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Expression of the ALK1 family of type I BMP/ADMP receptors during gastrula stages in *Xenopus* embryos

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ABSTRACT Multiple members of the transforming growth factor beta (TGFβ) family of secreted factors play central inductive and patterning roles during embryogenesis. During gastrulation in vertebrates, the bone morphogenetic protein (BMP) sub-family is linked to formation of the embryonic organizer, Spemann's organizer in *Xenopus,* and dorsal-ventral mesoderm patterning. Our knowledge regarding the BMP receptors mediating this signaling is still very incomplete. The BMPR1A (ALK3) and BMPR1B (ALK6) receptors are known to mediate the BMP4 signal. These receptors belong to the ALK1 subfamily of type I receptors that also includes ACVR1 (ALK2), and ACVRL1 (ALK1). We studied by qPCR and *in situ* hybridization the spatio-temporal expression patterns of ALK2 and ALK1 and compared them to ALK3 and ALK6, and to the main BMPs expressed during gastrulation, i.e., BMP4, BMP7, BMP2, and ADMP, in an attempt to establish a link between ligands and receptors. There is extensive overlap between *BMP4,* and *ALk3* and *Alk6* expression, supporting their functional interaction. Robust *Alk6* expression was observed from mid-gastrula. Animal region expression of both receptors shows co-expression with *BMP4* and *BMP7*. *Alk2* transcripts were detected within the organizer, overlapping with its proposed ligand, *ADMP,* suggesting a probable function within the organizer. *Alk1* is very weakly expressed during gastrula, but its transcripts were localized to the lateral marginal zone flanking the organizer domain. No receptor closely matched the maternal *BMP2* expression, although *Alk2, Alk3,* and *Alk6,* have transcripts of maternal origin. Our analysis shows that the BMP ligands and their receptors exhibit dynamic expression patterns during gastrula stages.

KEY WORDS: *TGFβ receptor, ALK receptor, BMP signaling, Xenopus embryo, anti-dorsalizing morphogenetic protein*

BMP signaling is a complex signaling network involving numerous ligands and receptors. The ligands function as homo– or hetero-dimeric proteins, while the receptors are hetero-tetrameric complexes comprised of two type I and two type II receptors (Heldin and Moustakas 2016). The type I family of TGFβ receptors, also known as activin receptor-like kinases (ALKs) has seven members while the type II family has five. Therefore, BMP signaling can exhibit a high level of complexity as a result of the multiple ligand possibilities and receptor combinations. This pathway is further regulated extracellularly by secreted antagonists, decoys, and proteases cleaving the precursor ligand proteins or the secreted antagonists. Intracellularly, the BMP signaling pathway mainly mediates its effects through phosphorylation of the Smad proteins, but alternative signaling possibilities also take place (Heldin and Moustakas 2016). This complexity allows for precise patterning of embryonic tissues by establishing morphogen gradients and regulating gene expression through dose-dependent responses (Dosch *et al.,* 1997; Marom *et al.,* 1999).

In *Xenopus* embryos BMP signaling plays a central role in the formation of Spemann's organizer, the embryonic organizer, and during dorsal-ventral patterning of the mesoderm (Dosch *et al.,* 1997; Marom *et al.,* 1999; Marom *et al.,* 2005). The important roles of BMP signaling during early embryogenesis prompted the

Abbreviations used in this paper: ADMP, anti-dorsalizing morphogenetic protein; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; ISH , *in situ* hybridization; qPCR, quantitative real-time reverse transcription PCR; TGF, transforming growth factor.

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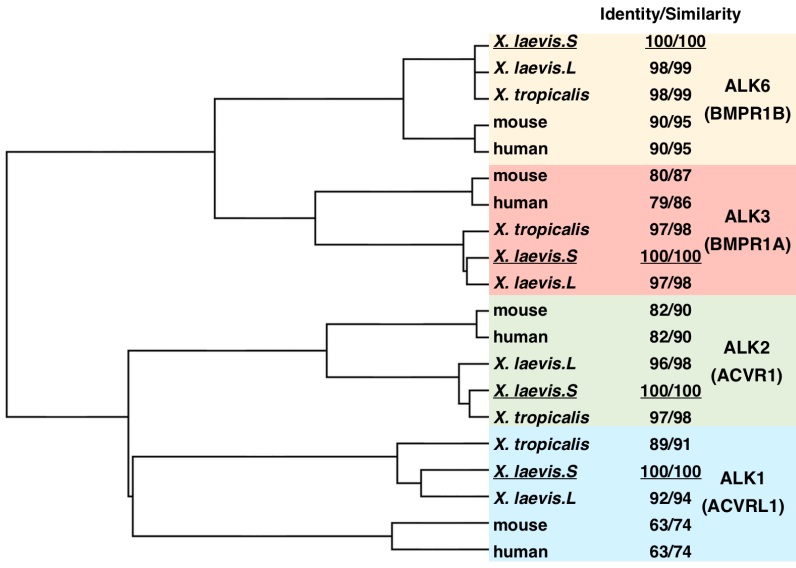

