A time-space translation mechanism for patterning the vertebrate anteroposterior axis

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Chapter 6

Summary and Discussion

During vertebrate embryonic development, structures along the anterior-posterior (A-P) axis or head-tail axis are generated progressively in a timed fashion: anterior structures are specified early and posterior structures are specified late (Eyal-Giladi, 1954; Gamse and Sive, 2000; Gamse and Sive, 2001; Nieuwkoop, 1952; Stern et al., 2006). Evidently, timing is involved in this process. In an attempt to understand how time and space are coordinated during A-P axis formation, previous research in our lab suggested a BMP/anti-BMP dependent time-space translation mechanism for patterning the trunk part of the axis (Durston et al., 2010; Wacker et al., 2004a). A key component of this mechanism is a BMP dependent timer, which causes sequential expression of Hox genes (Wacker et al., 2004a; Wacker et al., 2004b).

Hox genes have long been known to regulate the specification of positional identities along the A-P axis during development (Alexander et al., 2009; Deschamps and van Nes, 2005; Wellik, 2009). The most striking feature of Hox genes is collinearity: their 3' to 5' order on the chromosome matches their temporal expression sequence in development (temporal collinearity) (Dolle and Duboule, 1989; Duboule, 2007; Izpisua-Belmonte et al., 1991) and their spatial order of expression along the A-P axis (spatial collinearity) (Duboule and Dolle, 1989; Graham et al., 1989; Lewis, 1978). The sequential activation of Hox genes during early development has been found in many species to occur in a high BMP environment: the non-organiser mesoderm (NOM) in the frog (Kolm and Sive, 1995; Wacker et al., 2004a) and the posterior primitive streak in the chick (Denans et al., 2015; Gaunt and Strachan, 1994; Iimura and Pourquie, 2006) and mouse (Deschamps et al., 1999; Forlani et al., 2003). This is consistent with the finding that BMP signalling is required for Hox gene activation (Faial et al., 2015; Wacker et al., 2004b). In the high BMP environment, although Hox genes are sequentially expressed, their expression domains are largely overlapping with each other and show no spatial pattern. The time-space translation mechanism proposes that these domains will be sequentially exposed to anti-BMP signals from the Spemann organiser during gastrulation cell movement. This fixes Hox expression and translates the temporal pattern into a spatial pattern (time-space translation) (Wacker et al., 2004a). Through this way, positional identities are sequentially specified along the A-P axis. To test this mechanism further, my PhD project has been attempting to answer the following two questions: 1. How is Hox temporal collinearity achieved? 2. Is BMP/anti-BMP involved in head patterning?

Why Hox gene activation sweeps along the clusters has been a subject of great interest in developmental biology. The accepted wisdom is that Hox collinearity originates from evolutionary tandem duplication of an ur-Hox gene (Gehring et al., 2009), and is based on sequential opening of chromatin from 3' to 5' in the Hox clusters, sequentially making more and more Hox genes available for transcription (Chambeyron et al., 2005; Noordermeer et al., 2014). Considering that the occurrence of chromatin opening fits well with gene activation and gastrulation cell movement (Chambeyron et al., 2005), this explanation seems very attractive. However, if Hox temporal collinearity is solely due to chromatin opening, then it is difficult to understand how cells at a certain A-P position communicate with each other to ensure the same combination of Hox genes are expressed. Moreover, if at a posterior position, they also need to agree with each other to decide which anterior Hox genes to shut down. Due to these complexities, another layer of regulation is apparently required to synchronize Hox expression among different cells and tissues. There are two possible mechanisms whereby synchronization can be achieved: one is that Hox genes are regulated by other timing mechanisms (e.g. somitogenesis clock (Palmeirim et al., 1997; Pourquie, 2004) or cell cycle (Primmett et al., 1989; Stern et al., 1988)) to coordinate their expression; the other is that Hox genes could coordinate their expression by themselves, e.g. by Hox-Hox interactions (Hooiveld et al., 1999).

