Wnt, Vg1, BMP-4, and their roles in mesoderm patterning in *Xenopus* embryos

By

Yanzhen Cui

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Yanzhen Cui

has been approved

[redacted]

Professor in charge of thesis

[redacted]

Member

[redacted]

Member

[redacted]

Associate Dean for Graduate Studies

This dissertation is dedicated to my wife Lijie Qi, for her love and continuous support of my studies; and for Albert and Isaac, my two wonderful kids. Without their love and encouragement, I would not have accomplished this goal.

TABLE OF CONTENTS

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During the early years of my graduate studies, I very often received letters from my father, who, due to complications of diabetes, could not write in recent years. While I am working hard on my projects in the daytime, I fly back to my home essentially every night in my dreams. I feel sorry that I have not gone back to visit my parents and my family members in China, who have always been and will continue to be the most important source of support to me, and I find it difficult to describe how grateful I am to them.

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ABSTRACT

Three families of growth factors have been demonstrated to play essential roles in mesoderm induction and patterning in *Xenopus* embryogenesis: the transforming growth factor-ß (TGF-ß) family, the Wnt family, and the fibroblast growth factor (FGF) family. While FGF family members are important mainly in patterning the posterior mesoderm, the TGF-ß family member Vg1, and Wnt family members are believed to play crucial roles in initiating and determining formation of the dorsoventral axis. Other TGF-ß family members, such as the bone morphogenetic proteins (BMPs), are indispensable in patterning the ventral mesoderm at the gastrulation stages. This thesis will deal mainly with roles of Vg1, Wnt, and BMPs in embryonic pattering.

Vg1 is required for the process of mesoderm induction. The most compelling evidence for this is that a truncated activin receptor, which blocks Vg1 signaling, can inhibit differentiation of dorsal and ventral mesoderm when misexpressed in *Xenopus* embryos. Similarly, the Wnt signaling pathway is believed to be involved in specifying the dorsal axis. When transcripts encoding ß-catenin, a component of the Wnt pathway, are depleted in oocytes, the embryos derived from these oocytes lack dorsal structures.

In this thesis, I describe the isolation of a maternal Wnt, namely *Xwnt-8b*, which as shown by several criteria is potentially involved in dorsoventral axis determination. I further evaluate the roles of Vg1 and Wnt-signaling in this process, and find that while Vg1- signaling plays a general role in mesoderm induction, only Wnt signals can impart a dorsal fate on the mesoderm and endoderm that is formed.

BMPs, especially BMP-4, are another subject of this thesis. Like other members of TGF-ß family, BMP-4 is synthesized as a large protein precursor, which is subsequently processed to yield the mature ligand. The protease(s) responsible for cleaving BMP-4 have not yet been identified. I have used tissue-specific mis-expression of a furin-targeted inhibitor, α_1–Portland (α_1–PDX), in *Xenopus* embryos in order to identify these enzymes and found that two members of the proprotein convertase family (PCs), namely furin and PC6B, are implicated in cleaving BMP-4 during the embryonic development. Furthermore, I have identified a second cleavage site within the BMP-4 precursor, which is located 31 amino acid upstream of the primary processing sequence, and have shown that sequential processing of these two cleavage sites regulates the efficiency of cleavage of the BMP-4 precursor as well as the signaling range of the BMP-4 ligand.

INTRODUCTION

In *Xenopus*, the unfertilized egg consists of the animal hemisphere and vegetal hemisphere. The animal hemisphere will eventually differentiate into ectoderm, neural structures, and skin; the yolky vegetal hemisphere will give rise to gut and associated endodermal organs. The third primary germ layer, mesoderm, is derived from interactions between these two germ layers. This is a slow induction process, starting right after fertilization when signals from vegetal hemisphere (endodermal) cells instruct the ectodermal cells near the equator region (known as the marginal zone) to differentiate as mesoderm. These mesodermal cells provide a blueprint for setting up the body plan since they transmit relative positional information to the surrounding cells, so that the whole body plan can be laid out in an orderly manner (for representative stages of early *Xenopus* development, see Figure 1). Therefore, how mesoderm induction and patterning takes place in *Xenopus* embryos is a critical question, and this is one question pursued in this thesis.

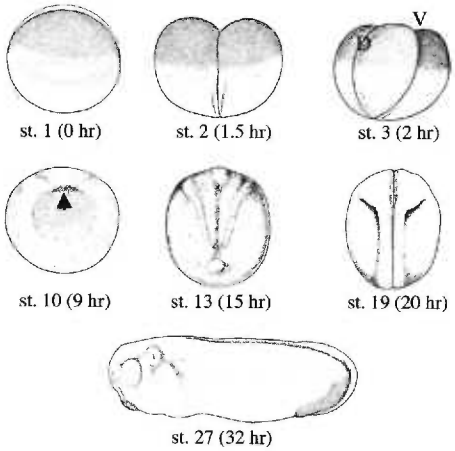


Figure 1. Representative developmental stages of *Xenopus laevis*. *Xenopus* embryos are staged according to Nieuwkoop and Faber (1956). These stages (st.) apply to development in room temperature (22°-24°) and start from fertilization (st. 1). Approximate hours after fertilization at each stage is indicated in parenthesis.

Major events in early *Xenopus* development: after fertilization (st.1), the one-cell embryo undergoes a cytoplasmic rearrangement called cortical rotation, which moves the dorsal determinant(s) to the future dorsal vegetal region (see text for more discussion). The dorsal-ventral difference (D,V) can be clearly seen at 4-cell stage (st.3). The embryo then goes through a series of rapid division without increasing its volume. This special cell division is called cleavage, which ends at st.6 (32-cell stage, not shown). Between st.6.5 and st.7 is the mid-blastula transition (MBT) stage, which marks the onset of zygotic gene transcription. Gastrulation starts at st.10, with the blastopore (arrow) formed in the dorsal side. Neural induction starts at about st. 13 and ends at st. 19-21, when the neural folds are fused. Starting from st. 26/27, the embryo shows muscular movement and it hatches at st. 35/36 (50 hr, not shown).

An overview of mesoderm induction

Mesoderm induction in *Xenopus* is part of a series of spatially localized signaling events that establish the body axes during development. Embryological analyses of these signaling centers have established the spatial and temporal pattern of information involved in mesoderm induction and patterning. Initially, a postfertilization rotation of the cortical region of the egg activates cytoplasmic dorsal determinants on the side opposite the sperm entry point. Specifically, it has been found that the shear zone of the egg (a region between the vegetal cortex and the deep cytoplasm) is filled with parallel microtubules, with which many pigmented granules are attached (Elinson and Rowning, 1988). These microtubules appear to provide the mechanical mechanism in the process of cortical rotation and are responsible for moving the dorsal determinants, which may include ß-catenin (Moon and Kimelman, 1998), up to the future dorsal side (Houliston and Elinson, 1991; Rowning et al., 1997), as treatments that are known to interfere with polymerization of microtubules also block this rotation (Elinson and Rowning, 1988). For example, ultraviolet (UV) irradiation of the vegetal hemisphere (which will give rise to endodermal cells) depolymerizes microtubules, it also blocks dorsal development. Such a treatment results in an embryo of

predominantly ventral character. Based on transplantation assays, a region of dorsal cytoplasm, which is capable of rescuing UV-irradiated embryos, is proposed to constitute the early blastula organizer (also known as the Nieuwkoop center). Embryological data indicate that, prior to the midblastula stage, this Nieuwkoop center signals equatorial cells to become dorsal mesoderm.

These data have been compiled into a model which states that three discrete signals are required for the induction and dorso-ventral patterning of the mesoderm. The first signal is activated by cortical rotation, emitted by dorsal vegetal cells (the Nieuwkoop center), and leads to formation of dorsal mesoderm with the Spemann organizer activity. The second signal comes from ventral vegetal cells and induces formation of purely ventral mesoderm. During gastrulation, a third signal emitted by dorsal mesodermal cells (the Spemann organizer cells) converts adjacent ventral mesoderm to more dorsal forms, such as muscle (Figure 2A).

While the rapid identification of growth factors (FGF, BMP, activin, Vg1, etc., see below for detail) which can induce ectoderm (animal pole tissue/cells) to differentiate as mesoderm *in vitro* led to the hope that the molecular basis of mesoderm induction would soon be understood, however, another significant concept in vertebrate development has been overlooked: that cell fate can be determined not only by the presence, absence or concentration of a morphogen, but by the competence of cells to respond to this information. The current studies return to the theme of competence as it applies to mesodermal patterning and suggest that agents involved in this process might be broadly grouped into two classes: inducing and modifying factors. Inducing agents direct cells to follow a given developmental pathway, while modifying agents do not affect cell fate themselves, but alter the competence of cells to respond to an instructive signal. For example, Xwnt-8, when expressed prior to the midblastula stage, cannot induce ectoderm to differentiate as mesoderm, but can modify

its response to bFGF, or low dose of activin. Specifically, while bFGF or low doses of activin themselves can only induce naive ectoderm to differentiate as ventral mesoderm *in vitro*, addition of a Xwnt-8 signal enables these cells to respond by forming dorsal, rather than ventral, mesoderm (reviewed by Christian and Moon, 1993; Moon and Christian, 1992).

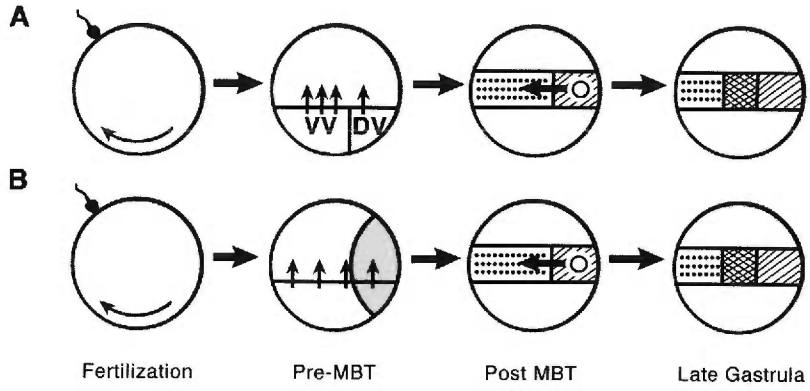


Figure 2. The three signal model (A) and synergistic model (B). A. The three signal model proposes that cortical rotation (curved arrow) after fertilization establishes a signaling center in dorsal vegetal (DV) cells. This signal leads to the formation of dorsal mesoderm (hatched) with Spemann organizer (O) activity. A signal from ventral vegetal (VV) cells leads to the formation of ventral mesoderm (stippled) in ventral and lateral cells of the embryo. During gastrulation, dorsalizing signals from the organizer convert the adjacent lateral mesoderm to intermediate mesoderm (diamond). B. In the synergistic model, it is hypothesized that cortical rotation (curved arrow) establishes the Nieuwkoop center activity (gray) on the dorsal side of the pre-midblastula transition (pre-MBT) stage embryo. This activity pre-patterns the competence of dorsal ectodermal cells to respond to a mesoderm inducing factor, or factors (vertical arrows) which are released from vegetal and equatorial cells across the dorsal-ventral axis. After the MBT, cells patterned by the Nieuwkoop center are competent to form dorsal mesoderm (hatched) with Spemann organizer (O) activity, while ventral and lateral cells are competent to form ventral mesoderm (stippled). The organizer (O) then emits a dorsalizing signal (horizonal arrow), such that lateral cells differentiate as intermediate forms of mesoderm (diamond).

The notion that restricted competence of responding cells may play a critical role in spatially organizing the mesoderm led to a synergistic model (Figure 2B) for mesoderm induction and patterning that represents an expanded and revised version of the original three signal model. In this model, the Nieuwkoop center, which is established in dorsal vegetal and equatorial cells by cortical rotation, is suggested to modify the response of dorsal ectoderm to one or more general mesoderm inducers, which are uniformly distributed along the dorsoventral axis. Noggin (Smith and Harland, 1992), a maternal Xwnt (such as Xwnt-8b, Cui et al., 1995), or Wnt signaling components such as ß-catenin (Heasman et al., 1994) may be components of the Nieuwkoop center, while activin and/or Vg1 may function as inducers. After the midblastula transition (MBT), which marks the onset of zygotic gene transcription, cells prepatterned by the Nieuwkoop center are competent to form dorsal mesoderm with Spemann organizer activity, while ventral and lateral cells are competent to form ventral mesoderm. Compelling evidence for involvement of Wnt, or a Wnt signaling component, ß-catenin, has come from the observation that when ß-catenin transcripts are depleted in oocytes, embryos derived from these oocytes lack dorsal structures (Heasman et al., 1994).

Vg1 as a mesoderm inducer

It should be noted that in both models described above, activin and/or Vg1 are hypothesized to play an important role in mesoderm induction, although the prepatterning model emphasizes that modifying factors may also be critical. Vg1 mRNA encodes a member of the TGF-ß family (reviewed by Vize and Thomsen, 1994), it is synthesized during early oogenesis and becomes tightly localized to the vegetal cortex of oocytes (Melton, 1987; Mowry and Melton, 1992; Yisraeli and Melton, 1988). As a result, Vg1 mRNA and protein become partitioned within vegetal pole blastomeres during cleavage (Dale et al., 1989; Tannahill and Melton, 1989).

Vg1, like other TGF-ß family members, is first synthesized as an inactive precursor. Whereas precursors for other TGF-ß family members are then proteolytically cleaved, releasing a mature bioactive C-terminal dimer (Massague et al., 1994), endogenous Vg1 protein accumulates as an unprocessed precursor and little if any mature Vg1 has been detected *in vivo* (Dale et al., 1989; Tannahill and Melton, 1989; Thomsen and Melton, 1993). Consistent with this observation, injection of embryos with Vg1 mRNA produces high levels of Vg1 precursors but no processed protein and, consequently, neither mesoderm induction nor developmental defects are observed (Dale et al., 1989, 1993; Tannahill and Melton, 1989; Thomsen and Melton, 1993).

Both activin and mature Vg1 (produced by misexpression of a chimeric BMP-Vg1 or activin-Vg1 protein, designated BVg and AVg hereafter, respectively) can induce pronounced morphogenetic movements and dorsal structures, such as muscle, in ectodermal animal caps. In whole embryos, activin has been shown to induce a partial dorsal axis when ectopically expressed in the ventral side of the embryo (Thomsen et al., 1990), and BVg can organize a full dorsal axis in UV ventralized embryos (Thomesen and Melton, 1993). A truncated activin type II receptor, which lacks the cytoplasmic serine/threonine kinase domain, has been demonstrated to block differentiation of dorsal and ventral mesoderm when misexpressed in whole embryos (Hemmati-Brivanlou and Melton, 1992). This was originally interpreted to mean that activin is required for mesoderm induction *in vivo* and for patterning the embryonic body plan. However, despite the potent activities observed for activin, it fails to fulfill a strong expectation for an endogenous mesoderm inducer: localization to the vegetal pole blastomeres that are responsible for mesoderm induction and patterning during normal development. Localization of maternal activin has yet to be observed (Fukui, 1993). Also, it has been recently shown that BVg activity can be blocked by the same truncated activin receptor, while follistatin, a protein which binds activin and inhibits its function (Ueno et al., 1987; Nakamura et al., 1990) does not block mesoderm

formation in whole embryos (Schulte-Merker et al., 1994). These results suggest that effects of the truncated activin receptors on *Xenopus* development could be explained by its inhibition of Vg1 activity. More recently, a mutant form of Vg1, which specifically blocks Vg1 signaling, has been shown to inhibit mesoderm formation and to affect endoderm development (Joseph and Melton, 1998), suggesting an *in vivo* requirement for Vg1 in normal development of *Xenopus* embryos. Thus, although activin cannot be definitively excluded as a candidate for the endogenous inducer, Vg1 emerges as the most likely one.

Wnts as patterning factors

It should be emphasized that, as suggested by the prepatterning model, not only inductive signals such as Vg1 but also modifying factors are required for mesodermal patterning. Wnts are proposed to play such a modifying role.

Wnts are a family of secreted glycoproteins related to the *Drosophila* segment polarity gene, *wingless*, and to the proto-oncogene, *int-1* (reviewed in Cadigan and Nusse, 1997). In *Xenopus*, at least 20 different *Wnts* are expressed during embryogenesis (Cadigan and Nusse, 1997). Overexpression of any of several Wnts (including Wnt-1, -3A, or -8) prior to midblastula stage, which is achieved by injection of *in vitro* transcribed Wnt mRNAs into ventral blastomeres of 4-cell embryos, results in a duplication of the embryonic axis (Smith and Harland, 1991; Christian et al., 1991; Sokol et al., 1991; Christian and Moon, 1993b). Although none of these Wnts are candidates for endogenous dorsalizing signals, since they are not expressed in dorsal cells of early embryos, this overexpression analysis has implicated a maternal *Xenopus* Wnt (Xwnt)-like activity in establishing the site of dorsal mesoderm formation. We have recently isolated a novel Wnt family member, Xwnt-8b, which possesses full dorsal axis-inducing activity when expressed at early *Xenopus* embryos (Cui et al., 1995). One of the most important features of Xwnt-8b is that its transcripts are present

in both oocytes and unfertilized eggs, consistent with its potential role in establishing dorsal ventral axis (Cui et al., 1995). These studies are described in Chapter 1 of this thesis.

The Wnt signaling pathway was initially deduced from genetic studies performed in *Drosophila*, which involved comparing particular mutant phenotypes with the *wingless* (the *Drosophila* ortholog of Wnt-1) mutant phenotype (i.e. the segment polarity defect). For example, *dishevelled* (*dsh*) mutant embryos have segment polarity defects similar to wg, but *zeste-white 3* (*zw3*) mutants have the opposite phenotype. These genes were ordered in a genetic pathway, and recent efforts have focused on studying the biochemical relationship between these proteins. Evidence accumulated from these genetic analyses in *Drosophila* and from studies of the vertebrate counterparts of these genes have been used to piece together the following view of the vertebrate Wnt signaling pathway.

Wnts are secretory proteins. After being secreted, Wnt protein acts on adjoining cells through binding to its receptor, frizzled (Bhanot et al., 1996). Wnt signals are transduced via activation of a novel cytoplasmic protein, dishevelled (DSH), which inhibits the activity of Xgsk3, the *Xenopus* homolog of zw3. Inactivation of Xgsk3 stabilizes the unphosphorylated, cytoplasmic pool of ß-catenin, which, when translocates into the nucleus, forms a complex with transcription factor 3 (XTcf3) (Molenaar et al., 1996) and activates expression of target genes such as *siamois* in the dorsal side of the embryos (for a review see Cadigan and Nusse, 1997).

There are two additional proteins that participate in this pathway, although it is not exactly clear what they do. One is adenomatus polyposis coli (APC), which activates Wnt signaling possibly through ß-catenin. The other is Axin, which is a cloned product derived from a naturally occurring mutation called *fused* (Zeng et al., 1997). Axin appears to inhibit ß-catenin by activating Xgsk3 or by acting on an intermediate, yet unidentified protein between ß-catenin and Xgsk3.

Therefore, both Axin and Xgsk3 must be inactivated by some mechanism in the dorsal side of the embryo. Consistent with this, misexpression of either Xgsk3 or Axin in the dorsal cells inhibits primary dorsal axis formation (He et al., 1995; Pierce and Kimelman, 1996; Zeng et al., 1997), and expression of dominant negative forms of either proteins in the ventral side results in a duplicated axis phenotype (He et al., 1995; Pierce and Kimelman, 1996; Zeng et al., 1997). Interestingly, overexpression of the dominant negative form of Xgsk causes ectopic axis formation even when expressed in vegetal cells, which do not themselves contribute to the induced axis (Pierce and Kimelman, 1995). These results suggest that the dorsal fate is actively repressed by this kinase in ventral cells and that GSK must be inactivated in dorsal cells, presumably by Wnt signaling, so that dorsal fate can be expressed.

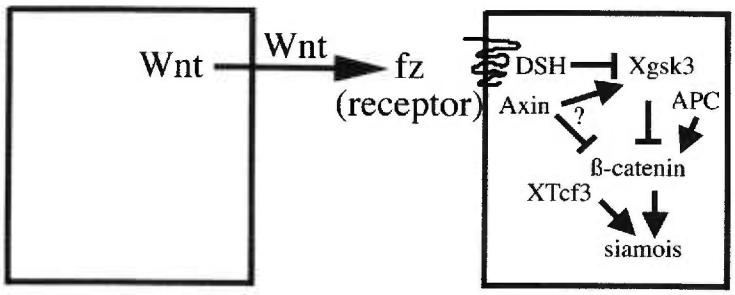


Figure 3. Wnt signaling transduction pathway. The Wnt protein is secreted from a cell (left) then acts on a neighboring cell (right) through its receptor frizzled (fz). In the target cell (right), dishevelled (DSH) is required to transduce the signaling, which inactivates Xgsk3. In the absence of the Wnt signaling, Xgsk3 phosphorylates and destabilizes ß-catenin. When the signaling is transduced into the cell, the cytoplasmic pool of ß-catenin is increased since Xgsk3 is inactivated. ß-catenin can then translocate into the nucleus, where it forms a complex with transcription factor 3 (XTcf3) and activates the Wnt target gene such as *siamois*. Adenomatus polyposis coli (APC) activates the Wnt signaling pathway upstream of ß-catenin, and Axin inactivates Wnt signaling, possibly by activating Xgsk3 or inactivating ß-catenin. Adapted from Cadigan and Nusse (1997).

Wnt induced secondary morphogen(s)

Considering the cytoplasmic nature of Xgsk, the fact that inactivation of this kinase in endodermal cells can cause overlying cells to form dorsal mesoderm suggests that Wnt signaling is mediated by activation of a secondary morphogen. This hypothesis is consistent with the recent finding that ventral endodermal cells made to misexpress Xwnt-8b express dorsal-specific genes and can induce conjugated ventral ectoderm to differentiate as dorsal mesoderm (notochord). In contrast, in the absence of ectopic Xwnt-8b, ventral endodermal cells express ventral-specific genes and induce formation of ventral mesoderm (Cui et al., 1996). Considering that a majority (~ 83% in case of wingless protein WG, Reichsman et al., 1996) of secreted Wnt protein is bound to the cell surface and associates with the extracellular matrix through specific, non-covalent interactions, it is unlikely that Xwnt proteins made in endodermal cells diffuse to, and act directly on, the ectodermal cells. It is therefore reasonable to assume that endodermal cells respond to Wnts by generating a secondary, non-cell-autonomous dorsalizing signal that instructs the prospective mesoderm to adopt a dorsal fate. Consistent with this possibility, when transcripts encoding ß-catenin, a component of Wnt signaling, are depleted in oocytes, the resultant embryos have no dorsal structures. Strikingly, expression of ß-catenin, a cytoplamic protein, in either the animal pole or vegetal pole of such embryos, completely rescue the dorsal structures (Wylie et al., 1996), suggesting that ß-catenin can generate a non-cell autonomous dorsalizing signal that diffuse into and act on mesodermal cells directly. Vg1 has been shown to be required for expression of dorsal-specific genes in endodermal cells (Cornell et al., 1995), and thus it is reasonable to propose that Vg1 is also required, in cooperation with Wnts, for generation of the non-cell-autonomous dorsalizing signal produced by endodermal cells. This possibility is examined in Chapter 2 of this thesis (see also Cui et al., 1996).

Consistent with the GSK and ß-catenin studies which suggest that dorsal endodermal cells play an important role in dorsal mesoderm induction, *Siamois*, a transcription factor which is expressed in dorsal endodermal cells shortly after the MBT, can dorsalize overlying mesoderm when misexpressed in ventral endoderm. This suggests that, contrary to the conventional models described above, zygotic genes encoding diffusible signals are activated by siamois in dorsal vegetal cells and can mediate induction of the Spemann organizer in the overlying mesoderm (Lemaire et al., 1995).

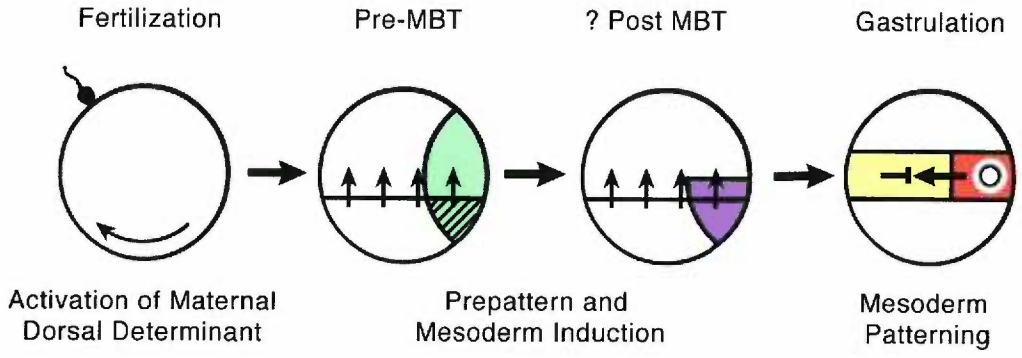


Figure 4. Secondary morphogen model for mesoderm induction and patterning. Cortical rotation (curved arrow) activates a maternal Xwnt, such as Xwnt-8b, or Wnt signaling component such as ß-catenin in both the animal and vegetal hemisphere in the presumptive dorsal side (green). Vg1 is evenly distributed in the vegetal hemisphere (vertical arrows). Vg1 and a Wnt signal constitute the components of the Nieuwkoop center on the dorsal side (hatched green), and induces expression of siamois (dark blue) shortly after the MBT. Siamois then activates zygotic gene(s) encoding diffusible morphogens that, probably in cooperation with Vg1 (vertical arrows), mediate the induction of Spemann's organizer (O) which differentiates as extreme dorsal mesoderm (red). The Spemann organizer then emits a dorsalizing signal (horizontal arrow). The ventral and lateral cells express ventralizing factors, attenuating the response of these cells (bar) to the dorsalizing signal and continue to differentiate as ventral and intermediate dorsal mesoderm, respectively (yellow).

Bone morphogenetic proteins (BMPs) and their expression

Recent studies have challenged the traditional view that signals from the organizer region dominantly specify dorsal fate, and that signals from the ventral region only passively attenuate these dorsalizing signals. As described below, growing evidence shows that BMPs, especially BMP-4, actively participate in the patterning of ventral mesoderm. Probably the most exciting finding in this regard is that overexpression of BMP-4 in *Xenopus* embryos leads to a ventralized phenotype, characterized by loss of dorsoanterior structures (Dale et al., 1992). Conversely, when BMP signaling is blocked by a dominant-negative BMP receptor, ventral mesoderm, which would otherwise form in normal embryos, develops as dorsal mesoderm instead, indicating that the ventral state must be actively maintained (Graff et al., 1994). Similarly, inhibition of BMP signaling by dominant negative ligands or antisense BMP-4 RNA results in mesoderm dorsalization (Hawley et al., 1995; Steinbeisser et al., 1995). Furthermore, when ectodermal cells are incubated with activin, a dorsal mesoderm inducer, these cells differentiate into dorsal mesoderm, as demonstrated by expression of chordin (a dorsal marker), MyoD, and actin (both muscle markers). When these same cells are treated with both activin and BMP-2 or BMP-4 proteins, expression of dorsal markers is suppressed as a function of BMP dose and expression of ventral markers, such as globin, is induced (Nishimatsu and Thomsen, 1998). These experiments demonstrated that BMPs can override the dorsalizing effect of activin and can convert dorsal mesoderm into ventral mesoderm. Collectively, these data suggest that BMPs, especially BMP-4, do not just passively attenuate dorsalizing signals, but instead actively, and dominantly, specify ventral fate.

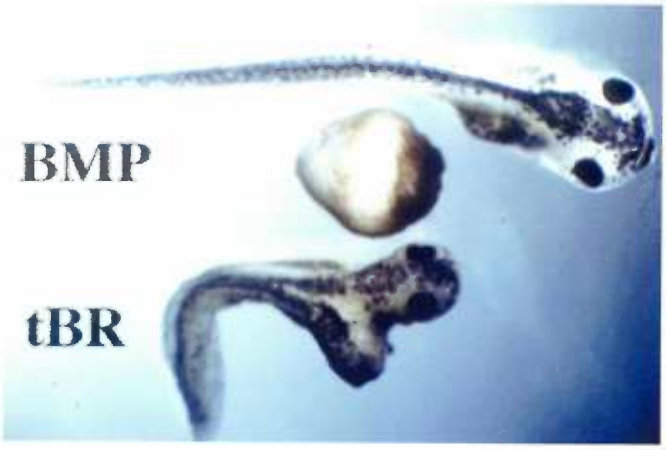


Figure 5. Developmental defects caused by gain- or loss-of-function of BMP-4 in *Xenopous* embryos. When BMP-4 activity is elevated in the dorsal side of the embryo, by means of overexpressing BMP-4 RNA, the embryo (BMP) is ventralized, as marked by loss of dorsal structures (head, notochord and muscle) in the embryo (compare to the normal embryo). Conversely, when endogenous BMP4 function is inhibited by means of a dominant-negative receptor, tBR (Graff et al., 1994), dominant-negative ligand (Hawley et al., 1995), downstream antagonistic signaling components (Nakayama, et al., 1998) or activity antagonists (Zimmerman, et al., 1996; Piccolo et al., 1996; Iemura et al., 1998), the ventral side of the embryo develops as dorsal structures instead, as indicated by formation of a secondary dorsal axis in the embryo (tBR).

