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Hox in frogs: xenopus reveals novel functions for vertebrate Hox genes

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General Discussion and summary

The ordered emergence of structures in a developing embryo is an amazing intellectual challenge and inspiring food for the imagination; this phenomenon has fascinated investigators since the ancient Greeks. The publication, in 1924 by Spemann and his student Hilde Mangold of their discovery of the organizer and of its ability to instruct a secondary axis when grafted into a host embryo excited the imagination of developmental biologists. This initiated a “gold rush”, which eventually (after more than 40 years) led to the identification of molecules responsible for the organizer’s properties. The surprise was that these were actually secreted antagonists for growth factors such as BMPs. In another breakthrough, attempts to understand patterning of the anterior-posterior (A-P) body axis led to the discovery of a family of transcription factors: the *Hox* genes. The *Drosophila Antennapedia* complex was the first *Hox* locus discovered. Gene cloning in *Xenopus* revealed that certain sequences in *Hox* genes are extremely conserved throughout evolution. However, despite extensive investigations, *Hox* genes remain a very challenging topic in the field of developmental biology.

The aim of this thesis was to get more insight in understanding some of the Spemann organizer and *Hox* gene functions during *Xenopus laevis* development. We questioned the role of the Spemann organizer in patterning the main body axis (Chapter2). Because *Hox* genes are key players in body patterning, we investigated several aspects of their function. (Chapters3-5) First, we investigated the role of the first *Hox* genes expressed in the frog by loss of function of the complete *Hox1* paralogue group. We found a very interesting connection between the loss of function of these *Hox* genes and segmentation as well as expression of segmentation genes such as *X-Delta-2*. Interestingly, the loss of function of the first paralogue group leads to a defect in segmentation as well as to downregulation of certain more posterior *Hox* genes, including *Hoxc6*.

Thus, I decided to investigate the role of some of these more posterior genes during development of *Xenopus*. Loss of function (and conversely gain of function) of several posterior *Hox* genes showed surprisingly little effect on segmentation or the expression of segmentation genes. However, *Hoxc6* appeared to give a segmentation phenotype similar to the loss of function of the whole *Hox1* paralogue group (Chapter5). In addition, it appeared that ectopically expressing or down regulating *Hoxc6* also induced specific phenotypes during primary neurogenesis, indicating a function in this process (Chapter3).

In the frog, *Hox* gene expression starts in gastrula mesoderm in a temporally sequential manner. During the course of gastrulation, this temporal sequence appears to be

translated to generate a spatial pattern (Wacker *et al.*, 2004a). A number of findings indicated that the mesodermal *Hox* gene expression sequence is somehow transferred to the gastrula's neurectoderm and that the time sequence of mesodermal movements in the gastrula causes mesoderm expressing early *Hox* genes to transfer their expression to a more anterior part of the neurectodermal axis, while mesoderm expressing progressively later *Hox* genes transfers expression to progressively more posterior parts of the neuraxis. In chapter 6, we have started to investigate this transfer of *Hox* gene expression from mesoderm to neurectoderm. We used gain and loss of function experiments in an *in vitro* system to confirm that the expression of the earliest expressed *Hox* gene, *Hoxd1*, in gastrula mesoderm actually is causal for the later expression of the same gene in neurectoderm. An unconventional *Hox* protein transfer was monitored for its possible relevance to the mechanism of this signaling process.