In **Chapter 2**, we did ectopic expression of 4 different Hox genes (*hoxd1*, *hoxb4*, *hoxa7* and *hoxb9*) in both *noggin*-injected embryos and wild-type embryos. As a BMP antagonist, *noggin* injection repressed Hox gene expression, because the initiation of Hox gene expression during gastrulation is BMP-dependent (Faial et al., 2015; Wacker et al., 2004b). Interestingly, ectopic expression of a Hox gene in *noggin*-injected embryos could only induce genes that are posterior to it, suggesting the existence of posterior induction --- induction of more posterior Hox genes by more anterior ones. This is consistent with overexpression studies in wild-type embryos, which showed that more posterior Hox genes were induced while more anterior ones were repressed (we call it here: posterior dominance --- the repression of anterior genes by posterior ones) in a gain-of-function situation. Therefore, there are both inductive and repressive interactions among Hox genes.

To dissect the different roles of these interactions in A-P patterning, we did a timing experiment by ectopic expression of *hoxb4* long before its initial expression during normal

development. Interestingly, this brought forward the Hox sequence without disrupting their temporal order. Moreover, since genes (*hoxb4*, *hoxb6/c6*, and *hoxb9*) brought forward by *hoxb4* overexpression also showed anteriorization in their expression domains at a later stage (st. 26), these results suggest a connection between temporal and spatial expression. This renders further support to the time space translation mechanism, which proposes that Hox temporal collinearity is required for generating a spatial pattern. Notably, the endogenous expression of *hoxd1* was not repressed by Hoxb4 until st.15, when the last paralogue group of Hox genes (*hox13*) are first expressed. Unlike posterior induction, which happens during gastrulation in the high BMP non-organizer mesoderm, this repression occurred in paraxial mesoderm and neurectoderm, where there are relatively low levels of BMP. These results suggest that the inductive and repressive Hox interactions serve different purposes during A-P patterning. The inductive Hox interaction (posterior induction) is required for driving the Hox timer. The repressive interaction (posterior dominance), on the other hand, is needed in dorsal paraxial mesoderm and neurectoderm where a spatially collinear Hox pattern develops. Consistent with this idea, the nested Hox expression zones in NOM mesoderm overlap fully with each other during gastrulation (Wacker et al., 2004a), whereas they show partial or no overlap starting from neurulation, when the last group of Hox gene (*hox13*) is initiated. It is likely that the inductive and repressive interactions between Hox genes set up a dynamic equilibrium which permits the genesis of dynamically metastable expression zones. That these zones have dynamical stability is shown by phenomena like pattern regulation.

In **Chapter 3,** we did Hoxc6 loss-of-function (LOF) using antisense morpholinos. The results further supported the findings in Chapter 2. In the *Xenopus* frog, depletion of this single Hox gene significantly truncates the body axis, and the truncation position is at the anterior boundary of *hoxc6* expression: the neck-thorax boundary. Hox genes 3' anterior to this boundary have increased or unaffected expression. Hox genes 5' posterior to it have their expression deleted. This effect is much more extreme than Hoxc6 LOF or even LOF for the whole *hox6* paralogue group in the mouse. One possible explanation for the difference is that the process of A-P axis specification is different in frog and mouse. In frog, the entire axis is specified early (Durston and Zhu, 2015), whereas in mouse the specification of the A-P axis happens in a much longer period due to axial growth (Steventon et al., 2016; Young et al., 2009). An alternative explanation may have to do with the discrepancies

between the outcomes of gene knockdown by anti-sense and knockout by genetic deletion, which have been observed in many species (De Souza et al., 2006; Gao et al., 2015; Kok et al., 2015). While these discrepancies are frequently attributed to off-target effects of the anti-sense reagents (e.g. morpholinos), a recent study, by comparing the proteomes and transcriptomes of the *egfl7* mutant and morphant, suggests that deleterious mutations but not gene knockdowns could trigger genetic compensation (Rossi et al., 2015). This could well be the reason that we observed a more severe phenotype in our Hoxc6 LOF experiment. Given that the effect of Hoxc6 LOF on Hox gene expression fits well with our gain-of-function results in Chapter 2, these findings strongly support our proposal that vertebrate axial patterning is mediated by collinear Hox-Hox interactions.