Fig. 1. The ALK1 family of type I bone morphogenetic protein (BMP) receptors. Phylogenetic tree of the ALK1family of type I BMP receptors. *Protein sequences from* Xenopus laevis *and* Xenopus tropicalis, *human and mouse, were included in the analysis. The analysis subdivided the ALK1 sub-families, ALK6, ALK3, ALK2, and ALK1. For each sub-family, the percent sequence identity to the relevant* Xenopus laevis *protein is shown.*

characterization of multiple components and interactions of this signaling pathway in frog embryos. While *BMP2* transcripts are present as a strong maternal contribution (Marom *et al.,* 2005), BMP7, BMP4 and ADMP are expressed mainly from zygotic transcription (Fainsod *et al.,* 1994; Moos *et al.,* 1995; Hawley *et al.,* 1995).

Our knowledge of the expression of the BMP receptors and their ligand specificity remains fragmentary. Here we describe the temporal and spatial pattern of expression of the ACVR1 (ALK2) and ACVRL1 (ALK1) type I receptors. We compare their expression patterns during gastrula stages to the well-studied BMP4 type I receptors BMPR1A (ALK3) and BMPR1B (ALK6) (Graff *et al.,* 1994; Schille *et al.,* 2016) and their putative ligands, BMP4, BMP2, BMP7, and ADMP. Our results show a very complex set of ligand and receptor expression patterns contributing to the regulated establishment of the organizer domain and subsequently the BMP morphogen gradient.

