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Activin ligands are required for the re-activation of Smad2 signalling after neurulation and vascular development in *Xenopus tropicalis*

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ABSTRACT The importance of Transforming Growth Factor β (TGFβ) signalling during early development has been well established. In particular, Nodal ligands have been shown to play essential roles for the specification and the patterning of the mesendoderm, axes formation and organogenesis. Activin ligands, like Nodal, signal by inducing the phosphorylation of the intracellular signal transducers Smad2 and Smad3. However, the roles of Activins during embryonic development are much less understood. Here, we report that during *Xenopus tropicalis* development two waves of Smad2 phoshorylation can be observed, first during gastrulation and then a second one after neurulation. Using a knock-down approach, we show that the second wave of Smad2 phosphorylation depends on *activinβa* (*actβa*) and *activinβb* (*actβb*) expression. Knocking down the expression of *actβa*, or treating the embryos with a chemical inhibitor inhibiting TGFβ receptor I (TGFβRI) activity after neurulation result in a decrease of the expression of endothelial cell markers and a lack of blood flow in *Xenopus* tadpoles. Taken together these data suggest that Activin ligands play an important role during vascular development in *Xenopus tropicalis* embryos.

KEY WORDS: *Activin, vasculogenesis, TGFβ, Xenopus, Smad*

Introduction

The vasculature is one of the most complex and important organs in the vertebrate body. It is essential for the transport of oxygen and nutrients and the removal of waste products throughout the organism. During embryonic development, vasculature formation starts by the de novo formation of blood vessels, or vasculogenesis. Firstly, hemangioblasts differentiate into angioblasts which finally give rise to endothelial cells (Patel-Hett and D'Amore, 2011). The angioblasts come from two distinct mesodermal tissues: the Dorsal Lateral Plate (DLP) and the Ventral Blood Island (VBI; Walmsley *et al.*, 2002). The endothelial cells of the VBI coalesce to form the Vascular Vitelline Network (VVN) which is necessary for the migration of red blood cells into the vasculature (Levine *et al*., 2003). At either side of the DLP, differentiated endothelial cells form the Posterior Cardinal Vein (PCV). Then the hypochord secretes angiogenic factors such as the Vascular Endothelial Growth Factor A (VEGFA) to instruct some cells of the PCV to form the Dorsal Aorta (DA) (Cleaver and Krieg, 1998). Eventually the two PCVs merge into one in the tail and connect to the DA setting up the first circulatory system in the tadpole. After this first phase of vasculogenesis, the subsequent blood vessels form by sprouting from existing vessels, a process known as angiogenesis (Herbert and Stainier, 2011). The Inter Somitic Vessels (ISVs) and the Dorsal Longitudinal Anastomosing Vessel (DLAV) then form, completing the circulatory system in the embryo (Levine *et al*., 2003).

Abbreviations used in this paper: Actβa, Activin βa; Actβb, Activin βb; DA, Dorsal Aorta; DLAV, Dorsal Longitudinal Anastomotic Vessel; ISV, Intersomitic Vessel; MO, Morpholino; NF, Nieuwkoop and Faber developmental stage; PVC, Posterior Cardinal Vein; TGFβ, Transforming Growth Factor β; TGFβR1, Transforming Growth Factor β Receptor I; VVN, Vascular Vitelline Network.

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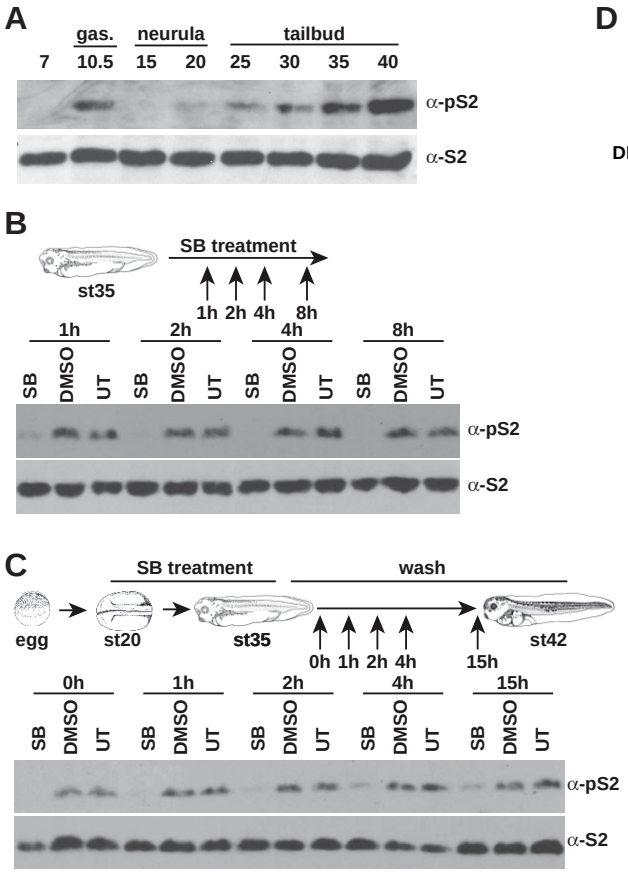

Fig. 1. The second wave of TGFβ signalling is necessary for establishing blood circulation in *Xenopus* embryos. **(A)** *Temporal time course of TGFβ activity during early* Xenopus *development. Total protein extracts from embryos at the indicated stages were analysed by Western blot. Activated Smad2 was detected using anti-phosphorylated Smad2 antibodies (α-pS2, upper panel). The membrane was stripped and reprobed with anti-Smad2 antibodies (α-S2).* **(B)** *The small molecule inhibitor SB505124 prevents Smad2 phosphorylation within an hour. Stage NF35 embryos were treated with SB505124 (SB), DMSO (as a control) or untreated (UT) for the indicated amount of time. After protein extraction, the level of Smad2 activity was assayed by Western blotting using antibodies against phosphorylated Smad2 (α-pS2, upper panel). The membrane was stripped and re-probed with anti-Smad2 antibodies (α-S2, lower panel).* **(C)** *The SB505124 inhibitor remains active 15h after treatment.* Xenopus *embryos were treated with the SB505124 inhibitor from stage NF20 to stage NF35 (about 12h for* Xenopus tropicalis *embryos), washed in 0.01X MMR and collected at the indicated times. After protein extraction, the level of Smad2 activity was assayed by Western blotting using antibodies against phosphorylated Smad2 (α-pS2, upper panel). The membrane was stripped and reprobed with anti-Smad2 antibodies (α-S2, lower panel). In all cases, the experiments were done at least three times and a representative blot is shown.* **(D)** *Overall phenotype of SB treated embryos. Embryos were treated with SB505124 (SB) or DMSO (as a negative control) from stage NF15 until stage NF35 and then raised in 0.01X MMR. Pictures were taken at the indicated stages.* **(E)** *Effect of the SB505124 treatment on blood flow. Embryos treated with the SB505124 inhibitor (SB, blue bar, n=95) or DMSO (as a negative control, red bar, n=105) were scored at stage 45 for the presence of blood cell circulation in the tail. The graph shows the compounded results of four independent experiments.*

