A role for Vg1/Nodal signaling in specification of the intermediate mesoderm

Britannia M. Fleming[1], Ronit Yelin[1], Richard G. James[2] and Thomas M. Schultheiss[1,*]

SUMMARY

The intermediate mesoderm (IM) is the embryonic source of all kidney tissue in vertebrates. The factors that regulate the formation of the IM are not yet well understood. Through investigations in the chick embryo, the current study identifies and characterizes Vg1/Nodal signaling (henceforth referred to as 'Nodal-like signaling') as a novel regulator of IM formation. Excess Nodal-like signaling at gastrulation stages resulted in expansion of the IM at the expense of the adjacent paraxial mesoderm, whereas inhibition of Nodal-like signaling caused repression of IM gene expression. IM formation was sensitive to levels of the Nodal-like pathway co-receptor Cripto and was inhibited by a truncated form of the secreted molecule cerberus, which specifically blocks Nodal, indicating that the observed effects are specific to the Nodal-like branch of the TGFβ signaling pathway. The IM-promoting effects of Nodal-like signaling were distinct from the known effects of this pathway on mesoderm formation and left-right patterning, a finding that can be attributed to specific time windows for the activities of these Nodal-like functions. Finally, a link was observed between Nodal-like and BMP signaling in the induction of IM. Activation of IM genes by Nodal-like signaling required an active BMP signaling pathway, and Nodal-like signals induced phosphorylation of Smad1/5/8, which is normally associated with activation of BMP signaling pathways. We postulate that Nodal-like signaling regulates IM formation by modulating the IM-inducing effects of BMP signaling.

KEY WORDS: Chick embryo, Intermediate mesoderm, Kidney, Mesoderm patterning, Nodal, Vg1

INTRODUCTION

The intermediate mesoderm (IM), a region of mesoderm that lies between the somites and lateral plate in the developing embryo, is the source of all vertebrate kidney tissue (Saxen, 1987). Although much has been learned recently about the inductive signals and transcription factors that regulate differentiation of IM into kidney tissue (Costantini and Kopan, 2010; Dressler, 2006; Dressler, 2009; Yu et al., 2004), much less is known regarding the factors that regulate formation of the IM itself.

Several previous studies have begun to characterize the molecular signals that lead to initial IM specification. Work from our laboratory and others established that BMP signaling plays a central role in IM formation (James and Schultheiss, 2005; Obara-Ishihara et al., 1999). Activation of the earliest IM genes, including those encoding the transcription factors Osr1 and Pax2, requires intermediate levels of BMP signaling, with higher levels generating lateral plate (LP) tissue and lower levels leading to paraxial mesoderm (PM) and somite formation (James and Schultheiss, 2005). These results are consistent with many studies that have described a central role for BMP signaling in patterning the dorsal-ventral axis of the mesoderm (or medial-lateral axis in flat embryos such as the chick) (DeRobertis and Sasai, 1996; Jones et al., 1996). In addition, studies in both *Xenopus* animal caps and chick embryos have found that kidney formation can be promoted by a combination of activin and retinoic acid (RA) signals (Moriya et al., 1993; Preger-Ben Noon et al., 2009). However, the modest results obtained thus far from attempts to activate IM genes in ES cells using bone morphogenetic protein (BMP), activin, RA and other

candidate signaling molecules (Bruce et al., 2007; Kim and Dressler, 2005) testifies to the need for greater basic knowledge regarding IM specification during embryogenesis.

The TGFβ family comprises a large group of signaling molecules, including BMPs, TGFβ, Nodal, Vg1, growth and differentiation factors (GDFs) and activin, that have multiple roles in development, physiology and cancer (Derynck and Miyazono, 2008; Schier, 2003). TGFβ family members signal primarily via Type I and Type II serine/threonine kinase receptor heterodimers and receptor Smad (rSmad) family intracellular signal transduction components that can recruit co-factors to activate or repress transcription. The TGFβ family can be divided according to the particular Type I receptors and rSmads used by the members of the family. BMP signals typically use Alk 3 and 6 Type I receptors and Smad 1, 5 and 8 adapters, whereas TGFβ, Nodal, Activin and some GDFs utilize Alk 4, 5 and 7 Type I receptors and the Smad 2 and 3 adapters. This second group can be further subdivided into those signals that require an EGF-CFC-family co-receptor, such as Nodal, Vg1 and GDF1 (which we will refer to as the 'Nodal-like signals'), and those that do not, such as activin and TGFβ (Cheng et al., 2003; Yan et al., 2002). TGFβ family members can also signal through non-Smad pathways, including mitogen-activated protein (MAP) kinases or Rho-like GTPases (Derynck and Zhang, 2003).

Nodal-like signaling has been found to play an essential role during several stages of vertebrate mesoderm formation and patterning. Nodal and/or Vg1 are required for mesoderm as well as endoderm formation in pre-gastrula and early gastrula stages of development (Conlon et al., 1994; Feldman et al., 1998; Schier and Talbot, 2001; Shah et al., 1997; Sirotkin et al., 2000), and Nodal signaling also plays a central role in initiating left-right patterning (Levin et al., 1995; Whitman and Mercola, 2001). However, data regarding whether Nodal-like signaling also plays a role in the establishment of particular types of mesoderm is less clear and somewhat conflicting. It has been reported that graded addition of Nodal to pre-gastrula *Xenopus* embryos results in formation of

[1]Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel. [2]Department of Pharmacology, University of Washington, Seattle, WA, 98195, USA.

*Author for correspondence (tschulth@tx.technion.ac.il)

progressively more dorsal types of mesoderm (Agius et al., 2000), and that exposure to Nodal for increasing lengths of time generates more marginal mesodermal cell types in zebrafish (Hagos and Dougan, 2007). However, in other studies, graded levels of Nodal signaling have been found to distinguish between prechordal mesoderm and notochord (Dougan et al., 2003; Gritsman et al., 2000; Harvey and Smith, 2009), but were reported not to influence formation of other types of mesoderm (Dougan et al., 2003; Gritsman et al., 2000).

One of the difficulties of studying potential roles of Nodal-like signaling in the formation of specific mesodermal tissues is the profound effect of Nodal manipulation on earlier stages of development, specifically the initial formation of the mesoderm itself. The avian embryo has the experimental advantage of being readily accessible to manipulation throughout development. In the current study, we have taken advantage of this feature and manipulated Nodal-like signaling in avian embryos at stages after initial formation of the mesoderm. Perhaps surprisingly, we find that during gastrulation, Nodal-like signaling promotes IM formation at the expense of paraxial mesoderm, which can be considered a form of ventralization. We also find that Nodal-like signaling interacts with BMP signaling to regulate the formation of IM. These observations expand our knowledge of the molecular mechanisms of IM formation, and advance our understanding of the role of Nodal-like signaling in mesodermal patterning.

MATERIALS AND METHODS
In situ hybridization
Whole-mount *in situ* hybridization was performed as previously described (Schultheiss et al., 1995), using digoxygenin-labeled RNA probes for chick *Osr1* (James and Schultheiss, 2005), *Pax2* (James and Schultheiss, 2005), *Lim1* (Tsuchida et al., 1994), *Paraxis* (James and Schultheiss, 2003) and *Tbx-6L* (Knezevic et al., 1997). Following whole-mount *in situ* hybridization, embryos were embedded in gelatin and cryosectioned (Leica) (James and Schultheiss, 2003). *In situ* hybridization was performed on explants after their dissection from the collagen gels in which they were grown, as described previously (James and Schultheiss, 2003).