Chapter 4 attempted to answer question 2: what mechanisms are involved in patterning the head? In our research, we observed that timed anti-BMP treatment of wild-type frog embryos by noggin injection at blastula and gastrula stages sequentially arrested the A-P axis at different positions, including the head part. Similar results were also observed in zebrafish (Tucker et al., 2008). The progressive arrest of the A-P axis by BMP inhibition is further supported by gene expression analysis, which shows that in zebrafish, timed anti-BMP treatment from mid-blastula to mid-gastrula stage sequentially regulates the expression of four anterior genes: *six3*, a forebrain marker (Kobayashi et al., 1998); *otx2*, a forebrain and mid-brain marker (Li et al., 1994; Mori et al., 1994); *gbx1*, a rostral hindbrain marker (Rhinn et al., 2003), and *hoxb1b* (Alexandre et al., 1996), the caudal hindbrain marker (Hashiguchi and Mullins, 2013). These results indicate that BMP signalling may be involved in regulating the timing of head patterning. In Chapter 4, this view was tested by applying timed anti-BMP treatment to BMP4-ventralized embryos. This sequentially fixed the expression of *six3*, *otx2*, *gbx2* and *hoxd1*, further reinforcing the above view. This gene sequence, with *hox1* being the last and most posterior component, is sequentially arranged along the A-P axis in the head and is spatially complementary to the Hox gene sequence in the trunk. However, the genes in this sequence are not expressed in a temporal order that can be continued by Hox genes. The sequential fixation of these genes by timed BMP inhibition is therefore likely to be due to an upstream BMP-regulated timing mechanism. This putative timing mechanism in the head and the mechanism regulating sequential Hox gene expression in the trunk may constitute an integrative timer, which can be stabilized by anti-BMP signals and translated into spatial patterns of gene expression.

In **Chapter 5**, we did a conceptual analysis on how the vertebrate A-P axis is patterned by a BMP/anti-BMP dependent time-space translation mechanism, and pointed out that there are several decision points involved in the transition between different axial blocks, e.g. extreme anterior domain (EAD), head, neck, thorax, abdomen, and tail. At these decision points, external signalling pathways are needed to interact with the axial timer. In summary, this thesis studies how BMP signalling patterns the vertebrate head-tail axis via its actions on sequentially expressed genes. In the trunk part of the axis, these genes are Hox genes, which show temporally and spatially sequential expression during head-tail patterning. The sequential activation of Hox genes is BMP dependent and dependent on collinear Hox-Hox interactions. The temporal information encoded by Hox genes can be stabilized by anti-BMP signals and translated into spatial information. In the head part of the axis, a currently unknown gene sequence imparts temporal information that could be fixed by BMP inhibition, resulting in the establishment of spatial patterns of gene expression along the head-tail axis.

References

Alexander, T., Nolte, C. and Krumlauf, R. (2009). Hox genes and segmentation of the hindbrain and axial skeleton. *Annu Rev Cell Dev Biol* **25**, 431-456.

Alexandre, D., Clarke, J. D., Oxtoby, E., Yan, Y. L., Jowett, T. and Holder, N. (1996). Ectopic expression of Hoxa-1 in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development* **122**, 735-746.

Chambeyron, S., Da Silva, N. R., Lawson, K. A. and Bickmore, W. A. (2005). Nuclear re-organisation of the Hoxb complex during mouse embryonic development. *Development* **132**, 2215-2223.