At the molecular level, many signalling molecules play important roles during the formation of the embryonic vascular system. The Vascular Endothelial Growth Factor (VEGF), Notch, Wnt, Angopoietin / Tie2 (or TEK), Eph-Ephrin and Transforming Growth Factor β (TGFβ) have all been shown to be important for the development of the vasculature (Herbert and Stainier, 2011; Walmsley *et al.*, 2008; Kume, 2012; Adams and Eichmann, 2010). TGFβ signalling has mainly been involved in the differentiation of smooth muscle cells lining the blood vessels and in the formation of the extracellular network of blood vessels in the yolk sac of mouse embryos (Carvalho *et al.*, 2007). Furthermore, TGFβ has been shown to have both pro and anti-angiogenic effects in the context of tumour growth, possibly depending on the level of TGFβ signalling (Pardali *et al.*, 2010).

The TGFβ superfamily of ligands is a major group of signalling molecules involved in multiple biological processes. TGFβ ligands signal by binding a type II serine/threonine kinase receptor which then recruits and activates a type I receptor. The type I receptor then phosphorylates its intracellular effector, the receptor regulated Smad (R-Smad). In the case of TGFβs, Activin and Nodal ligands, the R-Smads are Smad2 and Smad3, whilst Bone Morphogenetic Proteins (BMPs) signal through Smad1, 5 and 8 (Massagué, 1998). The R-Smads then form a complex with Smad4, translocate into the nucleus and in association with other transcription factors, regulate gene expression (Wu and Hill, 2009).

During early embryonic development, BMP and Nodal ligands are the main TGFβ superfamily members to be expressed. They are involved in mesendoderm specification and patterning and setting up of the dorso-ventral and left-right axes (Wu and Hill, 2009). Both the knock-out of *Smad2* in mouse (Nomura and Li 1998) and the knock down of Nodal co-receptors in *Xenopus* (Dorey and Hill, 2006) cause a lack of mesoderm tissues and a failure to gastrulate. Later during development, TGFβ ligands play a role in the morphogenesis of most organs (Goumans and Mummery, 2000). The knock-out of TGFβ1, TGFβ2 or TGFβ3 causes multiple defects leading to embryonic or perinatal lethality. Finally, Activin ligands are also expressed during embryogenesis. It was the first molecule isolated from a *Xenopus* cell line shown to be able to induce mesoderm (Smith *et al.*, 1990; Asashima *et al.*, 1990). In mammals, four Activin ligands have been identified: Inhba, Inhbb, Inhbc and Inhbe (also known as Actβa, Actβb, Actβc and Actβe). The knockout of these different genes have been generated leading to relatively mild phenotype for *actβa* and *actβb* or no observed phenotype in the case of *actβc* and *actβe* (Pardali *et al.*,

2010). This suggests that Activin ligands have a minor role during embryonic development or a high level of redundancy between the different family members. In *Xenopus*, recent evidence points to a possible role for Actβb during gastrulation (Piepenburg *et al*., 2004) by regulating the expression of genes involved in the control of the cell cycle (Ramis *et al*., 2007). The role of other Activin family members in *Xenopus* has not yet been explored. In vitro, Actβa has been shown to cooperate with VEGF to induce endothelial fate when applied to naïve ectodermic cells (Yoshida *et al*., 2005). However, a potential role of Activin in endothelial cells development *in vivo* has not been shown.

Here we show that during early embryonic development, two waves of TGFβ activity can be detected. One culminating at gastrulation which has been well characterised and a second one starting at the end of neurulation and increasing until tadpole stage. The second wave of TGFβ activity is due to Actβa and Actβb ligands and is required for the development of the vasculature. Using a chemical inhibitor and a morpholino approach, we show that the inhibition of TGFβ signalling causes a downregulation of endothelial marker genes expression. However, RT-PCR and *in situ* analyses indicate that the red blood cell or myeloid lineages are not affected by TGFβ signalling. Finally, Actβa is necessary for the formation of the vitellin plexus and the mature embryonic blood vessels. Actβa morphant embryos lack the DLAV and do not separate the DA from the PCV in the tail. Altogether, these data suggest that Activin-dependant TGFβ signalling is required for the differentiation or the maintenance of the endothelial cell population during vascular development.

Results

Two waves of TGFβ activity during early development

We first wanted to determine the temporal activity of the TGFβ pathway during early *Xenopus* development. To this end, we performed a time-course of Smad2 phosphorylation during *Xenopus tropicalis* development from stage NF7 (blastula stage) to stage NF40 (tadpole stage). As reported before, a short peak of Smad2 phosphorylation is detected during gastrulation (St NF10.5, Fig. 1A) which then returns to baseline by stage NF15. Smad2 starts to be phosphorylated again at stage NF20 and this