Immunofluorescence
Immunofluorescence was performed on cryosectioned chick embryos as previously described (James et al., 2006). The following primary antibodies were used: rabbit anti-Pax2 (1:250, Babco), mouse anti-Lim1 (1:10, Developmental Studies Hybridoma Bank), mouse anti-Pax7 (1:1000, Developmental Studies Hybridoma Bank), and mouse or rabbit anti-GFP (1:250, Molecular Probes). Sections were incubated with secondary antibodies (1:250, Jackson ImmunoResearch) for 1 hour, and were then washed with PBS and incubated with DAPI (1 μg/ml, Sigma) before coverslipping.

All steps for whole-mount immunofluorescence for pSmad1/5/8 were performed at 4°C. Embryos were fixed in fresh 4% paraformaldehyde (PFA) in PBS from 2 hours to overnight, washed three times with PBT (PBS with 0.1% Tween-20), dehydrated through a graded PBT:methanol series, and stored in 100% methanol at −20°C. For staining, embryos were rehydrated to PBT and blocked overnight with PBS containing 2% bovine serum albumin (BSA), 10% sheep serum, and 0.1% Tween-20. Embryos were incubated overnight with anti-pSmad1/5/8 (1:50, Cell Signaling Technology #9511) in blocking solution, rinsed with PBT for 8 hours, incubated with secondary antibody (1:250, Jackson #711-166-152) overnight, rinsed three times with PBT, and mounted with fluorescent mounting medium (Dako). Wide-field images were captured on a Zeiss Axioimager M1 upright microscope with a Qimaging Exi Blue monochrome digital camera. Confocal imaging was performed on a Zeiss LSM 700 upright confocal microscope.

Gene cloning into expression plasmids
The pMES expression vector (Swartz et al., 2001) was used to express genes of interest in chick embryos. pMES drives expression from a CMV/chicken β-actin promoter/enhancer, and expresses green fluorescent protein (GFP) from an internal ribosomal entry sequence (IRES) element. Full-length chicken Cripto was cloned from chick embryonic cDNA and subcloned into the pMES expression vector. Additional genes cloned into pMES included chick dorsalin-Vg1 (Shah et al., 1997), constitutively active Alk4 (Yeo and Whitman, 2001), mouse Lefty2 (Ishimaru et al., 2000) and *Xenopus* Tomoregulin-1 (Harms and Chang, 2003).

Electroporation
Electroporation and culture of chicken embryos was performed as previously described (James and Schultheiss, 2005). Briefly, embryos were collected onto a paper ring and suspended in PBS, dorsal side up, above a positive electrode. A solution containing DNA (0.6 μg/μl) and 0.05% Fast Green was injected into the space between the embryos and the vitelline membrane. A negative electrode (tungsten wire) was lowered above the embryos until it entered the PBS solution, and the embryos were pulsed three times (each pulse 100 mseconds at 12-15 V) using a BTX electroporator. Embryos were cultured intact, ventral side up, on agar-albumin culture dishes (50% albumin, 0.36% agar, 0.36% NaCl, 1.5% glucose) at 38°C for 24-48 hours, and were fixed in 4% PFA for future analysis.

Cell culture, transfection and transplantation
COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. The cells were transfected with a dorsalin-Vg1 expression plasmid [kindly provided by J. Dodd (Shah et al., 1997)], a Nodal expression plasmid [kindly provided by C. Stern (Bertocchini and Stern, 2002)], a Lefty2 expression plasmid [kindly provided by H. Hamada (Yoshioka et al., 1998)] or a cerberus-short expression plasmid [kindly provided by C. Stern (Bertocchini and Stern, 2002; Piccolo et al., 1999)] using Lipofectamine (Invitrogen). Cells transfected with pMES alone served as a control. Following transfection, the cells were cultured for 72 hours, and were grown in hanging drops containing 500 cells for an additional 48 hours. Cell aggregates were transplanted into embryos using a tungsten needle and mouth pipette. The embryos were cultured for 24-48 hours and fixed in 4% PFA for future analysis.

Explant culture
Regions of the primitive streak were dissected and cultured in collagen gels as previously described (James and Schultheiss, 2005). In selected cultures, BMP2, Activin, Noggin (all from R&D Research) or SB431542 (Sigma), were added to the medium at the indicated doses. Explants were processed for whole-mount *in situ* hybridization, as described above.

RESULTS
Nodal-like signaling promotes IM formation at the expense of paraxial mesoderm
In order to investigate a possible role for the Nodal-like branch of the TGFβ family during IM formation, we introduced pellets of Vg1-expressing COS cells into stage 4 (Hamburger and Hamilton, 1951) mid-gastrula chick embryos. We chose to focus on the Nodal/Vg1 sub-family of the TGFβ family because these molecules are known to be expressed and active during gastrulation stages (Conlon et al., 1994; Ishimaru et al., 2000; Joubin and Stern, 1999; Shah et al., 1997). As seen in Fig. 1 and Table 1, implants of Vg1-expressing cells induced a strong expansion of the IM in the area surrounding the implant (as evaluated by morphology and expression of the markers *Osr1*, *Pax2* and *Lim1*; Fig. 1A-L) and a corresponding contraction of the paraxial mesoderm (PM) (as evaluated by morphology and by the marker *Paraxis*; Fig. 1M-P). This effect was observed when the cells were placed adjacent to the primitive streak at a position ~20% of the distance between Hensen's node and the posterior end of the streak (see diagram in Fig. 1), a region that fate maps to the prospective paraxial mesoderm (Garcia-Martinez and
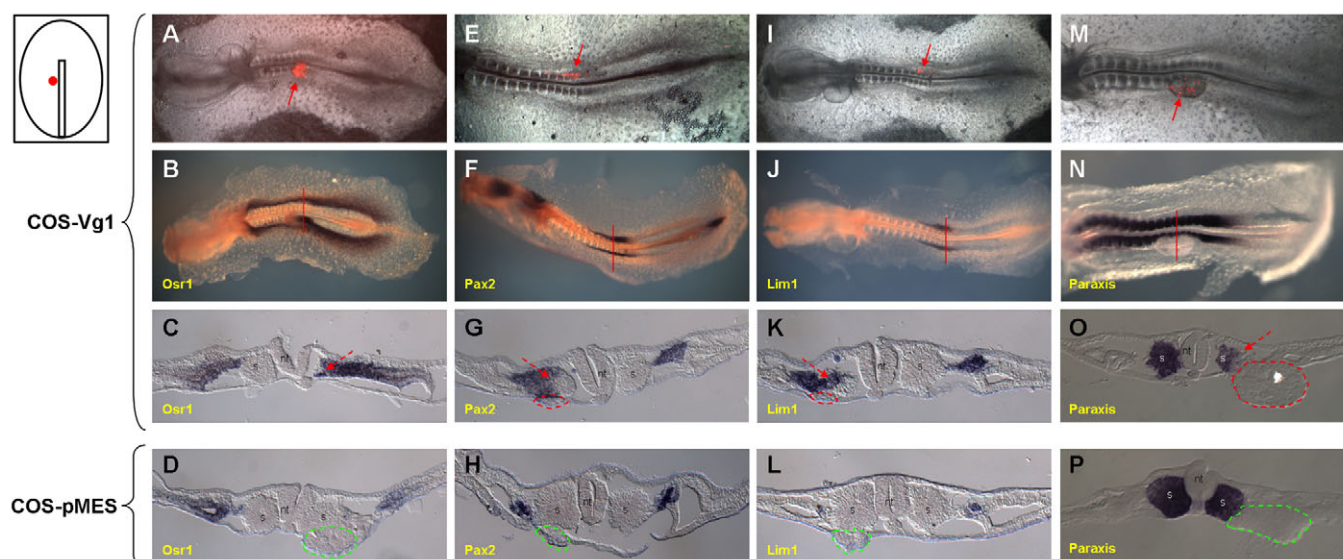
Fig. 1. Vg1 induces expansion of the intermediate mesoderm. COS cells transfected with Vg1 (A-C,E-G,I-K,M-O) or control pMES (D,H,L,P) were implanted into stage 4 chick embryos adjacent to the primitive streak at 20% streak length. COS pellets were marked with DiI (A,E,I,M) to identify their location. Diagram at upper left indicates position of cell pellets at the start of the experiment. Embryos are oriented with anterior towards the left, whereas the diagram is oriented with the anterior end up. Embryos were grown for 48 hours and analyzed by *in situ* hybridization for *Osr1* (**A-D**), *Pax2* (**E-H**), *Lim1* (**I-L**) or *Paraxis* (**M-P**). The red lines crossing the embryos in B, F, J and N indicate approximate planes of section shown in C, G, K and O, respectively. Locations of implants are indicated by arrows in whole embryos (A,E,I,M) and by dashed lines in sections (D,G,H,K,L,O,P). Dashed arrows denote expansion of IM markers into the somite compartment (C,G,K) or reduction in somite size and gene expression (O). nt, neural tube; som, somite.

