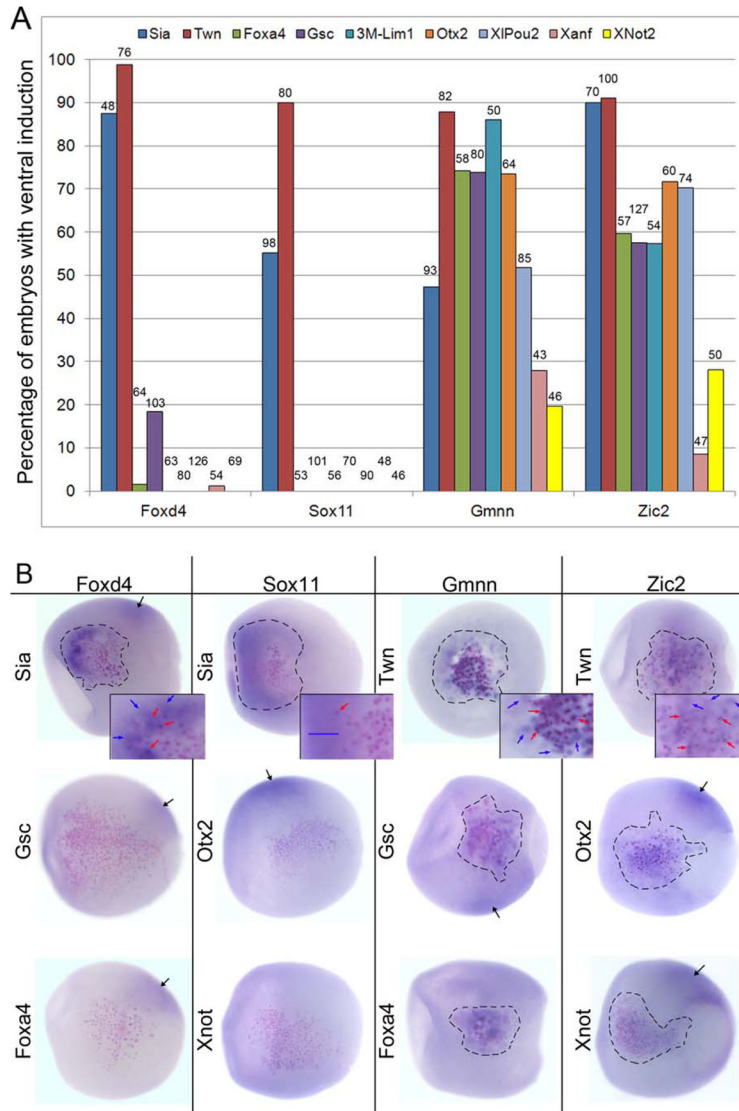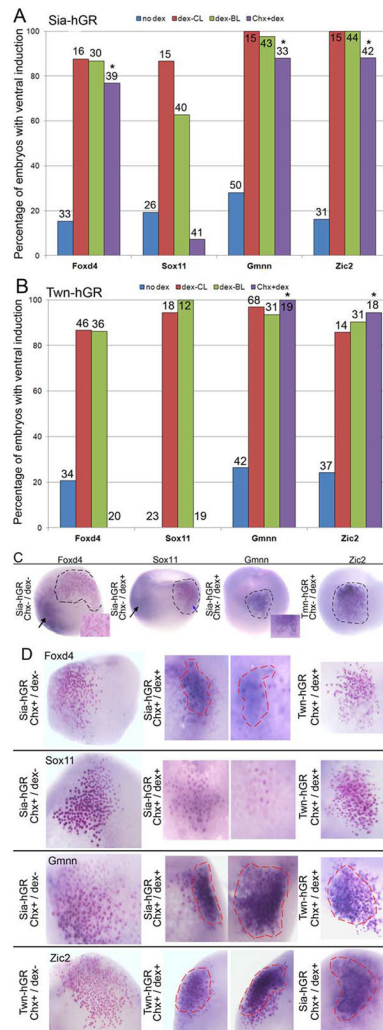
FIG. 1.