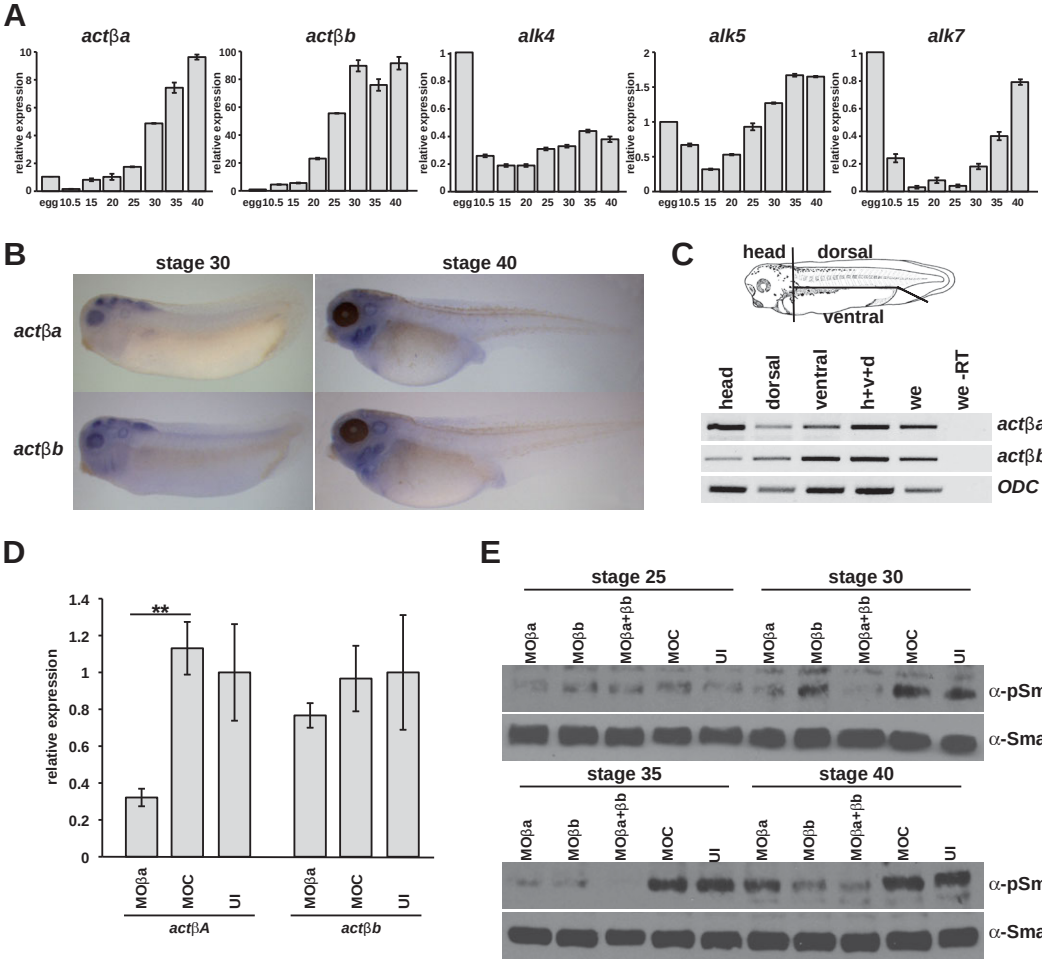

Fig. 2. Activinβa and Activinβb are responsible for the second wave of Smad2 phosphorylation. (A) *Total RNA from embryos at the indicated stages was isolated, reverse transcribed and subjected to real time qPCR for the indicated genes. The graphs show the mean and standard error of three independent experiments with the expression of each gene normalised to 1 at egg stage.* **(B)** *Spatial expression of* activin *genes.* In situ *hybridisation of stage NF30 and NF40* Xenopus *embryos using probes specific for* actβa *and* actβb. **(C)** Activin *ligands are also expressed in the ventral side of a tadpole. Stage NF35 embryos were dissected as indicated and RT-PCR perfomed using primers specific for* actβa, actβb *and* odc *as a loading control. h+v+d is a reconstituted sample from dissected head, ventral and dorsal parts of the embryo, we is whole embryo.* **(D)** *Efficacy and specificity of the morpholino against* actβa. Xenopus tropicalis *embryos were injected at the one-cell stage with 17ng of morpholino against* actβa *(MOβa), 17ng of morpholino control (MOC) or uninjected (UI) and raised until stage NF35. After extraction of total RNA and reverse transcription, the samples were subjected to real time qPCR using oligonucleotides specific for the indicated gene. The graph shows the mean and standard error of 4 independent experiments (** p<0.01).* **(E)** *Knock down of Actβa and Actβb expression causes a reduction of phosphorylated Smad2. Embryos were injected with 17 ng of Actβa (MOβa) morpholino, 17 ng Actβb morpholino (MOβb), 10 ng of each (MOβa+βb), 20 ng of MO control (MOC) or uninjected (UI). Embryos were collected at the indicated stage and the amount of phosphorylated Smad2 (α-pSmad2) assayed by Western blot. The membranes were stripped and reprobed for total Smad2 (α-Smad2). A representative blot from three independent experiments is shown.*

increases until stage NF40 (Fig.1, Zhang *et al.* 2014). The origin and role of the first wave of Smad2 phosphorylation has been well characterised (Whitman, 2001; Wu and Hill, 2009), however less is known about the function of the second wave of TGFβ activity. In order to understand the role of this second wave of Smad2 phosphorylation, we decided to test whether the TGFβRI chemical inhibitor SB505124 (SB) can inhibit Smad2 phosphorylation at tadpole stage in *X.tropicalis*. Stage NF35 tadpoles were incubated in 0.1 mM SB, DMSO (as a negative control) or untreated (UT) from 1h to 8h. After protein extraction, the samples were analysed for Smad2 phosphorylation (Fig. 1B). An hour treatment is sufficient to inhibit almost completely Smad2 phoshorylation and the inhibitor is still active after 8h (Fig. 1B). Because we wanted to completely prevent the second wave of TGFβ-like activity, we then treated embryos at stage NF20 (at the beginning of the second wave of Smad2 phosphorylation) until stage NF35. The embryos were then incubated in 0.01X MMR without inhibitior. Samples from stage NF35 (0h) to stage NF42 (15h after removing the inhibitor) were analysed by western blot to determine the state of Smad2 phosphorylation. Even 15h after removal of the inhibitor, only a faint band corresponding to pSmad2 was detected (Fig. 1C).

Inhibition of the second wave of pSmad2 causes vascular defects

Having a means to effectively prevent the second wave of Smad2 phosphorylation, we then analysed the phenotype of embryos treated with SB from stage NF20 (Fig. 1D). Multiple defects are apparent in these embryos: lack of pigment (both in melanocytes and eyes), defects in the gut looping and formation of an oedema in the heart (Fig. 1D). Very strikingly, almost all the embryos treated with the SB compound lacked blood flow (95% n=95 embryos from four independent experiments; Fig. 1E; supplementary movie 1) compared with only 2% of the DMSO control lacking blood flow (n=105 embryos from four independent experiments; Fig. 1E; supplementary movie 2 and 3 for DMSO and untreated embryo respectively).

Temporal and spatial expression of activin ligands in Xenopus embryos

The chemical inhibitor SB505124 inhibits Alk4, Alk5 and Alk7 TGFβ type I receptors (DaCosta Byfield *et al.*, 2004). We therefore wanted to determine which TGFβ superfamily member(s) were responsible for the second wave of Smad2 phosphorylation. Published literature and data mining indicate that Nodal-related ligands are unlikely to be involved in the second peak of Smad2 activation (Onuma *et al.*, 2006; Yanai *et al.*, 2011); http://kirschner.med.harvard.edu/*Xenopus*_Transcriptomics.html). Indeed, only *nr1* shows a peak of expression after gastrulation at stage NF23 but this has been shown to be important for setting up the left-right asymmetry in heart and gut looping (Onuma *et al.*, 2006). Interestingly, the expression of *activinβb* (or *inhbb*) increases of about 100 fold from stage NF15 to stage NF40 (Fig. 2A; Piepenburg *et al.*, 2004; Yanai *et al.*, 2011). We also analysed *activinβa* (or *inhba*) expression and it follows a similar pattern even if only a 10-fold induction was observed (Fig. 2A). However, *activinβc* (or *inhbc)* does not seem to be expressed in early *Xenopus* embryos (data not shown; Yanai *et al.*, 2011). All the type I receptors are expressed throughout early development, with *alk4* expression being constant from stage NF10.5 to NF40 whilst *alk5* and *alk7* expression increases after stage NF15 and NF30 respectively (Fig. 2A).