Schoenwolf, 1992; James and Schultheiss, 2003; Psychoyos and Stern, 1996). Under these conditions, mesodermal cells were exposed to the ectopic Vg1 signal during and shortly after their gastrulation. When the Vg1-expressing cells were placed further away from the streak, such changes in expression of PM or IM genes were not observed. Changes in PM or IM marker expression were also not observed if the Vg1 cells were placed adjacent to the posterior streak, the source of cells for the lateral part of the embryo (Garcia-Martinez and Schoenwolf, 1992; James and Schultheiss, 2003; Psychoyos and Stern, 1996). In summary, ectopic Vg1 produced expansion of the IM at the expense of the PM when cells were exposed to Vg1 around the time of their ingression through the primitive streak. These results are distinct from the effects of ectopic BMP signaling, which also produces a contraction of the PM but does not result in IM expansion (James and Schultheiss, 2005) (see Discussion).

Because the experiments described above used pellets of COS cells expressing Vg1, there remained the possibility that Vg1 itself was not acting on the embryo but was inducing the COS cells to express a factor which in turn affected the embryo. In order to address this issue, embryos were electroporated with a Vg1 expression construct. Electroporations targeted the primitive streak at 20% streak length (see diagram in Fig. 2) in order to target the prospective PM and were carried out at stage 3 in order to allow the electroporated plasmid to begin to be expressed by stage 4. Similar results were obtained with the expression construct as with the cell pellets, with expansion of IM markers into the PM region (Fig. 2; Table 1), indicating that Vg1 itself can produce changes in IM gene expression.

Promotion of IM gene expression by Vg1 is not cell-autonomous

We next examined whether mesodermal cells responded to Vg1 in a cell-autonomous manner. The Nodal-like branch of the TGFβ pathway signals primarily through the TGFβ Type I receptors Alk4, 5 and 7 (Reissmann et al., 2001). A mutated Alk4 receptor containing a T-to-E mutation in the transmembrane domain has been found to activate the Nodal-like pathway specifically and without the need for

Table 1. Summary of experimental results

	Treatment	Experimental	Control	*P*-value
1	Vg1 pellet (Fig.1)	59/88 (67%)	0/77 (0%)	$<1\times10^{-6}$
2	Vg1 electroporation (Fig. 2)	21/24 (88%)	*n*=22	N.A.
3	caAlk4 electroporation (Fig. 3)	13/23 (57%)	*n*=28	N.A.
4	Lefty2 pellet (Fig. 4)	17/32 (53%)	0/35 (0%)	$<1\times10^{-5}$
5	Cerberus pellet (Fig. 4)	20/26 (77%)	4/31 (13%)	$<1\times10^{-6}$
6	Cripto electroporation (Fig. 6)	12/21 (57%)	*n*=17	N.A.
7	Tomoregulin electroporation (Fig. 6)	14/27 (52%)	*n*=24	N.A.

For experiments with pellets, a positive result is defined as expansion (line 1) or reduction (lines 4 and 5) of IM gene expression on the side with the pellet compared with the side without the pellet in the same embryo. In electroporation experiments, because both sides of the embryo usually contained electroporated cells, a positive result is defined as an expansion (lines 2, 3 and 6) or reduction (line 7) of IM gene expression in treated embryos compared with that typically seen in control electroporations. For that reason, for electroporation experiments, the percent positive is given only for the experimental embryos, whereas for control embryos only the number of controls examined is given. Statistical significance for lines 1, 4 and 5 was calculated by the *Z*-test for comparison of two proportions. N.A., not applicable.
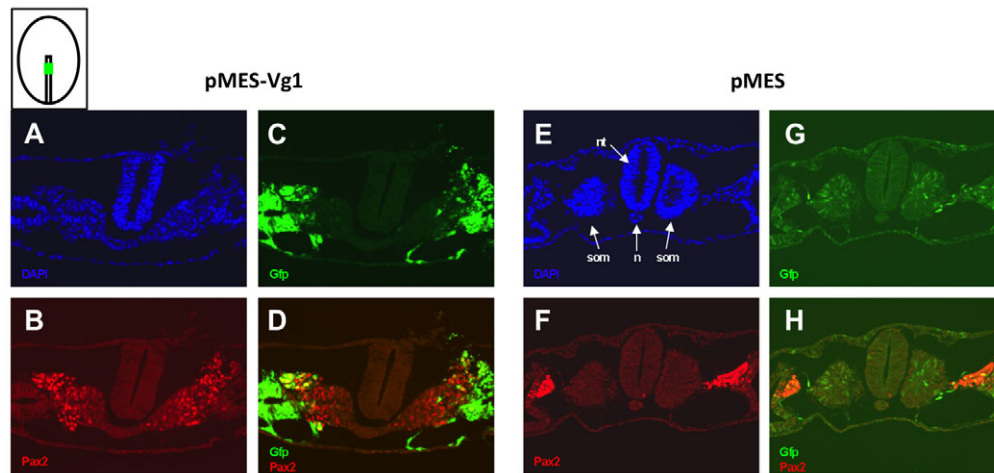
Fig. 2. Vg1 alone is sufficient to expand the IM. Electroporation was used to introduce a Vg1-expressing plasmid (A-D) or a control empty plasmid (E-H) into stage 3 chick embryos, targeted to 20% streak length. Diagram at upper left indicates location targeted by electroporation. Embryos were grown for 48 hours and stained with DAPI (**A,E**), and with antibodies to Pax2 (**B,F**) and GFP (**C,G**). Merged channels of B,C (**D**) and F,G (**H**) are shown. In Vg1-electroporated embryos, Pax2 expression reached the midline at the expense of the posterior somites and notochord, which did not develop properly (B,D). The control embryo electroporated with pMES alone did not induce ectopic Pax2 expression (F,H). n, notochord; nt, neural tube; som, somite.

a ligand (Chang et al., 1997). Electroporation of such a constitutively active Alk4 (caAlk4) construct (targeting the 20% streak level in stage 3 embryos, as in diagram in Fig. 2) resulted in ectopic activation of Osr1 and Pax2 in paraxial mesoderm (Fig. 3; data not shown; Table 1). Interestingly, the effect did not appear to be cell-autonomous. Ectopic Osr1 or Pax2 were found in cells adjacent to cells expressing high levels of GFP, but were typically not found in the high-GFP-expressing cells themselves. This suggests that high levels of Alk4/5/7 signaling might induce a second signal, which in turn induces IM genes (see Discussion). This is in contrast to results obtained with constitutively active BMP receptors Alk3 and Alk6, which can activate Osr1 and Pax2 expression in paraxial mesoderm in a cell-autonomous manner (James and Schultheiss, 2005).