In chapter 2, we analyzed the role of the Spemann organizer in the A-P patterning of the trunk in *Xenopus laevis*. In the classical publications (Spemann and Mangold, 1924, 1936), the organizer was shown to be able to induce a secondary completely patterned axis when it is grafted into the ventral side of an amphibian embryo. Researchers have since then been able to show that the organizer secretes molecules antagonizing respectively one of following three classes of growth factors: Bone Morphogenetic Proteins (BMPs), Wnts and Nodals. This antagonism leads to the neuralization of the ectoderm and the appearance of dorsal neural plate. Besides secreting these antagonising signals, the organizer also affects development by self-differentiating and by regulating morphogenetic movements during gastrulation (reviewed in De Robertis *et al.*, 2001). There is an evident function of the organizer in A-P patterning which has been proposed to reside in the existence of head, trunk and head organizers (Niehrs, 2004). On the other hand, neural induction, as a known major function of the organizer also connects to A-P patterning. It leads to the formation of anterior neural tissue (activation) which is progressively transformed into a more complete pattern including posterior neural tissue (transformation) (Nieuwkoop, 1952, De Robertis and Kuroda, 2004). Several signals have been shown to be implicated in this activation and transformation of neural tissue. Activation is believed to occur through signals from the organizer itself, while transforming signals appear to be derived from non-organizer mesoderm (Woo and Fraser, 1997; Kolm *et al.*, 1997; Muhr *et al.*, 1999; Wacker *et al.*, 2004b). However the actual role of the organizer in establishing a proper A-P axis is pretty unclear. One of our ideas in Chapter 2 was to abolish all organizer functions by UV treatment of the *Xenopus* zygote (which ventralises the very early embryo and thereby blocks any development of the organizer) and then to restore them separately. We also investigated some of the different functions of the organizer separately by using an *in vitro* assay: the wrap assay (Jansen *et al.*, 2007). This tissue recombination assay offers the possibility to manipulate different organizer signals and test their necessity for setting up an A-P pattern, in embryonic (neur)ectoderm.

It has been shown that if naive gastrula ectoderm (animal cap) is combined both with gastrula organizer mesoderm and with gastrula (*Hox* expressing) non-organiser mesoderm, this ectoderm expresses *Hox* genes and forms part of an A-P neurectodermal pattern. This system was used to analyse organizer function either by removing the organiser mesoderm and replacing it with molecular manipulations aimed at replacing specific organiser functions or by treating the system to block particular organiser functions. Neural activation by antagonizing BMP signaling, while the organiser and thus all of its other functions are absent, proved to permit the expression of A-P genes. Conversely, prevention of neural activation prevented the expression of several A-P genes tested. These results demonstrated that the role of the Spemann organizer is to induce competence in the neurectoderm to respond to transforming signals through its function of neural induction (activation) (**Chapter 2**). These data showed, for the first time, a clear function of the organizer in establishing the trunk A-P via an active process, neural activation. Our data also showed the capacity of a system such as the wrap assay as a paradigm for manipulating several sources of signals, and its usefulness for further investigations.

Axis formation, patterning and segmentation are intimately linked processes during development. It has been shown previously, in the mouse, that *Hox* gene expression is downregulated in a Notch effector mutant that is known to block segmentation (Zákány *et al.*, 2001). In addition, it is also known that Notch mutants exhibit homeotic like transformations. These data suggest a tight link between segmentation and patterning. In *Xenopus*, PG1 *Hox* genes are the first to be expressed during development. These genes could thus play a major role in setting up a proper pattern in the main body axis and coordinating this patterning with processes like segmentation. In **Chapter 4**, we knocked down the whole paralogue 1 group (PG1). This led to hindbrain segmentation defects as well as severe defects in trunk segmentation (Peres *et al.*, 2006). It was previously demonstrated that *Hox* gene function in the hindbrain is linked to its segmentation (Guthrie, 2007; Lumsden *et al.*, 2004; Dibner *et al.*, 2004). Here for the first time, we reported a complete paralogue group knockdown and its connection to segmentation. In former work, a double PG1 knock out in mouse (Rossel and Capecchi, 1999) and knockdown of two PG1 genes in zebrafish (McClintock *et al.*, 2002) had shown a progressive loss of rhombomeric identity. The triple PG1 knockdown in *Xenopus* showed a more severe hindbrain defect with the whole hindbrain being transformed to a rhombomere1 like identity with downregulation of several more posterior *Hox* genes belonging to PG's 2-6.

This loss of PG1 also induced a severe neural crest migration defects, presumably because members of PG1 need to be expressed in those migrating cells (Trainor and Krumlauf, 2001). More interestingly, loss of PG1 led also to downregulation of *X-Delta-2*, and because of this, caused segmentation defects (Peres and Durston, 2006). Loss of function of

Delta-like genes had previously been shown to trigger segmentation defects in mice and men (Turnpenny *et al.*, 2007; Kusumi *et al.*, 1998; Dunwoodie *et al.*, 1997). *X-Delta-2* is expressed from early during *Xenopus* development in an overlapping domain with *Hox* genes. It is interesting to see that the *Hox* genes down-regulated by PG1 loss are also expressed in the same general domain as *X-Delta-2*. I decided then to investigate the roles of those posterior *Hox* genes in segmentation.

