Maternal Mga is required for Wnt signaling and organizer formation in the early Xenopus embryo

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Maternal Wnt11 is both necessary and sufficient for the formation of Spemann organizer in Xenopus embryo. Xnr3 and Siamois have been identified as the direct target genes of maternal Wnt11/β-catenin during organizer induction. The depletion of maternal XTcf3 resulted in the ectopic expression of Xnr3 and Siamois, suggesting the activity of β-catenin/XTcf3 is strictly regulated in the early Xenopus embryos. Here, we show that Xenopus mga (Xmga) is a maternal gene required for dorsal axis formation. Overexpression experiments indicate that mouse Mga potentiates the activity of β-catenin in the induction of organizer-specific genes. Depletion of maternal Xmga results in the dramatic decrease of the expression of organizer genes and ventralization phenotype, indicating that Xmga is required for β-catenin function and organizer formation. Depletion of XTcf3 cannot rescue organizer gene expression and axis formation in Xmga-depleted embryos, suggesting Xmga is downstream of XTcf3 during organizer induction. We conclude that maternal Xmga is critical for the function of β-catenin during organizer formation and dorsal development of Xenopus embryo. To our knowledge, this is a report for the first time to implicated Mga in regulating Wnt signaling.

Keywords Xenopus; maternal genes; Mga; Wnt pathway; Spemann organizer; TCF4

Introduction

Spemann organizer induction is essential for the dorsal ventral axis development in the early Xenopus embryos. Our previous studies have shown that maternal XWnt11 is an essential component of maternal dorsal determinants [1]. Maternal XWnt11 mRNA is stored in the vegetal cortex of full-grown Xenopus oocyte [2]. After fertilization, as a result of cortical rotation XWnt11 mRNA is concentrated in one side of embryo from which organizer will be induced during mid- to late blastula stages. β-catenin is absolutely required for XWnt11-mediated organizer induction [1,3,4]. The function of organizer in dorsal development depends on the proper expression of a panel of organizer specific genes [5,6], among which Xnr3 and Siamois are well-characterized direct targets of maternal XWnt11/β-catenin signaling [7–10]. Xnr3 regulates the expression of Xbra in the organizer region and the convergent extension during gastrulation [11], and overexpression of Siamois can restore the dorsal axis formation in the embryos depleted of β-catenin [12].

Transcription factors of TCF3/LEF1 family are the primary nuclear targets regulated by nuclear β-catenin [13]. XTcf1, 3, and 4 are ubiquitously stored in the full-grown oocytes [14]. Depletion of maternal XTcf3 causes the over-activation of Xnr3 and Siamois in the organizer region and the ectopic expression of Xnr3 and Siamois in the non-organizer region, indicating XTcf3 primarily functions as a transcription repressor and is not necessary for the activation of Xnr3 and Siamois in organizer [15]. In the early development of zebrafish embryos, Tcf3 (headless) has also been shown to function as a transcriptional repressor [16,17]. Maternal XTcf1 can function as an activator or a repressor depending on the cellular context. Depletion of maternal XTcf1 results in the decreased expression of Xnr3 and Siamois in the organizer region, but the ectopic expression of these genes in the ventral side of early gastrula embryos. Maternal XTcf4 is required for the full activation of Xnr3, goosecoid, and chordin, but not for Siamois [14]. These results indicate that the activity of maternal Wnt/β-catenin signaling is under strict regulation by multiple transcriptional factors during organizer induction.

Mga was first characterized as a dual specificity transcription factor containing a typical T-domain in its N-terminal half and a bHLHZip domain in its C-terminal half [18]. Biochemical studies showed that Mga interacts with Max presumably through its bHLHZip domain. The T-domain of Mga is closely related to that of Brachyury (T). In vitro reporter assay showed that both T-domain and
Mga positively regulates Wnt signaling

bHLHZip domain are involved in its transcriptional activity. Mga is expressed in multiple tissues including developing mesoderm during mouse embryonic development [18]. Genes homologous to mouse Mga have been identified in all vertebrate model organisms, but not in invertebrates [19]. The function of zebrafish Mga during organogenesis has been reported to be required for the normal development of the head, heart, and gut [20]. However, the physiological role of maternal Mga in early vertebrates remained unknown until this report.