Results and Discussion

The BMP family of signaling factors binds mainly

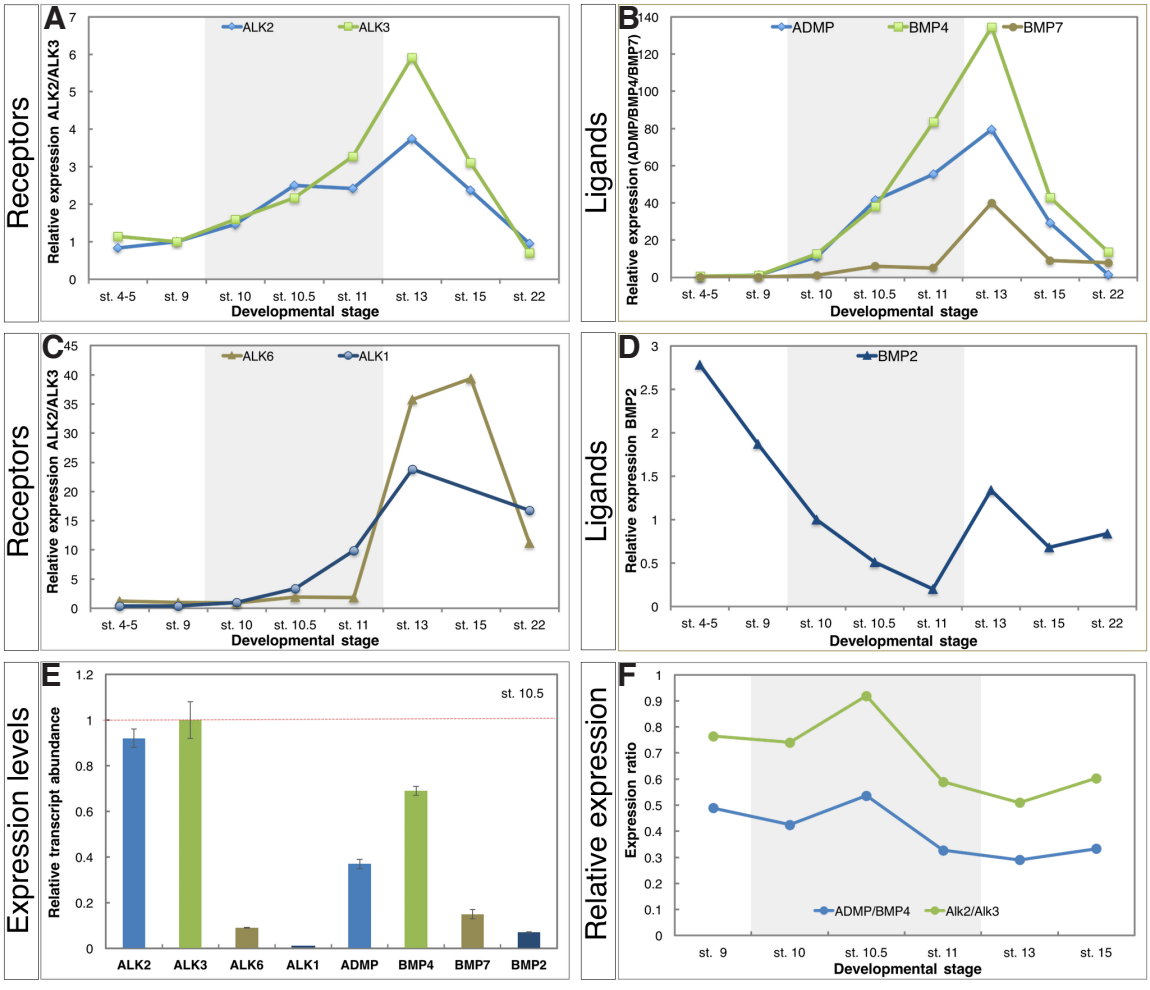

Fig. 2. Temporal expression of the *Alk1* receptor family and the main bone morphogenetic protein (*BMP*) ligands during gastrulation. *RNA samples from* X. laevis *embryos from early cleavage (st. 4-5) to late neurula (st. 22) stages were analyzed by qPCR. Temporal pattern of the receptor gene expression;* **(A)** Alk2 *and* Alk3, **(C)** Alk1 *and* Alk6. *Expression of the ligand genes as a function of developmental stage;* **(B)** BMP4, BMP7 *and* ADMP *and* **(D)** BMP2. **(E)** *Relative transcript abundance of the receptor and ligand genes during early/mid-gastrula (st. 10.5).* **(F)** *Comparison of the expression levels between* ADMP/BMP4 *and* Alk2/Alk3 *from late blastula (st. 9) to early neurula (st. 15) stages. The gray shading marks gastrula stages.*

to the ALK1 family of type I of TGFβ receptors (Yadin *et al.,* 2016). This family of receptors includes ALK1, ALK2, ALK3, and ALK6. In *Xenopus* embryos, the role of ALK3 and ALK6 as BMP receptors has been the focus of multiple of studies (Graff *et al.,* 1994; Schille *et al.,* 2016). In contrast, the characterization of ALK2 during early embryogenesis lags behind, and very little is known regarding ALK1 (Kondo *et al.,* 1996; Armes and Smith 1997; Chen *et al.,* 1997). To study the pattern of *Alk1* and *Alk2* expression, we determined their cDNA sequence through data mining of the GenBank and Xenbase databases. The sequence of ALK2 was previously reported (Kondo *et al.,* 1996; Chen *et al.,* 1997), and sequences of the *Alk1* cDNAs from *Xenopus laevis* (ACVRL1.L, XM_018247346; ACVRL1.S, XM_018248624), and *Xenopus tropicalis* (XM_002935116, XM_012957763) have been predicted (Xenbase.org). We corroborated the sequences of both receptors (Supplementary Fig. S1 A,B). Comparison of all four receptors emphasizes the similarities and differences between the receptors (Fig. 1). Comparison of the *Xenopus* sequences to the human and mouse receptors identified regions with a high degree of conservation (Fig. 1). We PCR cloned cDNAs of both genes (see Materials and Methods), and designed gene-specific primers for their characterization by quantitative real-time PCR (qPCR).

Temporal expression patterns of the ALK1 family of receptors and their putative ligands

The temporal expression patterns of *Alk1* and *Alk2* were determined by qPCR using RNA samples spanning from early cleavage stages (st. 4-5) to advanced neurula stages (st. 22). These temporal expression patterns were compared to the pattern of *Alk3* and *Alk6* expression using specific primers. *Alk2* transcription becomes apparent during late blastula (st. 9; Fig. 2A). *Alk2* transcripts continue to accumulate until mid-gastrula (st. 10.5), and then expression levels increase much slower until late gastrula/early neurula (st. 13) when they begin to decline (Fig. 2A). Comparison to *Alk3* expression shows that also this gene begins to be transcribed during late blastula, but transcripts of this gene continue to accumulate until the end of gastrulation (Fig. 2A).

Alk1 expression is reminiscent of the expression of *Alk2* and *Alk3* with the main difference being that *Alk1* expression begins with a slight delay such that transcript accumulation begins with the onset of gastrulation (st. 10; Fig. 2C). Using the same RNA samples, we also determined the temporal pattern of *Alk6* expression, whose zygotic expression is delayed until mid/late gastrula stages (st. 11; Fig. 2C). These patterns of *Alk3* and *Alk6* expression are in agreement with previously reported patterns (Graff *et al.,* 1994; Schille *et al.,* 2016).