NE genes are ectopically induced in the ventral epidermis by different sets of Organizer transcription factors. (**a**) The percentage of embryos in which an ectopic ventral patch of gene expression (*Foxd4, Sox11, Gmnn, Zic2*) was observed after mRNA encoding an Organizer transcription factor, indicated by colored bars (key is across the top), was injected into a ventral epidermis progenitor (V1.1; Moody, 1987). The number of embryos analyzed is shown over each bar. (**b**) Examples of ectopic ventral induction of the four NE genes in response to injection of Organizer transcription factor mRNAs. Induced clones are outlined in black. *Foxd4* and *Sox11* are ectopically induced by Sia, but not by Gsc, Otx2, Foxa4 or Xnot2, whereas *Gmnn* and *Zic2* are ectopically induced by each of these genes. Images are of the ventral sides of gastrula stage embryos, animal pole to the left. The cells expressing the Organizer transcription factor (noted to the left of each embryo) are identified by pink nuclei (nbgal staining). The NE genes, labeled at the top of each column, were detected by ISH (purple reaction product). Higher magnification insets in the top row show examples

of nβgal-positive cells that are also NE gene-positive (red arrows); for these cells the induction is likely cell autonomous. Blue arrows indicate cells that are nβgal-negative and NE gene-positive; for these cells the induction is non-cell autonomous. For *Sox11*, the blue bar indicates a broad region of ectopic induction in which there are no nβgal-positive cells, demonstrating that most of its induction is indirect. Black arrows indicate the endogenous expression domain of the NE gene on the dorsal side of the embryo.

FIG. 2.

NE genes are directly induced in the ventral epidermis by Sia or Twn. (**a**) The percentage of embryos in which an ectopic ventral patch of gene expression (*Foxd4, Sox11, Gmnn, Zic2*) was observed after injection of *hGR-Sia* mRNA. Embryos were either not treated with hormone (no dex, blue bars), treated with hormone at the 64-cell cleavage stage (dex-CL, red bars), treated with hormone at the stage 8 blastula (dex-BL, green bars), or pretreated at stage 8 with a protein synthesis inhibitor 40 minutes before hormone treatment (Chx+dex, purple bars). The high frequency of induction of ectopic gene expression with dex treatment alone at either cleavage or blastula stages indicates that the construct is hormone-inducible. *Foxd4, Gmnn* and *Zic2* are likely direct targets of Sia because in the presence of Chx they are induced at the same frequency as dex treatment alone, and at a significantly greater frequency compared to no dex embryos (*, $p < 0.001$); *Sox11* is not a direct target because it is not induced when protein synthesis is blocked by Chx. The number of embryos analyzed is shown over each bar. (**b**) The percentage of embryos in which an ectopic ventral patch of gene expression (*Foxd4, Sox11, Gmnn, Zic2*) was observed after injection of *hGR-Twn* mRNA. The data are presented as described in A. *Gmnn* and *Zic2* are likely direct targets of Twn (*, $p < 0.001$), whereas *Foxd4* and *Sox11* are not. The

number of embryos analyzed is shown over each bar. (**c**) When an embryo is injected with *Sia-hGR* mRNA and not treated with Chx or dex (Chx-/dex−) (left image), NE genes (in this case *Foxd4*) are not induced; the Sia-hGR expressing cells (outlined, and in the inset) have pink nuclei but no purple reaction product. When an embryo is injected with *Sia-hGR* or *Twn-hGR* mRNA and treated with only dex (Chx-/dex+), NE genes are induced (outlined). For *Sox11*, there is considerable non-cell autonomous induction (in area indicated by blue arrow), whereas for *Gmnn* nearly all the labeled cells have pink nuclei (inset). Black arrows indicate endogenous expression domain. (**d**) Examples of direct induction of NE genes by *hGR-Sia* or *hGR-Twn* mRNAs. The injected mRNAs (*Sia-hGR, Twn-hGR*) are indicated to the left of each image, and the assayed NE gene is indicated at the upper left of each row. Embryos treated only with Chx do not show ectopic induction (Chx+/dex−); only pink nuclei are visible. Two examples of responses to Sia-hGR and one example of responses to Twn-hGR direct induction at blastula stages (Chx+/dex+) are shown for *Foxd4*, *Sox11*, and *Gmnn;* two examples of responses to Twn-hGR and one example of response to Sia-hGR direct induction at blastula stages (Chx+/dex+) are shown for *Zic2*. The region of ectopic induction (purple) is outlined by red dashes in each case. Sia directly induces *Foxd4*, *Gmnn* and *Zic2;* Twn directly induces *Gmnn* and *Zic2. Sox11* is not directly induced by either.