Nodal-like signaling is required for IM gene expression

In order to evaluate whether Nodal-like signaling is required for IM gene expression, COS cells expressing the secreted Nodal inhibitor Lefty2 (Chen and Schier, 2002; Meno et al., 1999; Yoshioka et al., 1998) were implanted into stage 4 embryos adjacent to the mid-primitive streak, (30-40% streak length, which fate maps to the prospective IM). As seen in Fig. 4A-F and Table 1, the COS-Lefty2 cells produced a significant reduction of *Pax2* expression on the treated side. In a second loss-of-function approach, implantation at the same location of COS cells expressing a truncated form of the secreted molecule cerberus (cerberus-short), which has been reported to specifically inhibit Nodal signaling (Bertocchini and Stern, 2002; Piccolo et al., 1999), also inhibited IM gene expression (Fig. 4G-J; Table 1). The inhibition of *Pax2* expression by cerberus short was always partial, whereas Lefty2 occasionally produced complete inhibition of *Pax2* expression (as shown in Fig. 4A-F), although in most cases the effect of Lefty2 was also partial.

A third loss-of-function approach used an *in vitro* system for studying IM. Fate-mapping studies have determined the location of the prospective PM and IM when they are resident in the primitive streak (Garcia-Martinez and Schoenwolf, 1992; James and Schultheiss, 2003; Psychoyos and Stern, 1996). Based on these fate maps, prospective IM was dissected from the mid-streak region (30-

40% streak length) of stage 4 embryos and treated with 50 ng/ml SB431542, a specific chemical antagonist of Alk4/5/7-mediated signaling (Inman et al., 2002). As seen in Fig. 5, SB431542 strongly inhibited expression of the early IM marker *Osr1* in IM explants. [Under *in vitro* conditions, prospective IM reproducibly expresses *Osr1* whereas expression of the later markers *Pax2* and *Lim1* is more variable (James and Schultheiss, 2005) and thus the *in vitro* analysis here was restricted to *Osr1*.] The inhibition was specific to IM markers, as expression of the paraxial mesoderm marker *Paraxis* was maintained in the SB431542-treated explants (Fig. 5C; *Paraxis* is expressed in untreated 'IM' explants because the IM and PM regions are not yet distinct in the primitive streak at the stage that the explants were taken). Taken together, the data shown in Figs 4 and 5 indicate that Nodal-like signaling, and most likely Nodal itself, is required for normal levels of IM gene expression.

Levels of the EGF-CFC co-factor Cripto are rate limiting for IM formation

Nodal-like ligands, unlike activins, require the activity of the EGF-CFC co-receptor Cripto (Gritsman et al., 1999) in order activate downstream signaling pathways. Electroporation of a Cripto expression construct into chick embryos (targeting stage 3 embryos at the 20% streak level) resulted in marked expansion of IM markers (Fig. 6A-F; Table 1). Unlike the effect of electroporating Vg1 (see Fig. 2), ectopic Cripto resulted in an expansion of the IM without an accompanying contraction of the PM. This result suggests that levels of Cripto in the prospective IM might be rate limiting for the response to Nodal-like signaling. Electroporation of the molecule tomoregulin-1, which has been reported to have Cripto inhibitory activity (Harms and Chang, 2003), resulted in a decrease in IM gene expression (Fig. 6G-L; Table 1), consistent with a requirement of Cripto for IM gene expression.

The IM-inducing and left-right patterning activities of Nodal-like signaling are distinct and act during different time windows

The current results introduce a conundrum that needs to be addressed. We have found in these studies that Nodal-like signals regulate
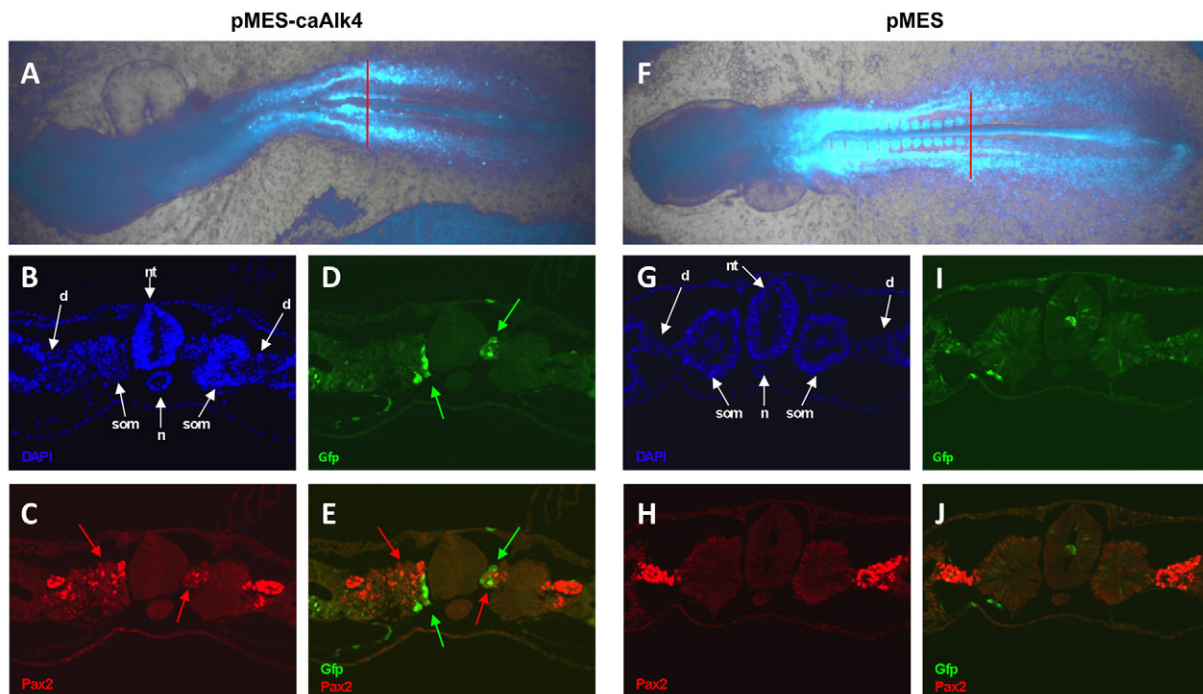
Fig. 3. Activation of IM markers by the Alk4 receptor is non-cell-autonomous. Electroporation was used to introduce a constitutively active Alk4-expressing plasmid (A-E) or a control empty plasmid (F-J) into stage 3 chick embryos, as in Fig. 2. Embryos were grown for 48 hours and stained with DAPI to mark nuclei (**B**,**G**), with antibodies to Pax2 (**C**,**H**) and with antibodies to GFP (**D**,**I**). Merged channels of C,D (**E**) and H,I (**J**) are shown. The red lines crossing the embryos (**A**,**F**) indicate approximate planes of section shown in B-E and G-J, respectively. The differences in GFP distribution in the caAlk4-electroporated embryo (A,D) and the control pMES electroporated embryo (F,I) suggested that somites were not capable of expressing high levels of caAlk4, resulting in groups of GFP-expressing cells being found mostly outside the somites, both medially and laterally (A, green arrows in D,E). In the caAlk4-electroporated embryo, ectopic expression of Pax2 was found in areas outside the IM, including the somites and groups of cells adjacent to the neural tube (red arrows in C,E). Cells expressing high levels of GFP did not express Pax2, but Pax2 was expressed in neighboring cells (red arrows in E), indicating non-cell-autonomous activation of Pax2 by caAlk4. Control embryos electroporated with pMES alone did not induce ectopic Pax2 expression (H,J). d, nephric duct; n, notochord; nt, neural tube; som, somite.