The temporal expression pattern of the main BMPs during early gastrula, BMP2, BMP4, BMP7, and ADMP (Fainsod *et al.,* 1994; Moos *et al.,* 1995; Hawley *et al.,* 1995; Marom *et al.,* 2005), was also determined using the same RNA samples. These BMPs are the putative ligands of the type I BMP receptors studied. ALK3 and ALK6 have been described as BMP4 receptors (Reversade and de Robertis 2005; Schille *et al.,* 2016). ALK3 also can bind BMP2 and BMP7. ALK2 has been shown to bind ADMP and BMP7. The ligand of ALK1 has remained elusive, and BMP9 has been identified as a ligand of this receptor. *ADMP* and *BMP4* exhibit almost identical temporal expression patterns with zygotic transcripts appearing during late blastula (st. 9) and continue to accumulate until early neurula (st. 13; Fig. 2B). Expression of BMP7 also begins during late blastula (st. 9) and continues to increase until late gastrula (Fig. 2B). Temporal analysis shows that the *Xenopus* embryo has significant maternal *BMP2* transcripts. The level of maternal *BMP2* transcripts decreases towards the onset of gastrulation, and during mid-, late gastrula (after st. 11) zygotic transcription ensues (Fig. 2D). A similar expression pattern has been previously described for *BMP2* (Marom *et al.,* 2005).

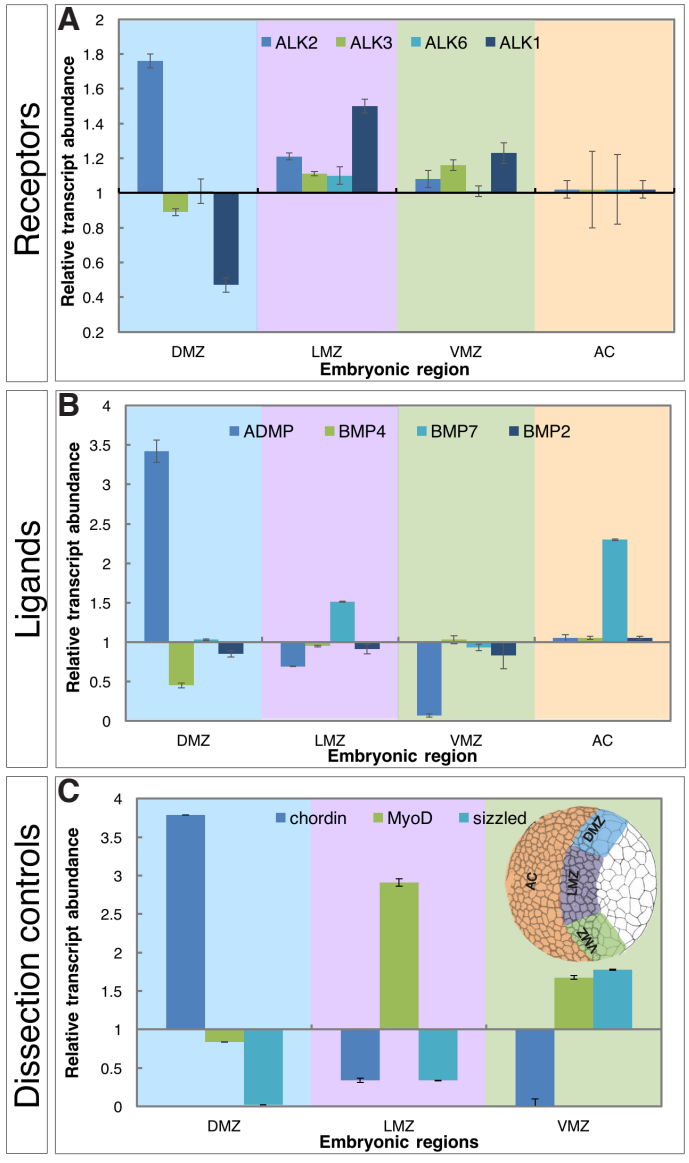

Fig. 3. Spatial localization of the *Alk1* family transcripts in gastrula embryos. X. laevis *embryos at early/mid-gastrula (st. 10.5) were dissected into four regions, the animal cap (AC), and the dorsal, lateral, and ventral, regions of the marginal zone (DMZ, LMZ, and LMZ, respectively). RNA from each embryonic region was analyzed by qPCR. Relative transcript abundance in each region was normalized for most genes to the AC level of expression, only for* BMP7 *the level of expression in the VMZ was used for normalization.* **(A)** *Relative transcript abundance of the ALK1 family receptors.* **(B)** *Relative spatial localization of the BMP ligand transcripts.* **(C)** *The transcript localization of the* chordin, MyoD, *and* sizzled *genes was analyzed to determine the dissection accuracy for the DMZ, LMZ+VMZ, and VMZ respectively. A schematic representation of the embryo is shown.*