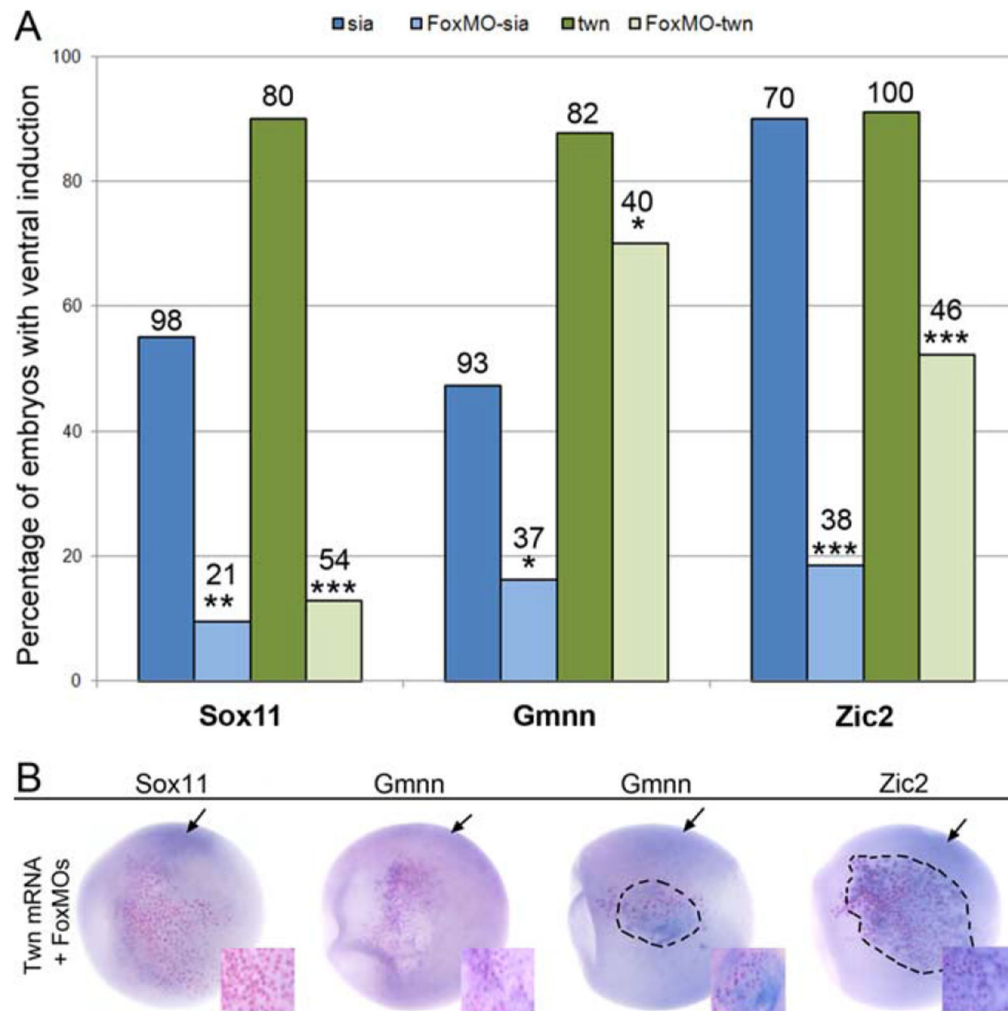
FIG. 3.

