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Cell fate specification and competence by Coco, a maternal BMP, TGFβ and Wnt inhibitor

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SUMMARY

Patterning of the pre-gastrula embryo and subsequent neural induction post-gastrulation are very complex and intricate processes of which little, until recently, has been understood. The earliest decision in neural development, the choice between epidermal or neural fates, is regulated by bone morphogenetic protein (BMP) signaling within the ectoderm. Inhibition of BMP signaling is sufficient for neural induction. Many secreted BMP inhibitors are expressed exclusively within the organizer of the *Xenopus* gastrula embryo and therefore are predicted to act as bona fide endogenous neural inducers. Other cell-autonomous inhibitors of the BMP pathway are more widely expressed, such as the inhibitory Smads, Smad6 and Smad7. In this report we describe the biological and biochemical characterization of 51-B6, a novel member of Cerberus/Dan family of secreted BMP inhibitors, which we identified in a screen for Smad7-induced genes. This gene is expressed maternally in an animal to vegetal gradient, and its expression levels decline rapidly following gastrulation. In contrast to known BMP inhibitors, 51-B6 is broadly expressed in the ectoderm until the end of gastrulation. The timing, pattern of expression, and activities of this gene makes it unique when compared to other BMP/TGFβ/Wnt secreted inhibitors which are expressed only zygotically and maintained post-gastrulation. We propose that a function of 51-B6 is to block BMP and TGFβ signals in the ectoderm in order to regulate cell fate specification and competence prior to the onset of neural induction. In addition, we demonstrate that 51-B6 can act as a neural inducer and induce ectopic head-like structures in neurula staged embryos. Because of this embryological activity, we have renamed this clone Coco, after the Spanish word meaning head.

Key words: Coco, BMP, Wnt, Nodal, Gastrula, *Xenopus laevis*

INTRODUCTION

Current models for neural induction propose that the initial specification of the neural territory takes place during gastrulation following a local inhibition of BMP signaling in the dorsal ectoderm overlaying the organizer region in amphibians (reviewed by Muñoz-Sanjuán and Brivanlou, 2002; Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1994). To this effect a large variety of secreted BMP antagonists are produced by the dorsal mesodermal cells of the organizer (Muñoz-Sanjuán et al., 2002). The expression and activity of these antagonists provided a molecular explanation for the initial observation that cells of the dorsal organizer region had the potential to induce the formation of a nervous system when transplanted to ectopic locations (Spemann and Mangold, 1924).

Although the cell-fate choice of ectodermal cells is traditionally thought to involve a decision to become epidermal or neural, it is known that ectodermal cells prior to gastrulation are pluripotent. In particular, ectodermal cells can adopt mesodermal fates if exposed to mesoderm-inducing signals during a defined window of time (‘competence’ window) that ends after gastrulation in *Xenopus*. Therefore, in order for correct ectodermal patterning to take place, cells in the prospective ectoderm must avoid exposure to mesoderm-inducing signals. The endogenous mesoderm-inducers are likely to be members of the TGFβ superfamily, in particular the nodal-related members (Harland and Gerhart, 1997; Schier and Shen, 2000). It is also thought that expression of vegetally localized maternal factors (VegT) act in the embryo to promote mesodermal gene expression through the activation of TGFβ signals (Heasman, 1997; Xanthos et al., 2002). Therefore it is likely that a TGFβ inhibitor would be expressed in the animal region of the early embryo (and ectoderm) in order to restrict the effect of diffusible nodal signals to the vegetal and equatorial regions of the embryo.

We describe the identification and characterisation of a novel member of the Cerberus/Dan/Gremlin superfamily of secreted BMP inhibitors (Bouwmeester et al., 1996; Hsu et al., 1998; Rodriguez Esteban et al., 1999; Stanley et al., 1998; Piccolo et al., 1999). This gene, which we have termed *Coco*, was initially identified as a gene differentially regulated by Smad7, a neural inducer, in ectodermal explants in a microarray-based screen (Muñoz-Sanjuán et al., 2002). *Coco* is expressed maternally in

an animal to vegetal gradient, and later on is restricted to the animal region of the embryo. *Coco* is expressed broadly within the ectoderm and this expression declines rapidly following gastrulation. We show that Coco can inhibit signaling mediated by BMP, TGFβ and Wnt ligands, and can act to inhibit mesoderm formation in vivo and in explants. In addition, expression of Coco in ectodermal cells changes their responsiveness to mesoderm-inducing signals. Based on these results, we propose that the expression and bioactivities of Coco are consistent with it being a bone fide inhibitor of mesodermal signals that acts within the animal region of the embryo to inhibit TGFβ signals.

MATERIALS AND METHODS

Embryo injections and preparation of RNA

Xenopus Coco RNA was made by linearising with *Asc*I and transcribing using the mMessage mMachine in vitro SP6 transcription kit from Ambion. Embryos were injected in either the animal pole or the ventral vegetal/VMZ with 1 ng of RNA. All injections were done with the *Xenopus* gene.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed on animal and DMZ/VMZ explants as has been described previously (Wilson and Melton, 1994). Ornithine decarboxylase (ODC) was used as a loading control.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisations were carried out as described previously (Harland, 1991). In situ probes were made as described elsewhere: *brachyury* (Smith et al., 1991), *emx1* (Pannese et al., 1995), *en* (Hemmati-Brivanlou et al., 1991), *Fgf8* (Christen and Slack, 1997), *goosecoid* (Cho et al., 1991), *hb9* (Wright et al., 1990), *nkx2.5* (Raffin et al., 2000) and *rx* (Mathers et al., 1997). Embryos were embedded in 20% gelatin/PBS and fixed overnight in 4% PFA at 4°C. Sections were cut at 100 μm using a vibratome.