An important observation from the qPCR data was an estimation of the relative expression levels of the different receptors and ligands. Our semi-quantitative comparison revealed that during early/mid-gastrula (st. 10.5) both *Alk3* and *Alk2* are expressed at very similar levels (Fig. 2E). At the same stage, the level of *ADMP* transcripts is about half the amount of BMP4 transcripts (Fig. 2E). Analysis of the other receptors and ligands revealed that they are expressed at even lower levels (Fig. 2E). The level of *Alk1* expression was unusually low in agreement with its temporal delay in expression compared to *Alk2* and *Alk3* (Figs. 2 C,E). Of particular interest was the comparison between *BMP4* and *ADMP* and their receptors, *Alk3* and *Alk2*, during gastrula stages as they appear to be the most abundant at these stages. Estimation of the relative transcript levels from late blastula (st. 9) to early neurula (st. 15), suggested that *ADMP* expression declines compared to *BMP4*, and *Alk2* expression becomes less abundant compared to *Alk3* (Fig. 2F). Based on the restricted area, the organizer, where *ADMP* is expressed (Moos *et al.,* 1995), these results suggest that the level of *ADMP* transcripts there is extremely abundant, and is similar or higher than *BMP4* transcripts throughout the rest of the embryo. The high level of *ADMP* transcripts within the dorsal region again questions its BMP-like anti-dorsalizing function.

Comparison of the ALK and BMP temporal expression patterns suggests that expression of all four type I receptors studied, partially overlaps in time with BMP4, BMP7, and ADMP. Our temporal analysis did not identify a type I BMP receptor recapitulating the temporal pattern of *BMP2* expression, in particular, its high maternal abundance and subsequent decline. The original description of *Alk3* reported high maternal transcript levels and a sharp decline during early development (Graff *et al.,* 1994). Analysis of recent high-throughput expression studies (Yanai *et al.,* 2011; Owens *et al.,* 2016) did not corroborate the high *Alk3* transcript levels, like our results, but otherwise suggested high maternal *Alk2* expression in *Xenopus tropicalis.* Also, in the high-throughput studies, the transcript levels of *Alk1* appear to be very low. Although the temporal analysis suggested possible receptor-ligand interactions, temporal expression overlap also requires spatial co-expression or some proximity.

Spatial localization of the ALK1 and BMP family expression

To further understand the overlap in the expression patterns of these receptors and ligands, we turned to a comparative analysis of their spatial pattern of expression during gastrulation. To this end, we manually dissected early/mid-gastrula stage embryos (st. 10.25-10.5) into dorsal, lateral and ventral marginal zone regions (DMZ, LMZ, and VMZ, respectively) and the animal cap (AC; Fig. 3). Care was taken to remove the animal cap tissue from the MZ explants. RNA was prepared from each region, and qPCR was performed to determine the transcript distribution of the BMP ligands, and the ALK receptors and the relative spatial abundance of the transcripts were estimated (Fig. 3). Among the receptors, most *Alk2* transcripts localize to the DMZ, while, *Alk1* is mainly expressed in the LMZ with little expression in the VMZ (Fig. 3A). *Alk3* and *Alk6* expression is widely distributed along the LMZ, and VMZ (Fig. 3A).

Parallel analysis of the BMP ligands revealed high DMZ abundance of *ADMP* transcripts (Fig. 3B) as previously described (Moos *et al.,* 1995). *BMP4* transcripts can be detected throughout the embryo avoiding only the DMZ (Fig. 3B) in accordance with the described pattern (Fainsod *et al.,* 1994). *BMP2* transcripts appear to be widely distributed in all embryonic regions (Fig. 3B) in agreement with its previously published whole-mount *in situ* hybridization (ISH) pattern (Clement *et al.,* 1995). Transcripts of *BMP7* appear to be mostly localized to the animal cap region with some accumulation in the MZ, in particular along the LMZ (Fig. 3B) as previously described (Hawley *et al.,* 1995). The qPCR patterns described, corroborate the previously described ISH patterns, and attest to the accuracy of our dissections. Also, we determined the transcript localization of *chordin*, *MyoD,* and *sizzled* as markers of the DMZ, LMZ+VMZ, and VMZ respectively (Fig. 3C).