Foxd4 mediates some of the ectopic induction of *Sox11, Gmnn* and *Zic2*. The percentage of embryos in which an ectopic patch of gene expression (*Sox11, Gmnn, Zic2*) was observed after injection of *Sia* (blue bars) or *Twn* (green bars) mRNA in a ventral epidermal lineage in which Foxd4 translation was knocked-down (FoxMO). *Sox11* induction by Sia and Twn was dramatically reduced in the absence of Foxd4. *Gmnn* and *Zic2* induction by Sia was more strongly affected by the absence of Foxd4 than was their induction by Twn (***,$p < 0.001$;**, $p < 0.01$;*, $p < 0.05$). The number of embryos analyzed is shown over each bar. Examples of ectopic ventral expression of *Sox11, Gmnn* and *Zic2* in response to injection of *Twn* mRNA in the presence of Foxd4 MOs. Black arrows point to the endogenous expression domains of the genes on the dorsal side. In most cases, there was no detectable *Sox11* expression at the site of Twn expression (pink nuclei without purple reaction product; inset). For *Gmnn* and *Zic2*, some embryos showed no induction in the absence of Foxd4 (*Gmnn* left embryo; pink nuclei without purple reaction product; inset), whereas in others there was robust induction despite the knock-down of Foxd4 (*Gmnn*, right embryo and *Zic2;* pink nuclei surrounded by purple reaction product; insets). All embryos are oriented with animal pole to left and vegetal pole to right.
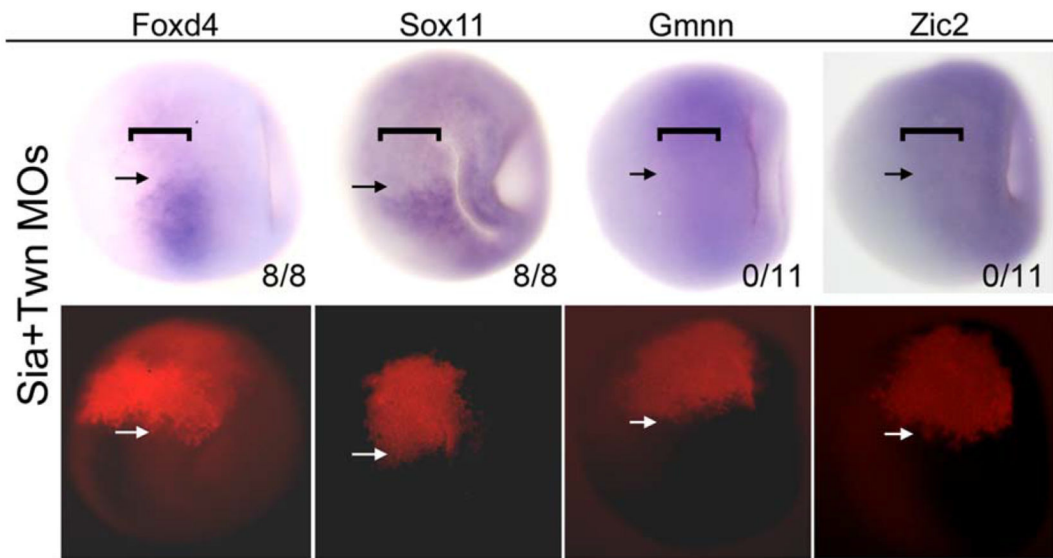
FIG. 4.

Sia/Twn are required for blastula expression of *Foxd4* and *Sox11*. Paired bright field (top row) and fluorescence (bottom row) images of blastula stage embryos in which Sia+Twn MOs were injected into a 16-cell blastomere that gives rise to the dorsal-equatorial blastula precursors of the Organizer mesoderm and neural ectoderm (marked by a bracket in the top row). The location of the MOs is visualized by red (lissamine) fluorescence on the left side of the embryo (above the white arrows in the bottom row). Normal *Foxd4* and *Sox11* expression is more restricted to these dorsal-equatorial cells (brackets), whereas *Zic2* and *Gmnn* normally are expressed throughout the animal hemisphere including the dorsal-equatorial cells (shown in right side of each embryo; below the black arrows). *Foxd4* and *Sox11* expression is greatly reduced in the region containing Sia/Twn MOs (above the black arrows in the top row). In contrast, *Gmnn* and *Zic2* expression appears unaffected. The numbers refer to the number of embryos in which gene expression was repressed by Sia/Twn knock down. Dorsal views of embryos are oriented with animal pole to the right.