Interaction of Coco with Xnr1, BMP4, Wnt8 at a biochemical level

Coco was flag-tagged in the C terminus by standard PCR methods. Flag-tagged *Coco* was co-injected into embryos at the 2-cell stage with BMP4-HA or Xnr1-HA. Protein extracts were made at stage 10-11, immunoprecipitated with an anti-HA polyclonal antibody, and probed with an anti-flag monoclonal antibody.

Inhibition of Wnt8 and BMP4 promoter activity by Coco

Injections were made in the animal pole of 4-cell stage embryos with 25 pg of reporter gene DNA, 10 pg *Wnt8* RNA (Hoppler et al., 1996) or 100 pg *Bmp4* RNA (Hata et al., 2000), with or without addition of 1 ng *Coco* RNA. Embryos were recovered at stage 9 for TOP-FLASH (a Wnt-responsive promoter) activity, and stage 10.5 for Bmp response element (BRE) activity. Luciferase transcription assays were performed with the Luciferase Assay (Promega Corp., Madison, WI) as described (Vonica et al., 2000).

Competence assay

Embryos were injected at the 2-cell stage with *Coco* RNA and animal cap explants were cut at stage 8. Activin-conditioned medium was added to uninjected and *Coco*-injected explants at stages 8, 9, 10 and 11. Explants that were beginning to heal were carefully reopened prior to addition of activin. Explants were harvested at stage 12/13 and analyzed for the induction of mesodermal markers by RT-PCR.

RESULTS

Coco is a member of the Cerberus/Dan/Gremlin superfamily of BMP inhibitors

We identified *Coco* as an upregulated transcript in a large-scale microarray-based screen aimed at identifying genes differentially regulated by Smad7 in embryonic ectoderm (Muñoz-Sanjuán et al., 2002). *Coco* encodes a 25 kDa protein with a predicted secretory signal sequence (Fig. 1A) with closest similarity to Cerberus and Caronte (Fig. 1B). The homology among these family members is low and resides mainly in the spacing of the 9 cysteines in the core domain (Fig. 1B). Using *Xenopus* Coco sequences to search NCBI and Celera databases, we identified human, mouse and *Fugu* homologs with closest homology to Coco in the genome (Fig. 1B). Human and mouse Coco map to 19p13.2 and 8, respectively, which are syntenic. Mouse Coco is a partial sequence derived from genomic searches, that lacks the 5′ region, as no full-length ESTs for this gene are available. A partial human Coco was assembled from an EST (GenBank BC025333) and genomic hits, as no full-length cDNA has been reported. Interestingly, as in other family members, the core cysteine-rich domain is encoded by a single exon. The Coco protein contains a putative signal sequence (Fig. 1A), and Coco is constitutively secreted following transfection into mammalian culture cells. Similarly to what has been reported for Cerberus protein (Piccolo et al., 1999), we have observed two distinct products in conditioned media, suggesting that Coco protein might undergo proteolytic cleavage in mammalian cells (not shown). Consistently with this observation, we found two putative cleavage sites (RRK; underlined in Fig. 1A) similar to the single site found in Cerberus, suggesting that proteolytic cleavage might be functionally important for Coco's bioactivities.

Coco is the earliest expressed BMP/TGFβ inhibitor in *Xenopus laevis*

Based on the sequence homology between Coco and related members, we postulated that Coco would be a BMP antagonist. However, based on sequence alone, we could not predict whether Coco would interact with other signaling factors of the Wnt and TGFβ families. In order to evaluate whether Coco could function in vivo in the context of BMP signaling, we analyzed its expression during early embryogenesis, when BMP signaling plays a critical role in dorsoventral patterning and neural induction (Muñoz-Sanjuán and Brivanlou, 2002).

As shown by in situ hybridisation and RT-PCR, *Coco* is strongly expressed maternally and at the gastrula stage, however levels of *Coco* sharply decline in the embryo after stage 12 (Fig. 2A-C). In the egg, *Coco* mRNA is expressed in the animal pole and overlaps with that of *Bmp4* mRNA expression (Fig. 2A, top panels). We did not detect expression of *Coco* in the vegetal pole, in contrast to *VegT* (Zhang and King, 1996) mRNA, which is most strongly expressed vegetally (bottom panel). This result suggests that *Coco* mRNA is maternally localized to the animal pole, and that a gradient of *Coco* message exists in the egg and early embryo. In order to independently evaluate this observation, we compared, by RT-PCR the expression of *Coco* and *Vg1* (Weeks and Melton, 1987), a member of the TGFβ family expressed vegetally. *Vg1* is expressed in the vegetal pole and to a lesser extent in the