Based on this analysis, both *Alk2* and *ADMP* transcripts are predominantly localized to the

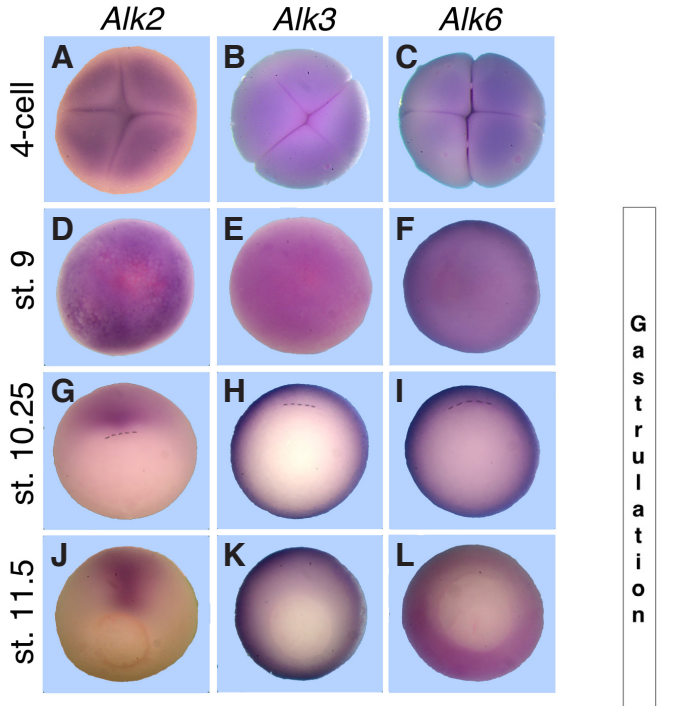

Fig. 4 (left). The *Alk2* gene encodes an organizer-expressed gene. *Whole-mount ISH analysis of the* Alk2, Alk3, *and* Alk6 *genes from early cleavage to late gastrula stages.* **(A-C)** *Four-cell embryos, animal view.* **(D-F)** *Late blastula stage (st. 9), animal view.* **(G-I)** *Early gastrula embryos (st. 10.25), vegetal view. The dorsal lip of the blastopore is marked with a dashed line.* **(J-L)** *Late gastrula (st. 11.5) expression, vegetal view.*

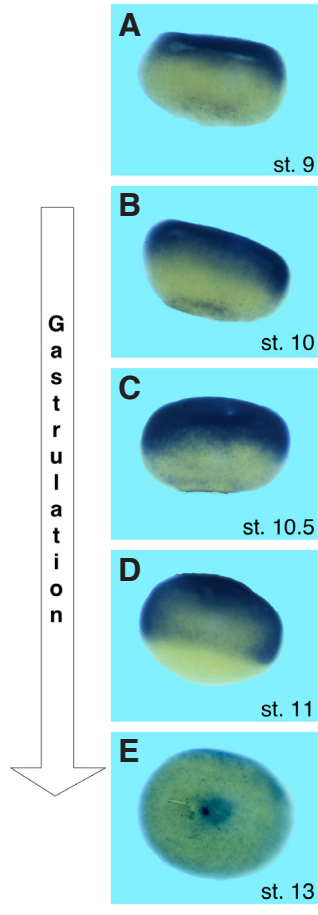

Fig. 5 (right). *Bone morphogenetic protein 7* (*BMP7*) expression marks the epiboly of the prospective ectoderm. *Whole-mount ISH analysis of* BMP7 *expression from late blastula to early neurula. Lateral views of st. 9* **(A),** *st. 10* **(B)**, *st. 10.5* **(C)**, *and st. 11* **(D)** *embryos, and posterior animal view of st. 13* **(E)** *embryo.*

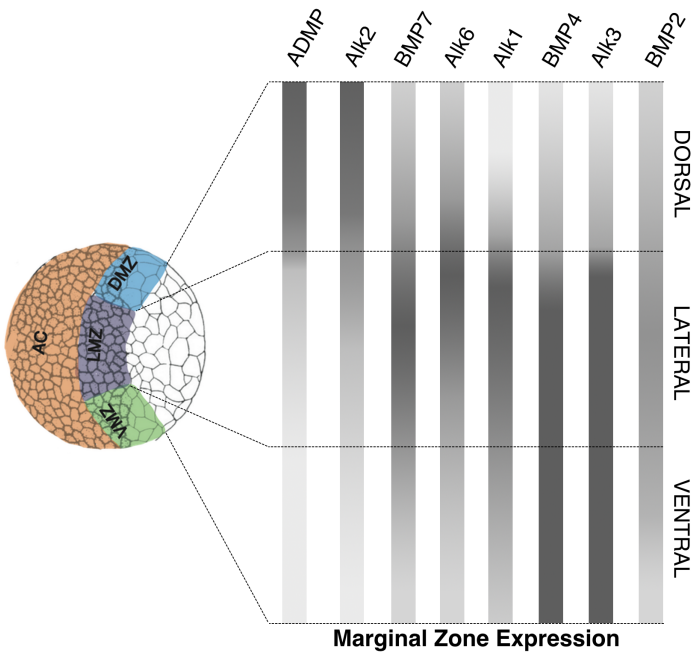

Fig. 6. Expression of the ALK1 receptor family and the bone morphogenetic protein (BMP) ligands during gastrula. *Schematic summary of the expression of the type I receptors* Alk1, Alk2, Alk3, *and* Alk6, *and the BMPs,* BMP2, BMP4, BMP7, *and* ADMP. *The summary focuses on the marginal zone expression of the different genes and relates to the dissection scheme.*