We then determined where *activin* ligands are expressed in the embryo using *in situ* hybridisation with probes specific for *actβa* and *actβb* (Fig. 2B). Both genes have a similar pattern of expression with strong staining in the eye, the brain and the branchial arches. This is consistent with the pattern of expression of *actβb* described in *Xenopus laevis* (Dohrmann *et al*., 1993). To further examine whether *activin* ligands are only expressed in the anterior part of the embryo, stage NF30 tadpoles were dissected in three parts: head, posterior dorsal and posterior ventral (Fig. 2C). After RNA extraction and generation of cDNA, the different samples were subjected to PCR using oligonucleotides specific for *actβa*, *actβb* and *odc* as a loading control (Fig. 2C). We could

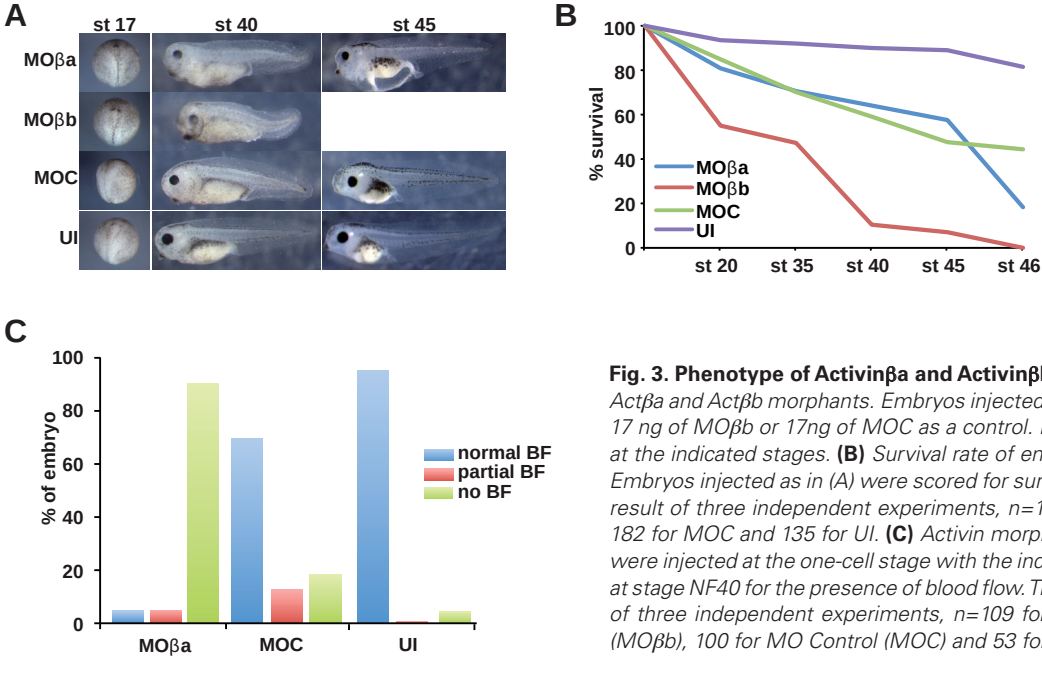

Fig. 3. Phenotype of Activinβa and Activinβb morphants (A) *Overall phenotype of Actβa and Actβb morphants. Embryos injected at the 1-cell stage with 17 ng of MOβa, 17 ng of MOβb or 17ng of MOC as a control. Pictures of typical embryos were taken at the indicated stages.* **(B)** *Survival rate of embryos morphants for Actβa and Actβb. Embryos injected as in (A) were scored for survival. The graph show the compounded result of three independent experiments, n=184 embryos for MOβa, 151 for MOβb, 182 for MOC and 135 for UI.* **(C)** *Activin morphants do not have blood flow. Embryos were injected at the one-cell stage with the indicated morpholinos as in (A) and scored at stage NF40 for the presence of blood flow. The graph shows the compounded result of three independent experiments, n=109 for MO Actβa (MOβa), 19 for MO Actβb (MOβb), 100 for MO Control (MOC) and 53 for uninjected (UI).*

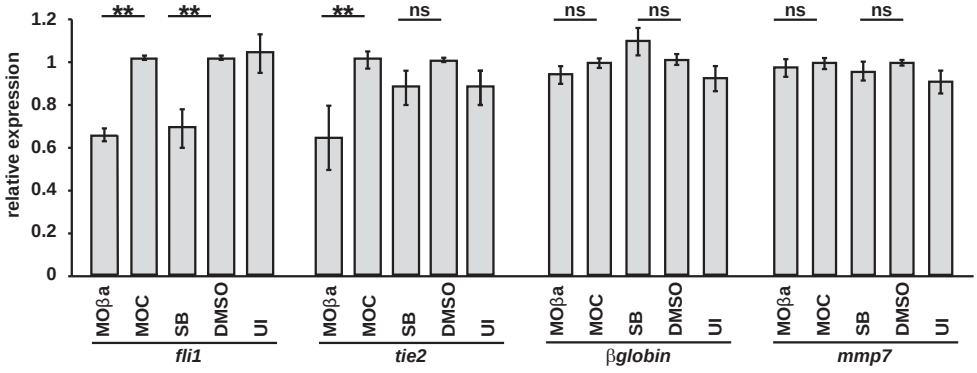

Fig. 4. TGFβ signalling is necessary for expression of endothelial cell markers. *Embryos were injected at the one-cell stage with MOActβa (MOβa) and a MO control (MOC) or treated at stage NF15 with 100μM of SB505124 (SB) and DMSO (as a negative control). At stage NF35, the embryos were harvested and total RNA isolated. Real time qPCR was then performed using specific primers for the indicated genes.* Fli1 *and* tie2 *are endothelial markers,* βglobin *is a red blood cell marker and* mmp7 *is expressed in myeloid cells. The graph represents the mean and standard error of three independent experiments. ns, non specific. ** p<0.01.*

detect transcripts of both *actβa* and *acβb* in all parts of the embryos indicating that their expression is enriched in the anterior part of the embryo, but they are also expressed in the dorsal and ventral part of the tailbud in a diffuse fashion not detectable by *in situ* hybridisation.