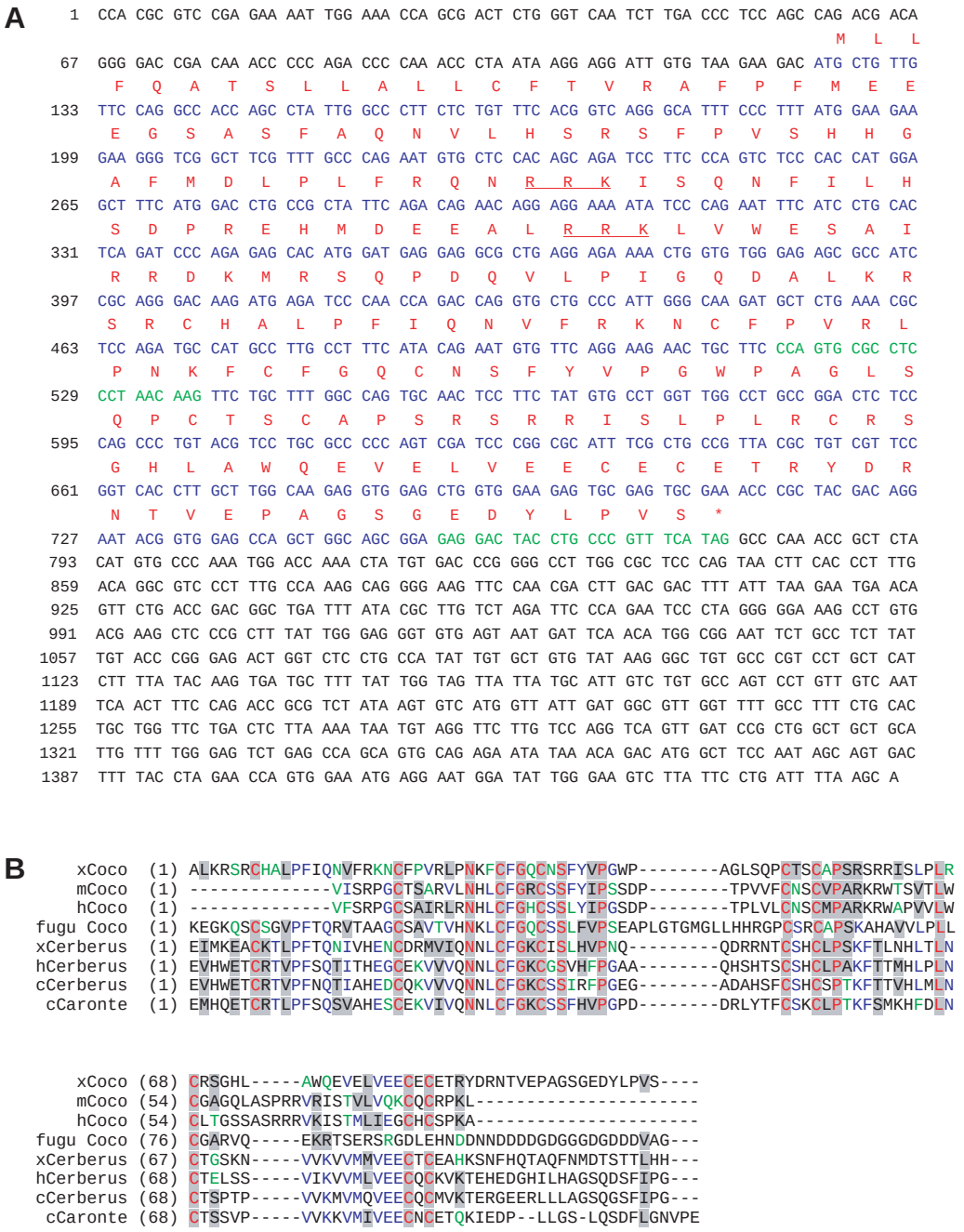

Fig. 1. Identification of Coco, a novel BMP inhibitor. (A) Nucleotide sequence of *Xenopus* Coco. ORF in blue and green. Green text indicates primers used for the RT-PCR. Translation is shown in red. RRK are putative cleavage sites similar to that found in Cerberus (Piccolo et al., 1999). (B) Alignment at the amino acid level of *Xenopus*, *Fugu*, human and mouse Coco and other family members, Cerberus and Caronte.

equatorial region. These results collectively suggest that there are two opposing gradients of maternal RNA localization in the egg, one animal to vegetal (exemplified by *Coco*), and the second vegetal to animal (exemplified by *VegT* and *Vg1*). Given Coco's biological activities, the animal-vegetal gradient of *Coco* RNA might act to restrict the activities of vegetally localized TGFβ ligands to the vegetal pole and equatorial region, where *Coco* expression is less pronounced.

At pre-gastrula embryonic stages, Coco is expressed in the animal pole exclusively (Fig. 2B). At gastrula, *Coco* mRNA transcripts are detected in both the dorsal marginal zone (DMZ; including the organizer, see *) and the ventral marginal zone (VMZ) and at very high levels in the animal cap ectoderm (Fig. 2D,E). There are also very low levels of expression in the vegetal pole (Fig. 2E). To date, Coco is the only known BMP inhibitor expressed maternally and ubiquitously within the ectoderm prior to neural induction. By contrast, Cerberus is expressed zygotically between stages 9 and 13 (Fig. 2C) (Bouwmeester et al., 1996) and is restricted to the anterior endoderm of the organizer at gastrula stages (Bouwmeester et