formation of the IM. However, beginning at stage 7 (1-somite stage) Nodal is expressed asymmetrically in the left LP, yet IM genes such as *Pax2* are not expressed asymmetrically. Because the manipulations of Nodal-like signals that resulted in changes in IM gene expression were performed at stage 4 (mid-gastrulation stage), we hypothesized that the ability of Nodal-like signaling to regulate IM genes might be confined to a specific stage of development. In order to test this hypothesis, Vg1 pellets were introduced into the right LP at stage 7. As seen in Fig. 7A,B, the implanted Vg1 cells caused ectopic activation of Nodal, as expected (Tavares et al., 2007), indicating that the implanted beads were functioning in a manner that could regulate left-right asymmetric gene expression. However, such beads did not induce expansion of the IM marker Pax2 (compare Fig. 7C with Fig. 1; Table 1). Similarly, beads expressing cerberus-short implanted in the left lateral plate at stage 7, where they would be expected to interfere with induction of the left-sided expression of Nodal, did not result in an inhibition of Pax2 expression (compare Fig. 7D with Fig. 4; Table 1). It thus appears that the competence of embryonic cells to regulate IM genes in response to Nodal-like signals is confined to developmental stages prior to the appearance of asymmetric left-sided Nodal expression in the LP.

Interactions between the Nodal-like and BMP signaling pathways during IM formation

Because the effects of Nodal-like signaling on IM gene expression bore some resemblance to the effects of BMP signaling (James and

Schultheiss, 2005) (see Discussion), we investigated whether these two pathways interacted in the regulation of IM genes. Explants were taken from the stage 4 anterior primitive streak (15-20% streak length, which fate maps to the PM) and treated with 10 ng/ml activin *in vitro*. Treatment of the anterior streak explants with activin resulted in strong activation of the IM marker *Osr1* (Fig. 8C), and this activation was blocked by addition of the BMP signaling antagonist Noggin (Fig. 8D) (Streit and Stern, 1999). [Activin was used instead of Nodal or Vg1 to activate the activin/Nodal-like signaling pathway because paraxial mesoderm downregulates the Cripto co-receptor necessary to respond to Nodal or Vg1 signaling (Colas and Schoenwolf, 2000).] In converse experiments, BMP (50 ng/ml) strongly activated *Osr1* expression in anterior streak explants (Fig. 8E), and this effect was weakly inhibited by the activin/Vg1/Nodal inhibitor SB431542 (50 ng/ml) (Fig. 8F). Thus the IM-promoting effects of activin signaling require an active BMP signaling pathway, and activin signaling appears to be able to modify the IM-inducing effects of BMP signaling.

In a further investigation of the link between Nodal-like and BMP signaling, Vg1-expressing cells were implanted into stage 4 embryos adjacent to the primitive streak at 20% streak length and checked after 12 hours for the presence of phosphorylated Smad1/5/8 (pSmad1/5/8), an indicator of active BMP signaling. As shown in Fig. 8G-J, Vg1 cells, but not control cells, induced phosphorylation of Smad1/5/8 in embryonic cells immediately surrounding the implanted cell pellet (3/5 Vg1-treated embryos
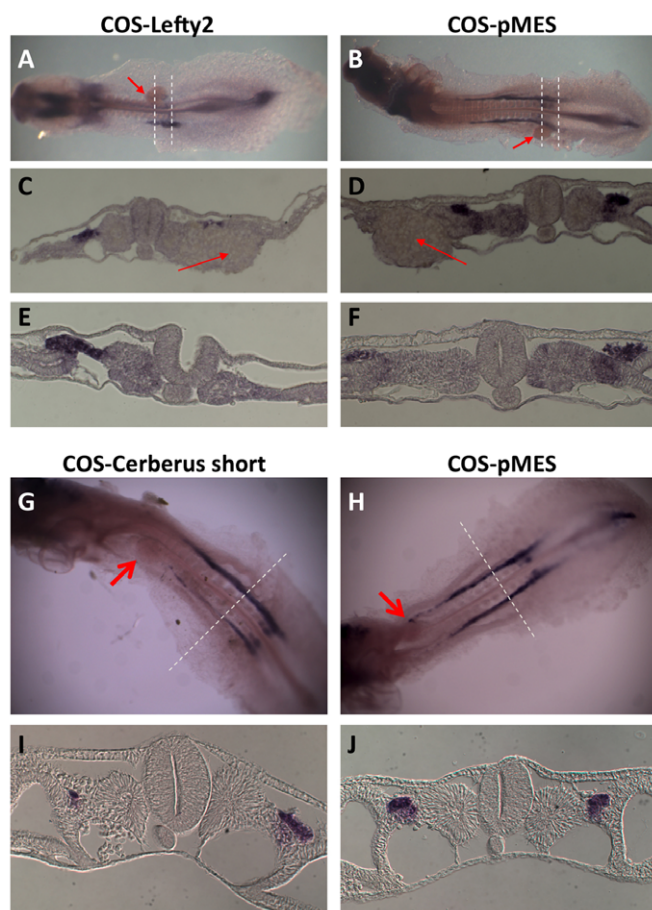
Fig. 4. Inhibition of Nodal-like signaling prevents IM gene expression. (**A-F**) Lefty2 reduces expression of IM markers. COS cells transfected with Lefty2 (A,C,E) or control pMES (B,D,F) were implanted adjacent to the primitive streak of stage 4 chick embryos at 30-40% streak length (prospective IM). Embryos were grown for 48 hours and analyzed by *in situ* hybridization for *Pax2*. The dashed lines crossing the embryos in A and B indicate approximate planes of section shown in C,E and D,F, respectively. Locations of implants are indicated by arrows (A-D). COS-Lefty2 cells produced diminished expression of *Pax2* on the transplanted side. (**G-J**) Inhibition of IM gene expression by cerberus-short. Stage 4 embryos were implanted with COS cells expressing cerberus-short (G,I) or control COS cells (H,J) and analyzed after 48 hours for expression of *Pax2*. Cerberus-short cells induced significant reduction of *Pax2* expression on the implanted side (G,I). The red arrows in G and H indicate pellet locations at the time of fixation. The dashed white lines in G and H indicate the plane of section in I and J, respectively.

showed clear pSmad1/5/8 induction, compared with 0/7 in controls). Interestingly, phosphorylation of Smad1/5/8 was only seen on the side of the Vg1 pellet facing the IM, whereas the side facing the somites did not show Smad1 phosphorylation, presumably because paraxial mesoderm and somite cells do not express Cripto (Colas and Schoenwolf, 2000) and thus cannot respond to Vg1. Thus, Nodal-like signals appear to be able to activate the Smad1/5/8 signaling pathway, providing a potential link between Nodal-like signaling and the known IM-inducing effects of BMP signaling.

DISCUSSION
Nodal-like signaling and IM formation
The current studies have identified a role for Nodal-like signaling in specifying the intermediate mesoderm (IM). We found that Nodal-

like signaling is required for IM gene expression, and that ectopic Nodal-like signaling produces marked expansion of the IM at the expense of the adjacent paraxial mesoderm (PM).