Here, we demonstrated that Mga plays a critical role for the organizer induction and dorsal axis formation in the early Xenopus embryo. Depletion of maternal Xmga resulted in significant reduction of the expression of Wnt target genes (Xnr3 and Siamois), strikingly similar to the effect of β-catenin depletion. Mouse Mga mRNA could partially rescue the organizer gene expression and the dorsal axis formation in the embryos depleted of Xmga, suggesting Mga plays a conserved role in the regulation of Wnt signaling in the embryonic development of vertebrates. We also reported here that the expression of Wnt11 target genes and organizer formation could not be rescued by simply attenuating the repressing effects of XTcf3 when maternal Xmga was depleted. To our knowledge, these findings for the first time strongly suggest that Xmga is an activator required for efficient Wnt/β-catenin signaling during organizer induction.

Materials and Methods

Oocytes and embryos
Xenopus laevis females and males were imported from the Nasco Inc. (Fort Atkinson, USA). Stage VI oocytes were manually defolliculated using a pair of watchmaker forceps and cultured in oocyte culture medium (OCM; 60% manually defollicuted using a pair of watchmaker forceps and 40% human chorionic gonadotropin-stimulated 

Materials and Methods

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Approximately one-fifth embryo equivalent of RNA was used for cDNA synthesis with oligo (dT18) primers followed by real-time reverse transcription-polymerase chain reaction (RT-PCR) and quantitation using Light-Cycler™ system 1.5 (Roche Diagnostic GmbH, Mannheim, Germany) as described by Kofron et al. [24]. Relative expression values were calculated by comparison with a standard curve generated by serial dilution of un-injected control cDNA. Samples were normalized to levels of ODC (ornithine decarboxylase), which was used as a loading control. The normalized expression levels of genes of interest were denoted as gene-N in all histogramical presentations of the quantitative RT-PCR results. Samples of water alone or controls lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products in all cases.

The primer pairs used for RT-PCR analysis in this study are listed in the Table 1.

## Results

**Mga potentiates the activity of β-catenin on the induction of organizer-specific genes**

Mouse Mga encodes a large nuclear protein containing a T-domain in its N-terminal part and a bHLHZip domain in its C-terminal part [18]. Sequence analysis indicates that Xenopus mga is 47% identical to mouse Mga protein. T-domains of Xmga and mouse Mga share 84% identity [Fig. 1(A)], and are closely related to those of Tbx6 sub-family members including VegT, Brachury (T), and Eomesodermin. In vitro analysis suggested that Mga functions as a dual specificity transcription factor [18]; however, its physiological function remains unknown. To investigate its function in the early development of Xenopus laevis, we first analyzed its expression pattern. Figure 1(B) shows that Xmga was maternally stored in the germinal vesicle of full-grown oocyte [Fig. 1(B,a)] and enriched in the animal half of fertilized egg (it’s common to call the pigmented half as animal half and the less pigmented half as vegetal half) and blastula stage embryo [Fig. 1(B,b,c)]. It continued to be expressed in the developing ectoderm and mesoderm during gastrulation and later development [Fig. 1(B,d,e)]. Quantitative RT-PCR data confirmed that Xmga is expressed throughout the early development [Fig. 1(C)].

We then injected synthetic mouse Mga mRNA into the Xenopus oocytes (0.5–2 ng/oocyte) and fertilized these oocytes through host transfer technique. Figure 2(A) shows that overexpression of Mga caused abnormal development in a dose-dependent manner. By the tadpole stages, overexpression of high-dose Mga mRNA (1–2 ng/embryo) caused the reduction of body length and a slight enlargement of anterior part, resembling the effect of XWnt11 overexpression in the oocytes [1]. To investigate whether