In **Chapter 5**, knocking down several posterior *Hox* genes didn't seem to trigger any particular segmentation defects even though genes like *Hoxb4* are expressed in somites and it has been reported that *Hoxb4* ectopic expression affects somitogenesis in *Xenopus* (Harvey and Melton, 1988). However, *Hoxc6* loss of function triggered a severe segmentation defect. Our results show a clear connection between *X-Delta-2* and *Hoxc6* similarly as previously reported between *X-Delta-2* and PG1 (Peres and Durston, 2006). Loss of function of *Hoxc6* already leads to downregulation of *X-Delta-2* at gastrula stages, and the opposite is also true. We show that *Hoxc6* is required for proper trunk segmentation in *Xenopus*. This is the second time that *Hox* genes and segmentation have been shown to be connected during *Xenopus* development. I am currently trying to see if the PG1 loss of function segmentation phenotype can be rescued by *Hoxc6*. The expectation is that the segmentation phenotype in PG1 genes loss of function is due to the severe downregulation of *Hoxc6*. A direct connection between a *Hox* mutant and segmentation has never so far been reported in the mouse.

However, it has been shown that the function of *Hox* genes is important within the presomitic mesoderm prior to somite formation (Carapuço *et al.*, 2005). The situation in *Xenopus* is similar: *Hox* genes are already expressed in mesoderm early in development (gastrulation), before somite formation (in the neurula at stage 17). Interestingly, *X-Delta-2*, a segmentation gene, is also expressed early in development at the same time as the first *Hox* genes (Peres *et al.*, 2006). Interaction between the somitogenesis clock and the “patterning clock” is suspected to occur prior to physical somite formation (Peres *et al.*, 2006). The methodology for *Hoxc6* loss of function by knockdown can explain the severity of the phenotype obtained in the frog in comparison with that resulting from the classical mouse *Hoxc6* mutant. Indeed, a morpholino approach does not disturb any regulation at the DNA level while physical disruption of the DNA by a cassette insertion leads to different regulation of neighbouring genes and hence is likely to trigger a phenotype not reflecting a clear knock out mutant (Montavon *et al.*, 2008). Moreover, the *Drosophila* homolog of *Hoxc6*, *Antennapedia*, is able to rescue the loss of segmentation that follows *Hoxc6* loss of function. This probably shows a more primitive function of a vertebrate *Antennapedia* orthologue. It would be interesting to see if *Drosophila labial* can rescue the phenotype of PG1 genes loss of function. In *Xenopus*, the first *Hox* gene expressed, *Hoxd1*, did rescue the downregulation of *X-Delta-2* upon PG1 loss of function but without restoring a

segmented pattern. This could reinforce the idea that segmentation defects in PG1 loss of function are somehow related to the downregulation of *Hoxc6*. Results from an attempt to rescue PG1 loss of function by *Hoxc6* could shed light on another function of a *Hox* gene beside its traditional patterning function.

Yet, another feature of *Hoxc6* morphans is a lack of response to the touch stimulus. Thus, I decided to monitor the expression of neural and neuronal markers in *Hoxc6* morphans. Our data show a severe loss of primary neurons when *Hoxc6* function is impaired. *X-Delta-2* is also a neuronal gene expressed in neurogenic regions from early stages of development (Peres and Durston, 2006). Extensive studies from Jessell's lab have provided strong evidence for a major role of HOX-C proteins in establishing the columnar structures within the spinal cord (reviewed in Di Sanguito *et al.*, 2008). It looks as if members of the HoxC cluster like *Hoxc6* play a very important role in neurogenesis in the frog. In addition, ectopic expression of *Hoxc6* triggers the ectopic expression of *N-tubulin* (Oschwald *et al.*, 1991). This result is very surprising, and shows for the first time a link between *Hoxc6* and formation of the primary neurons. I realized that *Hoxc6* and *N-tubulin* are already expressed in an overlapping domain during gastrulation. Indeed, previous work had shown that some genes belonging to the *N-tubulin* synexpression group were already expressed at very early stages (XPak3 another marker for primary neuronal differentiation is already expressed as mRNA in the egg for example, and XSeb-4 at early gastrula, Souopgui, thesis, 2002). Loss- and gain of function results place *Hoxc6* upstream of *N-tubulin* in the proneural cascade, although epistatic analysis with genes from the proneural pathway will answer this question definitively. It would very interesting to check a possible role of other *Hox* genes in neurogenesis in *Xenopus*. Indeed, a future line of research would be to knock down more *HoxC* genes and try to analyse neuron formation. It will be interesting to see if other *Hox* genes from other clusters will be able to affect the phenotypes of the neurons that are generated.