BMP-4 transcripts are expressed in ventral and lateral regions of the marginal zone at late blastula and early gastrula stages (Fainsod et al., 1994). In the mouse embryo, BMP-4 is similarly expressed in the ventral and posterior mesoderm, and it is essential for normal development (Winnier et al., 1995). Thus, the expression pattern of BMP-4 is consistent with a role in specifying ventral fate in vertebrate embryos. BMP-2 and BMP-7 are expressed zygotically in *Xenopus* and, like BMP-4, they have the ability to ventralize embryos. However, their transcripts are evenly distributed in ectodermal and mesodermal cells, including the organizer, at blastula and gastrula stages (Hemmati-Brivanlou and Thomsen, 1995).

BMP antagonists and mesoderm patterning

The ventralizing capacity of BMPs and their expression nearby, and even within, the organizer make necessary a mechanism by which the organizer can defend itself against being ventralized. Findings over the past several years demonstrate that the organizer produces factors that antagonize BMPs, and this antagonism takes place extracellularly and targets BMP ligands directly. Specifically, three molecules have been identified that act as extracellular BMP antagonists: noggin (Smith et al., 1993), chordin (Sasai et al., 1994), and follistatin (Hemmati-Brivanlou et al., 1994). Noggin and chordin were isolated in *Xenopus* based on their ability to partially mimic organizer function, and each encodes a unique secreted factor. Recent reports suggest that these two factors can each bind BMP ligands directly, with affinities similar to those with which BMPs bind cellular receptors (Zimmerman et al., 1996; Piccolo et al., 1996). Follistatin was originally identified as an antagonist of activin, a dorsal mesoderm inducer. It was subsequently demonstrated, however, that follistatin can dorsalize ventral mesoderm and induce neural tissue (Sasai et al., 1995). These findings are more consistent with the recent observation that follistatin can bind heterodimeric BMP-4/7 (Yamashita et al., 1995) and inactivate BMPs (Iemura et al., 1998). Very importantly, follistatin, like noggin and chordin, is expressed in the organizer and in axial mesoderm in the *Xenopus* embryos, consistent with its ability to induce neural tissue and dorsalize ventral mesoderm (Hemmati-Brivanlou et al., 1994).

Taking these antagonists into consideration, it is reasonable to suggest that limited or regulated diffusion of noggin, chordin and follistatin from the organizer might form a gradient of these secreted factors that trails off towards the ventral side. Since BMP-4 transcripts are evenly distributed in ventral and lateral mesodermal cells, this antagonist gradient might set up a complementary gradient of BMP-4 activity along

the dorsoventral axis, which directly specifies the different ventrolateral mesodermal fates.

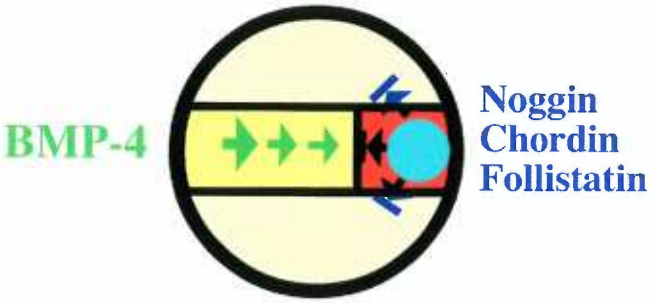


Figure 6. Roles of BMP-4 in mesoderm patterning. Transcripts encoding the secreted proteins noggin, chordin and follistatin are expressed in the organizer region (red) in gastrula stage *Xenopus* embryos. These proteins diffuse away (blue arrows) from the organizer and antagonize (blue bars) the activity of BMP-4, the major player in ventral mesoderm patterning. BMP-4 transcripts are expressed in ventral and lateral regions (yellow) but excluded from the dorsal side. Thus, due to the presence of the antagonizing factors around the organizer, BMP-4 activity is attenuated and forms a gradient that decreases from the ventral to the dorsal side (green arrows).

BMP-4 may be processed by furin-like proprotein convertases (PCs)

While considerable progress has been made toward understanding the role of BMPs in mesodermal patterning, detailed knowledge of how BMPs are assembled and activated is lacking. Like all other TGF-ß family members, BMP-4 is synthesized as an inactive precursor and is proteolytically activated by cleavage following the multibasic amino acid motif -Arg-Ser-Lys-Arg- to yield a carboxyl-terminal mature protein dimer (Aono et al., 1995; illustrated in Figure 7). This processing event has been proposed to regulate the secretion and/or diffusion of BMPs, thereby controlling the range over which these molecules signal during embryonic development (Jones et al., 1996). In general, very little is known about intracellular assembly, processing and secretion of BMPs.

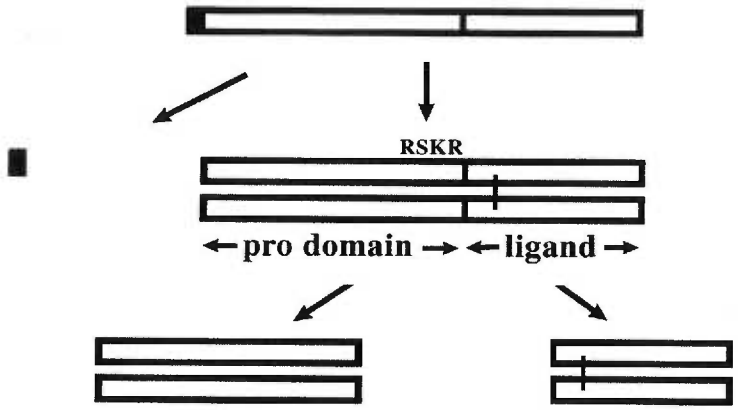
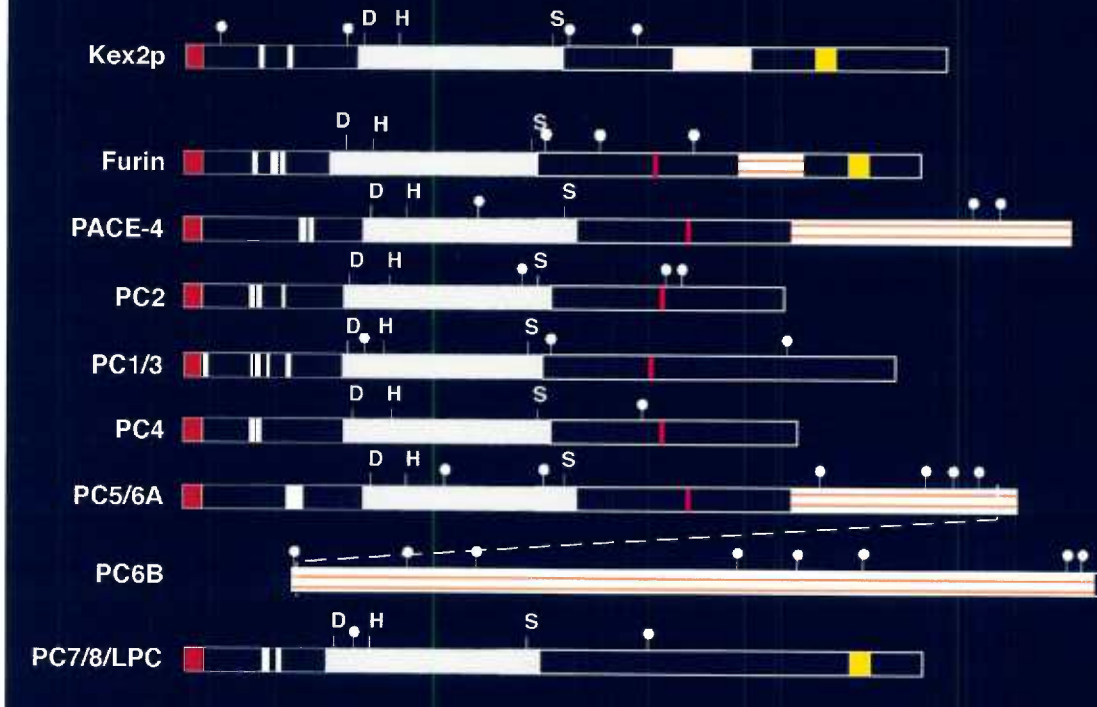


Figure 7. BMP-4 processing. BMP-4 is first synthesized in the endoplasmic reticulum (ER) as a precursor protein, where its signal peptide is cleaved (dark square). The precursor then pairs to form a disulfide-linked dimer. The precursor has a pro domain and ligand domain (ligand) and is then proteolytically cleaved following -RSKR- sequence (Aono et al., 1995) to generate the biologically active ligand. The cleavage most likely takes place in the trans golgi network (TGN), but the details are unknown.

Members of the proprotein convertase (PC) family (see Table 1; reviewed by Steiner et al., 1992) are good candidates for endogenous BMP convertases. In mammals, seven members of this family have been characterized and designated furin, PC2, PC1/3 (hereafter called PC3), PACE-4, PC4, PC5/6A and B (hereafter called PC6A and B), and LPC/PC7/PC8 (hereafter called PC7) (Table 1) (Seidah and Chretien, 1997). Individual PCs exhibit overlapping but distinct substrate specificity (Breslin et al., 1993; Creemers et al., 1993). The first member of this family that has been characterized, furin, is a membrane-associated, calcium-dependent serine endoprotease that proteolytically activates proprotein molecules at the carboxyl-terminal side of the consensus sequence -Arg-X-Arg/Lys-Arg-, or sometimes -Arg-X-X-Arg- (Molloy et al., 1992). Many precursor proteins, like BMP-4, share this cleavage site and therefore may be potential substrates for furin or furin-like PC members.

TABLE 1

THE PROPROTEIN CONVERTASES



		Ki*, nM		
		α1-PDX	α1-PIT	CMK
Kex2p	814 a.a.	ND	ND	ND
Furin	794 a.a.	1.4	>500	2.0
PACE-4	963 a.a.	>5,000	>500	3.6
PC2	638 a.a.	1,000	>500	0.36
PC1/3	753 a.a.	260	>500	2.0
PC4	638 a.a.	ND	ND	ND
PC5/6A	915 a.a.	ND	ND	ND
PC6B	1877 a.a.	2.3	>500	0.11
PC7/8/LPC	785 a.a.	>5,000	>500	0.12

*** Ki data are cited from Jean et al., 1998**

The central role of furin in precursor protein processing has led to the design of furin-specific inhibitors. One approach has been the construction of structural variants of α_1–antitrypsin (α_1–AT). Human α_1–AT is a 55-KDa serum protein that is a physiological inhibitor of elastase (reviewed by Perlmutter, 1989). It has been shown that mutations at the α_1–AT reactive site can change the specificity of this serpin (serine protease inhibitor) for different enzymes. For example, a naturally occurring mutation in the α_1–AT reactive site (M^{358}-R) changes the specificity of the serpin from an inhibitor of elastase to a potent inhibitor of thrombin (Lewis et al., 1978). Similarly, an α_1–AT variant (α_1–PDX) was generated such that it contains in its reactive site R^{355}-I-P-R^{358}, the minimal consensus sequence for efficient processing by furin. α_1–AT–PDX has been shown to be capable of blocking the processing of two furin substrates, pro-ß-nerve growth factor and human immunodeficiency virus (HIV) -1 gp 160 in transfected cells (Anderson et al., 1993). More recently, α_1–ATPDX has been demonstrated to be a selective inhibitor for furin and PC6, but not for PACE4 or PC7 (Table 1) (Jean et al., 1998).

We hypothesized that BMP-4 is a substrate for furin or a furin-like proprotein convertase, and tested this hypothesis by looking for specific developmental defects following mis-expression of the furin/PC6-targeted inhibitor, α_1–ATPDX, in *Xenopus* embryos (Cui et al., 1998). These studies are described in Chapter 3 of this thesis.

Summary

In summary, this thesis will consider issues ranging from assessing the roles of Wnt and Vg1 signaling in mesodermal induction and patterning (Chapter 1 and 2), to identifying enzymes responsible for activating BMP-4 in embryogenesis (Chapter 3). In addition, a further study was performed to characterize the role of a cis-acting element in the prodomain of the BMP-4 precursor in regulating the cleavage efficiency of the BMP-4 precursor and the signaling range of the ligand (Chapter 4).

Chapter 1

Cloning and characterization of *Xwnt-8b*: a novel member of the Wnt family which has a potential role in establishing the dorsoventral axis and patterning the nervous system

Yanzhen Cui, Jeffrey D. Brown*, Randall T. Moon * and Jan L. Christian

Department of Cell and Developmental Biology, Oregon Health Sciences University, School of Medicine, Portland, OR 97201, USA

*Department of Pharmacology and Howard Hughes Medical Institute, University of Washington, School of Medicine, Seattle, WA 98195, USA

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ABSTRACT

The molecular events that are involved in specifying the dorsoventral axis have been an area of intense interest. Progress made in recent years has established that dorsoventral asymmetry results from either dorsal-ventral differences in vegetally derived mesoderm-inducing signals, and/or differences in the competence of animal hemisphere, or the ectodermal cells, to respond to these signals. Previous studies have shown that some Wnt proteins can modify the response of ectodermal cells to mesoderm inducers, and that they can induce a secondary dorsal axis when misexpressed in whole embryos. However, none of these Wnts are present at an appropriate time to do so *in vivo*. Here we describe isolation and characterization of a novel Wnt member, *Xwnt-8b*, which, like its close cousin *Xwnt-8*, displays axis-inducing activity when misexpressed in early stage *Xenopus* embryos. Ectopic expression of Xwnt-8b completely rescues dorsal development of embryos which have been ventralized by exposure to ultraviolet light. However, in contrast to Xwnt-8, which is expressed too late in development to play a role in dorsal axis specification, Xwnt-8b transcripts are expressed maternally (in oocytes and unfertilized eggs) as well as zygotically. Specifically, during early cleavage stages, Xwnt-8b transcripts are confined primarily to animal hemisphere blastomeres, consistent with the hypothesis that it may be involved in patterning the dorsal mesoderm in response to inducing signals, thus initiating dorsal development. At neurula and tailbud stages, Xwnt-8b transcripts are restricted almost exclusively to a band of cells in the prospective forebrain, suggesting that it may play an essential role in patterning the nervous system of *Xenopus* embryos.

INTRODUCTION

In amphibians, establishment of dorsoventral asymmetry can be traced back to the very moment of fertilization, when sperm entry into the egg triggers a cytoplasmic rearrangement, which is referred to as cortical rotation. Cortical rotation is believed to activate an inductive signaling center, the Nieuwkoop center, on the side opposite the sperm entry point (SEP) and this side is determined to become the future dorsal side. When cortical rotation is blocked by exposure of the zygote to ultraviolet (UV) light, these embryos lack dorsal and anterior structures. Tissue recombination experiments have demonstrated that signals from the Nieuwkoop center induce the overlying marginal zone cells to initiate dorsal mesodermal development, while marginal zone cells that do not receive signals from the Nieuwkoop center form ventral mesoderm. Since the Nieuwkoop center operates prior to the time that zygotic gene expression begins at the midblastula stage, components of this signaling center must be derived maternally and, thus present in the egg prior to fertilization.

Strenuous pursuit of the molecular signaling events involved in mesoderm induction in the past two decades has led to identification of three classes of molecules that may be important in this process: members of the transforming growth factor (TGF-ß) family, fibroblast growth factor (FGF) family, and Wnt family. Of these, FGF family members are essential for patterning the lateral and posterior ventral mesoderm (Amaya et al., 1994). TGF-ß family members, especially activin and Vg1, have been hypothesized to play major roles in mesoderm induction. In support of this, when the endogenous activin/Vg1 signaling is inhibited, the embryos lose all mesodermal derivatives (Hemmati-Brivanlou and Melton, 1992; Joseph and Melton, 1998), and activin has been shown to induce full arrays of mesodermal differentiation in explanted ectodermal cells (Thomson et al., 1990).

While activin/Vg1 signaling is required for mesodermal induction, it turns out not to be the whole story. The earliest evidence that challenges this activin/Vg1 hypothesis comes from the observation that Wnt signaling may also be involved and may be able to modify the response of ectodermal cells to TGF-ß family as well as FGF family members (for review, see Christian and Moon, 1993).

Wnts are a family of structurally related cell-to-cell signaling molecules. The original member of this family, *Wnt-1*, was isolated as a proto-oncogene and thought to be involved in mammary tumorigenesis. Subsequently, at least 20 Wnt-1 related genes have been isolated (for review, see Cadigan and Nusse, 1997) in organisms ranging from mammals to the nematode C. elegans. All of these *Wnt* genes are expressed in unique, highly restricted patterns during development, suggesting that they may be involved in shaping and modifying distinct tissues of the embryo. Consistent with this hypothesis, ablation or misexpression of specific Wnts in mouse, fly, or *Xenopus* embryos leads to distinct patterning defects (Cadigan and Nusse, 1997). One striking phenotype following misexpression of a number of different Wnts in *Xenopus* embryos is that certain Wnts can induce a complete secondary axis in normal embryos, or a primary axis in embryos that have been ventralized by UV exposure. Lineage tracing experiments demonstrate that the ectopic axis induced by these Wnts is due to activation of a Nieuwkoop center-like signaling pathway, but not due to that of the later operating organizer-like activity. Specifically, axis induction is observed only when Wnts are expressed prior to the onset of midblastula transition, when the Nieuwkoop center is normally operative, but not after this stage (Christian and Moon, 1993).

While ectopic expression of Wnts leads to the formation of dorsal mesoderm, Wnts cannot directly induce the presumptive ectodermal cells to form dorsal mesoderm; rather, they seem to modify the response of these cells to inducing signals such that dorsal, but not ventral, mesoderm differentiates. Specifically, while ectodermal cells

tend to differentiate into ventral mesoderm in response to an added FGF signal, these same cells will form dorsal mesoderm when exposed to both an FGF signal and a Wnt reagent (Christian and Moon, 1993).

To date, *Xenopus* Xwnt-1, Xwnt-3A, and Xwnt-8 have all been shown to have full axis inducing activity when ectopically expressed in embryos; however, none of these Wnts are expressed in a pattern suggesting their involvement in the maternal steps of dorsal determination (Moon et al., 1993). Two maternally expressed Wnts, Xwnt-5A and Xwnt-11, have been reported, but neither of them can induce a complete axis in embryos (Ku and Melton, 1993; Moon et al., 1993).

Here we report the isolation and characterization of a novel member of the Wnt family, *Xwnt-8b*. During gastrula stage, zygotic Xwnt-8b transcripts are concentrated in prospective ectodermal cells of the animal hemisphere, while zygotically derived transcripts are restricted primarily to a narrow band of cells in the prospective forebrain. Ectopic expression of Xwnt-8b can completely rescue dorsal development of UV-irradiated embryos and can induce a complete secondary dorsal axis in normal embryos. This activity is observed only when Xwnt-8b is supplied prior to the onset of the zygotic gene transcription. The biological activity, together with the expression of Xwnt-8b in cells that will be induced to form the mesoderm, is consistent with the possibility that this novel Wnt may function to modify the response of ectodermal cells to mesoderm inducing signals, thereby initiating dorsal development. Furthermore, the expression of Xwnt-8b in the forebrain raises the possibility that it may also function in patterning the nervous system of *Xenopus* embryos.

Materials and Methods

Isolation of Xwnt-8b cDNA clones and plasmid construction

A partial length Xwnt-8b cDNA clone (Wolda and Moon, 1992) was used to screen a *Xenopus* neurula (stage 17) lamda gt 10 library (from D.Melton). The phage was subcloned, as two EcoRI fragments, into pGEM1 (Promega). However, neither of these two cDNAs contained the sequence encoding the amino terminus of Xwnt-8b. This was achieved by polymerase chain reaction (PCR)- based Rapid Extension of cDNA (RACE); and the full coding sequence of Xwnt-8b was obtained by recombining the original two cDNAs and the RACE-generated on 5'end using the Gene Splicing by Overlap Extension technique (Horton, 1993). The portions of the sequence that were generated by PCR were resequenced to verify that they did not contain any PCR-introduced errors. The full coding sequence was subcloned into the expression vector pSP64T (Kreig and Melton, 1984) to generate pSP64T-Xwnt-8b.

Ribonuclease protection and in situ hybridization

Total nucleic acids were isolated from staged embryos and RNA selectively precipitated in 4M LiCl. Ribonuclease protection analysis of 100 ug of total oocyte RNA, 50 ug of embryonic RNA and 10 ug of yeast RNA, was performed according to Ausebel et al. (1994). Levels of endogenous and ectopically expressed Xwnt-8b transcripts were analyzed by comparing these signals with those obtained with known amounts of Xwnt-8b transcripts synthesized *in vitro*. Protected bands were visualized with a Molecular Dynamics phorsphoimager and quantified with using the Macintosh IP lab gel program.

In situ hybridization of whole embryos to digoxigenin probe was performed as described by Harland (1991). Embryos were subsequently dehydrated, embedded in paraffin, sectioned as described by Kelly et al. (1991).

Analysis of RNA by reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted according to Chomczynski and Sacchi (1987). The first strand cDNA was prepared using an AMV reverse transcription kit (Life Sciences) according to manufacture's instructions. Standard 50 ul PCR reaction was carried out at 94°C for 4 minutes and was followed by three temperature of cycling: denaturation at 94°C, annealing at 54°C, and extension at 72°C, for 30 seconds at each temperature. Cycle number was determined empirically for each primer pair, so that PCR products were examined during the exponential phase of amplification. The sequences of primers used for RT-PCR are listed here: in 5' to 3'orientation. Xwnt-8b: (U) TGACTTGAACATCCATTCT, (D) TGGAGAAAGGAATCCTGTA. Xwnt8 (Christian and Moon, 1991) (U) AATGGAATTGAGGAGTGT, (D) GCTCCTCTGTTGTCAGC. Histone H4 (Niehrs et al., 1994) (U) CGGGATAACATTCAGGGTA, (D) TCCATGGCGGTAACTGTC. Xwnt-11 (Ku and Melton, 1993) (U) GAAGTCAAGCAAGTCTGCTGGG, (D) GCAGTAGTCAGGGGAACTAACCAG. Vg1 (Weeks and Melton, 1987) (U) CCCTCAATCCTTTGCGGTG, (D) CAGAATTGCATGGTTGGACCC. EF-1α (Hemmati-Brivanlou and Melton, 1994) (U) AATTGGTGCTGGATATGC,(D) ACTGCCTTGATGACTCCTAG.

Embryonic manipulation

Xenopus eggs obtained from mature frogs were fertilized *in vitro* and embryos were cultured routinely. All embryonic stages were according to Nieuwkoop and Faber

(1967). UV irradiation of zygotes was performed as described previously (Christian et al., 1991).

In vitro transcription and microinjection of synthetic RNA or expression plasmid DNAs

Capped synthetic RNA was generated by *in vitro* transcription of SP64T-Xwnt-8b, synthetic RNA or expression plasmid DNA was injected as described by Moon and Christian (1989).

RESULTS

Xwnt-8b is a member of the Xwnt-8 subfamily

Figure 1 shows the predicted amino acid sequence of Xwnt-8b, aligned with the sequence of the most closely related Wnt family member, Xwnt-8. Xwnt-8b contains a hydrophobic putative leader sequence, and a large number of characteristic, invariant cysteine residues, a diagnostic feature for Wnt proteins. The position of 19 out of 22 cysteine residues is shared between Xwnt-8b and members of the Xwnt family other than Xwnt-8. The amino acid sequence of Xwnt-8b is 56% identical to that of Xwnt-8, and the positions of all the 22-cysteine residues are conserved. In addition, both proteins possess a highly basic 20-22 amino acid carboxyl-terminal extension relative to other known Xwnts (residues 414-435, Figure 1). These data support the designation of Xwnt-8b as a submember of the Xwnt-8 family.

Alternative splicing of Xwnt-8b transcripts is developmentally regulated

The predicted amino acid sequence of Xwnt-8b includes a 73 amino acid insert (residues 84-156, Figure 1) which is not conserved among other known Wnts. Sequence encoding this insert is located at a predicted intron/exon junction (Nusse and Varmus, 1992) (Figure 2A). Ribonuclease protection analysis of embryonic RNA, using a probe which overlaps the boundary between conserved and nonconserved Wnt sequence (Figure 2B), revealed the developmentally regulated expression of two unique Xwnt-8b transcripts (Figure 2C). Messenger RNAs that protect an 139 nucleotide fragment of the probe used in this assay would be generated by splicing out the unique exon, and these mRNAs would encode a form of Xwnt-8b protein in which the non-conserved 73 amino acid insert in absent. Zygotic RNAs encoding this shorter form of Xwnt-8b were first detected near the onset of gastrulation (st. 10.5), increased in abundance by the end of gastrulation (st. 13), and peak levels were observed in

swimming tadpoles (st. 38; Figure 2C). A second class of transcripts, which would protect a 240 nucleotide fragment of the probe used in this assay, would be generated by splicing in the unique exon, and these would encode the relatively longer form the Xwnt-8b protein as shown in Figure 1. Zygotic transcripts encoding the longer form of Xwnt-8b were first detected at the end of gastrulation (st. 13), were present at fairly equivalent levels throughout the tailbud stages (st. 32) and then declined to barely detectable levels in tadpoles (st.38).

Maternally derived Xwnt-8b transcripts are enriched in animal blastomeres of cleaving embryos

To determine whether Xwnt-8b transcripts might be present prior to gastrulation, we analyzed expression of Xwnt-8b using the more sensitive reverse transcription-PCR (RT-PCR) assay, an assay more sensitive than the Ribonuclease protection assay. Maternal Xwnt-8b RNAs were detectable in eggs, although transcript levels increase after the onset of zygotic transcription (Figure 3A). For controls of maternally and zygotically derived genes, the same RNA pool was assayed for expression of Xwnt-8 transcripts, which are not detectable until the late blastula stage (Christian et al., 1991); for Xwnt-11 transcripts, which are known to be expressed both maternally and zygotically (Ku and Melton, 1993); for Histone H4, which serves as a loading control in each reaction, and for EF-1α, which is expressed at low levels maternally and upregulated strongly starting from the midblastula stage (Krieg et al., 1989).

We further asked whether Xwnt-8b transcripts are expressed in a localized pattern along the animal-vegetal axis by dissecting 8-cell embryos into animal and vegetal halves and RNA from each was analyzed by RT-PCR. As shown in Figure 3B, Xwnt-8b transcripts are clearly enriched in the animal half blastomeres relative to the vegetal half. To control for accuracy of the dissection, the same RNA pool was

assayed for expression of Vg1, whose transcripts are known to be localized to the vegetal, but not the animal half, and for Histone H4 transcripts, which are uniformly expressed along the animal-vegetal axis (Weeks and Melton, 1987).

To quantify the level of the endogenous Xwnt-8b transcripts, RNA from oocytes and neurula (st. 18) embryos were analyzed by an RNase protection assay, in which the probe was also hybridized to a series of synthetic Xwnt 8b RNA. The absolute level of Xwnt-8b transcripts was estimated, according to the standard curve derived from this assay, to be approximately 0.15 pg of RNA per oocyte, and about 1 pg per embryo at the neurula stage (Figure 3C).

Spatial distribution of zygotic Xwnt-8b transcripts

The localization of Xwnt-8b transcripts was analyzed by whole mount *in situ* hybridization of digoxigenin-labeled riboprobes to staged embryos. Xwnt-8b transcripts are first detected in late gastrula to early neurula stage embryos as diffuse staining in the prospective anterior neural ectoderm (data not shown). By mid- to late-neurula stage, the staining is concentrated in a single band of cells located at the predicted junction between forebrain and midbrain (Figure4A, B, arrowheads). In tailbud stage embryos, Xwnt-8b transcripts are detected in a thin, bilaterally symmetric band of cells in the dorsal diencephalon (Figure 4C, E-H, black arrowheads), and at the forebrain-midbrain boundary (Figure 4C, white arrowheads). When embryos were stained for prolonged periods, specific signal is observed in a few cells in the dorsal mesencephalon, just posterior to the forebrain (Figure 4C, right side, white arrow). In swimming tadpoles, an identical pattern of staining is observed, but the signal is much less intense (not shown). Staining is not observed in embryos hybridized with a sense Xwnt-8b probe (Figure4D).

Xwnt-8b has dorsal axis inducing activity when misexpressed prior to midblastula stage

Synthetic RNA encoding Xwnt-8b was injected into various blastomeres of 4-8 cell embryos to assay for potential functions of endogenous maternally derived Xwnt-8b. Injection of about 10 pg of Xwnt-8b RNA near the prospective ventral midline in the marginal zone or vegetal region led to the induction of a secondary axis, which in many cases are anteriorly complete (Table 1 and Figure 5A). Injection of the same amount of Xwnt-8b RNA into dorsal marginal zone cells did not produce observable phenotypic defects in most embryos (Table 1 and Figure 5B).

Given the observation that misexpression of Xwnt-8b can organize a secondary dorsal axis in normal *Xenopus* embryos, we further tested its dorsal inducing capacity by asking whether ectopically expressed Xwnt-8b could rescue dorsoanterior development in UV-ventralized embryos. Embryos that had been irradiated with UV light prior to first cleavage lacked dorsal axial structures, developing with an average dorsoanterior index (DAI; Kao and Elinson, 1988) of 0.27 (n=33). In contrast, sibling embryos, which were irradiated the same way and then injected with about 5 pg of Xwnt-8b RNA at the 4-cell stage, showed, in most cases, essentially indistinguishable form the normal embryos; the average DAI of Xwnt-8b RNA injected group was 4.2 (n=63).