DMZ (Figs. 3 A,B) in agreement with ADMP binding to ALK2 (Reversade and de Robertis 2005). To further corroborate the dorsal expression of *Alk2* we determined its spatial expression pattern by whole mount ISH. During early cleavage stages (4-cells), some *Alk2* transcripts are detected in the animal region, and this localization persists until late blastula stages (st. 9) (Figs. 4 A,D). Expression of *Alk2* during early gastrula shows a novel site of expression restricted to the dorsal blastoporal lip, i.e. Spemann's organizer (Fig. 4G). By late gastrula (st. 11.5) the transcripts of *Alk2* localize to the dorsal midline of the embryo from the blastopore to rostral regions (Fig. 4J). Our results show that both, ADMP (Moos *et al.,* 1995) and its receptor, ALK2, exhibit extensive expression overlap during gastrula stages, and suggest a possible signaling event mediated by this ligand-receptor pair within the organizer.

For comparison purposes, we also studied the spatial pattern of *Alk3* and *Alk6* expression. Similarly, both *Alk3* and *Alk6* transcripts can be observed in the animal region of 4-cell embryos and continue there until late blastula stages (Figs. 4 B,C,E,F). During early (st. 10.25) and late (st. 11.5) gastrula, the animal cap expression persists, and both *Alk3* and *Alk6* are expressed throughout the marginal zone (Fig. 4 H,I,K,L). In both cases, during gastrula stages, fewer transcripts appear to be present in the dorsal blastopore region, Spemann's organizer. These spatial patterns of expression together with the temporal analysis show that *Alk3* and *Alk6* expression extensively overlaps with the *BMP4* transcript localization (Fainsod *et al.,* 1994). This overlap further supports the ligand-receptor interaction between BMP4 and its receptors. The extremely low transcript levels of *Alk1* precluded the determination of its spatial pattern of expression by whole mount ISH.

The results of *BMP7* transcript localization based on embryo fragments placed the expression of this gene mainly to the animal region (Fig. 3B), corroborating the previously published ISH pattern (Hawley *et al.,* 1995). A more detailed analysis of *BMP7* expression during gastrula stages revealed a dynamic pattern. From late blastula to late gastrula, the expression of *BMP7* localizes mainly to the animal cap cells (Fig. 5 A-D). Interestingly, *BMP7* expression persists in the animal cap region as it envelops the embryo. As both, ALK3 and ALK6 are expressed in the animal cap and marginal zone during gastrula stages, they could be mediating some of the BMP7 signaling.

Based on their temporal and spatial synexpression (Fig. 6), we attempted to suggest possible ligand-receptor pairs between the main BMPs and type I receptors during gastrula stages. Based on their expression, ALK3 (BMPR1A) and ALK6 (BMPR1B) are poised to mediate the signaling induced by BMP4 and BMP7 (Fig. 6). This suggested ligand-receptor interaction has been identified in some instances (Little and Mullins 2009). The situation for BMP2 is less clear as several ALK1-family receptors seem to have some degree of maternal contribution, but none recapitulates the *BMP2* temporal expression pattern. From the expression analysis, the maternal *BMP2* transcript level from early cleavage to blastula stages could be equivalent to the level of *BMP4* during gastrula stages as we have previously suggested (Marom *et al.,* 2005). Then, relatively low levels of a receptor like ALK3 could mediate its signaling. ALK2 has been shown to mediate some BMP2 signaling, and it is also able to bind ADMP (Reversade and de Robertis 2005). The co-expression of *ADMP* and *Alk2* within Spemann's organizer supports this observation (Fig. 6), but suggests that ADMP might be performing some signaling in this region as ALK2 is known to be a functional, signal-transducing, receptor. The ALK1 receptor appears to be localized to the LMZ from early/late gastrula stages. This expression would place the ALK1 receptor in regions flanking Spemann's organizer. Our analysis of the main BMP ligands didn't identify a factor with a matching expression pattern, suggesting that, the relevant factor remains to be identified. This analysis of the BMP ligands and their putative receptors during gastrula stages revealed a very dynamic and complex signaling milieu observed in different vertebrate species and developmental stages.

