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Chapter 4

Timed interactions between BMP and anti-BMP are involved in head-tail patterning of the vertebrate embryo

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Abstract

Hox genes have long been known to regulate successive steps in trunk patterning, it is not clear how patterning of the head is regulated. In this study, we found that timed anti-BMP treatment of wild-type frog embryos in the blastula and gastrula stages sequentially arrested the A-P axis at different positions, including the head part. We further applied timed anti-BMP signals to BMP-overexpressed embryos, which contain a blob of tissue with no axis. This resulted in sequential fixation of four genes: *six3* (a forebrain marker), *otx2* (a forebrain and mid-brain marker), *gbx2* (an anterior hindbrain marker) and *hoxd1*(a posterior hindbrain marker). Since hoxd1, which is the most anterior component of the Hox sequence, is the last and most posterior component of this anterior gene sequence. Our results thus argue for a continuous BMP-dependent timer running from head to tail. However, these genes themselves (at least some of them) may not be part of the timing mechanism, as their endogenous expression did not follow a temporal order of expression. They are more likely to be regulated by an upstream timing mechanism that can be regulated by BMP signalling.

Introduction

During early development, the vertebrate embryo is patterned from anterior to posterior in a temporally progressive manner (Eyal-Giladi, 1954; Gamse and Sive, 2000; Gamse and Sive, 2001; Nieuwkoop, 1952; Stern et al., 2006): anterior tissues are specified early, and more posterior tissues are determined progressively later. Whereas coordination between temporal and spatial control of anterior-posterior (A-P) patterning is evident, a thorough understanding of the underlying molecular mechanisms is still lacking in vertebrates.

Recently, it has been proposed in the frog *Xenopus* that the coordination between time and space during A-P patterning of the trunk is achieved via a time-space translation mechanism involving *Hox* genes (Durston and Zhu, 2015; Wacker et al., 2004a). In the frog embryo, *Hox* genes are first expressed in a temporal sequence (*Hox* timer) from the beginning of gastrulation in the non-organizer mesoderm (NOM, high BMP) (Wacker et al., 2004b). It has been proposed that during involution, the NOM undergoes timed interactions with signals from the Spemann organizer (SO), and the *Hox* timer is arrested at different temporal values. As involution progresses, different *Hox* codes, which will later be copied to the neurectoderm (Bardine et al., 2014), are stabilized at different positional values. The putative SO signals that stabilize *Hox* codes are likely to be BMP antagonists, noggin (Smith and Harland, 1992), chordin (Sasai et al., 1994) etc., since they could mimic the behaviour of the organizer (Khokha et al., 2005; Smith et al., 1993). Moreover, noggin could rescue body axis formation and hence *Hox* pattern in ventralized embryos, which contain no SO tissue (Wacker et al., 2004a). Further support for the time-space translation mechanism comes from a recent study in chick that reported a role for noggin in inducing posterior primitive streak (ventral mesoderm; equals NOM) to become somites (Dias et al., 2014). Notably, in this research the *Hox* code in the ectopic somites is fixed by noggin treatment according to genes expressed at the time of explantation.

Although A-P patterning via time-space translation is better documented for the vertebrate trunk, recent evidence has led us to postulate that a similar mechanism also operates during head patterning. Using heat-shock inducible *chordin* transgene lines (Tg (*hsp70:chd*)), Hashiguchi et al. showed in zebrafish that the expression of *six3* (a forebrain marker) (Kobayashi et al., 1998), *otx2* (a forebrain and mid-brain marker) (Li et al., 1994; Mori et

al., 1994), *gbx1* (the counterpart of Xenopus *gbx2*; a rostral hindbrain marker) (Rhinn et al., 2003), and *hoxb1b* (a caudal hindbrain marker) (Alexandre et al., 1996) is sequentially induced by timed anti-BMP treatment from mid-blastula to early gastrula stages (Hashiguchi and Mullins, 2013). This is consistent with the observations that timed noggin injections in ventralized embryos rescued different portions of the A-P axis in frog (Wacker et al., 2004a), and that progressively later anti-BMP treatment resulted in progressively more posterior axis defects in zebrafish (Tucker et al., 2008). These findings suggest that a timing mechanism, which is likely to be BMP dependent, is also involved in patterning the head.