<table>
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<th>Gene</th>
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| BMP4     | U: 5'-ACCCATAGCTGCAAATGGAC-3'  
D: 5'-ACCCATAGCTGCAAATGGAC-3' | [15]           |
| Chordin  | U: 5'-AACCTGCCAAGAAGATGGAT-3'  
D: 5'-GGCAGGATTTAGGTTTTC-3'    | XenBase (http://www.XenBase.org) |
| Goosecoid| U: 5'-TTCCACCTTTTTGGGACTTTTCA-3'  
D: 5'-TTCCACCTTTTTGGGACTTTTCA-3' | [15]           |
| ODC      | U: 5'-GCCATTGTGAAGACAAACTGGA-3'  
D: 5'-GCCATTGTGAAGACAAACTGGA-3' | [24]           |
| Siamois  | U: 5'-CGTCCTCTACAAGAAGACTGCG-3'  
D: 5'-CATATGCTTTTGCTGGCC-3'    | [24]           |
| Xbra     | U: 5'-CTCTGGAAGGATGACATGAG-3'  
D: 5'-CTCTGGAAGGATGACATGAG-3'  | [10]           |
| Xmga     | U: 5'-ATGGACTGCAACCCCATTGC-3'  
D: 5'-ATGGACTGCAACCCCATTGC-3'  | Designed in this study |
| Xnr3     | U: 5'-CTCTGCACTGAGATTCCG-3'  
D: 5'-CTCTGCACTGAGATTCCG-3'    | [32]           |
| Xsox17a  | U: 5'-GCAAGATGGAGGGCCATTGC-3'  
D: 5'-GCAGATGGAGGGCCATTGC-3'   | [10]           |
| Xwnt8    | U: 5'-CTGAGTTTACCTCGTTC-3'  
D: 5'-CTGAGTTTACCTCGTTC-3'     | [28]           |

U, upstream; D, downstream.
Figure 1 Expression pattern of Xmga
(A) A schematic depiction of the peptide sequence identities between mammalian and Xenopus Mga. (B) In situ hybridization shows Xmga is stored in germinal vesicle of full-grown oocyte (a) and is enriched in animal half of fertilized egg (b) and blastula (c). Zygotic Xmga is enriched in mesoderm and ectoderm (d, e). (C) Quantitative RT-PCR data show Xmga is expressed throughout the early development of Xenopus embryo. Scale bar = 1 mm.

Figure 2 Mga enhances the activity of β-catenin in inducing dorsal gene expression
(A) Tailbud stage (st24) embryos show that overexpression of Mga in oocytes resulted in dorso-anteriorized phenotype in a dose-dependent manner. (B) Realtime RT-PCR data show the expression of organizer genes (Siamois and Xnr3) is increased by Mga overexpression. (C) Quantitative RT-PCR data show that in animal assay co-injection of Mga potentiates the activity of β-catenin to induce Xnr3 and Siamois. Scale bar = 1 mm.
overexpression of Mga changes the expression levels of early development regulatory genes, we performed quantitative RT-PCR analysis of the expression of organizer specific genes during gastrulation. Figure 2(B) shows that Siamois and Xnr3 were up-regulated in embryos injected with 1–2 ng Mga mRNA, suggesting that Mga may positively regulate the activity of \(\beta\)-catenin during organizer induction. The expression of BMP4, Xbra (a pan-mesoderm marker) and Xsox17a (a pan-endoderm marker) did not change in the embryos injected with Mga (data not shown).

To further demonstrate that Mga can potentiate the activity of \(\beta\)-catenin on organizer gene induction, we injected 10–50 pg of \(\beta\)-catenin mRNA with or without Mga mRNA (2 ng) into the animal pole at two-cell stage, and dissected animal caps at mid-blastula stage. The animal caps were then cultured to the sibling stage 10 and harvested for quantitative RT-PCR analysis. Figure 2(C) shows that co-injection of Mga increased the inducing activity of \(\beta\)-catenin on the expression of Xnr3 and Siamois.

Taken together, we conclude that Mga enhances the function of \(\beta\)-catenin during organizer formation in the early Xenopus embryo.