De Souza, A. T., Dai, X. D., Spencer, A. G., Reppen, T., Menzie, A., Roesch, P. L., He, Y. D., Caguyong, M. J., Bloomer, S., Herweijer, H., et al. (2006). Transcriptional and phenotypic comparisons of Ppara knockout and siRNA knockdown mice. *Nucleic Acids Res* **34**, 4486-4494.

Denans, N., Iimura, T. and Pourquie, O. (2015). Hox genes control vertebrate body elongation by collinear Wnt repression. *Elife* **4**.

Deschamps, J., van den Akker, E., Forlani, S., De Graaff, W., Oosterveen, T., Roelen, B. and Roelfsema, J. (1999). Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. *Int J Dev Biol* **43**, 635-650.

Deschamps, J. and van Nes, J. (2005). Developmental regulation of the Hox genes during axial morphogenesis in the mouse. *Development* **132**, 2931-2942.

Dolle, P. and Duboule, D. (1989). Two gene members of the murine HOX-5 complex show regional and cell-type specific expression in developing limbs and gonads. *Embo J* **8**, 1507-1515.

Duboule, D. (2007). The rise and fall of Hox gene clusters. *Development* **134**, 2549-2560.

Duboule, D. and Dolle, P. (1989). The Structural and Functional-Organization of the Murine Hox Gene Family Resembles That of Drosophila Homeotic Genes. *Embo J* **8**, 1497-1505.

Durston, A. J., Jansen, H. J. and Wacker, S. A. (2010). Review: Time-space translation regulates trunk axial patterning in the early vertebrate embryo. *Genomics* **95**, 250-255.

Eyal-Giladi, H. (1954). Dynamic aspects of neural induction in amphibia. *Arch Biol (Liege)* **65**, 179-259.

Faial, T., Bernardo, A. S., Mendjan, S., Diamanti, E., Ortmann, D., Gentsch, G. E., Mascetti, V. L., Trotter, M. W., Smith, J. C. and Pedersen, R. A. (2015). Brachyury and SMAD signalling collaboratively orchestrate distinct mesoderm and endoderm gene regulatory networks in differentiating human embryonic stem cells. *Development* **142**, 2121-2135.

Forlani, S., Lawson, K. A. and Deschamps, J. (2003). Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo. *Development* **130**, 3807-3819.

Gamse, J. and Sive, H. (2000). Vertebrate anteroposterior patterning: the Xenopus neurectoderm as a paradigm. *Bioessays* **22**, 976-986.

Gamse, J. T. and Sive, H. (2001). Early anteroposterior division of the presumptive neurectoderm in Xenopus. *Mech Dev* **104**, 21-36.

Gao, Y. B., Zhang, Y., Zhang, D., Dai, X. H., Estelle, M. and Zhao, Y. D. (2015). Auxin binding protein 1 (ABP1) is not required for either auxin signaling or Arabidopsis development. *P Natl Acad Sci USA* **112**, 2275-2280.

Gaunt, S. J. and Strachan, L. (1994). Forward spreading in the establishment of a vertebrate Hox expression boundary: the expression domain separates into anterior and posterior zones, and the spread occurs across implanted glass barriers. *Dev Dyn* **199**, 229-240.

Gehring, W. J., Kloter, U. and Suga, H. (2009). Evolution of the Hox Gene Complex from an Evolutionary Ground State. *Curr Top Dev Biol* **88**, 35-61.

Graham, A., Papalopulu, N. and Krumlauf, R. (1989). The Murine and Drosophila Homeobox Gene Complexes Have Common Features of Organization and Expression. *Cell* **57**, 367-378.

Hashiguchi, M. and Mullins, M. C. (2013). Anteroposterior and dorsoventral patterning are coordinated by an identical patterning clock. *Development* **140**, 1970-1980.

Hooiveld, M. H. W., Morgan, R., Rieden, P. I. D., Houtzager, E., Pannese, M., Damen, K., Boncinelli, E. and Durston, A. J. (1999). Novel interactions between vertebrate Hox genes. *Int J Dev Biol* **43**, 665-674.