To assay for potential functions of zygotic Xwnt-8b, a plasmid expression construct (CSKA-Xwnt-8b) consisting of the Xwnt-8b cDNA cloned downstream of the cytoskeletal actin promoter, was injected into different blastomeres of cleaving embryos. This promoter is transcriptionally active only after the midblastula transition (Harland and Misher, 1988). Injection of CSKA-Xwnt-8b DNA into the equatorial region of embryos at either dorsal or ventral side generated, at most cases, tadpoles indistinguishable from uninjected embryos, or from those injected with a control

plasmid, CSKA-CAT, which expresses the bacterial chloramphenical acetyl transferase (Christian and Moon, 1993). A small percentage of embryos injected dorsally with the Xwnt-8b plasmid showed a reduction in anterior structures similar to, but less severe than, that observed following misexpression of the related protein, Xwnt-8, in dorsal cells after the midblastula transition (Christian and Moon, 1993).

DISCUSSION

Zygotically derived Xwnt-8b may be involved in neural patterning

We have reported in this paper that alternatively spliced Xwnt-8b transcripts are present in *Xenopus* embryos both prior to and after the onset of zygotic gene transcription. Zygotically derived Xwnt-8b transcripts are restricted to a stripe of cells that presage the formation of a morphologically recognizable forebrain-midbrain boundary. Patterning molecules produced by cells in the boundary region, including members of the Wnt family, may be involved in establishing polarity within this neural field. The expression pattern of Xwnt-8b is consistent with the possibility that Xwnt-8b may participate in specifying polarity within the forebrain region.

Maternally derived Xwnt-8b may function as a dorsal determinant

The presence of maternal Xwnt-8b transcripts, coupled with our demonstration that Xwnt-8b can induce the formation of a complete dorsal axis when introduced prior to the midblastula stage, is consistent with the possibility that endogenous Xwnt-8b may be involved in establishing the dorsal axis.

While the expression pattern and biological activity of Xwnt-8b are consistent with its involvement in dorsal patterning, the quantity of endogenous maternal Xwnt-8b RNA is significant lower than the amount of synthetic RNA which is required to be injected for the formation of a secondary axis. However, the relative quantity of synthetic and endogenous transcripts does not necessarily reflect the protein levels since endogenous Xwnt-8b transcripts may be translated over a long period of time during oogenesis, whereas the injected transcripts have to be translated in hours prior to the midblastula transition.

In support of the hypothesis that an endogenous Wnt-like activity may be required for dorsal development in *Xenopus*, recent studies have shown that ß-catenin, a component of the Wnt signaling pathway, is required for dorsal induction. Specifically, when maternal ß-catenin transcripts are depleted by introducing antisense oligonucleotides into oocytes, dorsal mesoderm formation is blocked in the resulting embryos (Heasman et al., 1994), suggesting that ß-catenin is an essential component of Wnt-signaling and is required for formation of the embryonic axis.

So far, the strongest evidence against the involvement of Wnts in the maternal steps of dorsal axis formation has been the lack of a maternally expressed Wnt which possesses full-axis inducing activity. The biological activity of Xwnt-8b, together with the presence of Xwnt-8b transcripts in cells that will be induced to form the mesoderm, is consistent with the possibility that this Wnt may function to modify the response of ectodermal cells to mesoderm inducing signals, thereby participating in the establishment of the dorsal axis. Further studies involving the ablation of endogenous Xwnt-8b transcripts or activity will require to substantiate this hypothesis.

Acknowledgment

We thank Sharon Wolda for isolating and sequencing wnt-8b cDNA clones. This work is supported by a grant from the Medical Research Foundation of Oregon and NIH grant R29 HD31087 (JLC) and NIH grant HD 27525 (RTM).

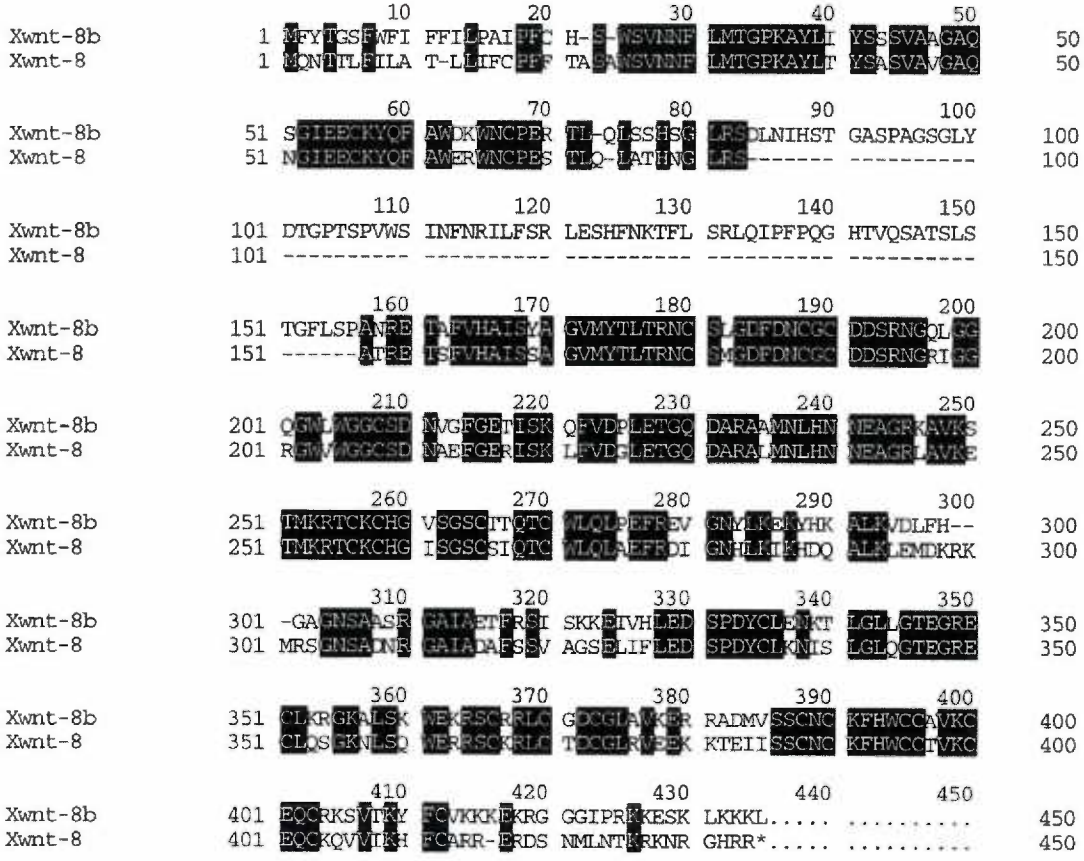

Figure 1. Comparison of predicted *Xwnt*-8b and *Xwnt*-8 protein sequences. Gaps introduced to align the sequences are shown as dashes and identical residues are shaded.

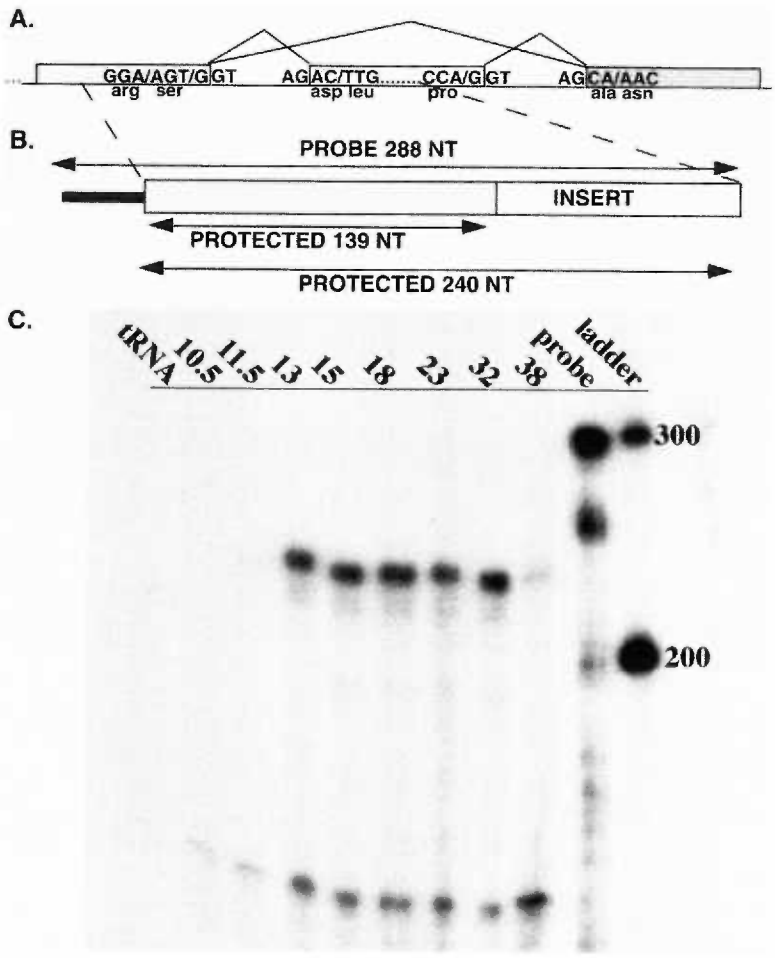

Figure 2. Developmental expression of two *Xwnt*-8b transcripts generated by differential splicing. (A) Schematic diagram of differential splicing event. Exons are shown as bars and introns as lines. Nucleotides at the intron/exon borders match conserved consensus sequences for eukaryotic splice sites (Mount, 1982) as shown. (B) Schematic diagram of cDNA which was transcribed to generate a probe for ribonuclease protection analysis of *Xwnt*-8b expression. Dotted lines indicate location of 5' and 3' limits of the probe template relative to alternatively spliced exon. Solid bar represents vector sequence included in unprotected probe. (C) Ribonuclease protection analysis of 10 μg of tRNA or 50 μg of total RNA isolated from embryos at the developmental stage indicated at the top of each lane (numbers refer to stages according to Nieuwkoop and Faber, 1967).

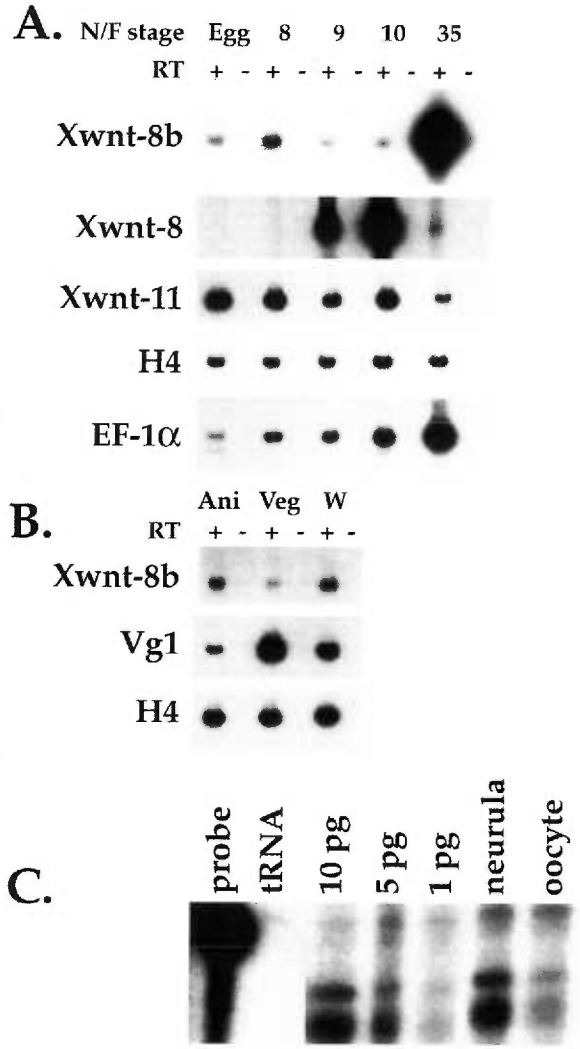

Figure 3. Expression of maternal *Xwnt*-8b transcripts in eggs and cleaving embryos. (A) Analysis by RT-PCR of the expression of *Xwnt*-8b, *Xwnt*-8, *Xwnt*-11, Histone H4 (H4) and EF-1α in unfertilized eggs (Egg) and embryos at the indicated stages (N/F: Nieuwkoop and Faber, 1967). (B) Analysis by RT-PCR of the expression of *Xwnt*-8b, Vg1 and Histone H4 in blastomeres isolated from the animal (Ani), vegetal (Veg) half, or whole embryo (W), of 8-cell embryos. For each RNA sample, PCRs were performed on duplicate aliquots incubated with (+) or without (-) reverse transcriptase (RT) as indicated above each lane. (C) RNase protection assay for *Xwnt-8b* in total RNA samples from oocytes (100 μg) and st. 18 neurulae (50 μg). Also included in the assay is *in vitro* synthesized *Xwnt-8b* RNA at the amounts indicated.

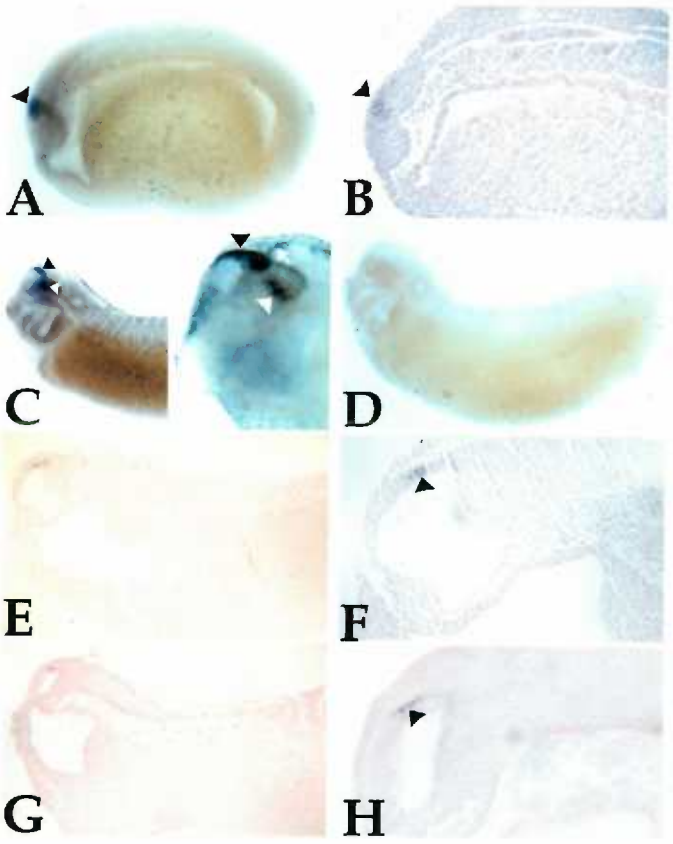

Figure 4. Localization of *Xwnt*-8b transcripts in neurula and tailbud stage embryos by whole mount *in situ* hybridization. (A) Neurula (st. 21), and (B) saggital section of neurula stage embryo hybridized with a digoxygenin-labeled antisense *Xwnt*-8b probe showing signal at junction between prospective prosencephalon and mesencephalon (arrowheads). (C) Low (left side) and high (right side) magnification views of whole tailbud (st. 33) embryos hybridized with antisense *Xwnt*-8b probe. Staining of dorsal diencephalon (black arrowhead), forebrain-midbrain border (white arrowhead) and dorsal mesencephalon (white open arrow) is indicated. Embryo on right was stained overnight to visualize midbrain staining. All of the blue signal with the exception of that indicated by arrows was also observed in embryos hybridized overnight with a sense probe (data not shown) and is thus nonspecific staining. (D) Tailbud (st. 33) embryo hybridized with sense *Xwnt*-8b probe. (E-H) Low (E, G) and high (F, H) magnification views of sagittal sections of tailbud (st. 33) embryos showing *Xwnt*-8b expression in the dorsal diencephalon (arrowheads).

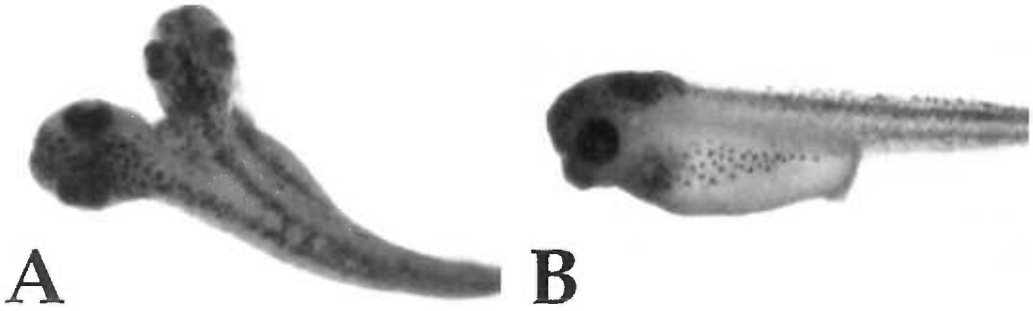

Figure 5. Injection of synthetic RNA encoding *Xwnt*-8b into ventral blastomeres of 4-cell embryos induces formation of an anteriorly complete secondary dorsal axis. Approximately 10 pg of *Xwnt*-8b RNA was injected near the marginal zone into ventral (A) or dorsal (B) blastomeres of 4-cell *Xenopus* embryos.

Table 1. Phenotype of *Xenopus* embryos injected with RNAs or cDNAs encoding *Xwnt*-8b.

RNA/DNA	Injection site	Phenotype			
		WT	2° Axis	Aceph/Micro	*n*
Xwnt-8b RNA	VMZ	6	94	0	137
Xwnt-8b RNA	DMZ	93	7	0	54
CSKA-X8b	VMZ	100	0	0	67
CSKA-X8b	DMZ	88	0	12	230
CSKA-CAT	DMZ	98	0	2	84

DNA was injected into the marginal zone region of 2 blastomeres of 4-cell embryos, near the prospective dorsal (DMZ) or ventral (VMZ) midline. Embryos surviving until stage 36 were scored as follows: Wild type (WT), showing no specific defects; Secondary (2°) axis, complete or partial duplication of dorsoanterior structures; acephalic (Aceph) or microcephalic (Micro), reduced or absent head. Numbers are expressed as percentages except for *n* which denotes sample size.

Chapter 2

Synergistic effects of Vg1 and Wnt signals in the specification of dorsal mesoderm and endoderm

Yanzhen Cui, Qi Tian and Jan L. Christian

Department of Cell and Developmental Biology

Oregon Health Sciences University, School of Medicine

Portland, OR 97201

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ABSTRACT

In amphibians, dorsoventral asymmetry is established by cortical rotation, a cytoplasmic rearrangement in the egg which activates a dorsal determinant on one side of the zygote. This determinant has been proposed to be either Vg1, an endodermally derived molecule that can directly induce ectoderm to form dorsal mesoderm, or a member of the Wnt family, which patterns the ectoderm such that it forms dorsal mesoderm in response to ventral inductive signals. In this study, we have investigated whether the endogenous dorsal determinant(s) functions as a direct inducer of dorsal mesoderm (Vg1-like), or whether it acts to pattern the response of ectoderm to inductive signals (Wnt-like). We report here that cortical rotation enhances both the dorsal inductive activity of endodermal cells and the response of ectodermal cells to endogenous inductive signals, and that both of these activities are required for notochord induction in ectoderm/endoderm recombinants. While ectopically expressed Xwnt-8b can substitute for the dorsalizing signals activated in either ectoderm or endoderm and can allow notochord formation in recombinants, Vg1 alone is not sufficient to induce notochord in ectodermal explants in the absence of signals activated by cortical rotation. Co-expression of Xwnt-8b along with Vg1 restores ectodermal competence to form notochord. Finally, in endodermal explants, ectopically expressed Xwnt-8b, but not Vg1, can divert the fate of ventral endodermal cells along a dorsal pathway. Thus, while Vg1 is most likely required for induction of mesoderm *in vivo*, our data suggest that a maternal Wnt-like signal acts synergistically with Vg1 to specify a dorsal fate not only in the mesoderm, but also in the endoderm.

INTRODUCTION

In amphibians, the dorsoventral axis is established during the first cell cycle by a cytoplasmic rearrangement, termed cortical rotation, that is believed to activate a dorsal determinant on one side of the zygote (reviewed by Gerhart et al., 1991). The mechanism of action and molecular nature of this putative determinant is unclear. One model suggests that cortical rotation activates a morphogen within endodermal cells, and that this morphogen directly induces overlying ectodermal cells to form dorsal mesoderm. A second hypothesis suggests that mesodermal induction and dorsal patterning are distinct events which require the cooperative action of separate signaling agents. According to this model, the determinant activated by cortical rotation is not a dorsal mesoderm-inducing factor, but is instead a patterning agent which modifies the response of presumptive mesodermal cells to ventral inductive signals, causing them to adopt a dorsal fate (reviewed by Christian and Moon, 1993).

Embryological evidence supports the existence of dorsal-ventral differences in endodermally derived mesoderm inducing signals. When dorsal endodermal cells are co-cultured with ectoderm, the recombinants form dorsal type mesoderm (e.g. notochord) while similar recombinants made with ventral endodermal cells form only ventral mesoderm (e.g. blood) (Nieuwkoop, 1969). Furthermore, when cortical rotation is blocked by ultraviolet (UV)-irradiation of the egg, mesoderm is still induced but it is purely ventral in nature (Gerhart et al., 1991). Dorsal mesoderm formation can be restored by transplanting single blastomeres from the dorsal vegetal or equatorial region of nonirradiated cleavage-stage donor embryos into the UV-irradiated hosts (Gimlich, 1986).

Molecular studies have identified Vg1, a member of the transforming growth factor-ß family, as a likely candidate for an endogenous dorsal mesoderm-inducing

agent. Consistent with this proposed role, Vg1 transcripts are present in endodermal cells of early embryos, although endogenous Vg1 protein has only been detected in its unprocessed, presumably inactive form (reviewed by Vize and Thomsen, 1994). When ectodermal explants are exposed to an *in vitro* synthesized mature form of Vg1, they differentiate as dorsal mesoderm (Kessler and Melton, 1995). In addition, when RNA encoding a bone morphogenetic protein (BMP)/Vg1 chimera is injected into ventral or UV-irradiated cells of *Xenopus* embryos, the chimeric protein is processed to produce mature Vg1, which in turn induces the formation of a partial (Dale et al., 1993) or complete (Thomsen and Melton, 1993) dorsal axis, respectively. This ability of processed Vg1 in and of itself to phenocopy the axis-rescuing ability of transplanted dorsal blastomeres is consistent with the possibility that Vg1 is the active cytoplasmic component of these cells.

Further evidence that Vg1 is required for dorsal mesoderm induction has come from studies in which both activin and Vg1 signal transduction pathways are blocked *in vivo* by ectopic expression of a mutant activin receptor. The resulting embryos lack both dorsal and ventral mesoderm (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994). In contrast, embryos in which activin signaling alone is blocked by overexpression of the activin binding protein follistatin develop normally.

In addition to differences in the inductive signals emitted by dorsal or ventral endodermal cells, a dorsoventral bias exists in the response of ectodermal cells to these inductive signals. Specifically, ectoderm isolated from prospective dorsal regions of blastula stage embryos will form dorsal types of mesoderm when exposed to exogenous inducing agents, while ectoderm from ventral regions, or from embryos in which cortical rotation is blocked, will form more ventral types of mesoderm (Sokol and Melton, 1991; Bolce et al., 1992). This bias in ectodermal response is evident prior to the time that mesoderm-inducing signals are first produced (Jones and

Woodland, 1987; Kinoshita et al., 1993), suggesting that the signals that establish this bias are distinct from inducing signals.

Members of the *Xenopus* Wnt (Xwnt) family of secreted proteins can substitute for the endogenous response modifying signal which is normally activated in dorsal ectodermal cells by cortical rotation. Certain Wnt proteins can completely rescue dorsal development when misexpressed in embryos ventralized by UV irradiation, but only if the Wnt signal is initiated prior to the onset of zygotic gene transcription at the midblastula transition (MBT) (reviewed by Christian and Moon, 1993). Although ectopic expression of Wnts in whole embryos leads to the formation of dorsal mesoderm, Wnts cannot induce isolated ectodermal cells to form dorsal mesoderm. Instead, Wnts modify or enhance the response of ectodermal cells to inductive signals such that dorsal, rather than ventral, mesoderm differentiates (Christian et al., 1992; Sokol and Melton, 1992). We have identified a maternally expressed *Xenopus* Wnt, Xwnt-8b, that is a candidate for an endogenous dorsal patterning molecule (Cui et al., 1995).

Compelling evidence that a Wnt-like activity is required for dorsal patterning in *Xenopus* embryos has been provided by the demonstration that depletion of maternal ß-catenin prevents the formation of dorsal but not ventral mesodermal derivatives (Heasman et al., 1994). ß-catenin is believed to be an essential downstream component of the Wnt-signaling pathway (reviewed by Peifer, 1995) and, consistent with this, dorsal development cannot be rescued by injection of Wnt RNA into these embryos. These studies suggest that Vg1 signals alone may not be sufficient to induce dorsal mesoderm.

In this study, we have used both tissue recombination and explant assay systems to test whether the endogenous dorsal determinant(s) activated by cortical rotation is generated by endodermal cells and acts as a direct inducer of dorsal

mesoderm (similar to Vg1), or whether it acts to pattern the response of ectoderm to inductive signals (similar to Wnts). Together, our data suggest that although Vg1 may be required for induction of mesoderm *in vivo*, a maternal Wnt-like signal acts synergistically with Vg1 to specify a dorsal fate not only in the mesoderm but also in the endoderm.

MATERIALS AND METHODS

Embryo culture and manipulation

Xenopus eggs were obtained and embryos were cultured as described by Moon and Christian (1989). All embryonic stages are according to Nieuwkoop and Faber (1967). UV irradiation of zygotes was performed as described by Christian et al. (1991). In all experiments, some embryos were allowed to develop to stage 28, at which time they were assigned a score of dorsoanterior index (DAI) as described by Kao and Elinson (1988). Only experiments in which the average DAI for UV-irradiated embryos was < 0.5 were included in the results. Ectoderm/endoderm recombinants were generated by isolating approximately one half of the ectoderm from the central region of the animal pole of stage 8 blastulae using sharpened tungsten needles, and sandwiching this tissue with endoderm isolated from the central portion of the vegetal pole of a sibling embryo. Conjugates of endoderm and ectoderm from control embryos were generated such that the relative dorsoventral polarity of endodermal and ectodermal tissues was maintained. All conjugates were cultured in agarose-coated Petri dishes containing one half strength normal amphibian medium (NAM/2, Slack et al., 1973).

Generation of Activin-Vg1 (AVg) chimeric construct

A cDNA encoding a chimeric activin-Vg1 (AVg) protein was generated using the polymerase chain reaction (PCR)- based gene splicing by overlap extention technique (Horton, 1993) and was subcloned into the transcription vector pT7TS (gift of P. Krieg) to generate pT7TS-AVg. The chimeric protein includes the entire preproregion and cleavage site of *Xenopus* activin ßB (gift of S. Sokol), which is known to be efficiently processed in *Xenopus*, fused in frame to the putative bioactive C-terminal domain of Vg1 (provided by K. Mowrey). Portions of the cDNA that were

generated by PCR were sequenced to confirm the absence of PCR-induced random mutations.

In vitro transcription of synthetic RNA and microinjection

Capped synthetic RNA was generated by *in vitro* transcription of SP64T-*Xwnt*-8b (Cui et al., 1995), pT7Ts-AVg, pCS2-ß-catenin (gift of R. Moon) or, in initial experiments, SP64T-*Xwnt*-8 (Christian et al., 1991). Synthetic RNA was injected into cleaving *Xenopus* embryos as described by Moon and Christian (1989). In all experiments AVg RNA was injected at 100ng/ul, Xwnt 8b RNA at 10ng/ul, and ß-catenin RNA at 20 ng/µl. Approximately 200 pg of AVg, 20 pg of Xwnt-8b or 50 pg of ß-catenin was injected per embryo. For lineage labeling experiments, rhodamine dextran was diluted in water and injected at a concentration of 5 mg/ml.

Immunostaining and histological analysis of whole embryos and recombinants

Whole mount immunocytochemical analysis using the muscle-specific monoclonal antibody 12/101 (Kintner and Brockes, 1984) or the notochord-specific monoclonal antibody Tor70 (Bolce et al., 1992; gift of R. Harland) was performed as described in Moon and Christian (1989). Following immunostaining, some recombinants were dehydrated in methanol, embedded in paraffin and 10 µm thick sections were cut and counterstained with hematoxylin-eosin. Differentiated cell types were identified by morphology as described by Green et al. (1990).

Analysis of RNA by reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis of RNA samples was performed as described previously (Cui et al., 1995) except that total nucleic acids were isolated as described by Moon and Christian (1989), and RNAs were selectively precipitated in 4M LiCl. Oligo-dT-primed first strand cDNA was prepared from RNAs of one whole gastrula stage

embryo, or six pooled explants or recombinants using an AMV reverse transcription kit according to the manufacturer's instructions (Life Science, Inc.). Negative controls in which reverse transcriptase was omitted were prepared in parallel for each sample using equivalent amounts of RNA. Twentyfive microliter PCR reactions were carried out using DNA polymerase (0.25 unit/reaction, Epicentre Technologies) and 1 X PCR buffer, with 100 μM dNTPs, 3 mM of $MgCl_2$, 0.5 μCi of (α-^{32}P)dCTP, 10 pmol of each primer, and 0.5 μl of first strand cDNA. The first PCR cycle involved denaturing at 94°C for 1.5 minutes, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds. This was followed by three-temperature cycling with 30 seconds at each temperature, followed by a final extension step at 72°C for 4 minutes. Cycle number was determined empirically for each primer pair so that PCR products were examined during the exponential phase of amplification. *Siamois* primers were used at 42 cycles, *Xwnt-8* primers at 30 cycles, *goosecoid* primers at 24 cycles, and *EF- 1 α* primers at 21 cycles. After amplification, 1/6 of each reaction was run on a 5% polyacrylamide gel which was subsequently dried. The amplified bands were visualized with a Molecular Dynamics phosphorimager and the Macintosh IP lab gel program.