Maternal Xmga depleted embryos are ventralized

To deplete the maternal store of Xmga mRNA, we designed and screened a group of eight antisense oligonucleotides as described previously [21,22]. Quantitative RT-PCR data show that oligo T3m efficiently depleted maternal Xmga mRNA in a dose-dependent manner. Injection of 9–12 ng of oligo T3m could deplete maternal Xmga mRNA to below 20% of control levels [Fig. 3(A)].
To determine the function of maternal Xmga, T3m oligo-injected oocytes were matured and fertilized 48 h after injection through host transfer technique. T3m-injected embryos underwent normal development through cleavage and blastula stages, and became abnormal during gastrulation. Figure 3(B) shows that when control embryos developed to late gastrula stage, the blastopore closure was delayed in embryos injected with lower doses (blue: 6 ng and purple: 9 ng) of T3m oligo. Embryos injected with high dose (red: 12 ng) of T3m oligo only attempted to form blastopore and never finished gastrulation. Shown in Fig. 3(C) are bisected embryos at late gastrula stage (st12) demonstrating that the dorsal lip and organizer failed to form in 9 ng and 12 ng of T3m oligo injected embryos. By tadpole stages, T3m-injected embryos showed progressive loss of head and dorsal structures in a dose-dependent manner [Fig. 3(D)]. Essentially, all 12-ng T3m oligo-injected embryos were completely ventralized and stayed in ball shape [Fig. 3(D), purple], strikingly resembling ventralization caused by β-catenin depletion [4,26]. The ventralization phenotype was high reproducible (n = 45, three independent experiments). These results indicate that maternal Xmga may play a critical role in organizer induction and dorsal axis formation in the early Xenopus embryos.

Xmga-depleted embryos fail to express organizer-specific genes Xnr3 and Siamois

To determine whether Xmga depletion affects organizer induction, we carried out quantitative RT-PCR analysis of the expression of a group of organizer-specific genes during gastrula stages (st9.5–st10.5). Figure 4(A) shows that the expression of organizer markers Xnr3 and Siamois was dramatically decreased in T3m-injected embryos. In contrast, the expression of ventral markers Xwnt8 and BMP4 was not significantly altered. The expression of pan-mesoderm gene Xbra and pan-endoderm gene Xsox17a was also reduced in the embryos depleted of maternal Xmga at late blastula stage, but recovered to the level comparable to that of control by the mid-gastrula stage, indicating the induction of mesoderm and endoderm was also compromised in the absence of maternal Xmga. These results suggest that organizer is not effectively induced in embryos depleted of maternal Xmga.

To determine the specificity of the ventralized phenotype from the maternal Xmga depletion, we performed rescue experiment. Oocytes injected with 9 ng of T3m oligo were culture for 36 h and then injected with capped synthetic mouse Mga mRNA (1.5 ng per oocyte). Control, Xmga depleted and rescue group of oocytes were then fertilized through host transfer technique. Figure 4(B,C) shows that reintroduction of mouse Mga mRNA could partially rescue the axis formation in Xmga-depleted embryos, suggesting the ventralized phenotype is specific due to the depletion of maternal Xmga mRNA. We further confirmed that the expression of organizer genes (Xnr3 and Siamois) was also partially rescued by mouse Mga mRNA injection [Fig. 4(D)].

Xnr3 and Siamois are direct target genes of maternal Wnt/β-catenin signaling. Therefore, we conclude that Xmga is required for Wnt signaling during organizer induction.

**XTcf3 depletion cannot rescue the ventralized phenotype from Xmga depletion**

Previous studies have shown that XTcf3 is a repressor for Wnt target genes during organizer induction, and depletion maternal XTcf3 results in the upregulation of organizer genes [14,15]. We then asked whether depletion of XTcf3 would rescue the ventralized phenotype from Xmga depletion. To answer this, we depleted maternal Xmga and XTcf3 each alone or simultaneously. Figure 5(A) shows that Xmga depletion caused ventralization (purple) and XTcf3 depletion resulted in dorso-anteriorization (red) as expected. However, Xmga and XTcf3 double-depleted embryos were still ventralized, suggesting that organizer cannot be induced by simply relieving the repression effects of XTcf3 when Xmga is absent. We also confirmed the expression of Siamois in those embryos. Figure 5(B) shows that the expression of Siamois was not rescued in the embryos depleted of both Xmga and XTcf3. These results further support our hypothesis that Xmga is required for Wnt/β-catenin signaling during organizer formation.