Iimura, T. and Pourquie, O. (2006). Collinear activation of Hoxb genes during gastrulation is linked to mesoderm cell ingression. *Nature* **442**, 568-571.

Izpisua-Belmonte, J. C., Falkenstein, H., Dolle, P., Renucci, A. and Duboule, D. (1991). Murine genes related to the Drosophila AbdB homeotic genes are sequentially expressed during development of the posterior part of the body. *Embo J* **10**, 2279-2289.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B. and Kawakami, K. (1998). Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973-2982.

Kok, F. O., Shin, M., Ni, C. W., Gupta, A., Grosse, A. S., van Impel, A., Kirchmaier, B. C., Peterson-Maduro, J., Kourkoulis, G., Male, I., et al. (2015). Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. *Dev Cell* **32**, 97-108.

Kolm, P. J. and Sive, H. L. (1995). Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. *Dev Biol* **167**, 34-49.

Lewis, E. B. (1978). A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-570.

Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). Expression of two zebrafish orthodenticle-related genes in the embryonic brain. *Mech Dev* **48**, 229-244.

Mori, H., Miyazaki, Y., Morita, T., Nitta, H. and Mishina, M. (1994). Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res Mol Brain Res* **27**, 221-231.

Nieuwkoop, P. D. (1952). Activation and organization of the central nervous system in amphibians. Part III. Synthesis of a new working hypothesis. *Journal of Experimental Zoology* **120**, 83-108.

Noordermeer, D., Leleu, M., Schorderet, P., Joye, E., Chabaud, F. and Duboule, D. (2014). Temporal dynamics and developmental memory of 3D chromatin architecture at Hox gene loci. *Elife* **3**.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* **91**, 639-648.

Pourquie, O. (2004). The chick embryo: a leading model in somitogenesis studies. *Mech Dev* **121**, 1069-1079.

Primmett, D. R., Norris, W. E., Carlson, G. J., Keynes, R. J. and Stern, C. D. (1989). Periodic segmental anomalies induced by heat shock in the chick embryo are associated with the cell cycle. *Development* **105**, 119-130.

Rhinn, M., Lun, K., Amores, A., Yan, Y. L., Postlethwait, J. H. and Brand, M. (2003). Cloning, expression and relationship of zebrafish gbx1 and gbx2 genes to Fgf signaling. *Mech Dev* **120**, 919-936.

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Holper, S., Kruger, M. and Stainier, D. Y. R. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* **524**, 230-+.

Stern, C. D., Charite, J., Deschamps, J., Duboule, D., Durston, A. J., Kmita, M., Nicolas, J. F., Palmeirim, I., Smith, J. C. and Wolpert, L. (2006). Head-tail patterning of the vertebrate embryo: one, two or many unresolved problems? *Int J Dev Biol* **50**, 3-15.

Stern, C. D., Fraser, S. E., Keynes, R. J. and Primmett, D. R. (1988). A cell lineage analysis of segmentation in the chick embryo. *Development* **104 Suppl**, 231-244.

Tucker, J. A., Mintzer, K. A. and Mullins, M. C. (2008). The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis. *Dev Cell* **14**, 108-119.

Wacker, S. A., Jansen, H. J., McNulty, C. L., Houtzager, E. and Durston, A. J. (2004a). Timed interactions between the Hox expressing non-organiser mesoderm and the Spemann organiser generate positional information during vertebrate gastrulation. *Dev Biol* **268**, 207-219.

Wacker, S. A., McNulty, C. L. and Durston, A. J. (2004b). The initiation of Hox gene expression in Xenopus laevis is controlled by Brachyury and BMP-4. *Dev Biol* **266**, 123-137.

Wellik, D. M. (2009). Hox genes and vertebrate axial pattern. *Curr Top Dev Biol* **88**, 257-278.