The sequences of *Xwnt*-8 (Christian et al., 1991), *goosecoid* (Kengaku and Okamoto, 1995) and *EF- 1 α* (Kengaku and Okamoto, 1995) primers have been reported previously. The sequence of the *siamois* primers used in this paper are listed as follows, in 5' to 3' orientation: upstream GGGATATTCCATGATATTC; downstream TCCTCTGCCTGAATCA.

RESULTS

Notochord induction requires dorsalizing signals in both endodermal and ectodermal cells

Our initial experiments were designed to ask whether the putative dorsal determinant that is activated by cortical rotation is generated by endodermal cells and possibly functions as a direct inducer of dorsal mesoderm (similar to Vg1), or whether it acts within ectodermal cells to modify their response to inductive signals (similar to Wnts). Embryos were irradiated with UV light during the first cell cycle in order to block cortical rotation, and thus to block any cortical rotation-mediated activation of dorsal determinants. Endoderm or ectoderm from these embryos was conjugated with ectoderm or endoderm, respectively, from control embryos as illustrated above Figure 1. Conjugates were cultured to the tailbud stage and analyzed for the formation of dorsal mesoderm by immunostaining for notochord. If, as hypothesized, Vg1 is proteolytically activated by cortical rotation (Thomsen and Melton, 1993) and is sufficient for dorsal mesoderm formation, then Vg1-producing endodermal cells from control embryos should be able to induce notochord formation, even in ectodermal cells from UV-irradiated embryos. In contrast, if Wnt-like signals which dorsalize the response of the ectoderm are sufficient for dorsal development, then ectoderm from control embryos should form notochord even when recombined with endodermal cells from UV-irradiated embryos.

As summarized in Table 1, notochord immunostaining was rarely detected in whole UV-irradiated tailbud stage embryos (Figure 1A), or in recombinants of ectoderm and endoderm isolated from such embryos (Table 1, UV/UV; Figure 1B). In contrast, when ectoderm and endoderm isolated from non-irradiated embryos was co-

cultured, most recombinants possessed immunoreactive notochord (Table 1, CON/CON; Figure 1E). When ectodermal cells from UV-ventralized embryos were conjugated with endoderm from normal embryos, the frequency of notochord formation was much lower (Table 1, UV/CON), and 50% of the positive cases represented isolated immunoreactive cells (Figure 1C) rather than well formed tubes. Recombinants in which ectodermal cells isolated from control embryos were placed together with UV-ventralized endoderm showed a further decrease in the frequency of notochord formation relative to control recombinants (Table 1, CON/UV), with approximately 70% of the immunoreactive notochords consisting of only a few cells as shown in Figure 1F. Notochord staining was never detected in ectodermal or endodermal explants cultured in isolation (data not shown). The results of these experiments suggest that neither a Vg1 signal, activated by cortical rotation in endodermal cells, nor a Wnt-like signal, activated in ectodermal cells, is sufficient for notochord formation. Instead, it appears that cortical rotation activates dorsalizing activity(s) in ectoderm and endoderm, and that both of these are required for notochord formation.

We also assayed ectoderm/endoderm conjugates for dorsal mesoderm formation by immunostaining with muscle-specific antibodies. Only 15% of whole UV-irradiated tailbud stage embryos possessed immunoreactive muscle (Figure 1H), and usually this consisted of small patches of staining. In contrast, when ectoderm and endoderm isolated from sibling UV-irradiated embryos at the blastula stage was conjugated and cultured to the tailbud stage, 84% of recombinants formed large blocks of immunoreactive muscle (Table 1, UV/UV; Figure 1I). Thus, blocking cortical rotation does not completely ablate dorsalizing signals *in vivo* but does prevent these signals from contacting, or acting upon, marginal zone cells in intact embryos.

Xwnt-8b can substitute for endogenous endodermal and ectodermal dorsalizing activities

We next tested whether addition of an ectopic Wnt signal could substitute for the endogenous dorsalizing activities that are activated by cortical rotation in ectodermal or endodermal cells. Zygotes were UV-irradiated during the first half of the cell cycle and a Wnt signal was subsequently introduced by injecting approximately 20 pg of synthetic RNA encoding Xwnt-8b into the animal or vegetal pole of both blastomeres at the two-cell stage. Embryos were cultured to stage 8, at which time Wnt-expressing ectoderm was conjugated with UV-irradiated endoderm, or vice versa, as illustrated above Figure 2. Recombinants were stained with notochord-specific antibodies at control stage 26.

Notochord formation was never observed in Wnt-expressing animal pole ectoderm (Figure 2A) or vegetal pole endoderm (Figure 2B) cultured in isolation. Although endodermal cells isolated from UV-irradiated embryos rarely induced notochord formation in ectoderm from UV-ventralized embryos (Table 1, UV/UV), these same cells were sufficient to induce most Wnt-expressing ectodermal explants to form full notochords (Table 1, WNT/UV; Figure 2C). Thus, consistent with previous studies showing that Wnt signals can modify responsiveness of ectodermal cells to exogenously applied ventral mesoderm inducing agents (Christian et al., 1992; Sokol and Melton, 1992), the current results demonstrate that Wnts can also modify the type of mesoderm formed in response to endogenous inductive signals.

Most conjugates of Wnt-expressing endoderm and UV-ventralized ectoderm also developed fully formed notochords (Table 1, UV/WNT; Figure 2D). This result might be explained as a secondary consequence of Wnt protein being secreted from endodermal cells and acting upon conjugated ectoderm to modify the pattern of mesoderm formed in response to ventral inductive signals as described above.

However, this is not an adequate explanation because recombinants were made from ectodermal cells explanted from late blastula stage embryos, which are no longer competent to be dorsalized by Wnt signals. Specifically, while ectopic expression of Wnts in cleaving embryos (by injection of synthetic RNA) dorsalizes the response of cells to induction, and leads to formation of secondary notochords, misexpression of Wnts after the MBT (by injection of plasmid expression constructs) ventralizes cell fate, leading to a loss of notochord (reviewed in Christian and Moon, 1993). Thus, any Wnt protein secreted by endodermal cells at the time that recombinants were made would have had no direct dorsalizing effect. Instead, our results suggest that Wnt signals can act directly within vegetal endodermal cells to promote dorsal development.

To further test the possibility that Wnt signals can act directly within endodermal cells, we ectopically expressed ß-catenin in endodermal cells in order to activate a Wnt signaling pathway in a cell autonomous fashion. ß-catenin is a ubiquitously expressed molecule which functions as an intracellular transducer of Wnt signals (reviewed by Peifer, 1995). Thus, ß-catenin injection provides a means of activating a Wnt signaling pathway within endodermal cells while excluding the possibility that the Wnt protein is secreted and acts directly upon conjugated ectodermal cells. UV-irradiated embryos were injected with ß-catenin RNA (approximately 50 pg/embryo) at the two-cell stage, and cultured to stage 8, at which time endoderm from these embryos was recombined with ectoderm from UV-irradiated embryos as described above. A fully formed, immunoreactive notochord differentiated in 77% of these recombinants (n=22) (Figure 2E), clearly demonstrating that activation of a Wnt signaling pathway within endodermal cells can promote dorsal development. Notochord formation was also observed when ectodermal cells made to overexpress ß-catenin were conjugated with endoderm from UV-irradiated embryos (data not shown), consistent with the notion that ß-catenin can transduce Wnt signals.

Since UV-ventralized endoderm possesses some dorsal inductive activity, as evidenced by its ability to induce muscle formation (Figure 1I), we next asked whether Wnt signals can promote dorsal development from within endodermal cells isolated from the ventral side of normal embryos, since these cells lack dorsal inductive activity (Dale and Slack, 1987). Ventral endoderm was isolated from Xwnt-8b RNA-injected embryos, or from uninjected embryos, at stage 8 and was recombined with UV-ventralized ectoderm. Recombinants were cultured to stage 26 and then assayed histologically, or by immunostaining, for muscle and/or notochord. While only 22% of non-Wnt-expressing recombinants possessed a small amount of immunoreactive muscle, and notochord was observed in only one of these (n=14), 100% of Wnt-expressing recombinants possessed muscle, and 67% formed a notochord that was similar in appearance to those shown in Figure 2C and 2E (n=15) .

Endodermal cells generate a non-cell autonomous dorsalizing signal in response to Xwnt-8b

To distinguish between the possibility that Wnt signals act within ventralized endodermal cells, causing these cells to emit a dorsal inductive or patterning signal that instructs overlying ectoderm to form notochord, or the possibility that Wnts make endodermal cells themselves competent to differentiate as dorsal mesoderm, we analyzed the lineage of notochord cells in conjugates of Wnt-expressing endoderm and rhodamine dextran-labelled ectoderm, both of which were derived from UV-irradiated embryos. In all cases, immunoreactive notochord was derived from rhodamine-labelled (ectodermal) cells, and not from the unlabelled (endodermal) cells (Figure 3). Together our results suggest that endodermal cells respond to Wnt signals by generating a secondary, non-cell autonomous dorsal patterning or inductive activity that is transmitted to ectodermal cells, causing them to differentiate as dorsal, rather than ventral, mesoderm.

Xwnt-8b and Vg1 can act synergistically to induce notochord formation in the absence of cortical rotation

Although endogenous Vg1 produced by dorsal endodermal cells is not sufficient to induce notochord to form in ectoderm from UV-ventralized embryos (Figure 1C), it is possible that elevated levels of Vg1 might be sufficient to do so. To test this possibility, we injected RNA (approximately 200 pg/embryo) encoding a chimeric Activin-Vg1 (AVg) protein near the animal pole of two-cell embryos and analyzed its ability to induce dorsal mesoderm in an animal cap assay, as illustrated above Figure 4. The chimeric AVg protein used in these studies is analogous to a previously reported Activin-Vg1 fusion protein, which has been shown to be efficiently processed to form bioactive Vg1 dimers *in vivo* (Kessler and Melton, 1995).

Consistent with previous observations (Kessler and Melton, 1995), we found that most animal caps from control embryos form both notochord (Figure 4B) and muscle (Figure 4F, Table 2), when made to express Vg1 protein. In contrast, most ectodermal explants isolated from UV-irradiated embryos did not differentiate as notochord in response to Vg1 (Figure 4C, Table 2), although they did form large blocks of muscle (Figure 4G). While some animal caps from UV-irradiated embryos did form immunoreactive notochord in response to Vg1 (Table 2), most of the staining was weak, and represented sparsely distributed, postively stained cells (Figure 4C, inset). Notochord or muscle staining was never detected in ectodermal explants isolated from uninjected embryos (data not shown).

Currently it is not well understood how UV irradiation leads to a ventralized phenotype in *Xenopus* embryos. Previous studies showing that Wnt signals can rescue the ability of FGF (Christian et al., 1991) or activin (Sokol and Melton, 1991) to induce dorsal mesoderm in ectoderm from UV-irradiated embryos, and the fact that exogenous Xwnt-8b can substitute for dorsalizing signals in either ectoderm or

endoderm (this study), suggest that Wnt signals can substitute for those that are inactivated by UV-irradiation. To test this possibility, we coinjected RNAs encoding Xwnt-8b and AVg near the animal pole of UV-irradiated embryos and assayed for notochord formation in ectodermal explants. Addition of Xwnt-8b largely restored the ability of UV-ventralized ectoderm to form notochord in response to Vg1 (Figure 4D, Table 2). Xwnt-8b alone did not lead to notochord or muscle formation in explants (Figure 2A and data not shown).

The ability of Vg1 alone, or in combination with Xwnt-8b, to promote dorsal development in ectodermal explants was further examined by analysis of early molecular markers of dorsal (Siamois: Lemaire et al., 1995; goosecoid: Cho et al., 1991) or ventral (Xwnt-8: Christian et al., 1991; Lemaire and Gurdon, 1994) specification. Each of these genes is expressed in both mesodermal and endodermal cells, although siamois transcripts are present primarily in endodermal cells. The results of all molecular analyses were confirmed in a minimum of two independent experiments. In all experiments, some explants were cultured to the tailbud stage and immunostained for notochord to determine whether markers of dorsal specification correlated with dorsal mesodermal differentiation.

As shown in Figure 5, Xwnt-8b alone did not induce expression of any mesoderm- or endoderm-specific genes in ectodermal explants (lanes 3, 9). In contrast, Vg1 alone induced expression of the ventral specific gene, Xwnt-8, as well as expression of the dorsal specific gene, goosecoid, in ectoderm isolated from control (lane 5) or UV-irradiated (lane 11) embryos. Since ectoderm from UV-irradiated sibling embryos did not form notochord in response to Vg1, this result suggests that goosecoid is not sufficient for notochord induction. Consistent with this possibility, Steinbesser et al. (1995) have shown that goosecoid may be dispensable for notochord formation *in vivo*. In addition, the ability of Vg1 to induce Xwnt-8 expression but not

notochord development in UV-irradiated ectoderm demonstrates that endogenous Xwnt-8 cannot synergize with Vg1 to promote dorsal development. This is presumably due to the fact that expression of endogenous Xwnt-8 is not induced until after the MBT, at which time it functions as a ventralizing rather than a dorsalizing factor (Christian and Moon, 1993). In contrast, if Xwnt-8 is ectopically expressed prior to the MBT, it can synergize with Vg1 to promote notochord formation in a fashion similar to that observed for Xwnt-8b (data not shown). Vg1 alone induced low level expression of the dorsal specific gene, siamois, in ectoderm from control embryos (Figure 5, lane 5), but did not induce appreciable expression of siamois in ectoderm from UV-irradiated embryos (lane 11). When Xwnt-8b RNA was co-injected along with Vg1 RNA, expression of Xwnt-8 was downregulated and a high level of siamois expression was induced, even in ectoderm isolated from UV-irradiated embryos (lane 13).

Together, our results show that Vg1 can act in the absence of signals activated by cortical rotation to specify ventral and some dorsal types of mesoderm and/or endoderm, as evidenced by muscle formation and induction of expression of Xwnt-8 and goosecoid. In addition, although Vg1 alone is not sufficient to induce high level expression of siamois or formation of notochord in ectoderm from UV-irradiated embryos, Wnt signals can synergize with Vg1 to respecify ventral mesoderm and/or endoderm as dorsal.

Ectopic expression of Xwnt-8b, but not Vg1, dorsalizes the fate of ventral endoderm

Having assayed the dorsal patterning activities of Wnts and Vg1 in the mesoderm, we next tested each molecule for dorsal patterning activity in endodermal cells that were isolated either from UV-irradiated embryos, or from the ventral side of normal embryos, as illustrated in Figure 6A. UV-irradiation had little effect on the

endodermal component of Xwnt-8 expression in isolated vegetal explants (Figure 6A, compare lanes 1 and 3), despite the fact that it has previously been shown to enhance mesodermal expression of Xwnt-8 (Smith and Harland, 1991). In addition, UV-irradiation did not significantly downregulate endodermal expression of siamois (Figure 6A, compare lanes 1 and 3).

To determine whether UV-irradiation downregulates expression of siamois in the mesoderm, we dissected total endoderm out of control or UV-irradiated blastulae (st. 9) and then cultured the endodermal fragments or the remaining tissue (which consists of prospective mesoderm and ectoderm) until the gastrula stage, at which time expression of siamois was analyzed by RT-PCR. Since siamois transcripts are not detected in ectodermal cells (Lemaire et al., 1995), expression of siamois in explants of ectoderm plus mesoderm reflects mesodermal expression. As shown in Figure 6B, expression of siamois is fairly equivalent in endoderm isolated from control and UV irradiated embryos. In contrast, UV irradiation blocks the mesodermal component of siamois expression. A similar finding has been reported by Cornell et al. (1995), in that dorsal mesodermal expression of goosecoid is absent in UV-irradiated embryos while endodermal expression persists. Thus, while UV-irradiation blocks dorsal pattern formation within the mesoderm, it does not eliminate endodermal expression of genes whose transcripts are normally restricted to dorsal cells.

Addition of a Wnt signal to endodermal cells from UV-irradiated embryos reduced expression of Xwnt-8 approximately 5-fold relative to endoderm from non-injected UV-irradiated embryos, and upregulated expression of siamois approximately 3.5-fold within these cells (Figure 6A, compare lanes 3 and 5). Notably, while overexpression of Vg1 in endodermal cells from UV-irradiated embryos increased levels of siamois transcripts by approximately 1.8-fold, it did not reduce expression of Xwnt-8 within the same cells (Figure 6A, compare lanes 3 and 7).

Endodermal cells from UV-irradiated embryos retain some dorsal inductive activity, as evidenced by their expression of siamois (Figure 6A, lane 3) and their ability to induce muscle formation in ectoderm-endoderm conjugates (Figure 1I). For this reason, we further assayed the dorsal patterning activities of Vg1 and Xwnt-8b in endodermal cells isolated from the ventral side of normal embryos, since these cells completely lack dorsal pattern and inductive activity (Dale and Slack, 1987). As shown in Figure 6A, ventral endodermal cells normally express Xwnt-8 but not siamois (lane 9). When these cells are made to misexpress Xwnt-8b, expression of siamois is induced while expression of Xwnt-8 is diminished to approximately 25% of control levels (lane 11). In contrast, overexpression of Vg1 in these cells did not induce expression of siamois, nor did it compromise expression of Xwnt-8 (lane 13). These results demonstrate that Wnt signals, but not Vg1, can impart a dorsal pattern on ventral endodermal cells.

DISCUSSION

Dorsalizing signals activated by cortical rotation are required in both ectodermal and endodermal cells

Taken together, our results demonstrate that dorsalizing signals generated by cortical rotation function both within responding (ectodermal) and within inducing (endodermal) cells during the process of mesoderm induction and patterning. Furthermore, tissue recombination experiments suggest that these signals are required within both cell populations for the formation of extreme dorsal mesoderm. Specifically, notochord is not formed if signals activated by cortical rotation are absent in both inducing and responding cells of tissue recombinants, and is infrequently and poorly formed when the signals are intact in only one of the two tissues.

This requirement for dorsalizing signals in both endoderm and ectoderm might be explained in several ways. First, it is possible that cortical rotation activates qualitatively different signals in dorsal endodermal and ectodermal cells (e.g. dorsal inductive and competence modifying signals), and that the synergistic action of both of these signals is required for dorsal mesoderm formation. A second possibility is that cortical rotation activates a single dorsalizing activity in ectodermal and endodermal cells, and that the degree of dorsal development is proportional to the quantity of active signal. Our observation that Wnt signals can substitute for either the endodermal or ectodermal dorsalizing signal activated by cortical rotation is consistent with this second hypothesis. *In vivo*, this signal may be present in a broad region along the dorsal midline but may be concentrated within prospective mesodermal cells during the late blastulae stage such that these cells are competent to form notochord. In contrast, the quantity of dorsalizing activity present in endoderm or ectoderm alone may be insufficient for notochord formation unless endoderm and ectoderm from non-irradiated

embryos is conjugated (providing an additive effect in terms of Wnt signal strength) or unless the Wnt signal is artifically elevated in either tissue by injection of Wnt RNA.

Although whole UV-irradiated embryos fail to form dorsal mesoderm, some dorsal inductive or patterning activity persists in the absence of cortical rotation. This is evident in that conjugates of endoderm and ectoderm from UV-ventralized embryos form muscle, while whole UV-ventralized embryos do not. Since muscle formation is completely blocked when either the Vg1 (Hemmatti-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994) or the Wnt/ß-catenin signaling pathway (Heasman et al., 1994) is disrupted, our results imply that both of these signaling pathways are operative at some level in UV-ventralized embryos. The observation that endodermal expression of the dorsal-specific genes siamois (this paper) and goosecoid (Cornell et al., 1995) is equivalent in UV-irradiated and non-irradiated animals further suggests that some dorsal endodermal pattern may be maintained in the absence of cortical rotation. These findings are consistent with the results of cytoplasm transfer experiments which revealed the existence of dorsal axis forming activity near the vegetal pole of UV-irradiated embryos (Fujisue et al., 1993; Holowacz and Elinson, 1993), and which suggest that cortical rotation serves to translocate this activity to the marginal zone. Whether this translocated activity consists of an active Wnt-like ligand, such as Xwnt-8b, will require further studies.

Vg1 is not sufficient for notochord formation in the absence of cortical rotation

Based on the expression pattern of endogenous Vg1, along with the observation that ectopically expressed Vg1 can induce dorsal mesoderm and can rescue dorsal structures in UV-ventralized embryos, it has been proposed that cortical rotation proteolytically activates Vg1 in dorsal endodermal cells, and that Vg1 then functions as the endogenous dorsal determinant (Thomsen and Melton, 1993; Kessler and Melton,

1995; Dohrmann et al., 1996). The results of our current studies do not support this hypothesis. Specifically, neither dorsal endodermal cells, which contain endogenous Vg1, nor ectopically expressed Vg1 is sufficient to induce notochord formation in the absence of cortical rotation. Instead, we show that activation of a Wnt signaling pathway in endodermal cells is sufficient to induce notochord to form in conjugated ectoderm.

While Wnts can substitute for cortical rotation-mediated dorsalizing signals in both endodermal and ectodermal cells, previous studies have established that Wnts are primarily patterning, rather than inductive agents (reviewed by Christian and Moon, 1993). Thus, any action exerted by Wnts is presumed to be dependent upon the presence of other inductive signals. The best candidate for such an endogenous synergistic inductive signal is Vg1. This statement is based upon previous studies which suggest that Vg1 is required for specification of both mesodermal (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994) and endodermal (Cornell et al., 1995; Henry et al., 1996) fates *in vivo*, along with our demonstration that Vg1 is capable of synergizing with Wnts in notochord induction assays. Our data, in combination with these previous studies, are consistent with the hypothesis that mature Vg1 functions as a general mesoderm and endoderm inducer across the entire dorsal-ventral axis, while a Wnt signaling pathway may be activated by cortical rotation in dorsal cells and may synergize with Vg1 to specify dorsal fate within both mesoderm and endoderm. This hypothesis is consistent with recent studies showing that the endogenous maternal dorsal determinant found in the vegetal cortex functions as a pattern modifier, similar to Wnts, and does not function like Vg1 to directly induce dorsal mesoderm (Holowacz and Elinson, 1995).

Although the ability of Wnts to synergize with growth factors in induction and dorsal patterning of the mesoderm has previously been documented (Christian et al.,

1992; Sokol and Melton, 1992), our results, and those of Gamer and Wright (1995) provide evidence that Wnts may be involved in dorsal endodermal patterning as well. In a previous study, Gamer and Wright (1995) observed that ectopic expression of either of two Nieuwkoop center morphogens, Xwnt-8 or noggin, led to upregulation of the dorsal-specific gene, XlHbox 8, in whole endodermal explants. Immunohistochemical analysis of such explants demonstrated that the XlHbox 8 expression domain was expanded in endoderm isolated from embryos made to misexpress Xwnt-8 or noggin, consistent with the notion that ventral endodermal cells had been repatterned as dorsal. However, based on their observation that noggin alone did not induce XlHbox 8 expression in ventral endoderm, along with the fact that activin alone induced expression of this gene in endoderm from UV-irradiated embryos, they proposed that specification of dorsal endoderm requires a high local concentration of activin (or a related TGF-ß-like molecule such as Vg1), in combination with Nieuwkoop center morphogens. While our results are consistent with theirs, in showing that Vg1 can upregulate expression of a dorsal-specific gene (siamois) in endodermal explants from UV-irradiated embryos, we show that Wnt signals, but not Vg1, can downregulate a ventral-specific gene (Xwnt-8) in these same cells, and that Wnts, but not Vg1, can respecify endoderm explanted from the ventral side of normal embryos as dorsal. Thus, our results suggest that high local levels of Wnt signaling on the dorsal side, in combination with Vg1 signals that are uniformly distributed across the dorsoventral axis, may be required for dorsal endodermal patterning.

Although Vg1 alone is not sufficient for extreme dorsal mesodermal or endodermal specification or differentiation, even when ectopically expressed at high levels, it is capable of inducing intermediate types of mesoderm, such as muscle, in UV-ventralized (this paper) and in ventral cells (Dale et al., 1993). Vg1 can also induce some markers of dorsal specification, such as chordin (data not shown) and goosecoid

(this paper), even in the absence of signals activated by cortical rotation. Expression of these genes, however, does not correlate with differentiation of extreme dorsal mesoderm (i.e. notochord).

Cooperation between Wnt and Vg1 signals may generate an endodermal dorsal organizing center

Our results, and the results of others, suggest that the dorsalizing effect of Wnt signaling may not be direct but may be mediated by a subsequent cell-cell interaction, possibly through the activation of a secondary dorsalizing morphogen. Specifically, we have shown that endodermal cells made to ectopically express Xwnt-8b can induce notochord formation in conjugated ectoderm isolated from UV-irradiated embryos. In addition, others have shown that when the Wnt signaling pathway is activated in ventral endodermal cells of whole embryos by injection of RNA encoding a dominant negative form of glycogen synthase kinase (a kinase that is normally inactivated in response to Wnt signaling, He et al., 1995; Pierce and Kimelman, 1995), or ß-catenin (Guger and Gumbiner, 1995), a dorsal axis is induced to form from overlying, non-endodermal cells. Finally, endodermal cells in which Wnt signaling is blocked by depletion of maternal ß-catenin are unable to transmit a dorsal inductive signal to conjugated ectodermal cells (Heasman et al., 1994). Thus, an active Wnt/ß-catenin signaling pathway appears to be required for generation of vegetal dorsalizing activity *in vivo*. Whether Wnts synergize with Vg1 to generate this dorsalizing activity remains to be tested.

While the above model focuses on the role of Wnt-induced endodermal signals in mesodermal patterning, it is possible that endodermal signals are dispensable for dorsal patterning *in vivo*. Specifically, it may be that interactions between Vg1 and Wnt signaling pathways within presumptive mesodermal cells themselves are sufficient to initiate the full complement of signals required for normal axial patterning, and that

signals from endodermal cells are redundant. Consistent with this possibility, goosecoid and Xwnt-8 can be activated in prospective dorsal and ventral mesodermal cells in the absence of cell-cell communication (Lemaire and Gurdon, 1994). However, the recent identification of a *Xenopus* homeobox-encoding gene which is expressed primarily in dorsal endodermal cells, and which can induce a complete secondary axis when misexpressed in ventral endodermal cells (Lemaire et al., 1995), supports an active role for a zygotic endodermally-derived morphogen in mesodermal patterning. The identity of this novel signaling agent remains to be established.

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Table 1. Dorsal mesoderm induction in the presence and absence of cortical rotation and Wnt signaling

	Ectoderm/endoderm conjugate						Whole embryos	
	UV/UV	CON/CON	UV/CON	CON/UV	UV/WNT	WNT/UV	UV	CON
Notochord	2% (50)	78% (55)	31% (68)	21% (63)	77% (69)	84% (73)	6% (104)	100%(78)
Muscle	84% (57)	93% (30)	100%(20)	88%(32)	98% (30)	96% (29)	15% (130)	100%(50)

Conjugates of blastula stage endoderm and ectoderm isolated from UV-irradiated (UV), non-irradiated (CON) or Xwnt-8b RNA injected (WNT) embryos were generated, cultured, and immunostained with notochord- or muscle-specific antibodies as illustrated above Figures 1 and 2. Data are expressed as percent of conjugates in which immunostaining was detected, followed by sample size (in parentheses).

Table 2. Synergism between Xwnt-8b and Vg1 in notochord induction.

	CONTROL	UV-IRRADIATED	
RNA Injected:	AVg	AVg	AVg +X8b
Notochord	80% (35)	15% (39)	82% (37)
Muscle	90% (30)	94% (36)	83% (36)

Ectoderm was isolated from UV-irradiated or non-irradiated (CONTROL) embryos which had been injected with RNA encoding AVg and/or Xwnt-8b (X8b) as indicated above each column. Explants were cultured until stage 26 or 22 and immunostained with notochord- or muscle-specific antibodies respectively. Data represent percentage of animal caps in which immunostaining was detected, followed by sample size (in parenthesis).