**Discussion**

Maternal genes are essential for the pattern formation in the early Xenopus development [5,10,29,30]. Both gain- and loss-of-function studies have shown maternal β-catenin is both necessary and sufficient for the induction of Spemann organizer and the establishment of dorsal ventral polarity in the early Xenopus embryos [3,4,26,30]. Maternal XWnt11 has been demonstrated to be required upstream of β-catenin for Spemann organizer induction and the dorsal axis development [1]. As a direct target of maternal XWnt11/β-catenin signaling [7], injection of Siamois can restore the dorsal axis formation in the embryos depleted of maternal β-catenin [12]. Therefore, that how the expression of organizer genes, Siamois in particular, is activated in the right place at the right time is of critical importance for understanding the fundamental mechanisms of early dorsal ventral patterning during the early Xenopus development.

Three members of Tcf/LEF1 family transcription factors, i.e. XTcf1, 3, and 4 are maternally expressed and have all been implicated in the regulation the proper expression of organizer-specific genes [5]. Specifically, XTcf3 has been defined as a repressor for the expression of organizer genes
and dorsal development [15]. XTcf4 is required as an activator for the expression of organizer genes \(Xnr3\) and \(Sia\). The expression of ventral marker genes \(Xwnt8\) and \(BMP4\) is not affected by \(Xmga\) depletion. The expression of pan-mesoderm (\(Xbra\)) and pan-endoderm gene (\(Xsox17a\)) is also reduced in high-dose T3m-injected embryos. (B) Tailbud stage (st22) embryos indicate that mouse Mga can partially rescue the dorsal axis formation. (C) Dorsal anteriorization index (DAI) [27] scoring shows the rescue effect of Mga. For normal embryos, DAI = 5; for completely ventralized embryos, DAI = 0; for embryos with small head and reduced eye, DAI = 3–4; for embryos without any anterior structures but having posterior dorsal structures such as the spinal cord, DAI = 1–2. (D) Quantitative RT-PCR data show that Mga partially rescues the expression of \(Sia\) and \(Xnr3\). Scale bar = 1 mm.

Figure 4 Xmga is required for Wnt/\(\beta\)-catenin-regulated organizer gene expression

(A) Quantitative RT-PCR data show that depletion of maternal Xmga specifically inhibits the expression of organizer genes \(Xnr3\) and \(Sia\). The expression of ventral marker genes \(Xwnt8\) and \(BMP4\) is not affected by Xmga depletion. The expression of pan-mesoderm (\(Xbra\)) and pan-endoderm gene (\(Xsox17a\)) is also reduced in high-dose T3m-injected embryos. (B) Tailbud stage (st22) embryos indicate that mouse Mga can partially rescue the dorsal axis formation. (C) Dorsal anteriorization index (DAI) [27] scoring shows the rescue effect of Mga. For normal embryos, DAI = 5; for completely ventralized embryos, DAI = 0; for embryos with small head and reduced eye, DAI = 3–4; for embryos without any anterior structures but having posterior dorsal structures such as the spinal cord, DAI = 1–2. (D) Quantitative RT-PCR data show that Mga partially rescues the expression of \(Sia\) and \(Xnr3\). Scale bar = 1 mm.
In addition, overexpression of Xmga could potentiate the inducing activity of both endogenous and overexpressed β-catenin on the expression of Xnr3 and Siamois, while overexpression of XTcf4 resulted in the decreased expression of these genes [14], further suggesting Xmga and XTcf4 function differently during organizer formation.

The molecular mechanisms by which Xmga regulates the activity of β-catenin in the induction of organizer genes are under investigation. Given the fact that the expression of Siamois responded differently to the depletion of Xmga and XTcf4, we are currently analyzing the potential interaction between Xmga and the promoter of Siamois [7]. Detailed analysis will also clarify the following questions: (i) whether the T-domain or bHLHZip domain is necessary for its function in the activation of Siamois; (ii) whether Xmga binds to β-catenin directly; and (iii) whether Xmga regulates the interaction between β-catenin and XTcf4.

The activity of β-catenin in transcription can also be regulated through its nuclear-cytoplasmic shuttling. For instances, the nuclear translocation of β-catenin has been reported to be negatively regulated by Chibby and positively by XTsh3 [25,31]. Further investigation will be necessary for testing the possibility that Xmga regulates the nuclear accumulation of β-catenin.

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