In the deuterostome embryo, the front-most portion of the A-P axis is not the head, but the extreme anterior domain (EAD), a region wherein ectoderm and endoderm are directly juxtaposed (Jacox et al., 2014). In the frog *Xenopus*, this region gives rise to three organs, the cement gland (CG), the primary mouth, and the anterior pituitary (Dickinson and Sive, 2007). Among them, the cement gland is an ectodermal organ that lies anterior to any neural tissue (Sive et al., 1989). The formation of CG can be affected by perturbations of the development of dorsal mesoderm (SO) (Kao and Elinson, 1988; Scharf and Gerhart, 1983), suggesting a requirement for SO signals in the formation of this anterior-most structure. Therefore, the formation of CG and EAD are also likely to be regulated by a BMP/anti-BMP dependent time-space translation mechanism.

In this study, we investigated the effect of timed BMP inhibition on A-P patterning. Timed anti-BMP treatment in ventralized embryos resulted in A-P structures and their corresponding genes: *six3*, *otx2* and *gbx2* and *hoxd1*, being sequentially fixed. These results argue for the involvement of a timing mechanism, which is BMP dependent and can be converted into spatial patterns by anti-BMP signals, in patterning the whole vertebrate A-P axis.

Materials and Methods

Microinjection

Frog embryos were harvested from naturally mated females and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). For timed anti-BMP treatment in wild-type embryos, 200nL 0.1μg/μL human noggin protein (Sigma H6416) was injected into the

blastocoel of embryos at stage 8, 10, 10.5 and 11.5. The embryos were then cultured to stage 28 for taking pictures. A similar approach has been used by others (Cooke and Smith, 1989; Wacker et al., 2004a). mRNA for injection was transcribed with mMessage mMachine Kit (Ambion, Life technologies, AM1340) from the following plasmids after linearization at the appropriate restriction sites: pSP64T-BMP4 (for *BMP4* RNA) (Nishimatsu et al., 1992) and pCS2-hSmad6GR (for *smad6GR* RNA) (Marom et al., 2005). To induce full ventralization, about 2ng *BMP4* RNA was injected to each embryo at 2-cell or 4-cell stage and cultured to stage 26. Timed anti-BMP treatment in BMP-ventralized embryos was achieved by combined injection of 2ng *BMP4* RNA and 2ng *smad6GR* RNA at 2-cell or 4-cell stage. The embryos were then treated with 10 μM dexamethasone for 2 hours at desired stages and cultured to stage 26.

Whole mount in situ hybridization

When reached desired stages, embryos were fixed overnight in MEMFA at 4°C. After dehydration in 100% methanol, they were stored in methanol at -20°C until use. Whole mount in situ hybridization (WISH) was performed as previously described (Wacker et al., 2004a). The probes for in situ hybridization were synthesized from the following plasmids after linearization: pVZ1-xcg1 (for *xcg-1* probe) (Gammill and Sive, 2000), pBSSK-Six3 (for *six3* probe) (Kenyon et al., 1999), pBluescript-KS-xotx2 (for *otx2* probe) (Blitz and Cho, 1995), pXgbx-2 (for *gbx-2* probe) (von Bubnoff et al., 1996), and pBluescript SK-xHoxLab1 (for *hoxd1* probe) (Sive and Cheng, 1991).

Results

Timed anti-BMP treatment arrests the A-P axis at different positions

In the time-space translation mechanism, the role of anti-BMP signals is suggested to fix the BMP-dependent timer at different time points, corresponding to different positional values. We therefore wanted to know if timed anti-BMP treatment could lead to progressive arrest of the A-P axis. To this end, the BMP antagonist, noggin protein, was injected to the blastocoel of the embryo at st.8, 10, 10.5 and 11.5 (Fig 1). The embryos injected with noggin at st.8 formed a ball of tissue with no clear head. However, the formation of the cement gland was clearly visible at the forefront of the embryo. When noggin was injected at st.10, a visible head was formed in the embryo. Injection of noggin at st.10.5 and 11 resulted in

the formation of more posterior structures, truncating the axis at the neck and thorax regions, respectively. Therefore, sequentially later application of noggin arrested the A-P axis at more and more posterior positions.