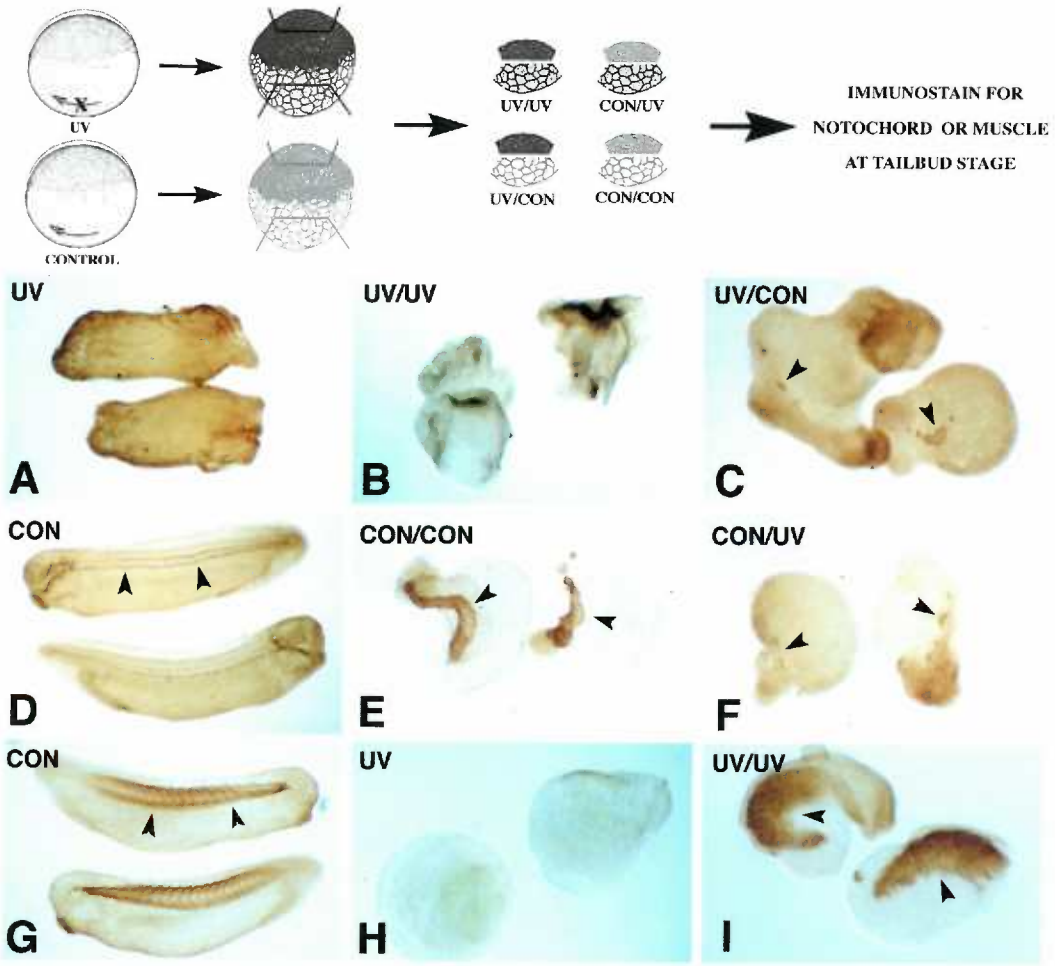

Figure 1. Dorsal mesoderm induction in the presence and absence of cortical rotation. Endoderm and ectoderm explanted from UV-irradiated (UV) and non-irradiated (CON) blastula stage (stage 8) embryos was conjugated as diagrammed at the top of Figure 1. Recombinants were cultured until sibling embryos reached tailbud stages and then immunostained with notochord-specific (A-F) or muscle-specific (G-I) antibodies. Arrows indicate specific notochord or muscle staining. Dark areas not denoted by arrows are non-specific background color due to unbleached pigment.

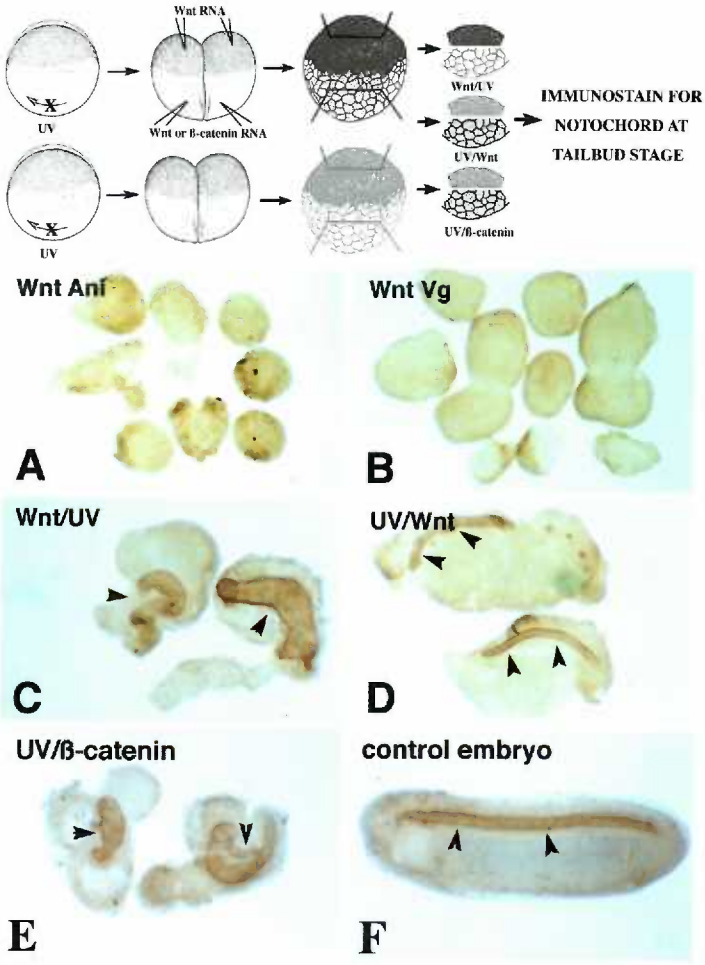

Figure 2. Xwnt-8b can substitute for dorsalizing signals activated by cortical rotation in either endodermal or ectodermal cells. Embryos were UV-irradiated during the first cell cycle and mRNA encoding Xwnt-8b or ß-catenin was injected into animal or vegetal blastomeres at the two-cell stage. Ectodermal (Ani) and endodermal (Vg) explants were isolated from embryos at the blastula stage and were cultured cultured in isolation (A,B) or were conjugated with endoderm or ectoderm isolated from uninjected embryos (C-E) as indicated above each panel. At stage 26, these explants or recombinants were immunostained with notochord-specific antibodies. Arrows indicate specific staining.

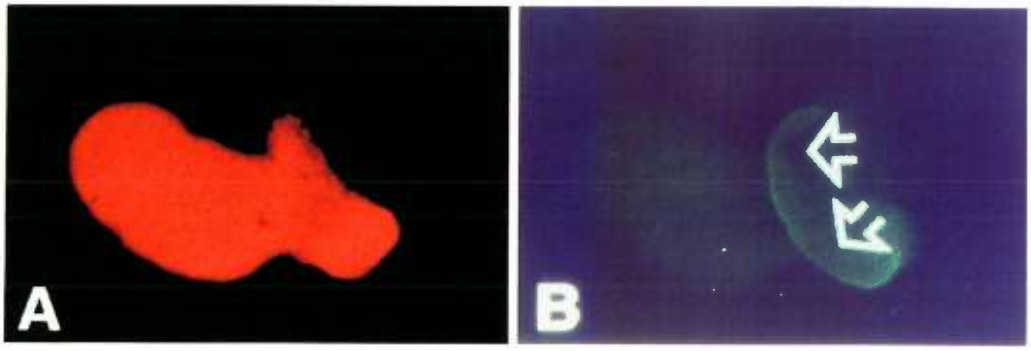

Figure 3. Notochord is derived from ectodermal cells in conjugates of Wnt expressing endoderm with ventral ectoderm from *Xenopus* blastulae. Ectodermal cells were lineage labeled by injection of rhodamine dextran and recombined with Wnt-expressing endodermal cells. Recombinants were fixed at stage 26 and immunostained with notochord-specific antibodies which were detected using fluorescein-conjugated secondary antibodies. Conjugates were examined under epifluorescence using appropriate filters for rhodamine (A) or fluorescein (B) excitation. In panel B, specific staining of the notochord is indicated by white arrows.

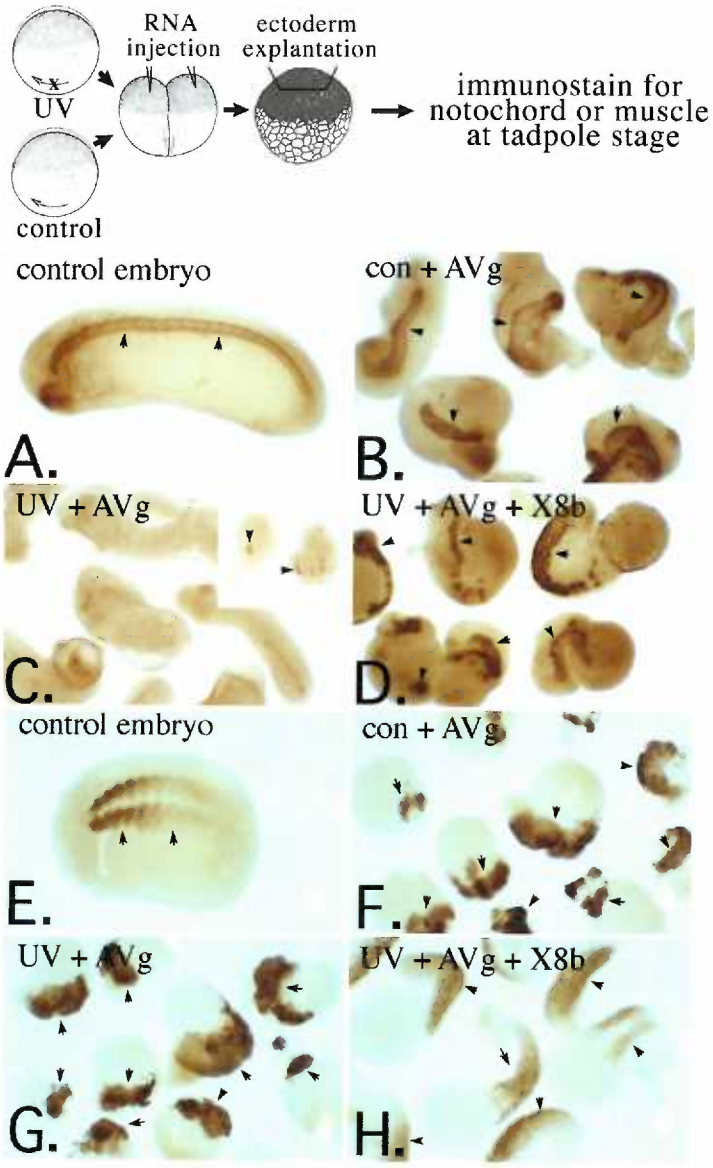

Figure 4. Wnts can synergize with Vg1 to induce notochord in ectodermal explants. RNA encoding AVg and/or Xwnt-8b was injected into animal pole blastomeres of UV-irradiated (UV) or non-irradiated (con) embryos. Ectodermal animal caps were isolated at the blastula stage (stage 8) as diagrammed at the top of the figure, cultured to stage 26 or 22, and immunostained for notochord (A-D) or muscle (E-H), respectively. Specific staining is denoted by arrows.

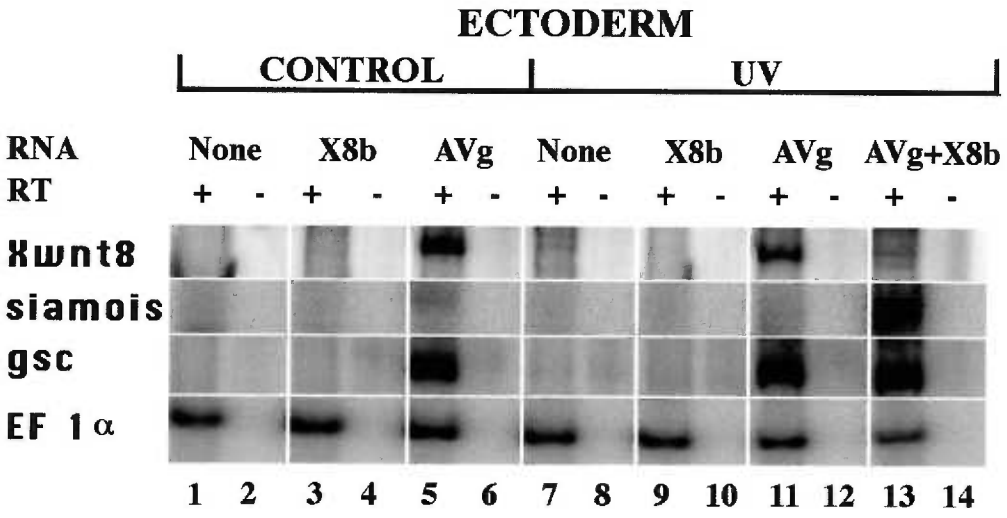

Figure. 5 Mesoderm induced by Vg1 is specified as dorsal in response to Wnt signals. Ectodermal explants were isolated from UV-irradiated (UV) or non-irradiated (CONTROL) uninjected embryos, or from embryos that had been injected with RNA encoding AVg and/ or Xwnt-8b as denoted above each lane. RNA was extracted from 6 explants at an early gastrula stage (stage 10.5) and expression of Xwnt-8, siamois, goosecoid (gsc) and EF-1α was assayed by RT-PCR. Equivalent aliquots of RNA from each group were analyzed in the presence (+) or absence (-) of reverse transcriptase (RT) to demonstrate that signal was not due to contamination of RNA samples with genomic DNA.

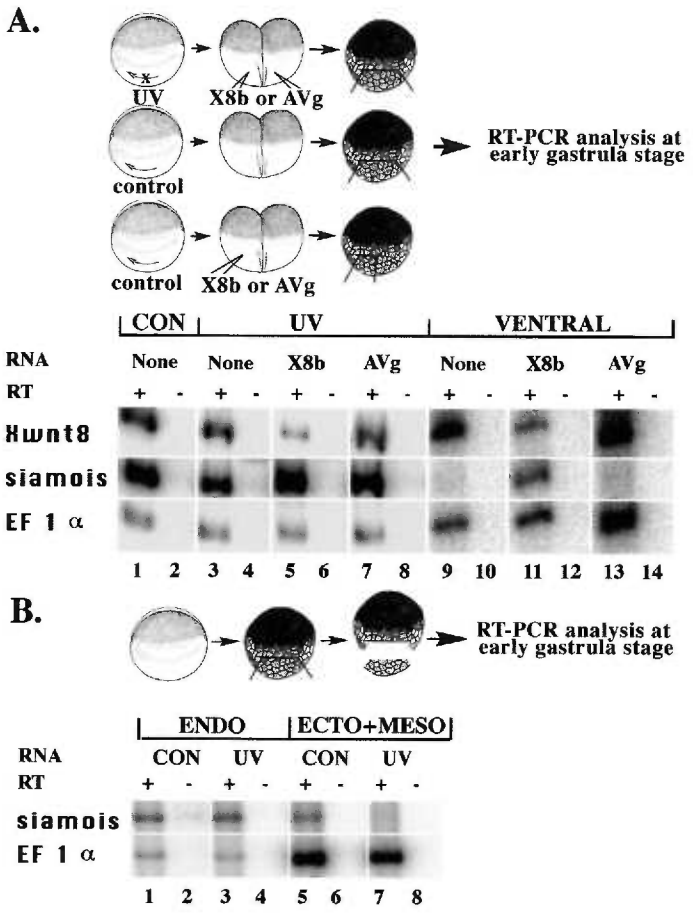

Figure. 6 Xwnt-8b, but not Vg1, can impart dorsal pattern on ventral endoderm. A. Endoderm was explanted at the blastula stage (stage 8) from UV-irradiated (UV) or non-irradiated (CON) uninjected embryos, or from embryos injected with RNA encoding Xwnt-8b- or AVg as denoted above each lane. Alternatively, endoderm was explanted from the ventral side of uninjected (none), AVg-, or Xwnt-8b (X8b)-RNA injected control embryos. Endoderm was cultured to stage 10.5 and RT-PCR analysis of siamois, Xwnt-8, and EF-1α expression was performed in the presence (+) or absence (-) of reverse transcriptase (RT). B. Endoderm (ENDO) was explanted at the blastula stage (stage 8) from UV-irradiated (UV) or non-irradiated (CON) embryos. Endoderm alone, or the remaining ectodermal and mesodermal portion of the embryo (ECTO + MESO), was cultured until sibling embryos reached stage 10.5 and RT-PCR analysis of siamois and EF-1α expression was performed in the presence (+) or absence (-) of reverse transcriptase (RT).

Chapter 3

BMP-4 is proteolytically activated by Furin and/or PC6 during vertebrate embryonic development

Yanzhen Cui[1], Francois Jean[2], Gary Thomas[2], and Jan L. Christian[1]

[1]Department of Cell and Developmental Biology and [2]Vollum Institute

Oregon Health Sciences University, School of Medicine

3181 SW Sam Jackson Park Road

Portland, Oregon 97201-3098 USA

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ABSTRACT

Bone Morphogenetic Protein-4 (BMP-4) is a multifunctional developmental regulator. BMP-4 is synthesized as an inactive precursor that is proteolytically activated by cleavage following the amino acid motif -Arg-Ser-Lys-Arg-. Very little is known about processing and secretion of BMPs. The proprotein convertases (PCs) are a family of seven structurally related serine endoproteases, at least one of which, furin, cleaves after the amino acid motif -Arg-X-Arg/Lys-Arg-. To examine potential roles of PCs during embryonic development, we have misexpressed a potent protein inhibitor of furin, α_1–Antitrypsin Portland (α_1–PDX), in early *Xenopus* embryos. Ectopic expression of α_1–PDX phenocopies the effect of blocking endogenous BMP activity, leading to dorsalization of mesoderm and direct neural induction. α_1–PDX-mediated neural induction can be reversed by co-expression of downstream components of the BMP-4 signaling pathway. Thus, α_1–PDX can block BMP activity upstream of receptor binding, suggesting that it inhibits an endogenous BMP-4 convertase(s). Consistent with this hypothesis, α_1–PDX prevents cleavage of BMP-4 in an oocyte translation assay. Using an *in vitro* digestion assay, we demonstrate that four members of the PC family have the ability to cleave BMP-4, but of these, only furin and PC6B are sensitive to α_1–PDX. These studies provide the first *in vivo* evidence that furin and/or PC6 proteolytically activate BMP-4 during vertebrate embryogenesis.

Keywords: bone morphogenetic proteins/furin/PC6/embryogenesis/proprotein convertase/α_1–antitrypsin Portland/*Xenopus*

INTRODUCTION

The process of embryonic induction, in which one population of cells influences the developmental fate of another, plays an essential role in establishing the basic body plan of all multicellular organisms. These inductive events, as well as subsequent patterning events, rely heavily upon cell-cell interactions mediated by secreted proteins, including members of the transforming growth factor-ß (TGF-ß) family.

Bone Morphogenetic Proteins (BMPs) are members of the TGF-ß family that have been implicated in the development of nearly all organs and tissues (reviewed by Hogan, 1996). One of the earliest and best documented roles for BMPs is in establishment of the dorsoventral axis (reviewed by Graff, 1997). In *Xenopus*, expression of BMP-4 is restricted to cells on the ventral side of gastrula stage embryos, where it plays a central role in specifying ventral mesodermal (e.g. blood) and ectodermal (i.e. skin) fates. When cells on the dorsal side of the embryo are made to misexpress BMP-4, they differentiate as blood, rather than notochord, or form skin, rather than brain. Conversely, when endogenous BMP signaling is blocked in ventral cells, by introduction of dominant interfering forms of either the BMP ligand or receptor, blood formation is eliminated and these cells form muscle instead. Furthermore, blockade of the BMP signaling pathway in explanted ectoderm causes these cells to differentiate as neural tissue. Thus, BMP-4 is required for ventral mesoderm formation and for the induction of epidermal fate at the expense of neural tissue.

As with all TGF-ß family members, BMP-4 is synthesized as an inactive precursor and is proteolytically activated by cleavage following the multibasic amino acid motif -Arg-Ser-Lys-Arg- to yield a carboxyl-terminal mature protein dimer (Aono

et al., 1995). This processing event has been proposed to regulate the secretion and/or diffusion of BMPs, thereby controlling the range over which these molecules can signal during embryonic development (Jones et al., 1996). In general, very little is known about intracellular assembly, processing and secretion of BMPs.

Members of a family of higher eukaryotic endoproteases, named proprotein convertases (PCs) (reviewed by Steiner et al., 1992), are good candidates for endogenous BMP convertases. In mammals, seven members of this family have been characterized and designated furin, PC2, PC1/3 (hereafter called PC3), PACE-4, PC4, PC5/6A and B (hereafter called PC6A and B), and LPC/PC7/PC8 (hereafter called PC7) (Seidah and Chretien, 1997). Individual PCs exhibit overlapping but distinct substrate specificity (Breslin et al., 1993; Creemers et al., 1993). The first member of this family to be characterized, furin, is a membrane-associated, calcium-dependent serine endoprotease that proteolytically activates proprotein molecules at the carboxyl-terminal side of the consensus sequence -Arg-X-Arg/Lys-Arg- (Molloy et al., 1992). Many precursor proteins, including those for TGF-ß (Dubois et al., 1995) and other growth factors, receptors, serum proteins, viral envelope proteins, and bacterial toxins, share this cleavage site and can be efficiently cleaved by furin in gene transfer and *in vitro* digestion studies (reviewed by Bresnahan et al., 1993; Nakayama, 1997).

Expression patterns of distinct PCs have been examined in a variety of species. In mammals, furin and PC7 are ubiquitously expressed throughout development (Constam et al., 1996). In contrast, PC2 and PC3 transcripts are confined to neuroendocrine tissues, and specifically cleave neuropeptides and other hormones (reviewed in Steiner et al., 1992), while PC4 is restricted to testicular germ cells (Nakayama et al., 1992). Interestingly, although PACE-4 and PC6A/B are expressed at low levels in all embryonic tissues, both genes display dynamic expression patterns throughout development and are upregulated in some tissues that exhibit high level

expression of BMPs (Constam et al., 1996; Zheng et al., 1997). This observation has led to the hypothesis that PACE-4 and PC6 may act in combination to locally modulate BMP activity (Constam et al., 1996). Direct evidence that BMPs are substrates for PC-like endoproteases, however, is lacking.

To begin to test the hypothesis that BMP-4 is proteolytically activated by a member of the PC family, we have used a protein-based inhibitor, termed α_1–PDX, to block endogenous PC activity *in vivo*. This inhibitor is a genetically engineered mutant form of the naturally occurring serine protease inhibitor, α_1–antitrypsin (Anderson et al., 1993). α_1–PDX contains in its reactive site the amino acids -Arg-Ile-Pro-Arg-: the minimal consensus motif for efficient processing by furin. This protein has been shown to be a potent inhibitor of furin and PC6B *in vitro* (Anderson et al., 1993; Jean et al., in press).

In the present study, we demonstrate that ectopic expression of α_1–PDX phenocopies the effect of blocking endogenous BMP-4 activity in *Xenopus* embryos, and can rescue ventralization caused by overexpression of exogenous BMP-4. Furthermore, α_1–PDX-mediated patterning defects can be blocked by co-expression of downstream components of the BMP-4 signaling pathway. These results demonstrate that α_1–PDX can inhibit BMP activity upstream of receptor binding, and suggest that α_1–PDX blocks the activity of the endogenous protease(s) responsible for proteolytic activation of BMP-4. Consistent with this hypothesis, α_1–PDX completely blocks cleavage of BMP-4 in an *in vivo* oocyte translation assay. Furthermore, we find that while furin, PACE-4, PC6B, and PC7 all have the potential to cleave BMP-4, only furin and PC6B are sensitive to inhibition by α_1–PDX. Taken together, these studies provide the first *in vivo* evidence that proteolytic maturation of BMP-4 is achieved by furin and/or PC6 and demonstrate the feasibility of using a selective protease inhibitor as a tool to investigate the developmental functions of PCs in a whole animal model.

Materials and methods

Embryo culture and manipulation

Xenopus eggs were obtained, embryos injected with synthetic RNAs and cultured as described (Moon and Christian, 1989). Embryonic stages are according to Nieuwkoop and Faber (1967). The coding regions of α_1–PDX and α_1–PIT cDNAs (Anderson *et al.*, 1993) were subcloned into the expression vector pSP64T (Krieg and Melton, 1984). A cDNA encoding the MH2 domain of Smad1 was generated by subcloning the AvaI fragment of pSP64TEN-Xmad1 (gift of Dr. D. Melton) into the expression vector pCS2+ (Turner and Weintraub, 1994) to generate pCS2+MH2. Capped synthetic RNA was produced by *in vitro* transcription of linearized pSP64T-α_1–PDX, pSP64Tα_1–PIT, pSP64T-tBR (Graff *et al.*, 1994), pCS2+MH2, DPC4(FL)/pSP64TEN (DPC4, gift of Dr. J. Massagué), and pSP64T-BMP-4Flag (gift of Dr. K.Cho). Embryonic explants were isolated and cultured as described in Cui *et al.* (1996).

Whole mount immunostaining

Whole mount immunostaining using the muscle specific antibody 12/101 (Kintner and Brockes, 1984) or the notochord specific antibody Tor70 (Bolce *et al.*, 1992, gift of R.Harland) was performed according to Moon and Christian (1989).

RT-PCR analysis

RT-PCR analysis of RNA samples was performed as described previously (Cui *et al.*, 1996). The sequences of BMP-4 (Fainsod *et al.*, 1994), XlHbox6 (Wright *et al.*, 1990), NCAM, EF-1α, OtxA, goosecoid and XAG (Kengaku and Okamoto, 1995) primers have been published previously. The sequence of the furin primers used in this paper are listed as follows, in 5' to 3' orientation: upstream

GTTATGTTGAGAAAATCG; downstream TAACATTAGCAGCAAAGT. Number of cycles of PCR was determined empirically to be in the linear range for each primer pair. Amplified bands were visualized with a Molecular Dynamics phosphorimager and quantified using the Macintosh IP lab gel program.

Oocyte injections and analysis of proteins

Ovaries were isolated from mature female frogs, and stage VI oocytes were manually defolliculated and injected with *in vitro* synthesized RNAs. Groups of ten oocytes were pooled and cultured in oocyte culture medium OR2 (50% L15 medium supplemented with 15mM Hepes, pH7.8, 1mM glutamine, 1mM BSA, 1μg/ml of bone pancreatic insulin, and 100 μg/ml of Gentamicin) in microtiter plates in the presence of 0.1 mCi/ml translabel (NEN) for 48 hours. ^{35}S-labeled oocytes (10 per group) were homogenized in RIPA buffer (Harlow and Lane, 1988) and BMP-4 FLAG protein was immunoprecipitated using the Flag specific antibody D8 (Santa Cruz Biotech) and protein A-sepharose as described (Harlow and Lane, 1988). Precipitated proteins were boiled in 1X SDS buffer and separated by electrophoresis on a 12% polyacrylamide gel. The gel was dried, and radiolabeled proteins visualized with a Molecular Dynamics 8500 phosphorimager.

For Western blot analysis, proteins were extracted from a group of ten oocytes as described by Moon and Christian (1989). Proteins were separated by electrophoresis on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. The Western blot was probed with an antibody directed against α_1–antitrypsin (Calbiochem) which was visualized by a chemiluminescence kit (Pierce) according to manufacturers instructions.

In vitro digestion assay

BMP-4Flag protein was immunoprecipitated from ^{35}S-methionine labeled oocytes which had been injected with RNAs encoding BMP-4 Flag (50 ng) and α_1–PDX (5 ng). Flag epitope-tagged furin, PC3, PC6B, PACE-4 and PC7 proteins were produced by infecting cultured cells with the corresponding vaccinia virus (VV) recombinant [VV:human fur713t/f (Molloy *et al.*, 1994; hereafter named hfurin/f), VV:mPC6B/f, VV:hPACE-4/f and VV:hPC7/f (secreted soluble flag-tagged human PC7; Jean *et al.*, in press). Secreted/shed enzymes were collected from culture media as described previously (Molloy *et al.*, 1992; Jean *et al.*, in press), concentrated [Biome filter, 30 kDa cut-off (Millipore)] and stored at -70°C until use. hfurin, mPC3, mPC6B and hPC7 were expressed in BSC-40 cells, while hPACE-4 was expressed in LoVo cells.

The activity of each purified PC was tested using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-methyl coumaryl-7-amide (PERTKR-MCA; Peptide International). Enzyme assay data were obtained using a FluoroMax-2 spectrofluorometer equipped with a 96 well plate reader (Instrument SA, Inc.) using excitation/emission wavelengths of 370/460 nm to measure released AMC (7-amino-4-methylcoumarin). Furin, PC6B, PC7, and PACE-4 assays were performed in 100 mM Hepes, pH 7.5, containing 0.5% Triton X-100 and 1 mM $CaCl_2$. PC3 assays were performed as described (Jean *et al.*, 1995). Each enzyme preparation was enzymatically pure based on the absence of PC activity in medium from replicate cells infected with wild-type VV (data not shown).

The concentration of each PC was determined by tight-binding titration using the active-site-directed irreversible inhibitor Dec-RVKR-CH_2Cl. PCs were incubated with increasing amounts of Dec-RVKR-CH_2Cl for 30 min at RT. pERTKR-MCA (100 μM) was added to determine residual PC activity. Values for E_0 were obtained by fitting the data (v and I) to the equation for equilibrium binding:

$$v = SA\ (E_0 - 0.5\{(E_0 + I + K_i) - [(E_0 + I + K_i)2 - 4E_0I]^{1/2}\})$$

(v, reaction velocity with the substrate concentration s; SA, specific activity; E_0, enzyme concentration and I, inhibitor concentration) by nonlinear regression (ENZFITTER, Elsevier-Biosoft, Cambridge, UK) (Knight, 1995; Jean *et al.*, in press). *In vitro* digestion of BMP-4Flag was conducted at RT using hfurin (5.0 nM), mPC6B (2.0 nM), hPC7 (2.0 nM), hPACE-4 (5.0 nM) and mPC3 (19 nM).