Materials and Methods

Embryo culture and treatments

Xenopus laevis frogs were purchased from NASCO (Fort Atkinson, WI). Experiments were performed after approval and under the supervision of the Institutional Ethics Committee. Embryos were obtained by *in vitro* fertilization, incubated in 0.1% MBSH, and staged according to Nieuwkoop and Faber (1967).

Embryo manipulation and dissection

Embryos were dissected into four parts: dorsal (DMZ), lateral (LMZ) and ventral marginal zone (VMZ) and animal cap (AC) at stage 10.25, in 1% MBSH buffer. Embryonic regions (15- 20 embryos for each region) were then processed for RNA extraction and qPCR.

cDNA clones and constructs

PCR cloning of *X. laevis Alk2*, *Alk6,* and *Alk1* was performed with the Herculase II Phusion DNA Polymerase (Stratagene) using cDNA samples from stages 10.5-11.

The primers used for cloning were:

Alk2, 5'AAGGATCCTGTCCTGCGGAATGG3',
5'CAGAATTCCTAACACAGTAATGGGAGAGGC3';
Alk6, 5'GCTTGCCTTTCACATTCTCTTC3',
5'TCCAGTTCCGACAGCTTACAT3';
Alk1, 5'TTCTGTGTCACCTGAAAACCC3',
5'GTCTGCCACTTTCATGCCTTT3'.

Since no sequence for *X. laevis Alk1* has been published, we took advantage of the predicted mRNA sequences (Xenbase.org) from the genomic data after extensive comparison to the *Xenopus tropicalis* published data and *Alk1* sequences from other vertebrates. All PCR products were verified by sequencing.

RNA extraction and quantitative real-time PCR

Total RNA from embryos was extracted with the PerfectPure RNA Tissue Kit with DNase (5 Prime, Hamburg, Germany). cDNA was synthesized using the Iscript cDNA Synthesis kit (Bio-Rad Laboratories).

Quantitative real-time PCR (qPCR) was performed using the Bio-Rad CFX384 thermal cycler and LightCycler 48 SYBR Green I Master (Roche). All samples were processed in triplicate. *GAPDH* expression levels were used for normalization. For each gene, the relative expression was calculated using the ΔΔct method, as described previously (Livak and Schmittgen 2001). All experiments were repeated at least three times. qPCR primers used:

BMP2: 5'ACACGGACAGCAGAAAACCA3',
5'AACAGCAGCAGGAGCAGAGA3'
BMP4: 5'GCAGCCCAGTAAGGATGT3',
5'CTTCTGTGCCTGGTAGATTC3'
BMP7: 5'TCTCCTTTGGACATACTTCTTGTG3',
5'CGCAACCTCCTCTGGATAAA3'
ADMP: 5'GCCTTCCGAGCAAGCTTACTT3',
5'CCTTGTGGCAACTGTATCTTATTTTTA3'
Chordin: 5'AACTGCCAGGACTGGATGGT3',
5'GGCAGGATTTAGAGTTGCTTC3'
MyoD: 5'CCCTGTTTCAATACCTCAGACAT3',
5'CGTGCTCATCCTCGTTATGG3'
Sizzled: 5'AACAAGGTCTGCTCCTTCCA3',
5'CTGTGGGTCTGGTCCGTATC3'
Alk1 (S+L): 5'GCTCTGGGGAAACTTGTGTT3',
5'CAACGCTCCTTTATGCTGTT3'
Alk2 (S+L): 5'TGTTATGGGCAGCAGTGTT3',
5'GATGTTCAAGTTACAGAGGTCACT3'
Alk3 (S+L): 5'TGGCTCAGGGCTACCATTATT3',
5'CACCTTCTCTCCTCTCCATTTTC3'
Alk6 (S+L): 5'ACAGCAGGAAGGAAGACACA3',
5'ACAGTGGTGGTGGCAGTAAC3'

Whole mount in situ hybridization

Whole-mount *in situ* hybridization (ISH) analysis of gene expression was performed as described previously (Marom *et al.,* 2005). Digoxigenin-labeled RNA probes were prepared from linearized plasmids transcribed *in vitro* using the RiboMax kit (Promega) and the digoxigenin RNA labeling mixture (Roche). Probes for ISH were transcribed from pSP64TNE BMPR clone for Alk3.L (Graff *et al.,* 1994). DNA templates utilized for transcription of *Alk2* and *Alk6 in situ* hybridization probes were generated using PCR amplification. The primers used: ALK2_DIG (S+L), 5'TGACCTCTGTAACTTGAACATCAC3', 5'ATTGCTGACCATCCGTCTG3';ALK6_DIG (S+L), 5'CGTTTCCTTGATTATGTTGCTATC3', 5'TATTTCAGTGTGTAGGTGGCAGA3'. The T7 promoter sequence (TAATACGACTCACTATAGGG) was added to the reverse primer for transcription.

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