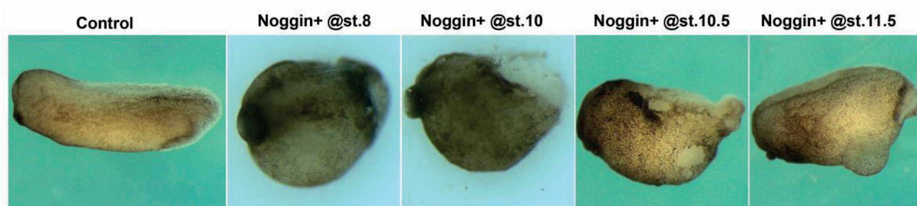

Figure 1. Timed noggin-injection resulted in progressive arrest of the A-P axis. 200nl 1ng/µL noggin was injected into the blastocoel of the embryo at different stages. Anterior is to the left and dorsal is up.

Timed anti-BMP treatment in ventralized embryos rescued different portions of the A-P axis

It has been reported that ventralized frog embryos (high BMP) contain the temporal *Hox* sequence, but lack a spatial pattern of expression (Wacker et al., 2004a). In contrast, dorsalized embryos (low BMP) have repressed or no *Hox* gene expression. These results suggest that the *Hox* timer is running in ventralized embryos. Different portions of the A-P axis and the Hox spatial sequence can be rescued by either organizer transplantation or anti-BMP treatments (e.g. noggin injection) at different developmental stages of these embryos (Wacker et al., 2004a). These results suggest that BMP and anti-BMP are involved in spatial patterning of *Hox* gene expression along the trunk part of the axis. We therefore wanted to know if earlier anti-BMP treatments could rescue the head part of the axis in ventralized embryos.

In *Xenopus*, ventralization can be achieved either by UV radiation (Scharf and Gerhart, 1983) or *bmp* overexpression (Clement et al., 1995; Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995). In our experiments, injection of the frog embryo with 2ng *bmp4* resulted in complete ventralization, showing a blob of tissue that had no axis (Fig 2). We then did anti-BMP treatments in ventralized embryos at different stages using a Smad6GR construct (Marom et al., 2005), which is an inhibitory Smad that can interfere with BMP pathway (Goto et al., 2007; Hata et al., 1998; Imamura et al., 1997). Timed Smad6 treatment fixed anterior markers sequentially at stage 26: it strongly fixed *six3* at stage 8 and 9, *otx2* at stage 9 and 10, *gbx2* at stage 10 and 10.5, and *hoxd1* at stage 10.5 (Fig. 3). The early

induction of cement gland led us to wonder whether the EAD is indeed induced early as would be predicted. In this experiment, one embryo (out of 17) developed staining for the cement gland marker *xcg-1* when treated with Smad6 at stage 8 (not shown). The fixation of *xcg-1* at stage 8 in this embryo fits well with the timing observed in Fig. 1, suggesting that EAD may also be involved in timed anti-BMP actions. However, since no other treated embryos later expressed *xcg-1*, this point will need further investigation. These results indicate that timed BMP/anti-BMP actions are involved in patterning the head.

Figure 2. *Bmp4* overexpression caused ventralization of the *Xenopus* embryo. Anterior is to the left; Dorsal is up.