The observed effects of Nodal-like signaling on IM and PM gene expression are perhaps surprising, as some previous reports have characterized Nodal and related signals as dorsalizing factors that promote dorsal/medial mesodermal cell fates, such as notochord and PM, at the expense of more ventral/lateral fates, such as IM and LP. In *Xenopus*, it has been reported that increasing levels of ectopic Nodal generate increasingly more dorsal types of mesoderm (Agius et al., 2000), and the related TGFβ family member activin, which signals through the same receptors as Nodal (Cárcamo et al., 1994), induces progressively more dorsal mesoderm in a dose-dependent manner (Green and Smith, 1990). However, Nodal has a well-described earlier role in generating mesoderm itself. In zebrafish, Nodal signaling specifies germ layers in a dose-dependent manner, with higher levels of signaling producing endoderm and lower levels generating mesoderm (Feldman et al., 1998; Schier and Talbot, 2001; Sirotkin et al., 2000). A requirement for Nodal-like signaling in mesoderm formation has also been documented in mouse embryos (Conlon et al., 1994). Because interventions in zebrafish, *Xenopus* and mouse embryos are typically carried out genetically or by manipulation of very early-stage embryos, it can be difficult to disentangle possible effects on mesodermal patterning from earlier effects on germ layer formation. Mouse and zebrafish embryos lacking Nodal signaling do not produce mesoderm, so it has not been possible to evaluate directly the effects of Nodal-like signaling on the formation of IM or other mesodermal fates in these systems (Conlon et al., 1994; Feldman et al., 1998). In avian embryos, manipulations can be carried out more easily at later embryonic periods, thereby allowing the study of later effects of Nodal-like signaling on mesodermal patterning. In the current study, we find that excess Nodal-like signaling during gastrulation actually promotes IM formation at the expense of the PM, which can be interpreted as a type of ventralization. Interestingly, Hagos and Dougan conducted experiments in which Alk4/5/7 signaling was inhibited after mid-blastula transition in zebrafish and found that somites require less exposure to Nodal-like signaling than more ventral tissues, such as blood (Hagos and Dougan, 2007). That study, together with the current findings, suggests that Nodal-like signaling should not be thought of as a dorsalizing activity, at least within the timeframe of blastula- and gastrula-stage embryos.

The IM-inducing and left-right patterning properties of Nodal-like signaling are distinct
The IM-inducing activities of Nodal-like signaling are confined to stage 4 (mid-gastrulation). This is important because beginning at somite stages (stage 7) Nodal has a well-established role in left-right patterning. The current study found that IM gene expression is not sensitive to manipulation of Nodal-like signaling at times when those same manipulations can influence left-right patterning. This study, taken together with the previous studies cited above, indicates that there are at least four separate time windows during which Nodal-like signaling produces distinct effects: (1) prior to primitive streak formation, a role in establishing the location of the future primitive streak; (2) slightly later, a role in the specification of the endodermal and mesodermal germ layers; (3) during gastrulation, a role in modulating the relative amounts of different types of mesoderm that are formed, as investigated in the current study; and (4) during somite stages, a role in the patterning of the left-right axis.
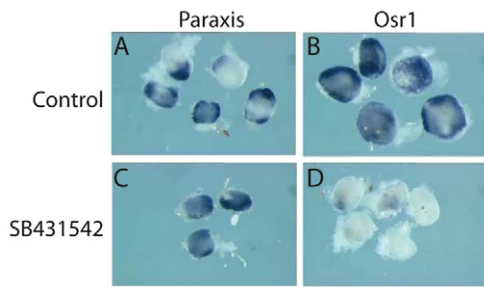
Fig. 5. *Osr1* expression requires Alk4/5/7 signaling. (**A-D**) Explants from the mid-primitive streak of stage 5 embryos were cultured with (C,D) or without (A,B) the Alk4/5/7 inhibitor SB431542 (50 ng/ml) and evaluated by *in situ* hybridization for expression of the IM marker *Osr1* (B,D) or the PM marker *Paraxis* (A,C). Treatment with SB431542 resulted in a strong inhibition of *Osr1* expression (D), whereas *Paraxis* expression was relatively unaffected (C). Note that the 'IM' explants express *Paraxis* because at the primitive streak stage the IM and PM domains are not well delineated, so explants of the mid-streak region contain precursors to both IM and PM.

The expression patterns of Nodal-like signaling components as reported in the published literature (and confirmed in our hands, data not shown) are consistent with a role for Nodal-like signaling in promoting IM formation. As depicted in Fig. 9A, during mid-gastrulation (stage 4), Vg1 and Nodal are expressed throughout the primitive streak with the highest concentration in the mid-streak, the co-receptor Cripto is expressed in the prospective PM and IM regions of the streak, and the Nodal-like signaling inhibitor Lefty is expressed in a gradient pattern with the highest levels in the most anterior portion of the streak (Ishimaru et al., 2000;

Joubin and Stern, 1999; Lawson et al., 2001; Levin et al., 1995; Seleiro et al., 1996). If these patterns are considered together, a picture emerges in which Nodal-like signaling activity during mid-gastrulation is predicted to be highest in the mid-primitive streak (the source of the IM), consistent with the current observation that Nodal-like signaling promotes IM formation during this stage of development.

Relationship between Nodal-like and BMP signaling in IM patterning

Our previous studies have reported that IM genes are activated at 'intermediate' levels of BMP signaling (James and Schultheiss, 2005). The current study made the surprising observation (Fig. 8) that ectopic Nodal-like signaling induces phosphorylation of Smad1/5/8, which is typically associated with activation of BMP signaling. This observation, together with the finding that Nodal-like signaling requires BMP signaling for its IM-promoting effects (Fig. 8), suggests that Nodal-like signaling exerts its IM-promoting effects by modulating BMP signaling. The model presented in Fig. 9B,C summarizes these results and illustrates how Nodal-like and BMP signals could be regulating mesodermal specification. Both BMP and Nodal-like signals can promote IM formation in tissue that would normally form PM. However, there is a difference in their effects. Ectopic BMP typically shifts both IM and LP regions medially, and thus does not result in an expansion of the IM (James and Schultheiss, 2005), whereas ectopic Vg1 expands the IM because it did not produce a medial shift of the border between the IM and the LP (Fig. 9B). Fig. 9C illustrates that these results could be obtained if ectopic Nodal-like signals produced a very local increase in BMP signaling, enough to shift the border between the PM and the IM, but not the border between the IM and the LP.
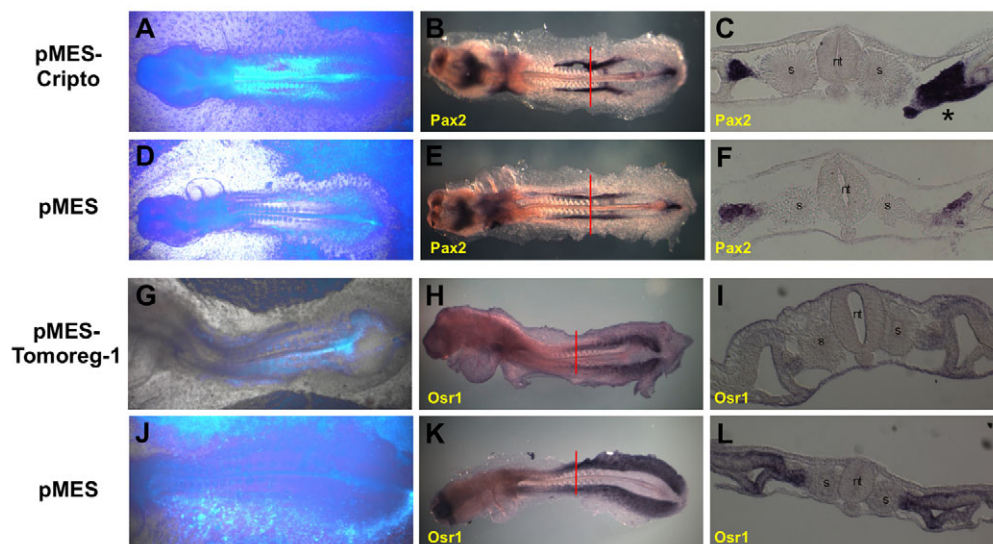