Activin ligands are responsible for the second wave of Smad2 phosphorylation

We next asked whether the effects we observed with the SB inhibitor were due to Activin signalling. To this end we designed antisense oligonucleotides (or morpholinos, MO) to prevent Actβa and Actβb expression. Because *actβb* gene is located at the end of the scaffold GL177808 on the assembly 4.2 of the *Xenopus tropicalis* genome (or scaffold_7691 of the assembly 7.1), it was not possible to design a splice morpholino and we therefore used an ATG morpholino (MOβb; Fig. S1). For *actβa*, we designed a morpholino at the junction of exon 1 and intron 1 (MOβA) and could estimate its efficacy by RT-qPCR (Fig. 2D). We were able to knock-down its expression by about 70% by injecting 17ng of MOβa. We also designed an ATG morpholino for *actβa* and observed the same phenotype as the splice morpholino (data not shown). Because Activin ligands induce phosphorylation of their downstream effector Smad2, we analysed the effect of MO injection on pSmad2 (Fig. 2E). We either injected 17ng of each individual morpholino or 10ng of MOβa and 10 ng MOβb in combination and compared with 20ng of MOC or uninjected embryos (UI). At stage 25, the level of pSmad2 is very low and only a weak downregulation was observed with MOβa. At stage 30, pSmad2 downregulation was more apparent when both MOβa and MOβb were injected together, almost completely preventing Smad2 phosphorylation. By stage 35 and 40, pSmad2 is weaker in MOβb injected embryos compared to MOβa injected ones (Fig.

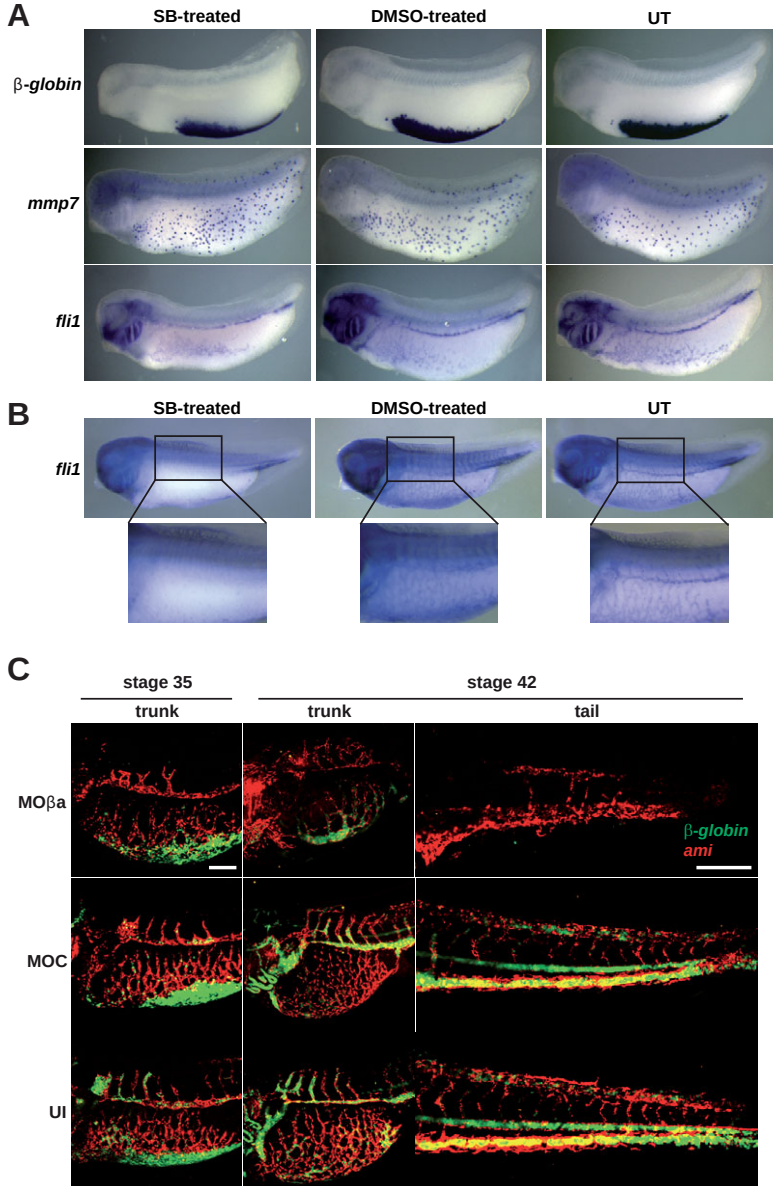

Fig. 5. Inhibition of TGFβ signalling affects endothelial gene expression. **(A)** In situ *hybridisation of stage NF30 embryos treated with the SB505124 inhibitor (SB-treated), DMSO (as a control) or untreated (UT) from stage NF15.* β-globin *is a marker for red blood cells,* mmp7 *is a primitive myeloid marker and* fli1 *labels endothelial cells.* **(B)** *Embryos were treated as in (A) but until stage NF35 and subjected for* in situ *hybridisation using a probe against* fli1. *The lower panels show a magnification of the black box.* **(C)** *Actβa morphant have a disrupted vasculature. Embryos injected with the indicated morpholinos were fixed at stage NF35 or NF42 and then processed for fluorescent* in situ *hybridisation using antisense probes against* β-globin *(a red blood cell marker, green) and* ami *(an endothelial cell marker, red). The samples were imaged using confocal microscopy and a maximal projection is shown. In all cases, anterior is left, dorsal up. The scale bar represents 200 μm.*

TABLE 1

PHENOTYPE OF MOACTβA INJECTED EMBRYOS

	DA and PCV[a]		Presence of the DLAV[b]		βglobin +ve cells in the tail[c]	
	Separated	fused	Yes	No	Yes	No
MOβa (n=10)	2	8	0	10	0	10
MOC (n=8)	8	0	8	0	8	0

Embryos were injected with MOβa or MOC and fixed at stage NF45. After *in situ* hybridisation for *ami* (endothelial cells marker) and β*globin* (red blood cells marker), the sample were sectioned and analysed by fluorescent microscopy. Three phenotypes were quantified [a]the fusion of the dorsal aorta (DA) and the posterior cardinal vein (PCV); [b]the presence of the Dorsal Longitudinal Anastomosing Vessel (DLAV) and [c]the detection of β-*globin* positive (+ve) cells in the tail. The quantification was done with the indicated number of embryos from three independent experiments.

2E), in accordance with the greater upregulation of *actβb* expression at these stages (Fig.2A). These results demonstrate that the main ligands responsible for the second wave of Smad2 phosphorylation during *Xenopus* development are Actβa and Actβb.