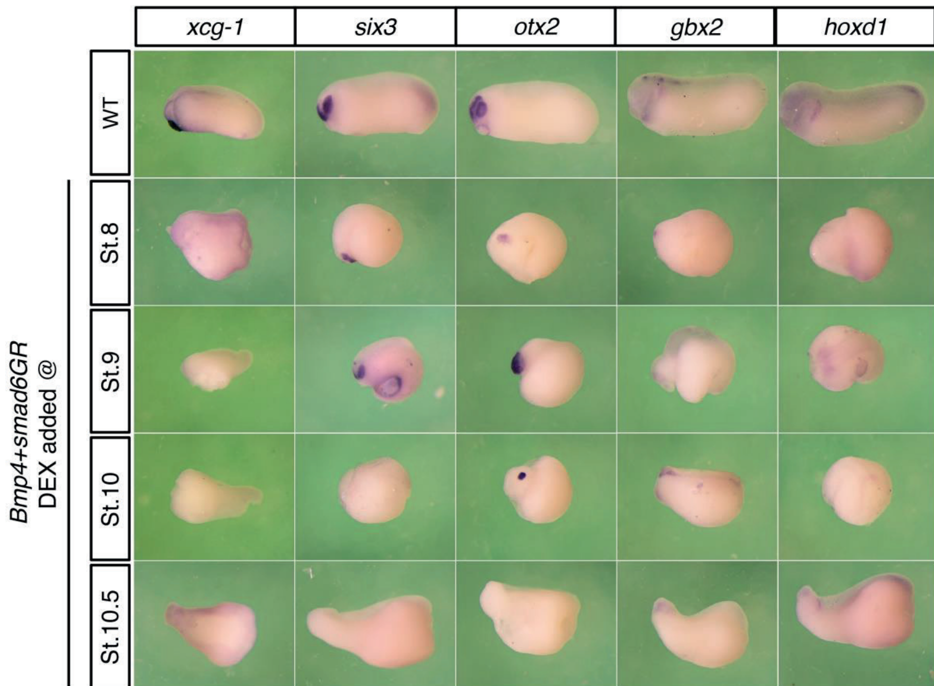

Figure 3. Timed anti-BMP treatment in ventralized embryos led to sequential fixation of anterior genes. The expression of *xcg-1, six3, otx2, gbx2,* and *hoxd1* in BMP-injected embryos that were subjected to Smad6 treatment at different stages.

Not all the molecular markers of A-P axial identity are involved in timing

The above results made us wonder if these anterior genes (*six3*, *otx-2* and *gbx2*) are sequentially expressed like *Hox* genes. Therefore, we examined the expression of these genes in wild-type embryos at different stages (Fig 4). Despite that these genes showed a spatial sequence of expression along the A-P axis (Fig 4, st.15), they were not sequentially expressed. For example, the activation of *six3* was much later than all the other genes. Moreover, unlike Hox genes, which are expressed in ventral and lateral mesoderm during gastrulation, the expression of *six3* and *otx2* were located at the dorsal side of the embryo.

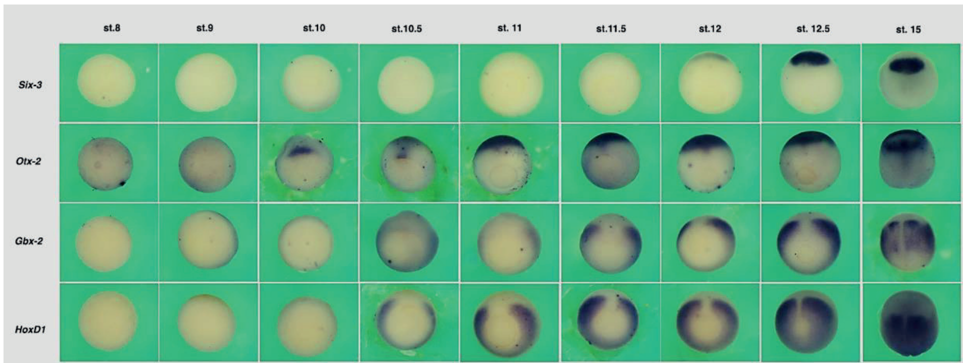

Fig 4. The expression of *six3, otx2, gbx2* and *hoxd1* at different stages in wild-type embryos. Embryos from st.8 to st.12.5 are vegetal views with dorsal to the top. St. 15 embryos are dorsal views with anterior to the top.

Discussion

Here we present evidence for a time-space translation mechanism that underlies patterning of the whole vertebrate A-P axis. A key component of this mechanism is the temporally sequential expression (timer) of A-P determinants, meaning that more anterior determinants are expressed earlier. Anti-BMP signals arrest the timer at different temporal points, which are correspondingly converted to different positional values. Through this way, the temporal sequence of gene expression is translated to a spatial sequence, imparting cells with axial identity along the A-P axis.

The time-space mechanism was first proposed in the frog to explain the patterning of the trunk by *Hox* genes (Wacker et al., 2004a), which are sequentially expressed during gastrulation in a tissue that contains high levels of BMP (NOM) and can be fixed by anti-BMP signals. We are interested to see if BMP and anti-BMP are also involved in the patterning of the head. Therefore, we injected noggin protein, an antagonist of BMP (Smith and Harland, 1992; Zimmerman et al., 1996), to the blastocoel of wild-type frog embryos at different blastula and gastrula stages. This sequentially arrested the A-P axis at different positions, including the head part. For example, embryos injected at stage 10 had more visible head structures than those injected at stage 8 (Fig. 1). Embryos injected at stage 11.5 showed more trunk structures. These phenotypes are similar to those observed in zebrafish after timed BMP inhibition. In zebrafish, sequential BMP inhibition results in axial defects at progressively posterior positions: later anti-BMP treatment affects posterior positions but

not anterior positions (Tucker et al., 2008). These results seem to suggest that the process involved in head-tail patterning can be sequentially stopped by timed BMP inhibition.