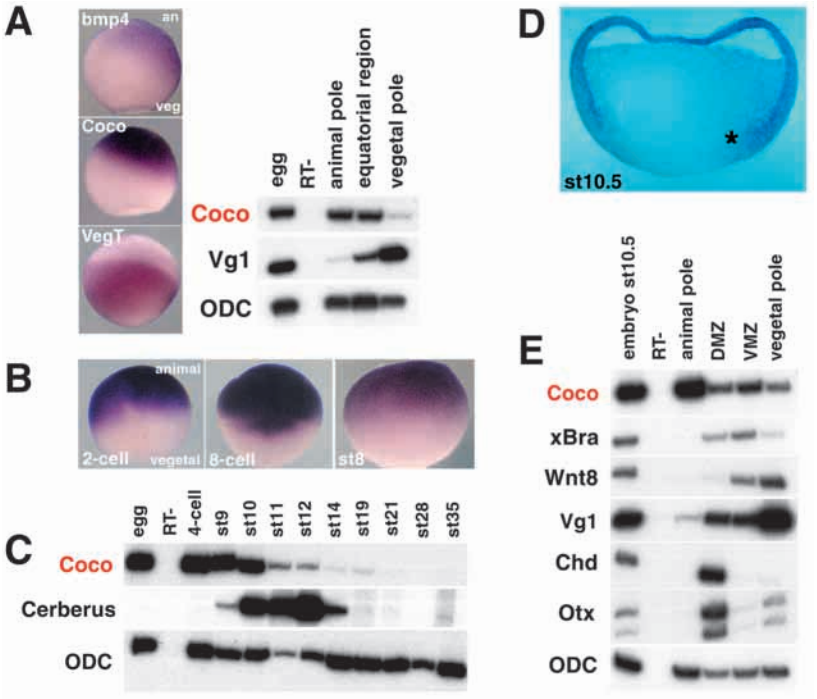

Fig. 2. Expression pattern of *Coco* mRNA during *Xenopus* development. (A) Whole-mount in situ hybridisation of *Coco*, *Bmp4* and *VegT* mRNA in the egg. Expression of *Coco* was also compared to *Vg1* by RT-PCR. (B) *Coco* is expressed strongly in the animal pole at the 2-cell, 8-cell stages and at stage 8. (C) *Coco* is strongly expressed maternally and then is downregulated at the post-gastrula/pre-neurula stages. In contrast *Cerberus* is first expressed at stage 9 and then downregulated after the onset of neurulation. (D,E) At gastrula stage *Coco* is detected at high levels in the ectoderm and marginal zones by (D) whole-mount in situ hybridisation and (E) as seen by RT-PCR at much lower levels in the vegetal pole. ODC was used as a loading control for the RT-PCR. * indicates the organizer.

al., 1996). The maternal expression of Coco, its widespread expression within the ectoderm, and the rapid decline in *Coco* mRNA levels following gastrulation prompted us to evaluate Coco's function in the context of BMP and TGFβ inhibition during ectodermal patterning.

Biological activities of Coco in early *Xenopus* development

As a first test for Coco's biological activities, we injected its mRNA into embryos at either the 2- or 4-cell stage. Our initial analysis was done at gastrula stages where *Coco* is expressed throughout the ectoderm and marginal zones. In the gastrula both *brachyury* and *Fgf8* are expressed in a ring of mesodermal cells around the vegetal pole (Fig. 3A,B, top panels). After injection of *Coco* in one of the two cells in the vegetal pole (Fig. 2), we found that both markers are repressed (Fig. 3A,B, lower panels), suggesting that Coco can inhibit mesoderm formation in vivo. Coco expands the size of the endogenous organizer as judged by the increase in expression of *Otx2* (Fig. 3C) and *Gsc* (Fig. 3D). In addition, the endogenous ectodermal expression of *Otx2* is increased (Fig. 3C, lower panel) and there are ectopic ectodermal patches of *Otx2* expression on the contralateral sides of the embryo (not shown), suggesting that Coco acts in cell non-autonomous manner. The inhibition of pan-mesodermal gene expression at gastrula stages suggests that Coco might inhibit mesodermal signals, probably through an inhibition of Nodal/Activin pathways (Schier and Shen, 2000), the presumed endogeneous mesoderm inducers.

The embryological consequences of expressing BMP inhibitors include expansion of dorsoanterior structures. In order to test the effects of *Coco* misexpression on dorsoventral patterning and anterior neural development, we analyzed *Coco*-injected embryos at tadpole stages. Overexpression of *Coco* in the animal pole results in embryos with expanded anterior structures and ectopic cement glands (compare Fig. 3E with 3F). In contrast, overexpression of *Coco* ventrally results in posterior truncations and the induction of extra anterior structures (75% of injected embryos have this phenotype; Fig. 3G). Very infrequently these extra structures also contain a single eye (5% of cases; not shown). Molecular analysis of these ectopic structures shows that they contain forebrain and midbrain tissue, as shown by the ectopic expression of the forebrain markers *Rx* (Fig. 3H), *Emx1* (Fig. 3I), *Otx2* (Fig. 3J), and the midbrain marker *En2 (*Fig. 3K). *En2* expression is detected where the ectopic head contacts the main dorsal axis of the embryo (see *, Fig. 3K, lower panel). By contrast, we failed to detect ectopic expression of *Hoxb9*, a marker of spinal cord (Fig. 3L). In addition, we have shown that there is no muscle tissue in the ectopic structures (Fig. 3N), although the heart marker *Nkx2.5* was strongly induced around the extra cement gland (Fig. 3M). However, we never detected ectopic hearts in the *Coco*-injected embryos, possibly because of the lack of endoderm formation in *Coco*-injected embryos (not shown and Fig. 4).

These phenotypes, ectopic anterior tissue including head structures, are consistent with an inhibitory activity of BMP and Wnt signaling by Coco (Glinka et al., 1998; Piccolo et al., 1999). In order to unravel the molecular mechanism underlying Coco's activity, we analyzed fate changes in embryonic explants by RT-PCR for a variety of molecular markers. When embryos were injected at the 2-cell stage in the animal caps, we failed to detect markers for the organizer or endoderm in gastrula-staged explants (not shown), but we observed a decrease in epidermal markers, and an increase in the early neural marker, β-tubulin at mid-gastrulation (Fig. 4A). This result suggests that Coco has the ability to inhibit BMP signaling. By stage 21, the pan-neural markers (*Ncam* and *nrp1*) and anterior-specific markers (*Otx* and *XAG*) are induced in explants expressing *Coco* (Fig. 4B), suggesting that Coco can neuralize ectodermal explants, consistent with an inhibition of BMP signaling (Wilson and Hemmati-Brivanlou, 1995). Similar to results seen with Cerberus overexpression