Fig. 6. Effects of addition and inhibition of the Nodal/Vg1 co-receptor Cripto on IM gene expression. (**A-F**) Increasing Cripto levels causes expansion of early IM markers. Electroporation was used to introduce Cripto, a Nodal/Vg1 co-receptor (A-C), or a control empty plasmid (D-F) into stage 3 chick embryos, targeted to the PM and IM regions (25-30% streak length). Embryos were grown for 48 hours and analyzed by *in situ* hybridization for *Pax2* (B,C,E,F). GFP expression in A,D indicates electroporated areas. The red lines crossing the embryos in B and E indicate approximate planes of section shown in C and F, respectively. *Pax2* staining was significantly increased in Cripto-electroporated embryos (B,C). Asterisk marks a expanded IM with strong *Pax2* expression (C) compared with the control (F). (**G-L**) Inhibition of Cripto reduces expression of early IM markers. Electroporation was used to introduce tomoregulin-1, an inhibitor of the co-receptor Cripto (G-I), or a control empty plasmid (J-L) into the prospective IM region (30-40% streak length) of stage 3 embryos. Embryos were grown for 48 hours and analyzed by *in situ* hybridization for *Osr1* (H,I,K,L). GFP expression in G and J indicates electroporated areas. The red lines crossing the embryos in H and K indicate approximate planes of section shown in I and L, respectively. Weak and patchy staining was observed in tomoregulin-1-electroporated embryos (H,I) compared with the control embryo (K,L). nt, neural tube; som, somite.
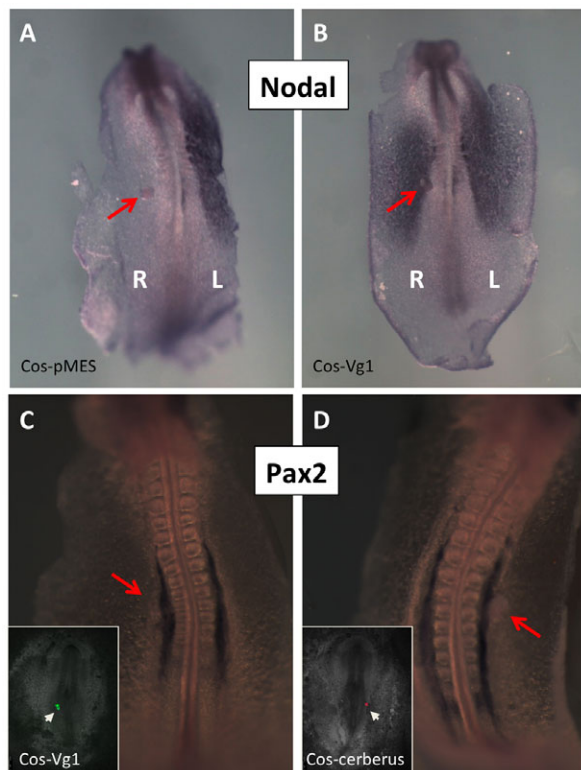
Fig. 7. Separate time windows for the IM-inducing and left-right patterning effects of Nodal-like signaling. COS cells expressing Vg1 (**B**) but not control COS cells (**A**) induce right-sided Nodal-expression if placed on the right side of the embryo at stage 7. COS-Vg1 implanted at stage 7 on the right side in a similar location as in B did not affect the expression of *Pax2* (**C**), nor did COS cells expressing cerberus-short implanted on the left side (**D**). The insets in C and D show the location of the COS cell pellets (white arrows) at the start of the experiment, and the red arrows indicate pellet locations at the end of the experiment.

The specific pathway by which Nodal-like signaling results in phosphorylation of Smad1/5/8 is not currently clear. One option, consistent with the observation that the IM-inducing effects of Nodal-like signaling are not cell-autonomous (Fig. 3), is that Nodal-like signaling might induce secretion of a BMP-like factor. We have found that ectopic Nodal-like signaling does not induce expression of Bmp2 or Bmp4 (data not shown), but it remains possible that secretion of another BMP-like molecule is induced by Nodal-like signals. An alternative, but not mutually exclusive, possibility is suggested by observations that cerberus can bind to and inhibit both Nodal and BMP, and that an increase in the concentration of one can result in the release of the other from inhibition by cerberus (Katsu et al., 2012). Under such a mechanism, whether through cerberus or another molecule with similar properties, ectopic Vg1 would result in an increase in free BMP, leading to local Smad1/5/8 phosphorylation and subsequent activation of IM genes.

The identity of the Nodal-like activity that patterns the IM

Several previous studies have reported that activin, which also signals through Alk4/5/7, promotes formation of kidney tissues. Studies in *Xenopus* embryos found that a combination of activin-like and retinoic acid (RA) signaling can promote kidney tubule formation from animal caps (Asashima et al., 2000; Moriya et al.,
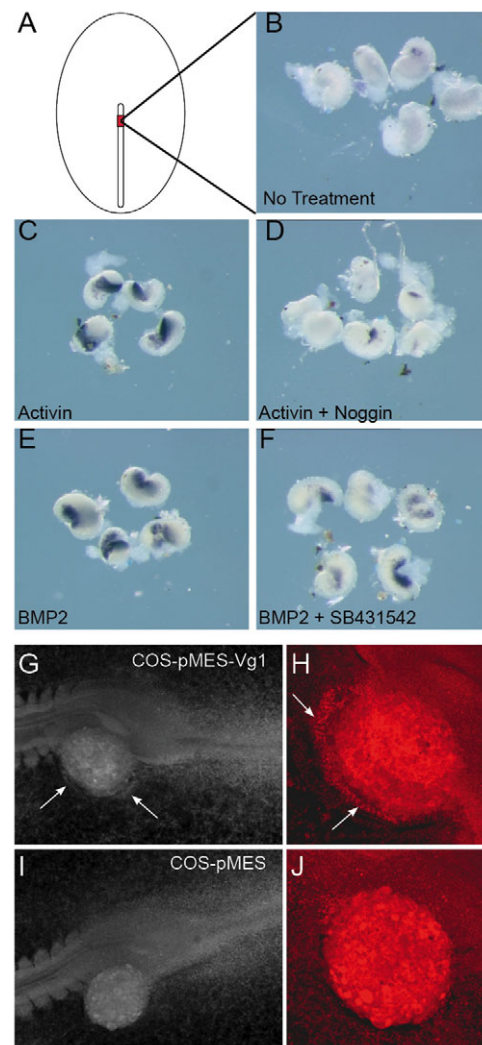

Fig. 8. Interactions of Nodal-like and BMP signaling in the regulation of IM gene expression. (**A-F**) Induction of *Osr1* by activin requires BMP signaling. Explants of stage 4 anterior-primitive streak (A) were grown in culture in control medium (B) or in medium supplemented with 10 ng/ml activin (C), 10 ng/ml activin and 1 µg/ml Noggin (D), 50 ng/ml BMP2 (E) or 50 ng/ml BMP2 and 50 ng/ml SB431542 (F) and stained by *in situ* hybridization for *Osr1*. Note strong inhibition of the *Osr1*-inducing effects of activin by Noggin (D), and partial inhibition of the *Osr1*-inducing effects of BMP2 by SB431542 (F). (**G-J**) Vg1 induces Smad1/5/8 phosphorylation. COS cells expressing Vg1 (G,H) or control COS cells (I,J) were placed in early stage 5 embryos, grown overnight, and analyzed at stage 9 by immunofluorescence for presence of phosphorylated Smad1/5/8. COS-Vg1 cells (G,H) but not control cells (I,J) induced a rim of pSmad1/5/8 adjacent to the cell pellet (arrows). G and I are wide-field microscope views, and H and J are confocal images.