The above observations are further supported by analysing the expression of different positional markers in the head: *six3* (a forebrain marker) (Kobayashi et al., 1998), *otx2* (a forebrain and midbrain marker) (Li et al., 1994; Mori et al., 1994), *gbx1* (an anterior hindbrain marker) (Rhinn et al., 2003), *hoxb1b* (a posterior hindbrain marker) (Alexandre et al., 1996). Using heat-shock inducible chordin transgenic lines, Hashiguchi et al. showed that timed anti-BMP treatment from mid-blastula to mid-gastrula stage sequentially expanded the expression domains of these genes (Hashiguchi and Mullins, 2013). The sequential regulation (stabilization) of these genes by anti-BMP may suggest that timing is also involved in patterning the head and is likely to do with BMP signalling. We therefore postulate that the "head timer" is BMP-dependent and can be sequentially fixed by BMP inhibition, resulting in positional values being sequentially specified. To test this, we used a Smad6GR construct to do timed anti-BMP treatment in BMP-ventralized embryos (Fig. 2) (Clement et al., 1995; Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995). This sequentially fixed the expression of *six3, otx2, gbx2* and *hoxd1*: *six3* was fixed at stage 8 and 9, *otx2* at stage 9 and 10, *gbx2* at stage 10 and 10.5, and *hoxd1* at stage 10.5. Since cement gland marks the most anterior part of the frog embryo, we also examined the expression of its marker gene *xcg-1*. However, fixation of *xcg-1* was only observed in one embryo (out of 17) treated with Smad6 at stage 8. This may seem at odds with the observation that most embryos injected with noggin at stage 8 later formed a clear cement gland and no other axial structures (Fig. 1). We also noticed that in most BMP injected embryos, applying anti-BMP treatment at stage 8 did not result in clear cement gland formation (Fig. 3). One possible explanation for these results is that BMP injected embryos contain only transient timed gene expression to trigger genesis of successive axial zones and the effect of anti-BMP treatment can only work within a small window of time. Therefore, a more detailed timing experiment might be needed for further examination, or a higher concentration of anti-BMP signals is required to conquer the high BMP background in these embryos. Nevertheless, since the fixation of *xcg-1* in the embryo mentioned above fits well with the timing of the anti-BMP actions, it is indicative that EAD formation may be part of the process regulated by BMP/anti-BMP. It is worth noting that, both in Hashiguchi's study and in this study, the last and most posterior component of the head gene sequence is *hox1*: *hoxb1* and *hoxd1*,

respectively. Since *hox1* is the most anterior component of the *Hox* sequence, the spatial arrangement of these head genes is clearly complementary to and continuous with the *Hox* gene sequence. However, it is not clear if the temporal sequence of these genes is also continued by that of *Hox* genes.

During trunk patterning, a collinear property enables that *Hox* genes are expressed in a 3' to 5' order, and that more 3' genes are expressed earlier and more anteriorly than/to more 5' ones (Duboule and Dolle, 1989; Graham et al., 1989; Lewis, 1978). The temporal collinear expression of *Hox* genes has been proposed to serve as a timer, which can be interpreted and translated into spatial information (Wacker et al., 2004a). Since the anterior genes are sequentially fixed earlier than Hox genes by anti-BMP treatment, it is natural to think that these genes are also expressed in a temporal sequence, which could complement the Hox sequence to constitute an integrative timer. Therefore, we examined the endogenous expression of these anterior genes at different stages in wild-type embryos. Unlike *Hox* genes, the activation of these genes did not strictly correspond to their spatial order along the A-P axis. For example, *six-3* demarcates the most anterior border of the developing neural plate (Oliver et al., 1995), but it is expressed at the end of gastrulation, much later than the other genes. The expression domain of *gbx2* is anterior to that of *hoxd1*, but it was also not expressed earlier than *hoxd1*. The expression kinetics of these genes make them less likely to be timer genes themselves (at least not all of them are). The only plausible explanation for the sequential stabilization of these genes by anti-BMP treatment is that they are downstream makers regulated by an upstream BMP-dependent timing mechanism.

In summary, the results in study suggest that there is a BMP-dependent timing mechanism in the head that could be continued by the *Hox* temporal sequence in the trunk. The two constitute an integrative timer which can be translated into spatial patterns of gene expression along the whole head-tail axis via a BMP/anti-BMP dependent mechanism.

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