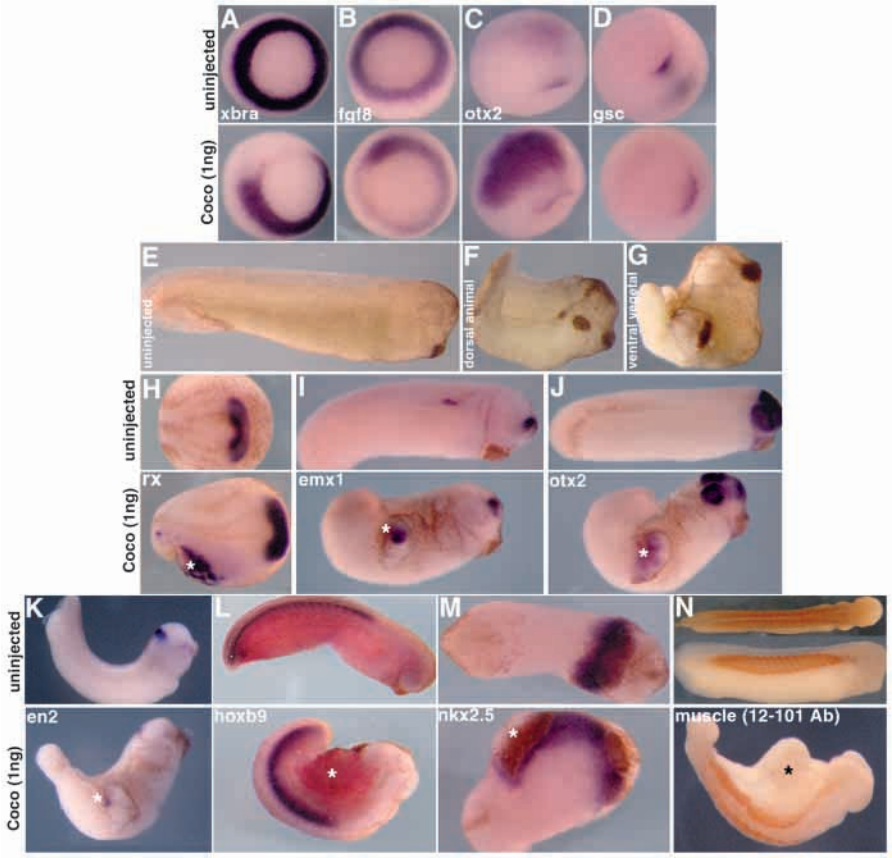

Fig. 3. Phenotypes resulting from *Coco* overexpression in *Xenopus* embryos. Analysis of injected embryos at gastrula (A-D), neurula (H) and early tadpole stages (E-G,I-N). In A-D and H-N, the top panels are uninjected embryos, the lower panels are embryos injected with 1 ng *Coco* vegetally in 1 cell at the 2-cell stage (A-D) or ventrally in one cell at the 4-cell stage (G-N). (A) *Xbra*/brachyury (a marker of mesoderm); (B) *Fgf8*, also a mesoderm marker, (C) *Otx2*, (the organizer and anterior ectoderm); (D) *gsc* (organizer); (H) *rx*, (forebrain); (I) *Emx1* (dorsal telencephalon); (J) *Otx2*, forebrain and midbrain; (K) *En2* (midbrain/hindbrain boundary); (L) *Hoxb9* (spinal cord); (M) *Nkx2.5* (heart) (N) 12-101 Ab (muscle). (E-G) Phenotypes resulting from (E) no injection (control embryo); (F) dorsal animal injection at the 2-cell stage; (G) injection in one ventral vegetal cell at the 4-cell stage. *indicates the extra head structures. A,B,D are vegetal views; C, lateral with animal pole to the top; E-G,I-K, lateral views; H,L,N dorsal views; M, ventral view. In E-N, anterior is to the right.

(Bouwmeester et al., 1996), Coco also induced *Nkx2.5* in this assay (Fig. 4B).

It has previously been shown that Cerberus can neuralize VMZ explants (Bouwmeester et al., 1996). We injected *Coco* mRNA into the VMZ at the 4-cell stage and analyzed its effects on cell fate determination in VMZ explants isolated at gastrula stages or for morphological changes at tadpole stages (stage 27) to test whether Coco can also neuralize ventral tissue (Fig. 4C-E). At the gastrula stage, the organizer markers *chordin* and *goosecoid* were weakly induced in the VMZ expressing Coco, whereas the expression of *brachyury* was suppressed (Fig. 4D), consistent with the in vivo results that Coco blocks mesoderm formation and neutralizes the embryo. At stage 27, the morphology of the VMZ+*Coco* explants were similar to that of the DMZ explants (Fig. 4C), and the injected explants contained anterior neural tissue and cement glands, but not dorsal mesodermal derivatives, such as muscle and notochord (Fig. 4C). During normal development at tailbud stages (stage 27), VMZ explants do not express the neural markers *Ncam*, *nrp1* and *Otx2*, or the cement gland marker *XAG*. In VMZ explants expressing *Coco* all of these markers are now induced (Fig. 4E), consistent with Coco blocking both mesoderm and ventral ectoderm (epidermis) inducing signals mediated by BMPs. The neural fate acquisition of VMZ explants expressing *Coco* and the absence of dorsal mesodermal markers strongly suggests that Coco acts to neuralize the explants, rather than having an effect on dorsalisation of the mesoderm. Therefore, although Coco can block BMP signaling, the lack of dorsal mesodermal gene expression highlights the notion that Coco efficiently blocks signaling by mesodermal inducers and might act endogenously to inhibit mesodermal gene expression in the ectoderm.