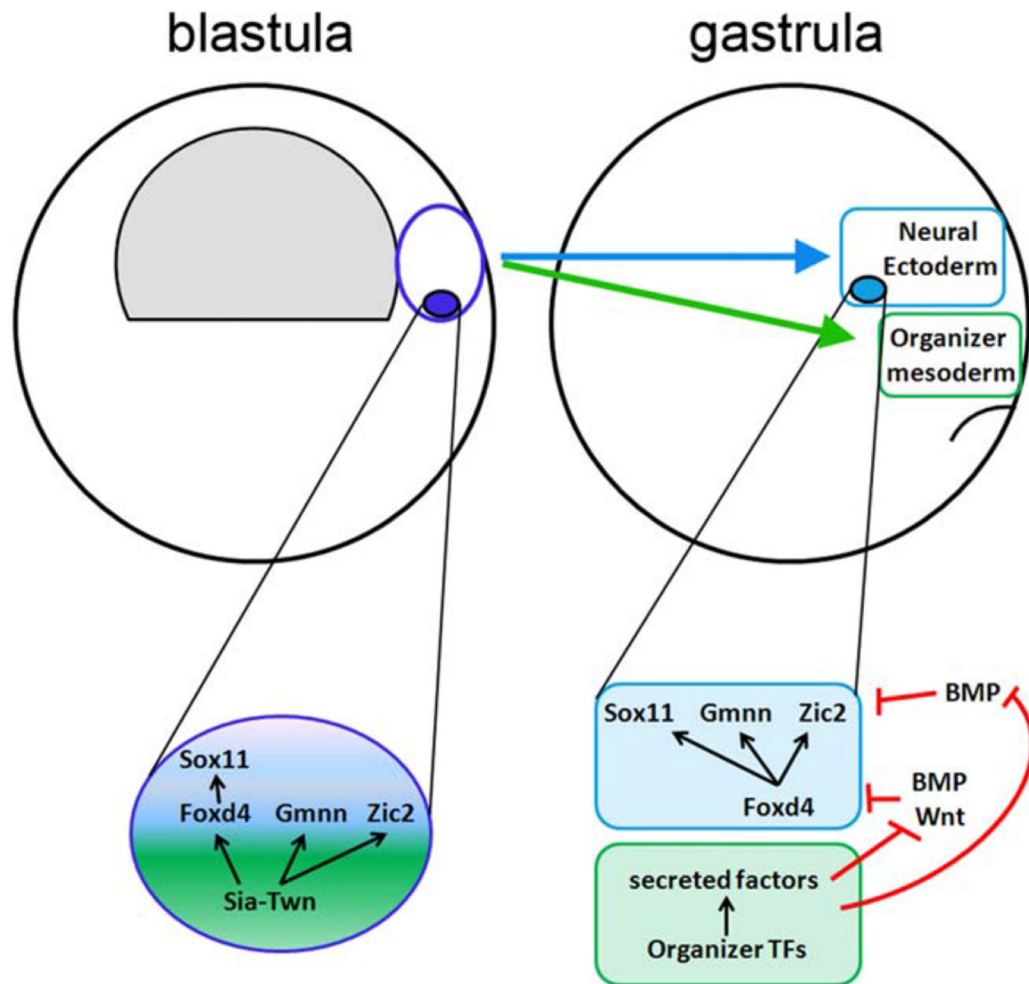
FIG. 5.

Summary of Sia/Twn time and location of induction of NE genes. At the blastula stage, cells that are fated to give rise to both Organizer mesoderm and neural ectoderm express Sia and Twn, which in turn directly activate *Foxd4, Gmnn* and *Zic2; Sox11* activation requires the presence of Foxd4. At the gastrula stage, when the descendants of these cells have segregated into the Organizer mesoderm and the neural ectoderm, NE genes are up-regulated indirectly by Organizer transcription factors that regulate the expression of secreted factors that inhibit the BMP and Wnt pathways. Previous studies show that *Sox11, Gmnn* and *Zic2* are expressed by inhibiting BMP, whereas *Foxd4* is best expressed when both BMP and Wnt signaling pathways are blocked (refs in text). In the neural ectoderm, Foxd4 directly activates *Sox11, Gmnn* and *Zic2* (Yan et al., 2009).