Phenotype of Activin morphants

Phenotypically, embryos injected with MO against *actβa*, *actβb* or both do gastrulate and neurulate normally (Fig. 3A and data not shown). However, at stage NF40 the embryos are shorter most notably for MOβb injected embryos. By stage NF45, less than 10% of the MOβb injected embryos survive (Fig 3B) whilst MOβa injected embryos display a lack of pigment and formed oedema (Fig. 3A). This is reminiscent of the phenotype observed in SB-treated embryos (Fig.1D). Because the phenotype observed in *actβb* morphants is more severe than SB-treated embryos, it suggests that Actβb may have an important role before stage 20 (when we start the SB treatment) or the SB treatment does not completely inhibit TGFβRI. However, MOβa injected embryos survive at the same level than MOC injected embryos (Fig. 3B). We also assessed whether MOβa injected embryos lacked blood flow. We scored stage 40 embryos for complete absence of blood flow or only partial blood flow (visible in the proximal part of the embryo only). Knocking down *actβa* expression causes a dramatic reduction in the number of tadpoles with blood flow (Fig. 3C, supplementary movies 4 and 5 for MOC and MOβA injected embryos respectively). This is also reminiscent of the phenotype observed when embryos are treated with the SB compound, indicating that Actβa is the ligand responsible for the observed phenotype.

TGFβ signalling and Activin regulate the expression of endothelial genes

To gain insight in the molecular mechanisms by which TGFβ signalling plays a role in vascular development, we analysed the level of expression of marker genes for the endothelial, myeloid and red blood cell lineages by RT-qPCR (Fig. 4). Embryos either treated with the TGFβR inhibitor, SB505124, or injected with MOβA were raised until stage NF35. After extraction of total RNA, we analysed the expression of two endothelial markers, *fli1* and *tie2*. The expression of *fli1* was downregulated significantly in MOβA injected and SB-treated embryos by about 40% when compared to control embryos (MOC and DMSO treated respectively, $p<0.01$). *Tie2* (also known as *tek*) expression was also significantly reduced in MOβA injected embryos ($p<0.01$) but only slightly in the case of the SB treatment ($p>0.05$). However, the expression of a red blood cells marker (β-*globin*) and a myeloid cell marker (*mmp7*) were not affected by the inhibition of TGFβ signalling (Fig. 4). These data indicate that the endothelial marker genes require TGFβ signalling and Actβa ligand for their expression *in vivo*.

TGFβ signalling and Actβa are necessary for vascular development

We then wanted to analyse the spatial expression of hematopoietic markers when TGFβ signalling is inhibited. To this end, we performed *in situ* hybridisation to analyse the expression of the different hematopoietic markers we used for the RT-PCR analyses (β-*globin* for red blood cells, *mmp7* for myeloid cells and *fli1* for endothelial cells). At stage NF30, *β-globin* and *mmp7* expression in SB-treated embryos is undistinguishable to that of control embryos (Fig. 5A). However, *fli1* expression is slightly weaker, the

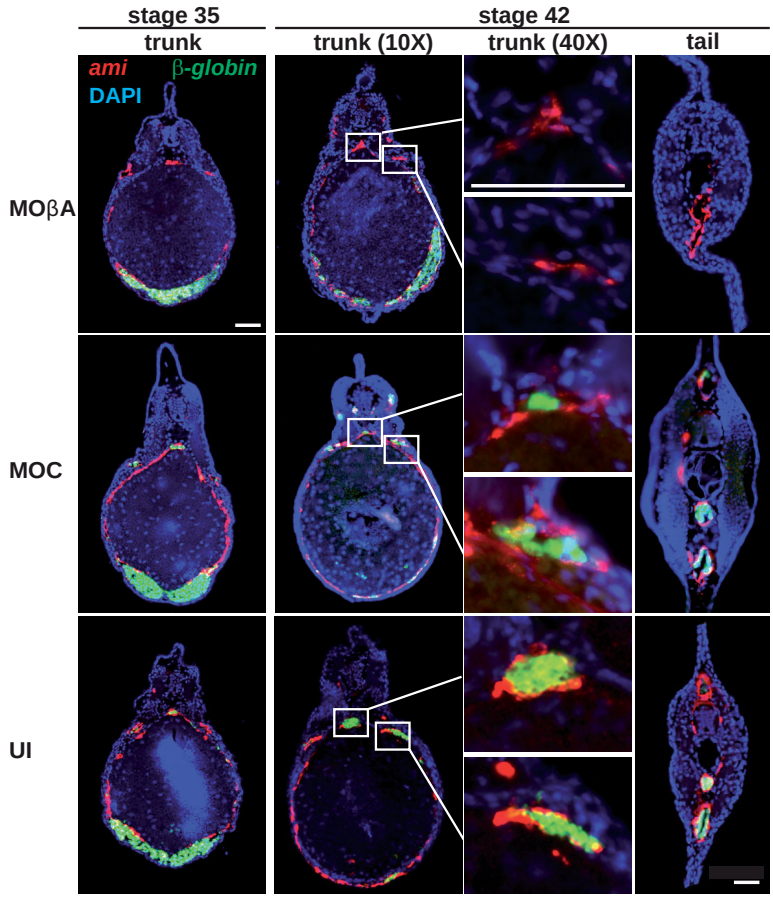

Fig. 6. The organisation of endothelial cells in disrupted in Actβa morphants. *Embryos injected with the indicated morpholinos were fixed at stage NF35 or NF42 and then processed for fluorescent* in situ *hybridisation using antisense probes against* β-globin *(a red blood cell marker, green) and* ami *(an endothelial cell marker, red). After embedding and cryosection, the sections were stained for DAPI (blue) to label the nuclei. The sections are at the level of the trunk and the tail. The white boxes show a close up of the dorsal aorta (DA) and the posterior cardinal vein (PCV). Dorsal is up and the scale bar represents 50mm.*

VVN is less developed and the posterior cardinal vein (PCV) is less defined. At stage NF35, the VVN, PCV and the intersomitic vessels (ISV) are almost undetectable using *fli1* as a marker (Fig. 5B). These data confirm and extend our RT-PCR analysis and indicate that Smad2 signalling is required for the establishment of the blood vessel network during early development.