To test the sensitivity of individual PCs to α_1–PDX, furin, PC6B, PACE-4 and PC7 were preincubated with α_1–PDX (1 μM final concentration) for 30 minutes at room temperature prior to addition of BMP-4 precursor. Reactions were allowed to proceed at 25°C for 6 hours, at which time cleavage of BMP-4 by each PC was essentially complete. Aliquots of each reaction were analyzed by SDS-PAGE and fluorography.

RESULTS

Misexpression of α_1–PDX respecifies the fate of ventral mesodermal cells

To begin to test the possibility that BMP-4 is a substrate for an endogenous furin-like endoprotease(s), we used a ventral marginal zone (VMZ) assay to ask whether misexpression of the PC inhibitor, α_1–PDX, can inhibit BMP-4 function in these cells. Overexpression of known antagonists of BMP-4, such as dominant negative receptors or ligands, can convert the fate of VMZ cells from blood and mesenchyme to more dorsal derivatives, such as muscle (reviewed by Graff, 1997). Thus, if α_1–PDX is sufficient to block the activity of an endogenous convertase that is required for proteolytic activation of BMP-4, overexpression of α_1–PDX should dorsalize the fate of VMZ cells.

Approximately 500 pg of synthetic RNA encoding either α_1–PDX or a dominant mutant, truncated BMP receptor (tBR, as a positive control; Graff et al., 1994) was injected near the ventral midline of four-or eight-cell embryos as illustrated above Figure 1. VMZ tissue was dissected out of early gastrula stage embryos, cultured in isolation until sibling embryos reached the tailbud stage (stage 26), and analyzed for the presence of dorsal mesoderm by immunostaining with muscle (Figure 1 A-D) or notochord (Figure 1 E-H) specific antibodies.

The majority of VMZ explants from uninjected embryos remained rounded and formed neither muscle nor notochord (Figure 1B, F; Table 1). An identical phenotype was observed in explants made to express α_1–PIT (Table 1 and data not shown), a naturally occurring mutant form of α_1–antitrypsin. α_1–PIT contains the residues -Met-Ile-Pro-Arg- in its reactive site, and can inhibit thrombin but not PCs (Anderson et al., 1993). In contrast, all of the α_1–PDX- or tBR-expressing VMZs formed

immunoreactive muscle (Figure 1 C-D, arrowheads; Table 1). Small patches of immunoreactive notochord were observed in some α_1–PDX-expressing explants (Figure 1G, arrowheads; Table 1) and, less frequently, in tBR-expressing explants (Figure 1H; Table 1).

These results demonstrate that misexpression of α_1–PDX in VMZ tissues phenocopies the effect of inactivating BMP-4 signaling, consistent with the possibility that α_1–PDX can inhibit the activity of the endogenous BMP-4 convertase(s).

Misexpression of α_1–PDX neuralizes the fate of ectodermal cells

In addition to inducing ventral mesodermal cells to adopt a dorsal fate, BMP antagonists can directly induce ectodermal cells to form neural tissue (reviewed by Graff, 1997). To further test the possibility that α_1–PDX can antagonize BMP-4 function, we misexpressed α_1–PDX in prospective ectodermal cells by injection of synthetic RNA (500 pg) as illustrated above Figure 2. Ectodermal explants (animal caps) were isolated at the blastula stage and cultured until sibling embryos reached the tadpole stage, at which point they were analyzed for gross morphology and for expression of neural-specific genes.

As shown in Figure 2, animal caps from uninjected, or α_1–PIT injected, embryos formed spheres of ciliated epidermis (Figure 2A) while animal caps from α_1–PDX-injected embryos elongated and formed an anterior ectodermal organ, the cement gland (Figure 2B, arrowheads). RT-PCR analysis revealed that α_1–PDX-injected animal caps, but not uninjected or α_1–PIT injected animal caps, expressed cement gland (XAG)-, anterior neural (OtxA)- and pan-neural (NCAM)-specific genes but did not express a posterior neural-specific gene (Xlhbox6) (Figure 2C). Animal caps explanted from α_1–PDX injected embryos did not form muscle, nor did they express the mesodermal gene, Xbra, demonstrating that neural induction is direct, i.e. it

occurs in the absence of mesoderm induction (data not shown). Specific blockade of the BMP signaling pathway within isolated ectodermal cells leads to an identical direct induction of anterior, but not posterior, neural tissue (reviewed by Wilson and Hemmati-Brivanlou, 1997).

α_1–PDX inhibits the activity of exogenously expressed BMP-4

To begin to determine whether the patterning defects caused by ectopic expression of α_1–PDX are due to blockade of endogenous BMP activity, we assayed for the ability of α_1–PDX to directly antagonize the activity of exogenous BMP-4. As previously shown (reviewed by Graff, 1997), microinjection of BMP-4 RNA (3 ng) into dorsal cells led to a complete loss of all anterior (Figure 3C) and dorsal mesodermal (e.g. notochord, Figure 3D) structures in most embryos (79% of embryos completely lacked immunoreactive notochord, n=47). Co-injection of RNA encoding α_1–PDX along with BMP-4 significantly rescued the formation of anterior structures (Figure 3E) as well as the formation of immunoreactive notochord (Figure 3F; 63% of embryos showed extensive immunoreactive notochord, n=51). In contrast, embryos co-injected with RNAs encoding α_1–PIT and BMP-4 appeared identical to those injected with BMP-4 alone (82% of co-injected embryos lacked notochord staining, n=36; data not shown). Expression of α_1–PDX alone did not inhibit notochord staining in any embryos (data not shown). This result supports the possibility that α_1–PDX inhibits an endogenous convertase that is directly required for proteolytic processing of BMP-4.

α_1–PDX-mediated neural induction is blocked by co-expression of intracellular transducers of BMP-4 signals

If neural induction mediated by α_1–PDX is due to inhibition of BMP processing, and therefore to antagonism of signaling upstream of receptor activation,

then co-expression of a downstream component of the BMP signaling cascade should block this phenotype. The intracellular protein Smad1 has been shown to transduce BMP signals from the membrane to the nucleus (reviewed by Massague et al., 1997). As with other Smads, Smad1 contains three domains (Figure 4A): an inhibitory (MH1) domain, a linker domain of unknown function, and an effector (MH2) domain. Previous work in other laboratories has shown that the MH2 domain in isolation is constitutively active, and can transduce BMP signals in the absence of ligand (reviewed by Massague et al., 1997). More recent work has shown that this activity can be augmented by co-expression of Smad4, presumably due to the fact that endogenous Smad4 is present in rate limiting amounts *in vivo* (Candia et al., 1997).

To determine whether activation of the Smad1 signaling cascade can block α_1–PDX mediated neural induction, RNA encoding α_1–PDX alone (500 pg) or together with RNA encoding Smad4 and the MH2 domain of Smad1 (500 pg each) was injected near the animal pole of two cell embryos. Animal caps were isolated at the blastula stage, cultured to the tadpole stage, and analyzed for expression of neural- or cement gland specific genes. As shown in Figure 4B, co-expression of the MH2 domain of Smad1, Smad4 and α_1–PDX nearly completely repressed α_1–PDX mediated induction of neural-specific genes in isolated animal caps and led to a 41-fold reduction in expression of the cement gland specific gene, XAG. Thus, α_1–PDX can inhibit BMP signaling upstream of receptor activation, consistent with the possibility that it directly blocks the function of an endogenous protease(s) required for cleavage of the BMP-4 precursor protein.

α_1–PDX blocks processing of Flag-tagged BMP-4 in *Xenopus* oocytes

An *in vivo Xenopus* oocyte translation assay was used to directly test the possibility that α_1–PDX blocks proteolytic activation of BMP-4. RNA encoding epitope (Flag)-tagged BMP-4 (50 ng) was injected into *Xenopus* oocytes either alone or

together with RNA encoding α_1–PDX or α_1–PIT (5 ng). Oocytes were labeled with 35S-methionine and BMP-4Flag protein was immunoprecipitated from oocyte lysates using a Flag-specific antibody. As shown in Figure 5A, α_1–PDX, but not α_1–PIT, completely inhibited cleavage of BMP-4 precursor protein. In this experiment, approximately 50% less BMP-4 precursor is synthesized in oocytes made to express α_1–PDX relative to control oocytes. However, the BMP-4 cleavage product remains undetectable in immunoprecipitates from α_1–PDX-injected oocytes even when the gel is overexposed, thereby demonstrating that the lack of detectable processing in these oocytes is not due to relatively lower levels of BMP-4 precursor.

To control for the possibility that the observed failure of α_1–PIT to inhibit BMP-4 processing was due to inefficient translation of α_1–PIT RNA relative to α_1–PDX RNA, Western blots of protein extracts isolated from injected oocytes were probed with an α_1–antitrypsin-specific antibody. As shown in Figure 5B, α_1–PIT and α_1–PDX proteins were expressed at comparable levels. Together, these results demonstrate that α_1–PDX, but not the related inhibitor α_1–PIT, can block proteolytic cleavage of BMP-4 in vivo, consistent with the hypothesis that BMP-4 is cleaved by an endogenous PC(s).

Cleavage of BMP-4 precursor by furin and PC6B, but not by PACE-4 or PC7, is blocked by α_1–PDX

An in vitro digestion assay was used to identify PCs that are capable of cleaving BMP-4. Radiolabeled BMP-4Flag precursor protein was incubated with purified PCs in solution and proteolytic cleavage of the precursor was assayed after six hours of incubation. As shown in Figure 6, BMP-4 precursor protein incubated in the absence of PCs (lane 1), or in the presence of the neuroendocrine-specific PC3 (lane 14), was not cleaved. In contrast, PC6B (lane 5) and PC7 (lane 8), cleaved the BMP-4

precursor protein to yield fragments of approximately 15 Kd (more readily visible on longer exposures) and 35 Kd, consistent with the predicted Mr of the mature bioactive BMP-4 protein and of the intact N-domain, respectively. The same 35 Kd band was observed following shorter (1 hour) incubations of the precursor with either furin or PACE-4 (data not shown). Intriguingly, furin (lane 2), PC6B (lane 5) and PACE-4 (lane 11), but not PC7 (lane 8), cleaved BMP-4 at a second site, most likely within the proregion, generating a 32 Kd fragment. The size of this product is consistent with cleavage at a minimal furin consensus sequence (-R-I-S-R-) located approximately 30 amino acids upstream of the primary cleavage site (-R-R-S-K-R-).

Since α_1–PDX can block proteolytic activation of BMP-4 in vivo, one criterion for candidate BMP-4 convertases is that they must be sensitive to inhibition by α_1–PDX. To determine whether furin, PC6B, PC7 or PACE-4 meet this criterion, a parallel set of in vitro digestions was performed in which purified PCs were preincubated with α_1–PDX for 30 minutes prior to assaying their ability to cleave radiolabeled BMP-4 precursor. Alternatively, purified PCs were preincubated with Decanoyl-Arg-Val-Lys-Arg-CH2Cl (CMK), an active-site directed inhibitor of all PC family members (Jean et al., in press), as a positive control for inhibition. As shown in Figure 6, CMK inhibited the ability of all PCs to cleave BMP-4 (lanes 3, 6, 9, 12), while α_1–PDX selectively prevented furin (lane 4) and PC6B (lane 7), but not PC7 (lane 10) or PACE-4 (lane 13), from efficiently cleaving BMP-4. These results, together with the observation that α_1–PDX inhibits processing of BMP-4 *in vivo*, argue that endogenous BMP-4 is proteolytically activated by furin and/or PC6.

Furin is ubiquitously expressed in *Xenopus* gastrulae

To begin to determine whether the spatial and temporal pattern of expression of furin is appropriate for an endogenous BMP-4 convertase, we used RT-PCR to detect furin transcripts in RNA isolated from developmentally staged embryos and in tissues

dissected from various regions of early gastrulae. Furin transcripts are present in oocytes, as previously shown (Korner et al., 1991), and throughout embryonic development (Figure 7A). Transcripts encoding furin are detected in all three germ layers of gastrulae (Figure 7B). RNA isolated from the DMZ and VMZ was analyzed for expression of the dorsal- and ventral-specific genes, goosecoid (gsc, Cho et al., 1991) and Xwnt-8 (X8, Christian et al., 1991), respectively, to confirm the accuracy of dissections (Fig 7C). The same RNA was analyzed for the presence of BMP-4 and furin transcripts. BMP-4 transcripts are enriched within ventral cells of early gastrulae (Figure 7C), and become restricted to ventral cells by mid-gastrula stages (Fainsod et al., 1994). Transcripts encoding furin are detected at fairly equivalent levels in dorsal and ventral cells of gastrulae when normalized to expression of EF-1α (Figure 7C). These results are consistent with previously published reports that furin is ubiquitously expressed throughout embryonic development in other vertebrates (Constam et al., 1996) and confirm that furin is present at an appropriate time and place to proteolytically activate BMP-4.

DISCUSSION

PCs as potential regulators of embryonic patterning

Members of the PC family of serine endoproteases are likely to be essential participants in the process of embryonic patterning, since many secreted or membrane bound developmental regulators derive from inactive precursors that require proteolytic maturation. Consistent with this possibility, most PCs are expressed ubiquitously throughout embryogenesis, although tissue specific enrichment of some transcripts has been observed (Constam et al., 1996; Zheng et al., 1997). An absolute requirement for proteolytic processing during development has recently been demonstrated in C. elegans, where disruption of the bli-4 gene, which encodes an PC-like endoprotease, leads to embryonic arrest (Thacker et al., 1995).

The wide tissue distribution of most PCs during vertebrate development, coupled with the essential embryonic roles of potential substrates, make it likely that targeted deletions of ubiquitously expressed PC genes in mice will produce complex phenotypes and early lethality. For this reason, conditional or tissue specific loss of PC function is an attractive strategy for investigating the developmental roles and substrate specificity of these endoproteases. We have taken advantage of a well characterized furin inhibitor, α_1–PDX, to block furin-like PC function within a subset of cells in early *Xenopus* embryos. Recent analysis of the ability of α_1–PDX to inhibit PC-mediated hydrolysis of a synthetic peptide substrate has shown that this protein is a potent and highly selective inhibitor of furin and PC6B, but not of other PCs (Jean et al., in press). Specifically, α_1–PDX inhibits both furin and PC6B at nanomolar concentrations (Ki = 0.6 nM and 2.3 nM, respectively) but is orders of magnitude (greater than 2,000 to 8,000 fold) less effective at blocking PACE-4 and PC7. Our studies provide the first *in vivo* evidence that proteolytic maturation of BMP-4 is

achieved by a member of the PC family, and demonstrate the feasibility of using α_1–PDX as a tool to investigate developmental functions of PCs in a whole animal model.

Taken together, our results demonstrate that the protease responsible for maturation of BMP-4 *in vivo* is most likely furin and/or PC6. Although we did not specifically assay PC2 or PC4 for their ability to cleave BMP-4, these enzymes are not appropriate candidate convertases since they are confined to neuroendocrine tissues (Zheng et al., 1994, 1997; reviewed in Steiner et al., 1992) or to adult testicular germ cells (Nakayama et al., 1992; Mbikay et al., 1997), respectively, while BMP-4 is widely expressed. PACE-4 has been suggested to be an endogenous BMP-convertase, based on the observation that it is co-expressed with BMPs in a number of embryonic tissues (Constam et al., 1996). Our results do not support a role for this convertase in processing BMP-4, since it is not sensitive to inhibition by α_1–PDX, but they do not rule out the possibility that it proteolytically activates other members of the BMP family.

One potential concern with our experiments is the possibility that we are overexpressing α_1–PDX at sufficiently high levels that the specificity of this inhibitor for PC6 and furin is lost. Several controls suggest that this is not the case. First, α_1–PIT is capable of inhibiting furin if it is expressed at very high (micromolar) levels (Anderson et al., 1993). In our studies, ectopically expressed α_1–PIT is inactive against the endogenous BMP-4 convertase, whereas the same concentration of α_1–PDX efficiently inhibits this enzyme, suggesting that we are not achieving micromolar concentrations of either inhibitor. Second, we have characterized activin as a substrate for PACE-4 and have shown that levels of α_1–PDX that inhibit processing of BMP-4 *in vivo* do not inhibit processing of activin (Cui and Christian, manuscript in preparation). Taken together, our studies strongly support the hypothesis that BMP-4

is proteolytically activated by furin and/or PC6. While the spatial and temporal pattern of expression of furin is appropriate for this proposed role, we do not currently have the necessary reagents to determine whether PC6 is co-expressed with BMP-4 in *Xenopus*. Given, however, that patterns of expression of other PCs have been shown to be conserved across species, we anticipate that PC6 is ubiquitously expressed throughout early development in *Xenopus* as it is in other vertebrate species (Constam et al., 1996; Zheng et al., 1997).

In addition to BMP-4, a number of other candidate PC substrates, including Vg1, activin ßA, lunatic fringe and *Xenopus* nodal-related (Xnr) proteins, are activated by proteolytic cleavage after the furin consensus motif, -Arg-X-Arg/Lys-Arg-, and have been proposed to play essential roles in patterning the early embryo. Blockade of endogenous activin and/or Vg1 signaling, for example, leads to a complete loss of mesoderm (Hemmati-Brivanlou and Melton, 1992; Kessler and Melton, 1995). Vg1 has additionally been hypothesized to function as a dorsal determinant that is proteolytically activated in dorsal, but not in ventral cells (reviewed by Vize and Thomsen, 1994). Lunatic fringe has also been implicated in the process of mesoderm induction, and, like Vg1, its activity has been proposed to be restricted to certain regions of the embryo by regulated proteolytic processing (Wu et al., 1996). Finally, the nodal-related proteins Xnr1 through Xnr4 are restricted to dorsal cells of gastrulae and have been suggested to function as neural- or mesoderm-inducing molecules or as dorsalizing factors (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997). Interestingly, global misexpression of α_1–PDX in one cell *Xenopus* embryos does not perturb mesoderm induction and misexpression of α_1–PDX in dorsal blastomeres does not lead to a loss of dorsal or neural fate (Cui and Christian, unpublished). Because the process of mesoderm induction is initiated at very early stages (Jones and Woodland, 1987), it is conceivable that activin, Vg1 and/or lunatic fringe protein(s) is/are processed during oogenesis and that pre-cleaved forms of these molecules are present

and ready for secretion at the one-cell stage, prior to the time that injected α_1–PDX is active. In contrast, Xnrs function during gastrulation and thus should be sensitive to processing inhibitors. Our results suggest that Xnrs are either proteolytically activated by an endoprotease which is insensitive to α_1–PDX or that they are not required for induction or patterning of the mesoderm or central nervous system. While the substrate specificity of most PCs is not well established, our findings that PC3 does not cleave BMP-4, and that PC7 fails to recognize a second cleavage site within BMP-4 that is efficiently cleaved by PACE-4, PC6 and furin (Figure 6), suggest that individual PCs have unique roles in proteolytic activation of discrete signaling molecules. Furthermore, the identification of a potential second cleavage site within the pro-domain of BMP-4 raises the possibility that proteolysis liberates a novel, bioactive peptide that is distinct from BMP-4 itself, or that this second cleavage in some way regulates the bioactivity of BMP-4.

Proprotein processing: a novel mode of regulating BMP-4 activity?

Consistent with its multifunctional nature, BMP-4 activity is regulated at both transcriptional and post-transcriptional levels (reviewed by Hogan et al., 1994). Post-transcriptionally, two secreted proteins (noggin and chordin) have been identified which bind BMP-4 with high affinity and thereby block BMP-mediated activation of cognate cell-surface receptors (reviewed by Hogan, 1996; Graff, 1997). BMP-4 function may also be limited by competition with related signaling pathways for shared components of the intracellular signal transduction cascade, as has been shown to be the case with Smad4 (Candia et al., 1997). Finally, Smad-related proteins have been shown to function within BMP-responsive cells to downregulate the amplitude and/or duration of BMP signaling (Nakayama et al., 1998; reviewed by Heldin et al., 1997).

One final level at which BMP activity may be regulated is at the level of proprotein processing. While furin and PC6 are ubiquitously expressed during embryogenesis, it is not known whether they are constitutively active in all cells at all times. Indirect evidence that ectopically expressed BMPs are not cleaved until the gastrula stage (Candia et al., 1997), despite the presence of transcripts encoding furin many hours prior to this time, supports the possibility that the activity of these convertases is regulated post-transcriptionally. Our results provide a framework for future studies into the substrate specificity and regulation of activity of members of the PC family during vertebrate development.

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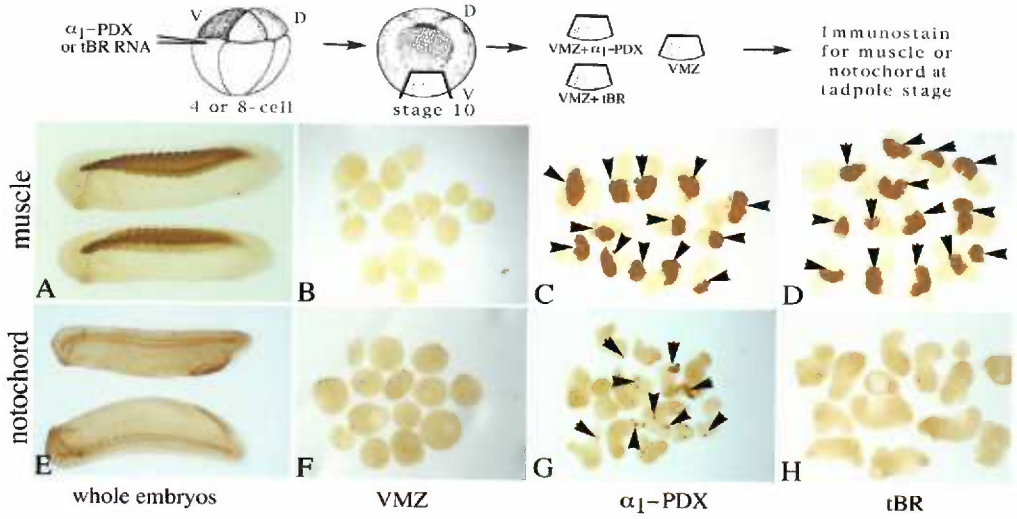

Figure 1. Dorsal mesoderm formation in α_1–PDX and tBR-expressing ventral marginal zones (VMZs). RNAs encoding α_1–PDX or tBR (approximately 500 pg) were injected into VMZs of four- to eight-cell embryos, and VMZs were explanted at stage 10 as illustrated at the top of the figure. VMZs (B-D, F-H) were cultured until sibling embryos (A,E) reached tailbud stages at which time they were immunostained with muscle- (A-D) or notochord-(E-H) specific antibodies. Specific staining is indicated by arrowheads.

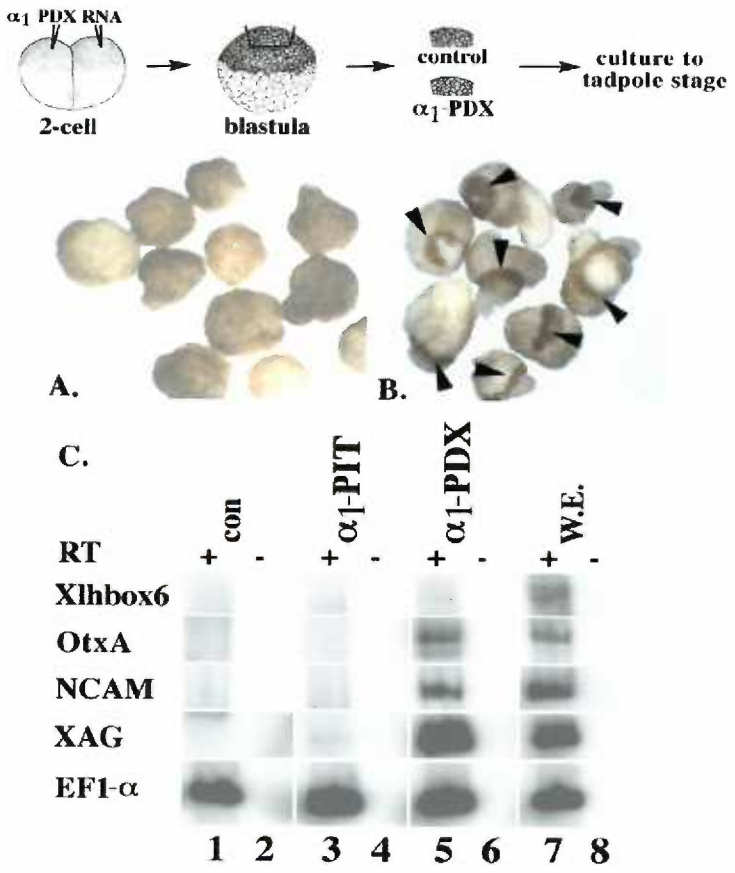

Figure 2. Neural induction in α_1–PDX expressing ectodermal explants.

RNA encoding α_1–PDX or α_1–PIT was injected near the animal pole of two-cell embryos, animal caps were explanted at the blastula stage and cultured until sibling embryos reached the tadpole stage as shown above the figure. Animal caps from control embryos (A) retain an epidermal morphology while animal caps from α_1–PDX-expressing embryos (B) form cement gland (arrowheads). (C) RNA samples from tadpole stage animal caps or whole embryos (W.E.) were analyzed for expression of neural (Xlhbox6, OtxA, NCAM)- or cement gland (XAG)-specific genes by RT-PCR in the presence (+) and absence (-) of reverse transcriptase (RT). The faint XAG signal in α_1–PIT expressing animal caps is not reproducible. EF-1α is a control for equivalent amounts of RNA in each sample. Note OtxA was consistently detected as double bands.

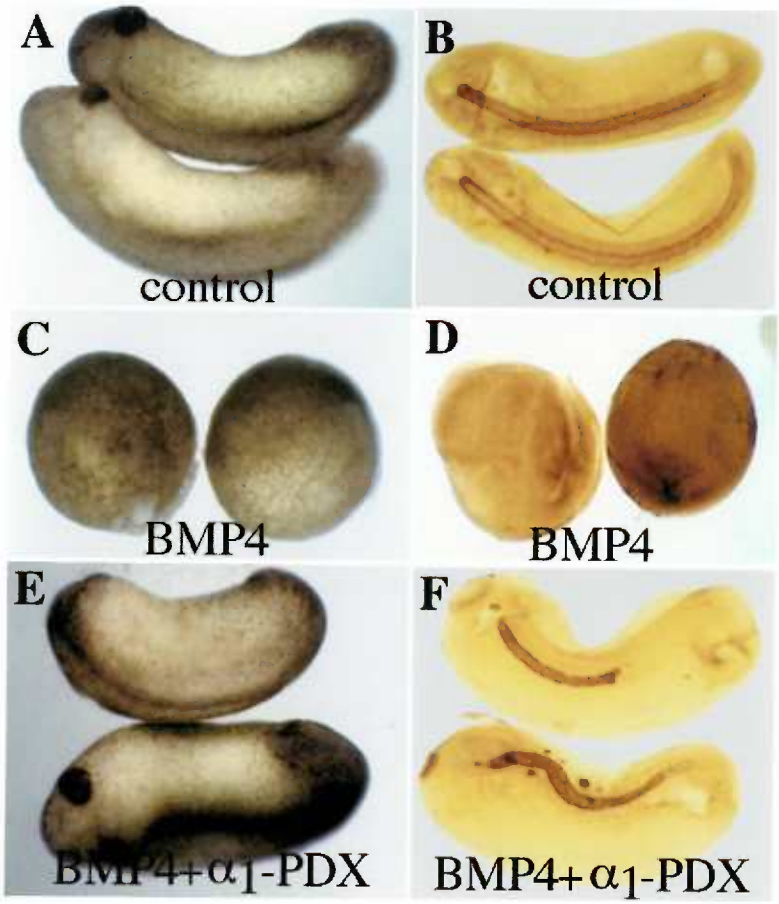

Figure 3. α_1–PDX inhibits the activity of exogenously expressed BMP-4. RNA encoding BMP-4 was injected alone (C,D), or in combination with α_1–PDX (E, F), into dorsal blastomeres of four-cell embryos. Injected embryos were cultured until control siblings (A,B) reached the tailbud stage at which time they were scored for gross morphology (left panels) or for the presence of immunoreactive notochord (right panels).