Coco can inhibit signaling by BMP, Nodal, Activin and Wnt signaling

In order to test the inhibitory interactions of Coco with BMPs, TGFβ members and Wnts, we co-injected *Coco* for animal cap assays with RNAs of BMP4, Xnr1 [nodal-related factor-1 (Hyde and Old, 2000)], Wnt8 (Sokol and Melton, 1991) or Activin (Fig. 5) (Smith et al., 1990), and monitored the expression of immediate response genes normally activated by these signaling molecules in the ectoderm. For instance, it has been shown that both *Xbra* and epidermal *keratin* expression are upregulated in animal caps following overexpression of BMP4. In this assay, Coco blocked induction of these markers (Fig. 5A). In similar assays, Coco could also block Wnt8 induction of *Xnr3* and *siamois* expression (Fig. 5B) (Sokol and Melton, 1991) and Nodal and Activin (Smith et al., 1990; Sokol and Melton, 1991) signaling, as detected by the inhibition of the expression of Chordin, Brachyury and Wnt8 induced by *Xnr1* and Activin (Fig. 5C,D).

Based on the expression and biological activities of Coco, we propose that an endogenous role of Coco might be to regulate fate determination in the ectoderm through an inhibition of TGFβ signals. In order to test whether Coco can interact with BMP/TGFβs proteins, we co-injected synthetic RNAs encoding tagged Coco protein together with tagged BMP4 or Xnr1 constructs into animal caps and tested for direct binding in immunoprecipitation experiments (Fig. 5E,F). Indeed, we found that we can detect biochemical binding and

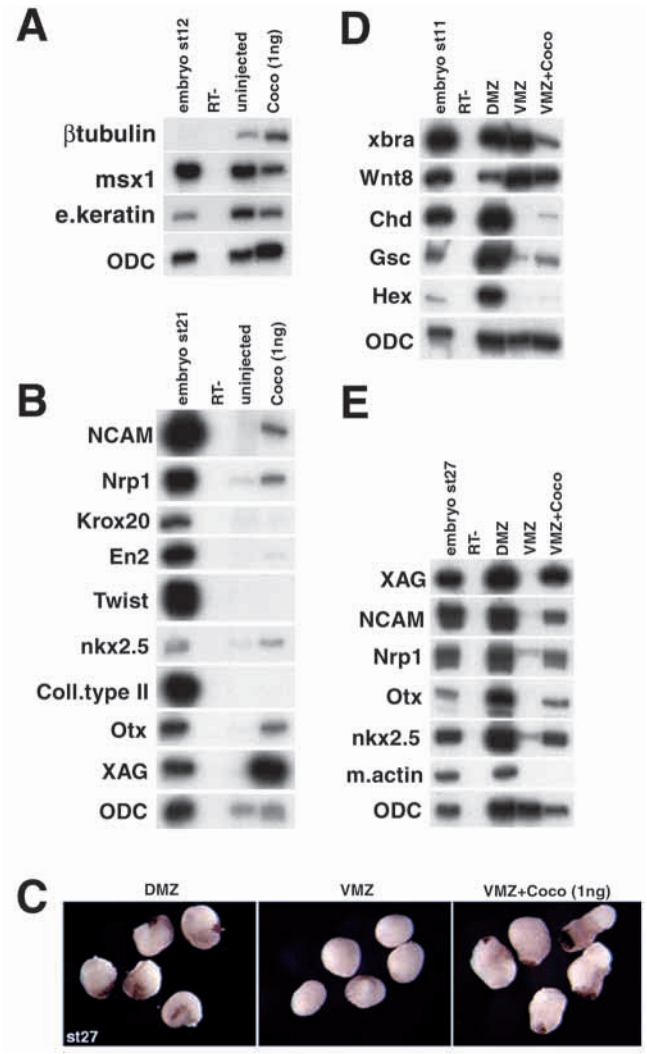

Fig. 4. Dorsalization effects of Coco in embryonic explants. (A) Animal caps injected with 1 ng *Coco* at the 2-cell stage and analyzed for the expression of epidermal, mesodermal and neural markers. (B) Animal caps analyzed for neural induction at early tadpole stages. Both the general neural markers *Ncam* and *nrp1* have been induced as well as anterior markers *Otx* and *XAG*. (C) Morphology of the VMZ+Coco explants compared to control DMZ and VMZ. (D) RT-PCR analysis of VMZ explants injected with 1 ng *Coco* compared with DMZ and VMZ of uninjected explants at gastrula stages. The organizer markers *chordin* and *goosecoid* are induced in the VMZ+Coco explants. (E) Analysis at tadpole stages. The VMZ+Coco now expresses dorsal molecular markers. ODC was used as a loading control.

immunoprecipitate Coco protein with BMP4 and Xnr1 in this assay. We postulate that this interaction is likely to inhibit the signaling input of the two TGFβ ligands. In addition, and as an independent way to assess whether Coco's bioactivities are due to direct interference with the BMP and Wnt signaling pathways, we tested whether Coco could prevent the transcriptional activation of Wnt and BMP-responsive promoters (Fig. 5G,H). Embryos were injected the Wnt-responsive promoter TOP-FLASH (Hoppler et al., 1996) together with *Wnt8* RNA or co-injected with *Wnt8* and *Coco* RNAs (Fig. 5H). Indeed, we found that there was a significant repression of this promoter by Coco. Similarly, Coco was able to completely inhibit the activation of the *Bmp4* responsive promoter [Bmp Response Element, BRE4 (Hata et al., 2000)] by BMP4 (Fig. 5G).

In addition we tested whether Coco could inhibit the activity of another signaling molecule, FGF. We cultured animal caps with or without *Coco* in the presence of FGF and analyzed them at neurula stages for mesoderm formation. We found that in this assay Coco could not prevent mesoderm formation, suggesting the activity of Coco is specific for selected signaling molecules (data not shown) and is not promiscuous.