1993). However, in those studies, it was difficult to distinguish between the known mesoderm-inducing effects of activin and the specific effects it had on the IM. In addition, the basis of the synergy between activin and RA signaling was not explained. More recently, studies in the avian embryo have reported that activin-like signaling is required for early IM gene expression, and that Hox genes, which are regulated by RA, control the competence of cells to respond to activin-like signals and to activate IM genes, thus providing a potential explanation for the synergy between RA and activin-like
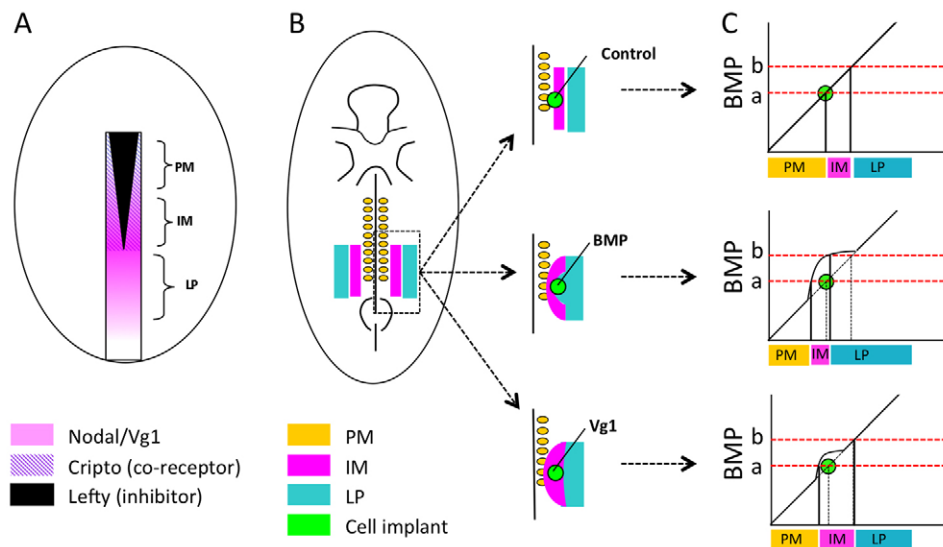
Fig. 9. Model of the effects of Nodal/Vg1 signaling on IM patterning. (**A**) Summary of the expression patterns of Nodal-like signals, inhibitors and co-receptors in chick stage 4 embryos. At stage 4 (mid-gastrula), Nodal and Vg1 are expressed throughout the primitive streak with highest concentrations in the mid-streak (pink); the co-receptor Cripto is expressed primarily in the anterior part of the streak (diagonal purple lines); and the Nodal-like inhibitor Lefty is expressed in a gradient with strongest expression at the anterior end of the streak (black, with width of the black domain indicative of expression level). PM, IM and LP indicate the region of the primitive streak containing prospective paraxial mesoderm, intermediate mesoderm, and lateral plate mesoderm, respectively. Whereas both the prospective PM and IM express Nodal-like signals and Cripto co-receptor, the Lefty1 inhibitor is expressed at higher levels in the prospective PM, consistent with a model in which effective Nodal-like signaling is highest in the prospective IM region. (**B**) Comparison of the effects of BMP and Nodal-like signaling on mesodermal patterning. The left-hand diagram depicts a stage 10 chick embryo. The right-hand diagrams summarize the effects of addition of a pellet of control cells (top), cells secreting BMP (middle) or cells secreting Vg1 (bottom). The dashed box in the left-hand figure shows the area that is expanded in the diagrams on the right. Excess BMP produced a medial shift and contraction of both PM and IM, with a corresponding expansion of the LP. Excess Vg1 produced an expansion of the IM at the expense of the PM, but the LP was unaffected. See text for further discussion. (**C**) Illustration of how the observation that Nodal-like signaling induces pSmad1 could explain the results summarized in panel B. The y-axis denotes levels of BMP signaling, with 'a' denoting the threshold of BMP signaling that distinguishes PM from IM, and 'b' denoting the threshold of BMP signaling that distinguishes IM from LP. Addition of a BMP source (middle) results in a broad elevation of BMP signaling centered around the source, with a resulting shifting of the PM-IM and the IM-LP borders medially and reduction in the size of the IM and PM. Addition of a Vg1 source (bottom) results in a localized rise in BMP signaling, which moves the PM-IM border medially but does not affect the IM-LP border, resulting in a broadened IM. Black dotted vertical lines in the middle and bottom panels indicate the borders between PM, IM and LP in control embryos (as taken from the top panel).

signals (Preger-Ben et al., 2009). However, as all of these studies used reagents that affect all signals that act through the Alk4/5/7 receptors, it was not established whether activin itself was the relevant signal for the observed effects.

The current study suggests that the Nodal-like branch of the TGFβ signaling pathway is likely to be mediating these previously reported pro-IM effects *in vivo*. Like activin, Nodal-like molecules also signal through Alk4/5/7 receptors and are inhibited by pan-Alk4/5/7 inhibitors such as SB431542 (Schier, 2003). However, the current study found that IM gene expression is sensitive to levels of Cripto, which is a required co-factor for Nodal-like but not activin signaling (Cheng et al., 2003). In addition, Nodal and Vg1 are expressed in the primitive streak at the time when IM genes are sensitive to Alk4/5/7 inhibition or activation (Ishimaru et al., 2000; Joubin and Stern, 1999; Lawson et al., 2001) (see Fig. 9A). The ability of cerberus-short, a specific inhibitor of Nodal (Bertocchini and Stern, 2002; Piccolo et al., 1999), to inhibit IM gene expression suggests that Nodal itself is required for normal IM gene expression. However, because the inhibition by cerberus-short was only partial (Fig. 4), it is still possible that other Nodal-like signals, such as Vg1, are also required *in vivo* for IM gene expression. Knockout of Nodal strongly disrupts mesoderm formation prior to IM patterning (Conlon et al., 1994). In mice, two Vg1 homologs, GDF1 and GDF3, appear to share the Vg1 activity (Andersson et al., 2007),

with GDF1 being the ortholog of chick Vg1 (UCSC Genome Browser). *Gdf1-Gdf3* double knockout mice also show severe disruptions in mesoderm formation, but specific effects on IM generation have not been evaluated (Andersson et al., 2007). In summary, we suggest that Nodal-like signaling is likely to be the endogenous pathway responsible for the observed IM-inducing effects of activin. A similar situation appears to be the case with mesoderm induction; early reports of mesoderm-inducing effects of activin were later shown to be attributable to the endogenous activity of Nodal-like signals (Green and Smith, 1990; Sirotkin et al., 2000).

Understanding the combination of signals that generates IM *in vivo* is an important prerequisite for attempts to generate kidney tissue in the laboratory. Previous attempts to activate IM and kidney markers in embryonic stem cells and other stem cells based on current knowledge of kidney regulatory molecules have thus far yielded limited results (Kim and Dressler, 2005). A major reason for such modest progress might be the incomplete knowledge of the parameters that regulate IM formation *in vivo*. The current study adds Nodal-like signaling as a regulator of IM formation and also emphasizes that the IM-inducing effects of Nodal-like signaling are very specific with respect to time and location in the embryo. These restraints could be important for attempts to generate kidney tissue *in vitro*.

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