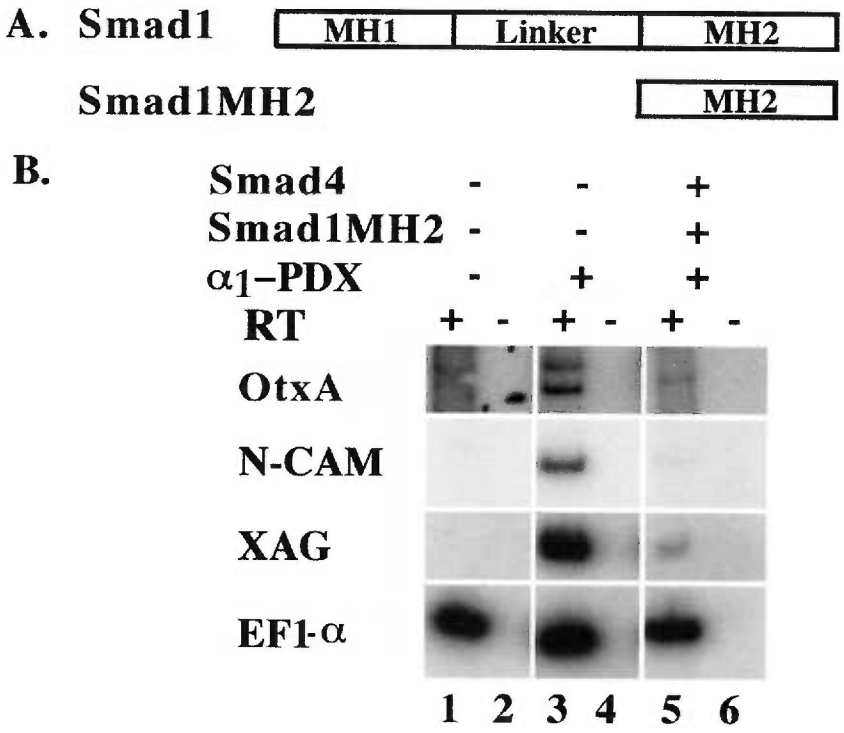

Figure 4. Activation of the intracellular BMP-4 signal transduction cascade represses α_1–PDX mediated neural induction. (A) Schematic diagram showing the domain structure of wild type Smad1 and the deletion mutant, Smad1MH2. (B) α_1–PDX RNA alone or in combination with Smad1MH2 and Smad4 RNA, was injected near the animal pole of two cell embryos. Ectoderm was explanted at blastula stages as diagrammed above Figure 2, cultured until siblings reached the tadpole stage and analyzed for expression of neural (OtxA, NCAM) or cement gland (XAG)-specific genes by RT-PCR in the presence (+) or absence (-) of reverse transcriptase (RT). Specific, reproducible bands corresponding to OtxA are observed only in ectodermal cells made to express α_1–PDX alone.

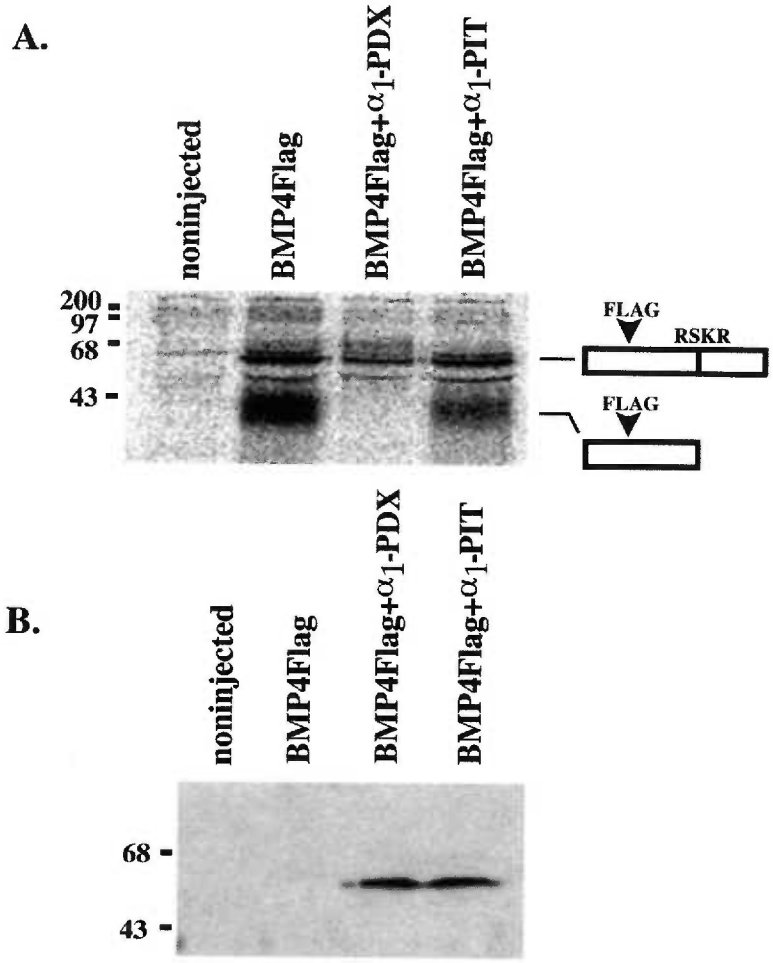

Figure 5. α_1–PDX blocks proteolytic processing of BMP-4 precursor *in vivo*. (A) RNA encoding BMP-4 flag alone, or in combination with α_1–PDX or α_1–PIT, was injected into stage VI oocytes. Oocytes were labeled with 35S-methionine and the lysates were subjected to immunoprecipitation with a flag specific antibody. The position of uncleaved BMP-4 precursor protein and the position of the cleaved proregion are illustrated schematically on the right side of the gel. (B) Western blot of protein extracts from uninjected oocytes, or from oocytes injected with the RNAs indicated above each lane, probed with an antibody which recognizes α_1–antitrypsin.

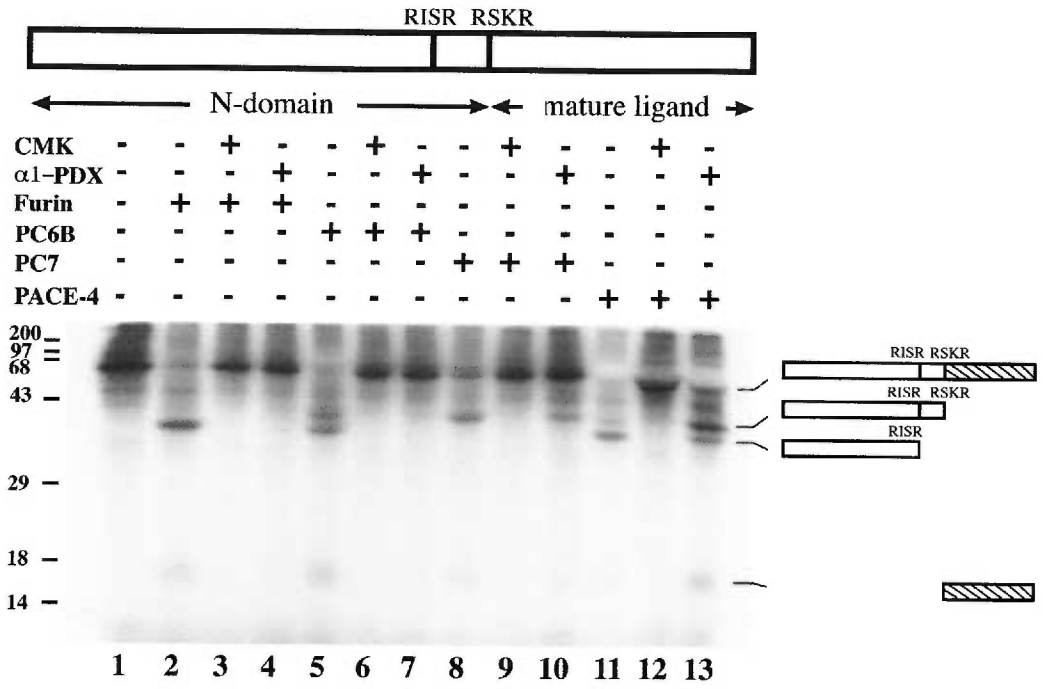

Figure 6. α_1–PDX inhibits the ability of furin and PC6B, but not PACE-4 or PC7, to cleave the BMP-4 precursor *in vitro*. Radiolabeled BMP-4 precursor protein was incubated for six hours with purified PCs, or with PCs that had been preincubated with purified α_1–PDX protein or Decanoyl-Arg-Val-Lys-Arg-CH2Cl (CMK) as indicated above each lane. Aliquots of each reaction were separated electrophoretically on a 12% polyacrylamide gel and proteolytic fragments were detected by fluorography. Bands predicted to correspond to the uncleaved precursor, intact amino (N)-terminal domain following cleavage at the -RRSKR- site (illustrated schematically above the figure), N-terminal domain following cleavage at the -RIKR- site, and mature polypeptide are indicated to the right of the gel.

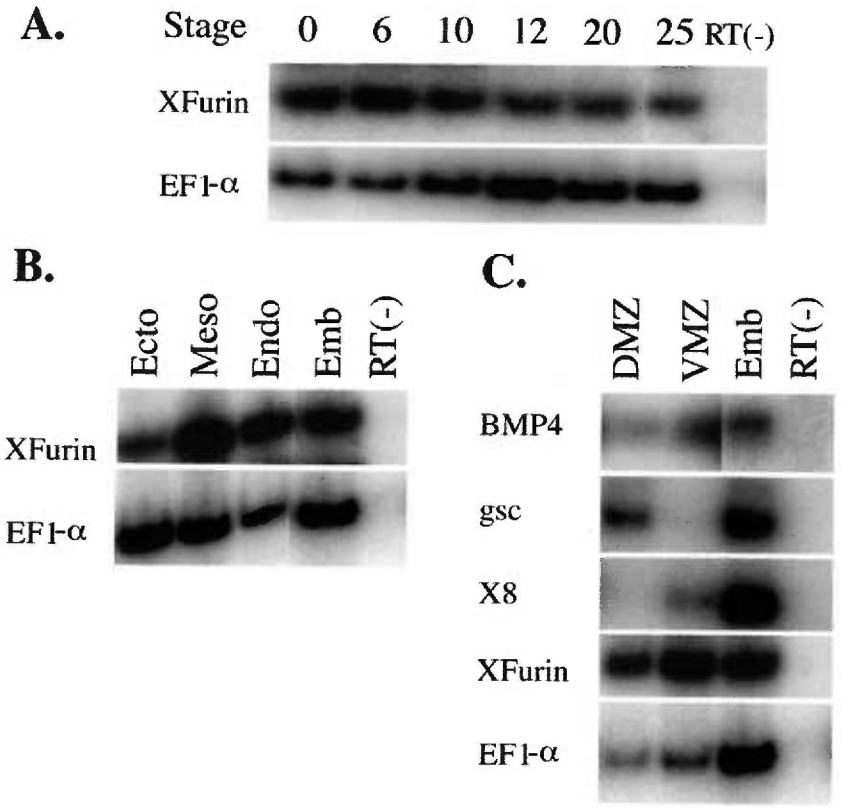

Figure 7. Furin is ubiquitously expressed in gastrula stage embryos.

(A) RNA isolated from oocytes (stage 0), blastulae (stage 6), early- or late gastrulae (stage 10 and 12, respectively), neurulae (stage 20) or tailbud stage embryos (stage 25) was analyzed for expression of *Xenopus* furin (Xfurin) by RT-PCR. (B) RNA isolated from ectodermal (Ecto), mesodermal (Meso) or endodermal (Endo) portions of gastrulae (stage 10), or from whole stage 10 embryos (emb) was analyzed for expression of *Xenopus* furin (Xfurin) by RT-PCR. (C) The dorsal marginal zone (DMZ) or ventral marginal zone (VMZ) region was dissected from gastrulae at stage 10 and RNA isolated from each group of explants, or from whole embryos (emb), was analyzed for expression of BMP-4, goosecoid (gsc), Xwnt-8 (X8) or furin by RT-PCR. In all panels, EF-1α serves as a loading control and representative samples were analyzed in the absence (-) of reverse transcriptase [RT(-)] to demonstrate absence of genomic contamination.

TABLE I. α_1–PDX-expressing ventral cells form muscle and notochord

EXPLANT	RNA INJECTED	MUSCLE	NOTOCHORD
VMZ	NONE	7 (28)	0 (12)
VMZ	α_1–PDX	100 (18)	50 (36)
VMZ	tBR	100 (20)	10 (32)
VMZ	α_1–PIT	ND	0 (19)
DMZ	NONE	ND	98 (31)

The dorsal marginal zone (DMZ) or ventral marginal zone (VMZ) region was isolated from stage 10 embryos that had been injected with synthetic RNAs as indicated above the table. These explants were cultured until sibling embryos reached stage 26 and were immunostained with muscle- or notochord-specific antibodies. Data are expressed as percentage of explants showing positive staining for muscle or notochord, as indicated at the top of the table, following by the number of explants examined, in parentheses. ND: not determined

Chapter 4

A novel cleavage site within the prodomain of the BMP-4 precursor regulates the activity and signaling range of mature BMP-4

Yanzhen Cui[1], Francois Jean[2], Gary Thomas[2], and Jan L. Christian[1]

[1]Department of Cell and Developmental Biology and [2]Vollum Institute

Oregon Health Sciences University, School of Medicine

3181 SW Sam Jackson Park Road

Portland, Oregon 97201-3098 USA

YC acknowledges that FJ provides recombinant PCs in experiments described in this chapter.

ABSTRACT

Bone morphogenetic protein-4 (BMP-4) plays an essential role in many aspects of embryonic patterning and its activity is tightly regulated at multiple levels (Cho and Blitz, 1998). Proteolytic maturation of pro-BMP-4 is required to generate an active cell-cell signaling molecule (Hogan, 1996) and this intracellular processing step has been implicated in regulating the stability and/or diffusion of BMP family members (Jones et al., 1996; Kessler and Melton, 1995). Here we show that the BMP-4 precursor is cleaved in an ordered manner: an initial cleavage occurs at a previously identified site adjacent to the mature ligand domain, and this allows for subsequent cleavage at an unexpected upstream site within the inactive prodomain. Mature BMP-4 synthesized from genetically engineered precursors in which the upstream site is non-cleavable, or in which both sites are cleaved simultaneously, has more biological activity and signals over a greater range *in vivo* than does BMP-4 cleaved from the native precursor. Differential use of the upstream cleavage site in the precursor protein may represent a novel mechanism for tissue-specific regulation of the activity and signaling range of BMP-4.

RESULTS AND DISCUSSION

The BMP-4 precursor is cleaved by furin, PC6B and/or PACE-4 (Cui et al., 1998; Constam and Robertson, 1999) following a multibasic amino acid motif (-RSKR-) (Aono et al., 1995) that matches the preferred furin recognition sequence (-RXKR-; Molloy et al., 1992). When BMP-4 precursor was incubated *in vitro* with recombinant furin for one hour, it was cleaved to produce fragments of ~15 kDa and ~35 kDa, consistent with the predicted M_r of mature BMP-4, and of the intact proregion, respectively (Figure 1a). A third cleavage product of ~32 kDa was also observed (Figure 1a) (Cui et al., 1998) , consistent with cleavage at a minimal furin consensus motif (-RISR-) (Molloy et al., 1992) located ~ 30 residues upstream of the primary cleavage site. Upon longer incubation, the 35 kDa prodomain fragment became undetectable while the 32 kDa fragment became more distinct. Identical results were obtained following incubation with PC6B or PACE-4 (data not shown) whereas precursor incubated in the absence of furin (Figure 1a, -Furin) was not cleaved.

The above results suggest the possibility that the BMP-4 precursor is sequentially cleaved, first at an optimal furin consensus motif (designated the S1 site), and subsequently at a minimal furin consensus motif (the S2 site) within the prodomain. To test this possibility, we assayed cleavage of mutant forms of BMP-4, in which the furin consensus motif at either the S1 or the putative S2 site had been destroyed. BMP4(mS2G) precursor protein, which lacks a furin motif at the S2 site, was cleaved to generate a single ~35 kDa prodomain fragment (Figure 1b, lanes 1-4), indicating that the ~32 kDa proteolytic fragment requires the presence of the upstream -RISR- motif. BMP4(mS1) precursor protein, in which the furin motif at the S1 site had been destroyed, was completely resistant to cleavage by furin (Figure 1b, lanes 5-8). Thus, cleavage at the S1 site, which generates the mature ligand, is required for cleavage at the upstream S2 site.

To further test whether a minimal furin recognition sequence is required for sequential cleavage of BMP-4, we analyzed maturation of a mutant precursor protein [BMP4(mS2K)] in which the S2 site was converted to an optimal furin motif. When native BMP-4 was incubated with furin for one hour, proteolytic fragments corresponding to the intact prodomain and to the amino (N)-terminal prodomain fragment generated by cleavage at the S2 site were observed (Figure 1c, lane 2) and by three hours, cleavage at the S2 site was complete (lane 3). In contrast, BMP4(mS2K) was cleaved within one hour to generate the N-terminal prodomain fragment and a single mature fragment (Figure 1c, lane 6) that migrated with an M_r identical to that of mature BMP-4 cleaved from the native precursor, suggesting that cleavage had occurred at both the S1 and S2 sites. A fragment corresponding to the intact prodomain of BMP4(mS2K), generated by cleavage of the S1 site alone, was barely detectable following a very short (20 minute) incubation with furin (lane 5). Thus, introduction of an optimal furin consensus motif at the S2 site disrupts sequential cleavage of the BMP-4 precursor and allows both sites to be cleaved simultaneously.

A *Xenopus* oocyte translation assay was employed to ask whether the BMP-4 precursor is cleaved at both S1 and S2 sites *in vivo*. Native BMP-4 precursor was cleaved *in vivo* to generate two prodomain fragments (Figure 2a, black dots, lane 3) that migrate with the same M_r as the intact and N-terminal prodomain fragments generated by *in vitro* digestion (lane 2). In contrast, *in vivo* cleavage of BMP4(mS2G) produced a single prodomain fragment corresponding to cleavage at the S1 site alone, while cleavage of BMP4(mS2K) produced only the N-terminal prodomain fragment, consistent with simultaneous cleavage at S1 and S2 sites. Notably, *in vivo* maturation of the mutant BMP-4 precursors appeared to be modestly more efficient than that of native BMP-4 as evidenced by the approximately 1.3- or 3-fold higher levels of prodomain generated by cleavage of BMP4(mS2G) or BMP4(mS2K), respectively (Figure 2a), following injection of equivalent amounts of RNA encoding each

precursor into oocytes (Figure 2b). Our results are consistent with the hypothesis that the endogenous BMP-4 precursor is sequentially cleaved *in vivo* at an optimal (S1) and then a minimal (S2) furin consensus motif, and that this sequential cleavage limits the rate of proprotein maturation.

To determine whether sequential cleavage of the BMP-4 precursor is necessary to generate a functional ligand, we analyzed the ability of S2 cleavage mutants to activate the BMP-4 target gene, Xbra, in *Xenopus* animal pole explants. Injection of RNA encoding BMP4(mS2G) or BMP4(mS2K) reproducibly led to an approximately two- or six-fold higher induction of Xbra expression, respectively, than did injection of an equal or greater amount of RNA encoding native BMP-4 (Figure 3). The increased efficiency of maturation of the cleavage mutant precursors (Figure 2a) correlates with, but cannot fully account for, the enhanced activity of BMP-4 cleaved from mutant precursors. These results demonstrate that cleavage at the S1 site is sufficient to generate a biologically active ligand, and suggest that sequential cleavage of the BMP-4 precursor limits the activity of the mature ligand *in vivo*.

Primary and upstream furin consensus cleavage motifs are conserved in all known vertebrate BMP-2 and BMP-4 precursor proteins (representative examples shown in Figure 4) and in the *Drosophila* BMP-2/4 ortholog, decapentaplegic (DPP). Other members of the BMP superfamily, such as BMP-7 (Jones et al., 1994), possess only a single -RXXR- motif immediately preceding the ligand domain (Figure 4). Mature BMP-2, BMP-4 and DPP bind tightly to the extracellular matrix and cell surface when expressed in cultured cells (Panganiban et al., 1990; Israel et al., 1992; Hazama et al., 1995) and function as short range signaling molecules in some developmental contexts (Jones et al., 1996; Panganiban et al., 1990). In contrast, homodimers of BMP-7 are freely diffusible (Hazama et al., 1995). The observation that poorly diffusible BMPs possess two conserved consensus cleavage motifs whereas diffusible

BMPs have only a single cleavage site raises the possibility that sequential cleavage of the BMP-4 precursor can limit the range of action of the mature ligand.

We used an *in vivo* assays to compare the signaling range of BMP-4 generated by maturation of native and S2 cleavage mutant precursors. In this assay (illustrated above Figure 5), a single animal pole blastomere of 32-64 cell embryos was co-injected with RNAs encoding ß-galactosidase and native or mutant BMP-4. These embryos were cultured to stage 11, and stained for ß-galactosidase activity and expression for the BMP-4 target gene, Xbra. When cells were made to express the native BMP-4 precursor, Xbra staining (diffuse purple stain) was detected only in cells adjacent to, or within, BMP-4 producing cells (marked by punctuate turquoise ß-galactosidase stain, Figure 5, BMP4). In contrast, when cells were made to express mutant BMP-4 precursors in which the S2 site is either non-cleavable [BMP4(mS2G)] or is cleaved simultaneously with the S1 site [BMP4(mS2K)], Xbra staining was evident many cells distant from the BMP-producing cells (Figure 5, BMP4(mS2G), BMP4(mS2K)). As a control, embroys expressing ß-galactosidase alone never activated Xbra expression (Figure 5, ß-gal). Thus, in *Xenopus* embryos, mature BMP-4 generated by proteolytic processing of either of the S2 cleavage mutants was able to signal over a greater range than that generated by processing of native BMP-4.

BMP-4 and DPP have been shown to function as morphogens in that they affect the development of target cells in a concentration dependent manner. In some contexts, these molecules diffuse freely from a localized source and establish a morphogenic gradient of protein, whereas in other contexts they act as short range signals, and a morphogenic activity gradient is established by the effects of long range diffusible inhibitors (Neumann and Cohen, 1997; DeRobertis and Sasai, 1996). DPP, for example, acts over long range to specify cell fate along the anteroposterior axis of limb (Neumann and Cohen, 1997), but signals at short range between germ layers of the developing gut (Panganiban et al., 1990; Immergluck et al., 1990; Reuter et al., 1990)

and in the dorsal embryonic ectoderm (St. Johnston et al., 1987; Ferguson et al., 1992). Similarly, *Xenopus* BMP-4 acts only within the immediate environment of its synthesis in ectodermal explants (Jones et al., 1996) but can act as a longer range signal within the embryonic mesoderm (Dosch et al., 1997; Jones and Smith, 1998). Our finding that the signaling range of BMP-4 is regulated by cleavage at an unpredicted site within the proregion suggests a possible mechanism by which the spread of BMP protein might be differentially controlled. Specifically, it is possible that cleavage at the upstream (S2) site is regulated in a spatially and/or temporally restricted fashion, perhaps by the tissue-specific availability of endoproteases or other components of the processing machinery. If so, cells that are capable of cleaving the BMP-4 precursor at both S1 and S2 sites would generate a less active, short range signaling molecule, whereas those in which the precursor is cleaved only at the S1 site would generate a long range, diffusible molecule with enhanced biological activity. This may represent a novel mechanism for differentially tailoring the activity of BMPs, and possibly related signaling molecules, to suit specific developmental needs.

Methods

cDNA constructs

cDNAs encoding S2 cleavage mutant forms of the BMP-4 precursor were generated by polymerase chain reaction (PCR)-based amplification of native cDNAs using primers carrying appropriate point mutations. Regions of cDNAs generated by PCR were sequenced to verify that only the intended nucleotide substitutions had been introduced. The S1 cleavage mutant was provided by Ken Cho.

Embryo culture and manipulation

Xenopus embryos were obtained, microinjected, and cultured as described (Moon and Christian, 1989). Generation of embryonic explants and recombinants, lithium treatment and histological analyses were performed as described (Cui et al., 1996).

Oocyte injections, immunoprecipitation and in vitro digestion

Oocytes were isolated, injected with RNAs, labeled with trans-label (NEN) and proteins immunoprecipitated from lysates as described (Cui et al., 1998) with the exception that monoclonal anti-FLAG antibody M2 (Sigma) was used in some experiments. Radiolabeled BMP-4 precursor was isolated, digested *in vitro* with recombinant furin, and analyzed by SDS-PAGE as described in Chapter 3 (Cui et al., 1998).

RNA analysis

Whole mount *in situ* hybridization and Northern blot analyses were performed as described (Christian and Moon, 1993).

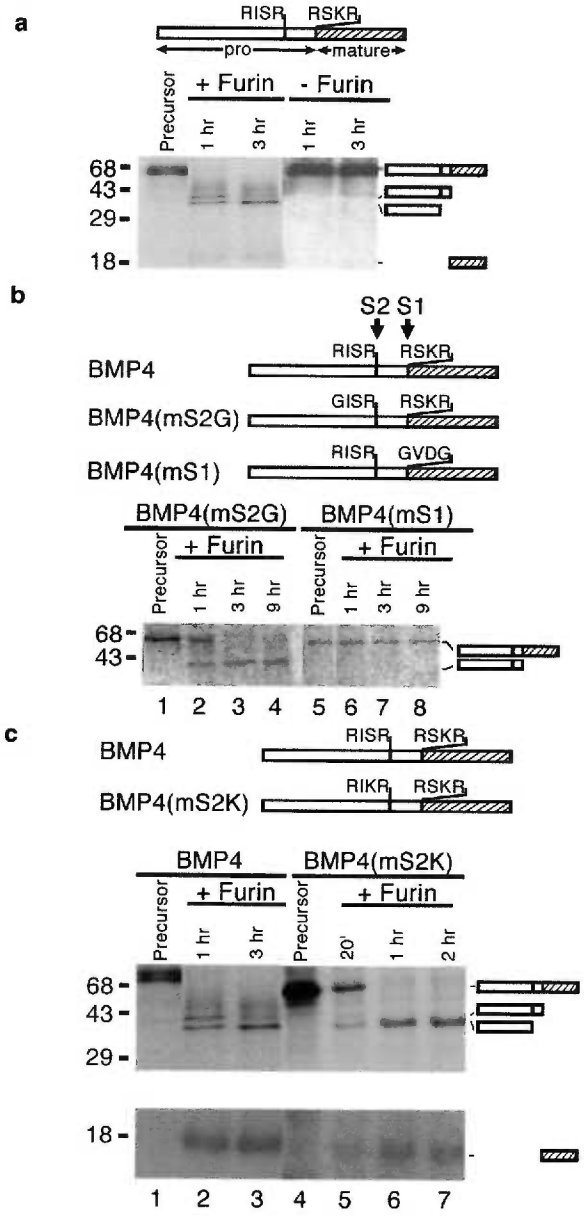

Figure 1. The BMP-4 precursor is sequentially cleaved *in vitro* at two sites within the prodomain. Radiolabeled native (**a**) or cleavage mutant forms (**b**, **c**) of the BMP-4 precursor were incubated with recombinant furin for varying amounts of time as indicated. Bands predicted to correspond to uncleaved precursor, intact prodomain following cleavage at the S1 site, N-terminal prodomain fragment following cleavage at the S2 site and mature BMP-4 are indicated to the right of each gel. The region of the gel containing mature BMP-4 has been enhanced in (**c**).

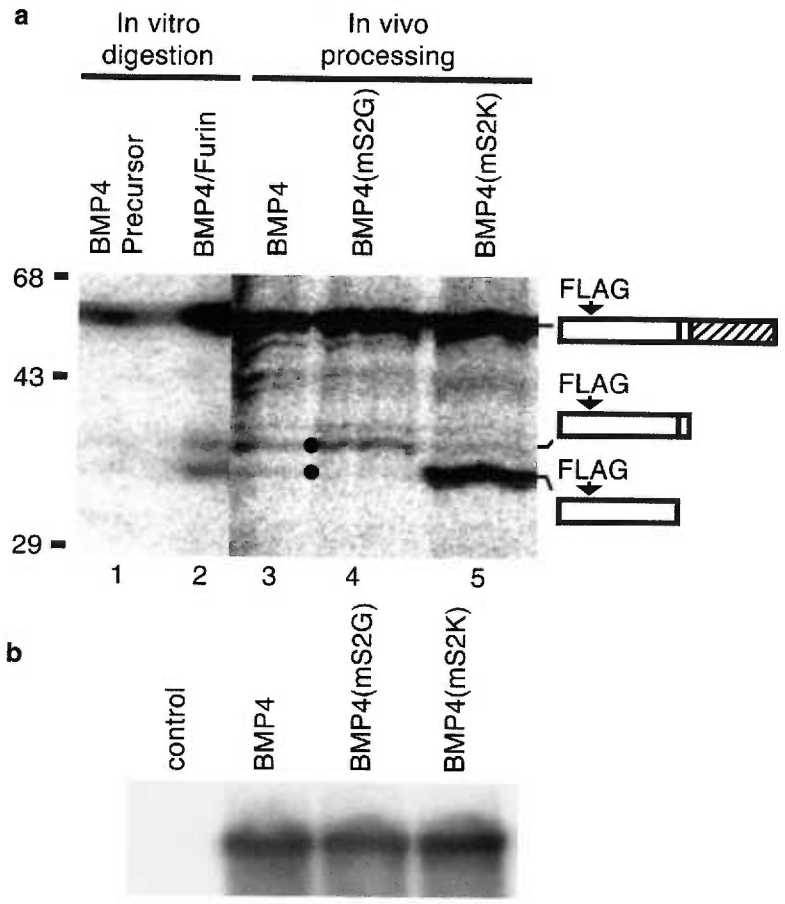

Figure 2. *In vivo* cleavage of BMP-4 precursor occurs at two sites within the prodomain. (a) FLAG-tagged native or cleavage mutant BMP-4 precursors were expressed in *Xenopus* oocytes and immunoprecipitated using an anti-FLAG antibody. Bands corresponding to uncleaved precursor, intact prodomain following cleavage at the S1 site and N-terminal prodomain fragment following cleavage at the S2 site are indicated (b) Northern blot analysis of injected BMP-4 transcripts demonstrates that each precursor protein was translated from an equivalent amount of injected RNA.