Involvement of Coco in ectodermal competence

The maternal expression of *Coco* makes it a unique gene among the large family of BMP inhibitors. Several BMPs, Wnts and Nodal-related factors (Cui et al., 1995; Hemmati-Brivanlou and Thomsen, 1995; Onuma et al., 2002) are inherited maternally. However, no inhibitors are reported to be expressed during these early stages. Therefore, a potential function of *Coco* might be to block maternal signaling by these molecules in the prospective ectodermal space. *Coco* is widely expressed in the ectoderm until the end of gastrulation in *Xenopus* at stage 12. The timing of the decline of the mRNA coincides with the loss of competence of ectodermal cells to respond to mesoderm-inducing signals (Green et al., 1990; Domingo and Keller, 2000).

Therefore, we investigated whether the presence of *Coco* in the ectoderm at specific stages could inhibit mesoderm formation or affect the competence of the ectoderm to respond to mesoderm-inducing signals. Animal cap explants can respond to activin and become mesoderm, although the responsiveness of the explants declines over time until stage 12, at which point they are no longer able to respond (Green et al., 1990). We therefore tested whether *Coco* RNA would alter this responsiveness temporally or qualitatively, by exposing dissected caps to activin protein at different time points (Fig. 5I). Indeed, animal caps expressing *Coco* responded differently to Activin over time (Fig. 5I). In contrast to control explants, *Coco*-injected caps failed to express mesodermal markers following Activin exposure at earlier stages, suggesting that Coco can indeed change the timing of the responsiveness of ectodermal cells to Activin. It is noteworthy that the levels of *Coco* RNA used in this experiment are not sufficient to block mesoderm induction following exposure to activin protein from the blastula stages (Fig. 5I). However, if Activin was added to *Coco*-injected caps at or after stage 10 (beginning of gastrulation), no mesoderm induction was observed compared to control explants. These results strongly suggest that Coco changes the responsiveness of the ectoderm to mesoderm inducing signals, and that the effect might not necessarily be due to direct binding exclusively. Although it is possible that Coco induces a prior fate change in the ectoderm, which would lead to an altered responsiveness of the explants to Activin, this result is consistent with the in vivo inhibition of mesodermal gene expression by Coco. Furthermore, the secondary structures induced by *Coco* expression lack axial tissues (Fig. 3N), and the primary axis shows a loss of axial muscle tissue, further suggesting that Coco inhibits mesoderm formation in vivo.