Hox genes are first expressed in marginal zone mesoderm in the *Xenopus* gastrula (Wacker *et al.*, 2004a). During gastrulation, the mesoderm cells involute and move under the gastrula ectoderm. Then, somehow the mesodermal *Hox* pattern is transferred to the overlying ectoderm: the presumptive neurectoderm shortly after these two tissues have been in contact (Wacker *et al.*, 2004a). It has been shown that there are signals from the underlying mesoderm (called 'vertical signals') that act on the neurectoderm to pattern this tissue layer (Proznanski and Keller, 1997; Wacker *et al.*, 2004a). However, the exact nature of such signals is not well understood. It is noteworthy though that twenty years ago, Prochiantz'lab had demonstrated that the Antennapedia protein is able to cross cell membranes of several cell types under conditions excluding any endocytosis (Joliot *et al.*, 1991). This peculiar behaviour seems to be a common feature of homeoproteins because of the existence of a signal peptide within the third helix of the homeodomain, the

penetratin peptide (see **chapter 6**; Joliot and Prochiantz, 2004). It was very tempting to imagine that this unconventional protein transfer could be happening *in vivo* and could be the basis of the vertical signalling responsible for posteriorizing the neurectoderm. It has been shown that hosted proteins are able to retain their activity, and can regulate targets in the host cell (Brunet *et al.*, 2005; reviewed in Prochiantz, 2003). We started the analysis of this question using the wrap assay because of the possibility to manipulate different components in this tissue recombination assay and because it mimics the embryonic situation. We demonstrated for the *Hox* gene *Hoxd1* that the expression of this *Hox* gene within the mesoderm is necessary and sufficient to induce its expression in the neurectoderm. These results pushed us to see whether the protein itself is involved in this process. We took advantage of recombinant protein technology to see whether some *Hox* proteins could indeed travel from cell to cell. We started the analysis with the first *Hox* gene expressed in *Xenopus*, *Hoxd1*. As previously reported for HOXB4, HOXD1 is able to cross an intact epithelium in *Drosophila* imaginal discs. Moreover, mutations of two amino-acids within the penetratin sequence abolished its capacity to translocate in accordance with previous *in vitro* data (Brunet *et al.*, 2005). In addition, the full HOXD1 protein is able to translocate into *Xenopus* cells when injected into the blastocoel and then triggers a phenotype similar to that caused by its mRNA injection. This showed that our recombinant protein seems to retain its activity. The main difficulty with this project has been the limited knowledge of **direct** HOX targets (required to demonstrate activity of translocated *Hox* proteins) (Svingen and Tonissen, 2006). Our data about *Hoxc6* loss- and gain of function suggested that *X-Delta-2* is a very good candidate for being a possible direct target of *Hoxc6*. Thus, I am currently checking *X-Delta-2* expression after HOXC6 injection into the embryo, as well as after injection of an antibody recognizing the endogenous protein, and thus blocking the transfer. We have also incubated embryos with the recombinant HOXC6 protein and checked *X-Delta-2* and *N-tubulin* expression. Immunohistolocalization on slides is currently being used to follow the possible transfer of a tagged protein from mesoderm to ectoderm in the wrap system and in the whole embryo. I am really looking forward to the results of these investigations to see if *Hox* proteins could indeed play a “messenger protein” function as predicted by Prochiantz.