To address the specific role of Actβa during vascular development, embryos were injected at the 1-cell stage with 17ng of MOβa, 17ng of MOC or UI. At stage NF35 or NF42, the embryos were fixed in MEMFA and subjected to double fluorescent *in situ* hybridisation with probes recognising *β-globin* (for red blood cells) and *ami* (an endothelial marker; Inui, Asashima, 2006). In control embyos at stage NF35, most of the *β-globin*-positive cells are still located in the ventral blood island, the VVN is already well formed and the PCV and ISVs are visible (Fig. 5C). In MOβa injected embryos, the VVN is reduced and whilst the PCV is visible, it is not continuous and the ISVs are malformed (Fig. 5C). This is reminiscent of the phenotype seen in SB-treated embryos (Fig. 5B). At stage NF42, the *β-globin*-positive cells have now entered the vasculature, the VVN covers all the ventral part of the embryos, the PCV, DA and DLAV are formed and the ISVs link the DA and DLAV in control embryos (Fig. 5C). In contrast, MOβa injected embryos display *β-globin* staining in the VBI, the VVN is disorganised, and whilst some *ami* staining is visible in the tail, the different blood vessels have not formed properly (Fig. 5C).

To analyse this phenotype in more details, we then performed cryosection of the whole mount *in situ* hybridisation shown in Figure 5C (Fig. 6, Table 1). At stage NF35, most of the *β-globin* expressing cells reside in the ventral side of the embryo even if some red blood cells are already localised in the PCV and DA. The *ami*-positive cells are forming a single cell-layer and the DA and PCV already have a lumen. In MOβa injected embryos, much fewer *ami* positive cells are visible and whilst some are localised where the PCV and DA should be, they do not form a lumen. At stage NF42, the phenotype is much more severe with only few *ami*-positive cells present where the DA and the PCV is located (Fig. 6). In the tail, the DA, PCV and DLAV are all visible in control embryos. In contrast, morphant embryos display a fused DA-PCV and the DLAV is absent (Table 1).

Discussion

Here we show a novel role for Activin in blood vessel formation during *Xenopus tropicalis* development. In particular, we present evidence that the downstream effector of the TGFβ pathway, Smad2, is activated in two waves during early development: firstly during gastrulation due to Nodal activity (Dorey and Hill, 2006; Luxardi *et al.*, 2010) and then after neurulation due to *actβa and actβb* expression (Figs.1A and 2E). Preventing the second wave of Smad2 phosphorylation results in defects in the formation of the vasculature of the embryos and the downregulation of endothelial genes expression such as *tie2* or *fli1*.

TGFβ – like activity during embryonic development

The role of TGFβ signalling during gastrulation has been extensively studied. It is essential for the specification and the patterning of the mesendoderm (Wu and Hill, 2009). Here, we first analysed the temporal phosphorylation status of the intracellular effector of TGFβ signalling, Smad2. We show that Smad2 phosphorylation decreases dramatically after gastrulation and becomes detectable again after the neural tube has closed (stage NF20) and increases until stage NF40. This is similar to what has been reported in *Xenopus laevis* (Zhang *et al.,* 2014). We then decided to determine which of the TGFβ superfamily of ligands are responsible for this second wave of Smad2 activation. Using published literature (Piepenburg *et al*., 2004) and recent transcriptomics dataset (Yanai *et al.*, 2011) accessible via Xenbase (http://www.xenbase.org/), we could restrict the number of TGFβ ligands possibly responsible. In particular, *tgfβ1*, *tgfβ2*, *actβa* and *actβb* all show a substantial increase in expression between stage NF12 and stage NF33. Using a morpholino knock-down approach, we show that *actβa* and *actβb* are required for the second wave of TGFβ-like signalling. Our analysis indicates that at stage NF30, most of the pSmad2 is Actβa-dependent, with only a small contribution from Actβb. At stage NF35, both Activins seem to contribute to the phoshorylation of Smad2 equally. Finally, at NF40, pSmad2 is mainly Actβb-dependent (Fig. 2E). These data correlate with the RT-PCR data showing that both *actβa* and *actβb* expression increase at the same time, but the relative expression of *actβb* increases at much higher level (Fig. 2A). Unfortunately, we do not have absolute levels of mRNA expression, precluding a direct correlation between level of expression of the ligand and activation of the downstream effector. We did not investigate the role of Tgfβ1 and Tgfβ2 in this study but this will be interesting to do so in the future.

Role of Activin during early embryonic development

Activin was first isolated as a potent mesoderm inducer (Smith *et al*., 1990) and has been extensively studied for its morphogenetic activities (Green and Smith, 1990). However, the role of Activin during embryonic development is still unclear. In zebrafish, there has been suggestion that maternal Activin is required for mesoderm and axis formation (Wittbrodt, Rosa, 1994), however this study relies exclusively on the over-expression of dominant-negative forms of Activin. More recently, the expression of Actβb was knock-down in *Xenopus laevis* embryos resulting in a lack of axial development and a reduction in the expression of dorsal mesodermal genes (Piepenburg *et al*., 2004). Finally, in mouse embryos, the role of Activin ligands has been well established in gonadal sex development, follicle cells development and in the differentiation of stem cells to immune cells (Xia, Schneyer, 2009); (Vallier *et al.*, 2005). Knockout of *Actβb* leads to defects in eyelid development and female reproduction (Vassalli *et al*., 1994). Mice knocked out for *Actβa* die within a day after birth showing multiple defects in craniofacial development (Matzuk *et al*., 1995). There is probably no redundancy between Actβa and Actβb function as the double knockout produce an additive effect rather than a synergetic one (Matzuk *et al*., 1995). However, the situation might be complicated by the existence of other Activin family members, which have not yet been fully characterised.

Here, we used the frog *Xenopus tropicalis* and its genomic information to address the role of Activin during early embryogenesis. To this end an ATG morpholino (preventing translation) and a splice morpholino (preventing splicing) were designed for *actβa*. Unfortunately, the genomic information for *actβb* did not allow us to design a splice morpholino and we therefore only used an ATG morpholino. Knocking down the expression of Actβa, Actβb or both has no effect on the morphology of the embryo until after neurulation. Later during development, Actβb morphant embryos

are shorter, with less pigment, form an oedema and show poor survival. By contrast, Actβa morphants develop fairly normally and their main phenotype is a decrease in pigmentation and a lack of blood flow. TGFβ signalling has been involved in epithelial-mesenchymal transition (EMT) in different contexts including during melanocyte differentiation (Nishimura *et al.*, 2010). This may explain the decrease in pigmentation observed in SB-treated embryos and in MOβa and MOβb injected embryos. To understand further the lack of blood flow phenotype, we performed *in situ* hybridisation studies using probes recognising red blood, myeloid and endothelial cells. At early stage of development (until stage NF30), we do not observe major differences between control and Actβa morphant embryos. From stage NF35, the ISVs, DA and DLAV do not form correctly in Actβa morphants. This correlates with a reduction in the expression of endothelial cells markers such as *tie2* and *fli1*. Finally, the red blood cells do not leave the ventral blood islands, indicating that the VVN is probably not functional. Importantly, treatment of tadpoles with a dose of tricaine able to block heart beat does not cause this phenotype (data not shown), ruling out a possible heart defect as the primary cause for the malformation of blood vessels. This is further confirmed by the fact that embryos SB-treated or MOβA-injected have a beating heart (see Supplementary movies 1 and 5).