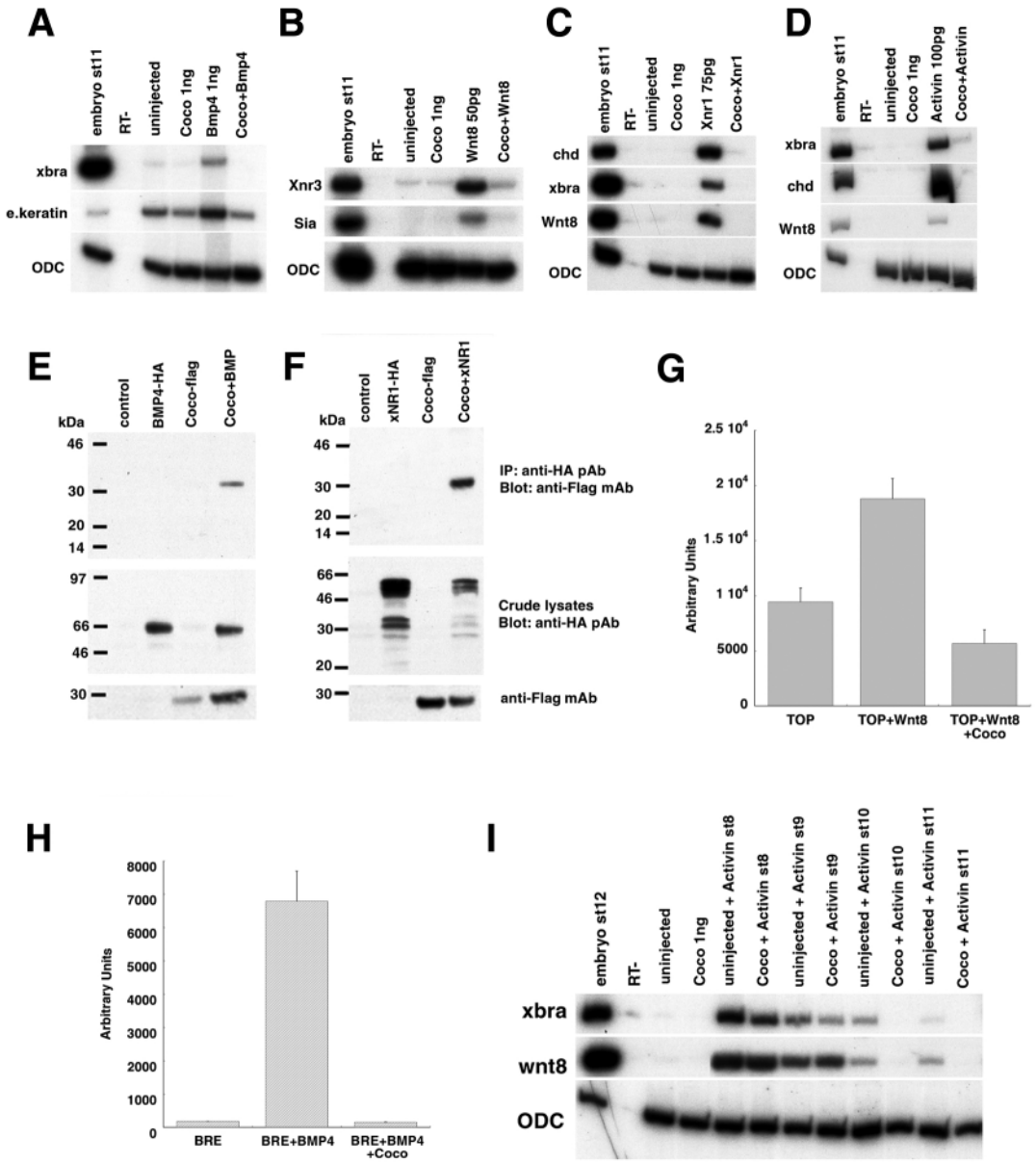

Fig. 5. Inhibitory effects of Coco on BMP, TGFβ and Wnt signaling. (A) *Bmp4* and *Coco* RNAs were injected separately, and together, into embryos at the 2-cell stage. Animal caps were analyzed at gastrula stages for the presence of Xbra and epidermal keratin. Coco blocked the induction of both these markers by BMP4. (B) *Wnt8* and *Coco* were injected into animal caps and the markers Xnr3 and Siamois analyzed. Coco blocked the induction of these markers by Wnt8. (C) Inhibition of nodal signaling by Coco. Coco blocked the induction of chordin, Xbra and Wnt8 by Xnr1. (D) Inhibition of activin signaling by Coco. (E,F) Direct binding of Coco to BMP4 and Xnr1. Flag-tagged Coco was co-injected with HA-BMP4 (E) and HA-Xnr1 (F). Coco inhibited the activation of both the Wnt8-responsive promoter TOP-FLASH (G) and the BMP response element (H). (I) Ectodermal competence assay. Ectodermal explants, either uninjected or *Coco*-injected, were exposed to activin-conditioned medium at different stages. Notice that in the presence of Coco, explants are unable to respond to activin from stage 10 onwards.

DISCUSSION

Ectodermal fate determination is thought to occur through an initial modulation in overall levels of BMP signaling prior to neural induction (Muñoz-Sanjuán and Brivanlou, 2002). The initial specification of the neural territory in *Xenopus* probably takes place as a consequence of the effects of the secreted BMP antagonists localized to the organizer region. Although the ultimate fates of ectodermal cells following gastrulation encompass epidermal and neural derivatives, the ectoderm has the potential to become mesoderm, as has been shown by a variety of in vivo (Green et al., 1990) and in vitro assays (Domingo and Keller, 2000). In essence, ectodermal cells are pluripotent prior to gastrulation, this competence being lost at a very discrete window of time in development that coincides with the end of gastrulation in amphibians, and with the downregulation of *Coco* mRNA expression. Therefore, it is predicted that inhibitors of mesoderm formation must act

locally within the ectoderm to ensure that ectodermal cells will adopt either an epidermal or a neural fate. However, no candidate genes that were expressed in the appropriate spatial and temporal domains had been found. We propose that Coco is such a factor, given its biological activities and expression patterns.

The induction of mesoderm in vivo is thought to occur as a consequence of TGFβ signaling (Smith et al., 1989; Harland and Gerhart, 1997; Schier and Shen, 2000). In particular, the role of Nodal signaling in mesoderm specification is strongly supported by biochemical and genetic evidence (Schier and Shen, 2000). Therefore, it is predicted that Nodal signals must be blocked to allow ectoderm to be appropriately patterned (Thisse et al., 2000) during gastrulation. This suggests that a function of Coco might be to inhibit mesoderm-inducing signals operating in the ectoderm. We have shown that Coco can bind and inhibit Xnr1, as well as BMP4, Activin and Wnt8. These results, combined with the overall inhibitory effects of Coco on mesodermal gene expression in vivo and in explants, suggests that Coco's bioactivities are largely due to its inhibitory effects on nodal and activin signaling. Amongst the known TGFβ inhibitors, Coco is the only member whose expression is consistent with a role in inhibiting mesodermal signals in the ectoderm. By contrast, the other two known Nodal inhibitors, Antivin (Tanegashima et al., 2000) and Cerberus (Piccolo et al., 1999), and the Wnt inhibitor Dkk (Glinka et al., 1998) are not expressed during those stages in the ectoderm. Further experiments are required such as loss of function of Coco to confirm this role of Coco in embryonic patterning.

The TGFβ inhibitory activities of Coco might also act to restrict the mesodermal domain to the characteristic ring of cells prior to involution. The animal-to-vegetal gradient of *Coco* RNA in the egg and early embryo suggest that two opposing gradients of TGFβ activity might act to shape the future mesodermal domains in the embryo. It has been well established that the vegetally localized gradients of VegT and Vg1 expression act to promote mesoderm formation. Therefore, Coco activity might act to restrict the activity of Vg1 and potentially other TGFβ ligands to the vegetal and equatorial regions of the embryo, and ensure a tight domain of mesodermal gene expression.

Altogether, we have identified a maternal BMP, TGFβ and Wnt inhibitor, whose expression and biological activities are consistent with a role in the regulation of ectodermal competence, to ensure proper ectodermal patterning during gastrulation. *Coco* expression in the ectoderm might also act to lower overall levels of BMP signals, so that additional BMP inhibitors expressed in the organizer can induce the formation of the nervous system. Therefore, expression of *Coco* in the entire ectodermal region prior to gastrulation might act to prevent fate specification in the ectoderm and ensure the maintenance of the stem-cell-like properties exhibited by ectodermal cells (Tiedemann et al., 2001). Interestingly, the mouse and human homologs of Coco are also expressed in undifferentiated, multipotent stem cells suggesting that this potent new inhibitor might fulfil similar functions during mammalian embryogenesis.

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