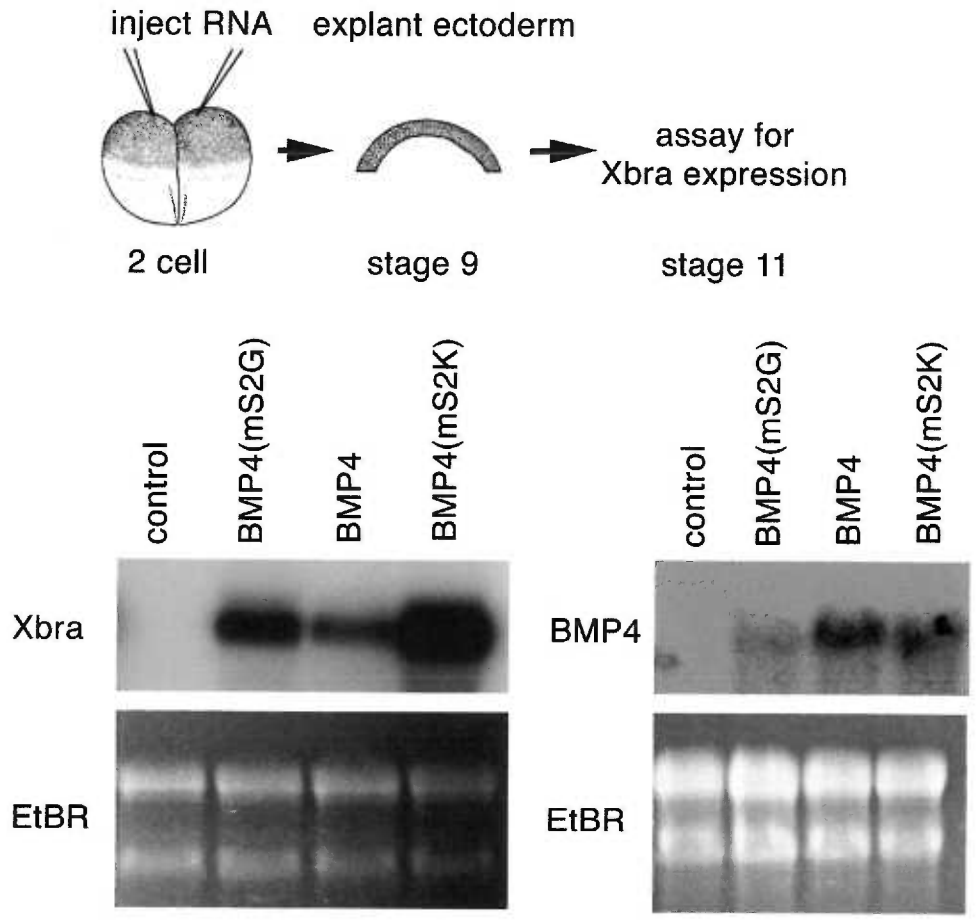

Figure 3. Enhanced biological activity of BMP-4 generated by maturation of S2 cleavage mutants. Ectodermal cells explanted from *Xenopus* embryos that had been made to express native or S2 cleavage mutant forms of the BMP-4 precursor were cultured to stage 11. Levels of expression of the BMP-4 target gene, Xbra (left panel), and levels of injected BMP-4 transcripts (right panel) were analyzed by Northern blot hybridization. Gels were stained with ethidium bromide (EtBr) prior to transfer as a loading control.

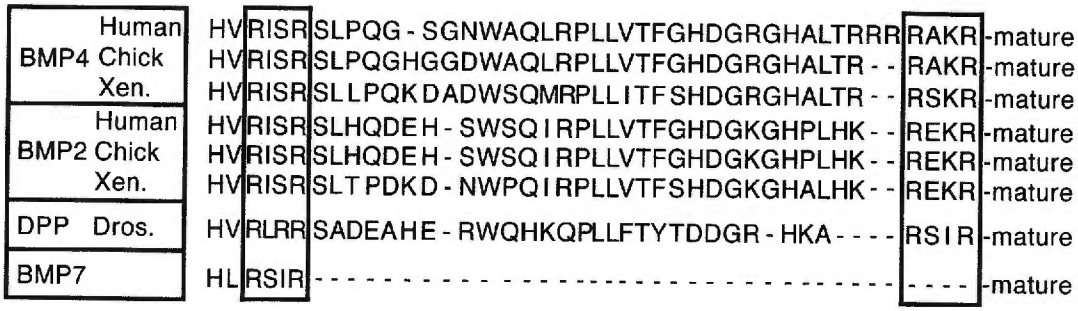

Figure 4. S1 and S2 cleavage sites are conserved in BMP-2 and BMP-4 from all species. Alignment of sequence surrounding the cleavage site(s) of BMP-2, -4 and -7 from human, chick, *Xenopus* (Xen) and *Drosophila* (Dros.) is shown.

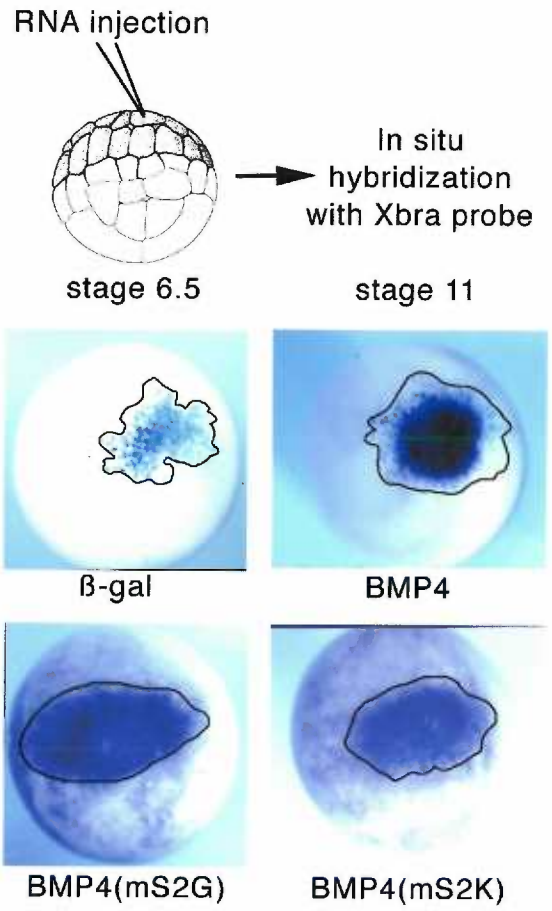

Figure 5. BMP-4 generated by maturation of S2 cleavage mutant precursors signals over a greater range in vivo than does BMP-4 cleaved from native precursor.

Xenopus embryos made to express native or mutant BMP-4 precursors together with nuclear ß-galactosidase (ß-gal) were stained for ß-galactosidase activity (turquoise nuclear stain) and Xbra transcripts (by in situ hybridization, diffuse purple stain) as illustrated. Black lines indicate ß-galactosidase or BMP-4 producing cells.

Chapter 5

THESIS SUMMARY

Synergistic effects of Wnt and Vg1 in the formation of dorsal mesoderm and endoderm

In the first two chapters of this thesis I have described findings in my graduate training that have aided our understanding of the mechanisms underlying dorsoventral axis specification. Specifically, I have investigated why some cells differentiate into dorsal mesoderm, while adjacent cells differentiate into ventral mesoderm. This is a fascinating question that has attracted the attention of embryologists for many generations. In fact, similar studies can be traced back to 1924, when Spemann and Mangold found that the dorsal lip of the blastopore of gastrula stage embryos has the ability, when grafted to the ventral side, to induce the surrounding cells to adopt an entirely different fate and produce a remarkable twin-embryo phenotype (Hamburger, 1988). Based on this inductive capability, these dorsal cells are called Spemann's organizer.

In addition to the Spemann organizer, there exists another dorsal inductive center, namely the Nieuwkoop center, which operates earlier in development. Tissue transplantation experiments demonstrate that components of the Nieuwkoop center must be maternally derived (Gimlich, 1986), but the identity of this dorsal determinant(s) remains a matter of debate. Specifically, while some argue that mature Vg1 functions as the endogenous dorsal determinant (Thomsen and Melton, 1993; Kessler and Melton, 1995; Dohrmann et al., 1996), there are also growing arguments that a maternal Wnt or Wnt signaling component such as ß-catenin plays this role. The

first hypothesis is based upon the expression pattern of endogenous Vg1, along with the observations that mature Vg1 can induce ectoderm to form dorsal mesoderm and can rescue dorsal structures in UV-ventralized embryos. More recently, it has been shown that endogenous Vg1 signaling is required for the development of dorsal mesoderm and endoderm (Joseph and Melton, 1998). Several lines of evidence, however, argue against the hypothesis that Vg1 alone specifies dorsal fate and favor a role for the Wnt signaling pathway in this process.

Firstly, the endogenous dorsal determinants in the vegetal cortex of the egg or the dorsal vegetal cells of blastula stage embryos have a Wnt-like dorsal patterning activity rather than a Vg1-like mesoderm inducing activity. Specifically, while injection of egg-derived vegetal cortical cytoplasm into UV-ventralized embryos partially rescues dorsal axial structures, animal caps isolated from these injected embryos produce only epidermis. Therefore, vegetal cortex on its own cannot induce mesoderm, whereas Vg1 can (Holowacz and Elinson, 1995). Furthermore, in an animal-vegetal reversed embryo, the animal cap, which contains the dorsal determinants, expresses the Wnt target genes Xnr3 and Siamois but does not express the Vg1 target gene, Mix.1 (Marikawa et al., 1997). Secondly, when endogenous Vg1-like activity is assayed *in vivo* using Vg1/activin response element from the goosecoid promoter, dorsal-ventral differences in Vg1 activity are not observed (Watabe et al., 1995). Thirdly, when transcripts encoding ß-catenin, a downstream component of the Wnt signaling pathway, are depleted in oocytes, embryos derived from these oocytes lack dorsal structures (Heasman et al., 1994). Thus, Vg1 alone is not sufficient for dorsal development.

How do we reconcile these two views? The answer may lie in part in the findings presented in Chapter 2, which demonstrate that Wnt, but not Vg1, can

substitute for the endogenous dorsalizing activities and that Vg1 alone is not sufficient for dorsal mesoderm induction in the absence of a Wnt signal.

While Wnts may be involved in patterning both mesodermal cells and endodermal cells, Wnts cannot induce mesoderm to form (reviewed by Christian and Moon, 1993). Thus, any action exerted by Wnts must be dependent upon the presence of other inductive signals. The best candidate for such an endogenous synergistic inductive signal is Vg1. This statement is based upon previous studies which suggest that Vg1 is required for specification of both mesodermal (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994) and endodermal (Cornell et al., 1995; Henry et al., 1996) fates *in vivo,* and our data that Vg1 is capable of synergizing with Wnts in notochord induction (Chapter 2). Therefore, we suggest a synergistic model in which we propose that mature Vg1 functions as a general mesoderm and endoderm inducer across the entire dorsal-ventral axis, while a maternal Wnt such as Xwnt-8b, or a Wnt signaling pathway component such as ß-catenin, is activated by cortical rotation in dorsal cells. The combinatorial activities of Wnt and Vg1 signaling may constitute the components of the Nieuwkoop center. Wnt signaling may further synergize with Vg1 in specifying formation of the organizer and endoderm (Cui et al., 1996; Moon and Kimelman, 1998). Consistent with this hypothesis, a maternal Wnt, Xwnt-8b, which possesses full dorsal axis-inducing capability when introduced into early stages of embryos, has been characterized (Chapter 1). In further support for a role of Wnt signaling in dorsal axis specification, it has been shown that ß-catenin is required for formation of the endogenous axis, and that complexes of ß-catenin and XTcf3 in the nucleus directly activate expression of the dorsal specific gene siamois (Moon and Kimelman, 1998). On the other hand, inhibition of TGF-ß signaling prevents formation of the primary axis and expression of the organizer-specific genes (Hemmati-Brivanlou and Melton, 1992; Cornell et al., 1995). Therefore, both Wnt and Vg1 are required for the development of dorsal mesoderm and endoderm.

While embryological studies suggested that mesoderm induction occured prior to the mid-blastula transition (MBT), a recent report challenged this view. Zhang et al.(1998) showed that a member of the T-box transcription factor family, VegT, is crucially involved in this process. VegT contains a T-box DNA-binding motif and its maternal transcripts are localized to the vegetal hemisphere of egg and embryos. When the maternal VegT transcripts are depleted by means of antisense oligonucleotides, the resultant embryos cannot form endoderm, and vegetal pole cells isolated from such embryos have lost the ability to secrete mesoderm inducing signals but instead form mesoderm themselves (Zhang et al., 1998). Since VegT is a transcription factor, any of its effects must be mediated by products of its downstream gene(s), which cannot be transcribed prior to the MBT, thus it appears that mesoderm induction takes place at a time that is significantly later than that originally proposed. It will be interesting to see whether VegT regulates secretion or cleavage of the mesoderm inducing signals, especially Vg1, within endodermal cells.

Role of furin-like proprotein convertases (PCs) in activating BMP-4 and other molecules

In Chapter 3 I described our findings which suggest that two members of the proprotein convertase (PC) family may be required to proteolytically activate the BMP-4 precursor.

Members of the PC family may be essential for embryonic development, since many secreted and membrane-bound developmental regulators derive from inactive precursors and require proteolytic activation. Consistent with this, most PCs are expressed ubiquitously throughout embryogenesis, although tissue-specific expression of some enzymes has also been observed (Constam et al., 1996; Zheng et al., 1997). The wide tissue distribution of PCs during vertebrate development, coupled with the essential embryonic roles of potential substrates, make it likely that targeted deletions of

these PC genes in mice will produce complex phenotypes and early lethality. For instance, mouse embryos lacking furin, the best characterized member of this family, die between days 10.5 and 11.5 with severe ventral closure defects; these embryos also have defects in heart tube fusion and looping morphogenesis (Roebroek et al., 1998). While these phenotypes highlight the important roles of furin in vertebrate embryonic development, they shed little, if any, light on the mechanisms underlying its actions. For example, one cannot draw any conclusion from these knock-out studies about which signaling pathway is crucially involved in patterning the heart or ventral mesoderm, since conceivably many important developmental regulators could be substrates for furin. These studies also do not address the issue of substrate specificity. For this reason, conditional or tissue-specific loss of PC function is a more attractive strategy for investigating the developmental roles and substrate specificity of these endoproteases. As described in Chapter 3, we have taken advantage of a well characterized furin inhibitor, α_1–PDX, to block furin-like PC function within a subset of cells in early *Xenopus* embryos and to demonstrate that the protease responsible for maturation of BMP-4 *in vivo* is most likely furin and/or PC6.

Proprotein processing may be one important level at which the activity of BMP-4 and other TGF-ß family members is regulated. While furin and PC6 are ubiquitously expressed at the transcript level, it is not known whether these transcripts are translated into proteins in all cells or if the enzymes are active in all cells after translation. For example, furin is initially synthesized as an inactive zymogen, which requires a set of ordered autoproteolytic cleavages at two distinct cleavage sites in order to function (Anderson et al., 1997). Furthermore, the trafficking and localization of the active form of furin are also subject to regulation by a number of proteins (Molloy et al., 1999), which may determine where furin actually cleaves its substrates. These observations support the hypothesis that the activity of these convertases is regulated

post-transcriptionally, and it will be interesting to see if they are developmentally regulated.

Many other developmental regulators, in addition to TGF-ß family members, need to be cleaved by furin-like PCs in order to function. Xolloid and BMP1 are two examples of such molecules. Xolloid and BMP1 are astacin-like metalloproteases. Xolloid has been shown to cleave and inactivate chordin, an inhibitor of BMP-4. This cleavage releases active BMP-4 from an inactive chordin/BMP-4 complex (Piccolo et al., 1997). Although BMP1 has not yet been shown to cleave chordin, it most likely can, since both Xolloid and BMP1 appear to indirectly upregulate BMP-4 activity (Goodman et al., 1998). The evidence for this is that overexpression of either of these enzymes in embryos leads to a ventralized phenotype, characterized by a smaller or absence of head and diminished dorsal structures. This phenotype is similar to, although considerably less severe than, that of embryos made to overexpress BMP-4. Interestingly, both Xolloid and BMP1 are first synthesized as zymogens which need to be cleaved after -RVRR- or -RERR-, respectively (Goodman et al., 1998), to release the active form of the enzyme. Since their cleavage sequences fit the furin consensus motif, Xolloid and BMP1 may be substrates for furin-like enzymes. Subsequent studies with the furin specific inhibitor α_1–PDX as well as *in vitro* digestion assays support this hypothesis. Specifically, our digestion assays indicate that while furin and PC6 can cleave Xolloid, PC6 and PACE4 cleave BMP1 *in vitro* (Cui and Christian, unpublished data). *In vivo* studies support this finding, since expression of α_1–PDX in *Xenopus* embryos completely rescues phenotypes caused by ectopic expression of Xolloid but cannot rescue phenotypes caused by BMP1 (Cui and Christian, unpublished data) . This suggests that furin/PC6 may be required to activate the Xolloid metalloprotease, while PACE4 is the most likely candidate for maturation of BMP1 in *Xenopus* embryos.

In summary, our studies demonstrate that furin-like proprotein convertases can regulate BMP-4 activity at two independent levels. First, furin and/or PC6 directly cleave the BMP-4 proprotein, which releases the biologically active ligand, hence regulating the pool of active ligand. Second, fuirn/PC6 and PACE4 activate Xolloid and BMP1, respectively. The active Xolloid and BMP1 enzymes then cleave and inactivate chordin, the BMP-4 inhibitor, thereby releasing BMP-4 from the BMP-4-chordin complex. In this way, the activity of the BMP-4 ligand pool that is available is finely tuned to fit its physiological needs. Conceivably, activities of other members of the BMP family, such as BMP-2 and BMP-7, which are believed to play crucial roles in mesoderm patterning, neural induction, and in patterning organs such as the heart and eye, are also regulated through complicated cascades involving furin-like convertases. The roles of this group of endoproteases in vertebrate embryogenesis, therefore, cannot be overemphasized. Further studies of the subcellular localization and tissue/species-specific expression and establishment of substrate-specificity of these enzymes will surely shed light on the mechanisms underlying their actions.

Diffusion of the BMP-4 protein

In addition to proteolytic activation, which is one level of posttranslational regulation of BMP-4 function, there are other mechanisms to regulate BMP-4 activity. For instance, the actual biological response of a cell is dependent upon, and proportional to, the concentration of active BMP-4 ligands that are available to that responding cell. Thus, the distance between the source cell and responding cell is critical and the response may be dependent on how fast the ligand can diffuse.

Numerous studies have demonstrated that extracellular matrix (ECM) components can indirectly regulate a particular signal transduction pathway by restricting or promoting movement of that growth factor within the extracellular environment. *Wingless* (WG, the *Drosophila* ortholog of Wnt-1) signaling, for

example, is disrupted in *Drosophila* embryos carrying mutations in the gene encoding UDP-glucose dehydrogenase (UDP-GDH). This enzyme is essential for synthesizing proteoglycans, which are components of the ECM. *UDP-GDH* deficient embryos show segment polarity defects, and overexpression of WG partially suppresses this phenotype. Thus, proteoglycans are thought to regulate wg signaling by promoting WG distribution from cell to cell (for a review see Cumberledge and Reichsman, 1997). On the other hand, purified BMP-3 (osteogenin) has been shown to bind avidly to heparin and type IV collagen. This is demonstrated by the observation that fractions eluted from a type IV collagen affinity column, but not unbound protein fractions, show strong bone-inducing activity (Paralkar et al., 1990). Similar examples of growth factor activity being regulated by ECM components include fibroblast growth factor (FGF) binding to syndecan, a heparan sulfated proteoglycan (Klagsbrun and Baird, 1991), and TGF-ß binding to betaglycan (Lopez-Casillas et al., 1994). It is likely that these growth factors are not freely diffusible but are, instead, sequestered on proteoglycans.

BMP-4 protein likely interacts with ECM components in the same way that other growth factors do, and thus ECM components regulate BMP-4 signaling. Consistent with this possibility, BMP-2, which is the closest homolog of BMP-4 in the BMP family, exists mainly attached to the cell surface when expressed in cultured mammalian cells (Israel et al., 1992). However, it is a matter of dispute as to whether BMP-4 is a diffusible morphogen. Specifically, when expressed in a mouse osteoblastic cell line, a large portion of the osteogenic differentiation-inducing ability, the characteristic feature of BMPs, remains in the cells (Hazama et al., 1995); and in *Xenopus* ectodermal cells, BMP-4 functions as a local morphogen (Jones et al., 1996). In contrast to these findings, however, BMP-4 appears to have a longer signaling range in mesoderm cells (Dosch et al., 1998), where a graded response to BMP-4 can be achieved by the presence of a complementary concentration gradient of BMP-4

antagonists such as chordin, noggin and follistatin (Thomsen, 1997). Thus, the diffusion and signaling range of BMP-4 may be determined by the cellular context in which the protein resides. Consistent with this possibility, DPP, the *Drosophila* homolog of BMP-2/4, has been shown to function as a long range morphogen in the wing imaginal disc but to diffuse for a very limited range in the midgut. Specifically, DPP in the wing imaginal disc has been demonstrated to act on responding cells directly and at a distance, and its effect is concentration-dependent (Nellen et al., 1996; Lecuit et al., 1996). In contrast, extracellular DPP protein does not move into regions of the visceral mesoderm that do not make DPP mRNA (Panganiban et al., 1990a). In agreement with this, in a *Drosophila* cell culture system, DPP protein has been found to associate with other proteins on the surface of cell culture dishes (Panganiban et al., 1990b).

Regulation of BMP-4 diffusion by a cleavage site within its prodomain

While the signaling range of BMP-4 appears to be dependent on its cellular context, the mechanism underlying this behavior is not understood. Vg1 ligand generated by a chimeric construct AVg (with the activin prodomain fused to the Vg1 ligand) is highly secreted, while the same ligand generated by a BVg construct (BMP-4 prodomain fused to the Vg1 ligand) is not secreted or diffusible (Kessler and Melton, 1995; Jones and Smith, 1998). This suggests that the prodomain may be important in determining how long the mature ligand can signal, but does not address the question of which part of the prodomain is critical in this regard. Our *in vitro* digestion assay has uncovered a second cleavage site within the BMP-4 prodomain which is cleaved following cleavage at the primary site. This regulated cleavage of the BMP-4 protein appears to limit the signaling range of the ligand.

How does the sequential cleavage regulate signaling range of BMP-4 ligand? We propose that the ECM proteins as discussed above, especially heparin, may play very important roles in determining how far the ligand can signal since, like other members of the BMP family, BMP-4 ligand has heparin binding sites with its N-domain (Ruppert et al., 1996). Heparin binding sites are groups of basic amino acids, with which it has been known that proteins can interact with the negatively charged moiety of heparin. Interaction of growth factors with heparin may provide very important physiological mechanisms to regulate that particular signaling pathway. This is demonstrated by the studies which show that binding of basic fibroblast growth factor (bFGF) to cell surface, heparin-like molecules is required for binding to its cognate, high affinity receptor (Yayon et al., 1991).

BMP-7 (OP-1) and BMP-2 both have heparin binding sites at N-domains of their ligands, yet they behave differently in terms of diffusibility. BMP-7 ligand, on one hand, preferentially associates with its prodomain as a complex after proteolytic cleavage, and this purified complex is more soluble in physiological buffers than the purified mature BMP-7 ligand (Jones, et al., 1994). BMP-2 ligand, on the other hand, associates with the extracellular matrix as well as the surface of cells in CHO cell cultures (Israel, et al., 1992). These studies suggest that while the heparin binding sites in BMP-2 interact with heparin on the cell surface, those in BMP-7 do not. It is possible that the heparin binding sites on BMP-7 are masked by the associated prodomain fragment, as illustrated in Figure 1, while those on BMP-2 remain accessible.

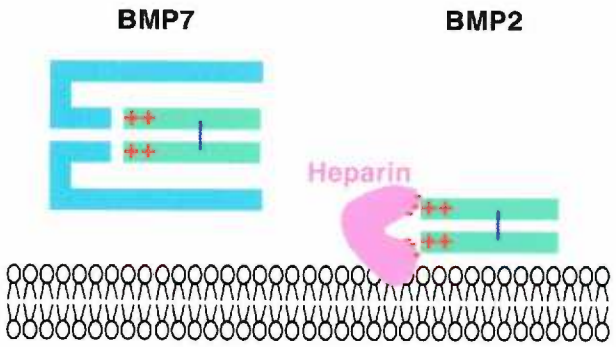

Figure 1. Interaction of BMP-7 and BMP-2 with heparin. After proteolytic processing, BMP-7 is secreted as a complex between the ligand and its prodomain. The heparin binding sites are masked by its prodomain in such a complex so that the ligand cannot associate with heparin and is highly diffusible. In contrast, BMP-2 is secreted as the ligand itself, not as a ligand-prodomain complex. With its heparin binding sites exposed, the ligand can associate with heparin thus it cannot diffuse away from the cell.

Interestingly, we noticed that the BMP-7 precursor protein possesses only one cleavage site while the BMP-2 and BMP-4 precursor proteins have two (Chapter 4). The sequential cleavage of BMP-4 protein appears to limit the signaling range of the BMP-4 ligand, and this is likely to be true for BMP-2 as well. Taking this into consideration, we propose a model with which to describe how sequential cleavage of the BMP-4 precursor may regulate cell attachment of the ligand (Figure 2).

We propose that the BMP-4 precursor protein, after being synthesized in the endoplasmic reticulum (ER), dimerizes and is sequentially cleaved at the S1 and then S2 sites, perhaps in different subcellular compartments. The two cleavage events generate different comformations. After the S1 site is cleaved, the ligand is able to bind to the prodomain. Subsequent cleavage at the S2 site may prevent this complex from forming. As a result, the ligand is secreted as the ligand proper, like BMP-2, not as a ligand-prodomain complex. In this form, BMP-4 binds to heparin on the cell surface

and cannot diffuse away (Figure 2). The mutant BMP-4 precursor BMP4(mS2G), which cannot be cleaved at the S1 site, may behave like BMP-7, since the ligand can associate with the prodomain and is secreted as a complex. For BMP4(mS2K), the two cleavage sites are cleaved simultaneously, and the linker protein between the ligand and the prodomain may diffuse away immediately after the sites have been cleaved. This may allow the ligand generated from this mutant to form a complex with the prodomain and thus be diffusible after secretion. It will be interesting to see if the ligand domain remains associated with the prodomain following in vivo cleavage of the BMP-4 cleavage mutant precursors [BMP4(mS2G) and BMP4(mS2K)] but does not remain associated following cleavage of the native precursor as predicted by this model.

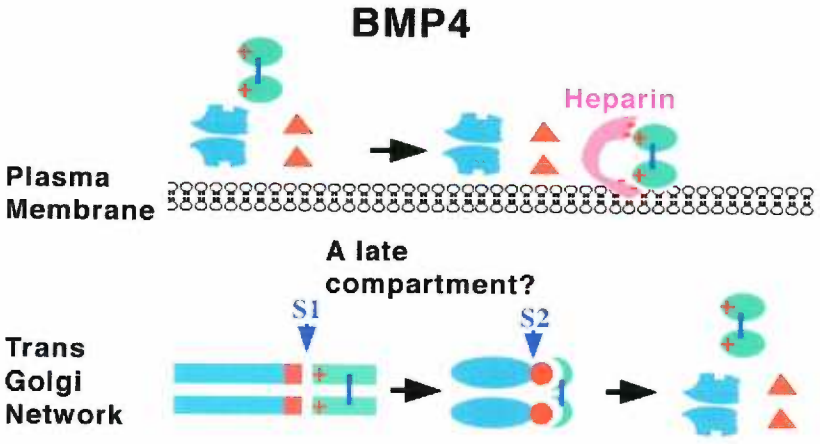

Figure 2. Sequential cleavage regulates the signaling range of BMP-4 ligand. Dimerized BMP-4 protein precursor is cleaved sequentially in different compartments, with the S1 site possibly cleaved in the trans golgi network (TGN). Each cleavage generates different protein conformations as indicated by different cartoons in this model. While cleavage at the S1 site permits formation of the ligand-prodomain complex, cleavage at both sites (first at S1 then at S2) prevents this complex from forming. Thus the ligand is secreted free of the prodomain and attaches to heparin on the cell surface through its heparin binding sites. See text for discussion of the mutant forms of BMP-4.

Our finding that cleavage at the second site regulates the BMP-4 signaling range could potentially explain the observations that BMP-4 produced in ectodermal cells signals over short range (Jones et al., 1996) while that produced in mesodermal cells signals over long range (Dosch et al., 1997). Specifically, we hypothesize that cleavage at the second site is temporally and/or spatially regulated, so that the BMP-4 precursor is sequentially cleaved at both sites in ectodermal cells, which limits the signaling range of the ligand, while only the primary site is cleaved in mesodermal cells, where the ligand can diffuse for a longer distance. Therefore, regulated cleavage of the BMP-4 protein may be a key mechanism to determine its signaling range. As a result, its activity can fit the requirements of its microenvironment and correct patterning of different tissues can be achieved.

Concluding remarks

In this thesis, I have described findings and achievements in my graduate training. These include the characterization of a new Wnt family member, *Xwnt-8b*, roles of Wnt and Vg1 in dorsoventral mesoderm specification and identification of furin-like proprotein convertases in proteolytic activating BMP-4. Finally, I have provided evidence showing that a second cleavage site within the BMP-4 prodomain appears to regulate the signaling range of the ligand.

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