Possible roles of Activin in blood and vasculature development

Activin family members have been shown to play important roles during wound repair and inflammation in mice (Werner and Alzheimer, 2006) and in fin regeneration in zebrafish (Jaźwińska *et al.*, 2007). However, no specific role of Activin in angiogenesis have been established in these cases (Wankell *et al.*, 2001; Antsiferova, Werner, 2012). In a xenograft model, transformed cells expressing ActivinA form tumours with fewer blood vessels compared to control cells (Panopoulou *et al*., 2005), suggesting a negative role of Actβa in inducing blood vessels formation. Interestingly, treatment of injured cornea with Actβa induces VEGF expression and increase neo-vascularisation (Poulaki *et al*., 2004). Taken together, these data indicate that the influence of Activin on blood vessel formation seems to be complex and context dependant.

In *Xenopus laevis*, treatment of naïve ectodermic explants, or animal caps, with various growth factors has shown that Activinβa can induce erythrocyte differentiation (Miyanaga, 1998). In the context of the whole embryo, Actβa morphants do not display any defects in red blood cells differentiation, indicating that this role is probably not conserved in the context of a whole organism. Interestingly, treatment of animal caps with low concentration of Actβa potentiates the ability of VEGF to induce *flk1* and *tie2* expression (Yoshida *et al*., 2005). This is consistent with our data showing a reduction of *fli1* expression in embryos treated with the TGFβ inhibitor or injected with MOβA. Taken together, these data would suggest that Activinβa acts as a potentiator for VEGF to induce the programme leading to the formation of the vasculature in early embryos. This effect might be mediated by the canonical Smad2/3 or by the activation of p38 and p42/44 downstream of the TGFβ receptor (Poulaki *et al*., 2004).

Materials and Methods

Embryo manipulations and morpholino injections

Xenopus tropicalis embryos were obtained as previously described (Collu *et al*., 2012). For chemical treatment, the TGFβR inhibitor SB505124 (Sigma) was used at 100μM final. DMSO diluted at 1:300 was used as a negative control. Morpholinos (Gene-Tools) were injected at the 1-cell stage with the indicated concentration. The morpholinos used were actβa e1i1 (MOβa) 5'TTACTGTCTCACAAACTCACCTGAT3', actβa atg (MoβaATG) 5'CTCCTTTCAGCAAGCGTGCAGGCAT3', actβb atg (MOβb) 5'CCACAAGCAGTAACAGGAGAGCCAT3' and the standard human βGlobin control (MOC) from GeneTools 5' CCTCTTACCTCAGTTACAATTTATA3'.

Western blot

Total embryo lysates were prepared as described previously (Dorey and Hill, 2006) and the equivalent of three embryos were loaded per lane. The antibodies used were anti-phospho Smad2 (clone A5S, Millipore) and anti Smad2/3 (Molecular Probes). After fractionation by SDS-PAGE, the proteins were transferred on a PDVF membrane (Millipore) and immunoblotted using standard techniques.

In situ hybridisation

Probes against *Xenopus tropicalis β-globin*, *mmp7*, *fli1* were described elsewhere (Costa *et al*., 2008). Probes against *ami* (Tneu054o06) and *actβb* (Tneu142f12) were isolated from the EST full-length clone library (accessible at http://genomics.nimr.mrc.ac.uk/online/xt-fl-db.html). Constructs for a probe against *actβa* was generated by amplifying the coding sequenced from cDNA using the following primers actβa fwd 5'ATGCCTGCACGCTTGCTGAAAGGAG3' and actβa rev 5'TTACGAGCAGCCACATTCCTCCAC3', cloned in pCRII TOPO vector and verified by sequencing.

Chromogenic *in situ* hybridisation were performed essentially as described (Harland, 1991) using BM purple as a substrate. Double fluorescent *in situ* protocol was recently published (Lea *et al*., 2012). For fluorescent in situs, whole mount were cleared in benzo-benzoate solution and image on a Olympus Fluoview FV100 confocal microscope. The images presented are maximum z-projection of stacks. For cryosections, the embryos were embedded in 25% fish gelatin / 15% sucrose after the *in situ* and subsequently sectioned at 20μM thickness. Images were acquired on a Nikon Eclipse 80i microscope.

RNA extraction and RT-PCR

Total RNA was extracted using QiaShredder (Qiagen) and RNAeasy mini columns (Qiagen). cDNA was generated from 1μg of total RNA using AMV reverse transcriptase (Roche) according to the manufacturer's instructions.

Semi-quantitative real-time PCR was performed using SYBR green (ABI). The data for each sample was normalised to the expression level of *odc* and calculated by the $2^{-\Delta\Delta Ct}$ method. The following primers were used for real-time qPCR *fli1* fwd 5'GGCGGATCTACTTGTTCTGG3' *fli1* rev 5'CTCCAAGAGAAACTGCCACA3'; *tie2* fwd 5'GCGTGAGAAGCCATATGAAA3' *tie2* rev 5'AAACCTATGCAGCCTCCTCA3'; *actβa* fwd 5'AGACAGTGCCGGCTCTTCCTAA3' *actβa* rev 5'TCCACGTTGGTCTTTCTGACCTCT3'; *actβb* fwd 5'GGGAATAGCGCCGAGAGTAG3' *actβb* rev 5'GATGGCGAAGGAGAGTCG3'; *alk4* fwd 5'CCTTCAGCCTCCTTCTCTTTGG3' *alk4* rev CCATCCGTAACACAGGTGAA; Xt *alk5* fwd 5'CGTGCTCTTGTTGATTGCAC3' *alk5* rev 5'TAAGCACAATCCATCGGTCA3'; Xt *alk7* fwd 5'GAATCCTAGGGTAGGGACCAA3' *alk7* rev 5'TCTGAGCATTTGAACGAGTCA3'. As an internal control, *odc* was used (*odc* fwd 5' CATGGCATTCTCCCTGAAGT3' *odc* rev 5' TGCTGGCAGTAGGACAGATG3'). Oligonucleotides for *mmp7* and *β-globin* were previously published (Costa *et al*., 2008).

Statistical analysis

To test the equality of variance between samples, an F-test was performed using Excel. All other analyses (normality test, one-way ANOVA and Dunn's post-hoc tests) were done using Prism. ** <0.01

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