Hox Temporal Collinearity: Misleading Fallacy or Essential Developmental Mechanism?

Abstract

Kondo and collaborators recently reported the absence of *Hox* temporal collinearity in *Xenopus tropicalis*. They found none in the initiation of accumulation of *Hox* transcripts (detected via RNA seq). And none in the initial expression sequence of primary unprorocessed transcripts (Identified by using qRT-PCR against introns or intron-exon boundaries). Nor in the initial acquisition by *Hox* gene DNA of a mark for active chromatin. These findings are in conflict with the idea that temporal collinearity has to do with the initiation of *Hox* gene transcription or with the opening of and a progression from repressed to active states in Hox chromatin. But collinear acquisition of the same active chromatin mark has been shown by others in murine 5' *Hoxd* cluster genes.The reason for this difference is unknown . This careful study thus indicated that the initiation phase of *Hox* expression shows no temporal collinearity in X. tropicalis. A previous study in *X. laevis* from the same group also showed   that the sequence of times for reaching (normalised) half maximal *Hox* expression showed no temporal collinearity. These conclusions are likely to be correct. These authors do however also conclude that "experimental evidence for the temporal collinearity hypothesis is not strong" There is however strong evidence that Hox temporal collinearity does occur in early vertebrate embryos.   Below. I present and discuss 3 lines of evidence to resolve the present conflict   I argue that *Hox* temporal collinearity actually does exist and that it is part of a central mechanism in early development.

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Keywords: hox genes; temporal collinearity; axial patterning; gastrulation; xenopus

1/ Introduction

Kondo and collaborators[1] recently reported the absence of *Hox* temporal collinearity in *Xenopus tropicalis*. They found none in the initiation of accumulation of *Hox* transcripts (detected via RNA seq). And none in the initial expression sequence of primary unprorocessed transcripts

(Identified by using qRT-PCR against introns or intron-exon boundaries). Nor in the initial acquisition by *Hox* gene DNA of a mark for active chromatin[1]. These findings are in conflict with the idea that temporal collinearity has to do with the initiation of *Hox* gene transcription or with the opening of and a progression from repressed to active states in Hox chromatin. But collinear acquisition of the same active chromatin mark has been shown by others in murine 5' *Hoxd* cluster genes[2].The reason for this difference is unknown . This careful study thus indicated that the initiation phase of *Hox* expression shows no temporal collinearity in X. tropicalis. A previous study in *X. laevis* from the same group[3] also showed   that the sequence of times for reaching (normalised) half maximal *Hox* expression showed no temporal collinearity[3]. These conclusions are likely to be correct. These authors do however also conclude that "experimental evidence for the temporal collinearity hypothesis is not strong" There is however strong evidence that Hox temporal collinearity does occur in early vertebrate embryos.   Below. I present and discuss 3 lines of evidence to resolve the present conflict   I argue that *Hox* temporal collinearity actually does exist and that it is part of a central mechanism in early development.

2/ Temporal Collinearity in the Literature

Several groups have presented impressive evidence that *Hox* temporal collinearity actually does exist in different early vertebrate embryos, including *Xenopus*[4-15] For discussion, see[16] Particularly important is that some studies used in situ hybridisation as the method of monitoring *Hox* expression. This enabled specific examination of *Hox* expression in tissues that show temporal collinearity, while excluding background from those that do not. These studies therefore give the clearest data. For details, see[6-8, 10, 11].

3/ An Evaluation of Temporal Collinearity in *X. Tropicalis*: Direct Comparison with the Study by Kondo et al.

Investigation using the same principal data source as Kondo et al[1] (a publicly available RNA seq. database[17]) shows that there is substantial *Hox* temporal collinearity in *X. tropicalis*. This tendency towards temporal collinearity is seen during the first major increase in *Hox* expression, leading to the first main *Hox* expression peak, between Stages 10.5 (10h) and St.25 (25h). The *Hox* transcript numbers at these stages are potentially enough to mediate a developmental function[18].   In the *X. tropicalis Hoxd* cluster (most temporally collinear), 8 out of the 8 *Hox*

genes expressed, are potentially all in a clear temporally collinear sequence for most of this early trajectory. Two (*Hoxd8, Hoxd9*) are out of sequence for some of the time and in sequence for the rest due to having unusually early initiation times combined with a prolonged period of low level expression before they take their place in the temporal collinearity sequence and two more (*Hoxd4, Hoxd9*) are ambiguous. They are each expressed close to expression of a neighbouring *Hox* gene (*Hoxd3* and *Hoxd10*, respectively). Sometimes one gene is ahead. Sometimes the other. The other *X. tropicalis Hox* clusters show temporal collinearity as well as exceptions. In the *Hoxa* cluster, *Hoxa1, Hoxa2. Hoxa3, Hoxa4, Hoxa5, Hoxa11, Hoxa13* show potential temporal collinearity. *Hoxa9, Hoxa10, Hoxa6, Hoxa7* are out of sequence and *Hoxxa4, Hoxa5* are ambiguous. In the *Hoxc* cluster, *Hoxc6, Hoxc9, Hoxc10, Hoxc11, hoxc12*, show potential temporal collinearity; *Hoxc4, Hoxc5, Hoxc8 Hoxc13* are out of sequence   In the *Hoxb* cluster *Hoxb1, Hoxb3, Hoxb4, Hoxb8, Hoxb9* are potentially in a collinear temporal sequence. , are out of sequence. *Hoxb4, Hoxb8* are ambiguous. In total, 25 out of the 38 *Hox* genes examined show potential temporal collinearity and 13 do not. It is clear that most but possibly not all Hox genes probably show temporal collinearity in this phase of development. There is thus clearly at least a subclass of Hox genes that show early temporal collinearity.

This tendency for temporal collinearity above is thus apparently not absolute. There are exceptions. These may relate to the fact that RNA seq. is not the ideal method for recording *Hox* expression. Previous studies using a suitable method (in situ hybridisation) show 100% temporal collinearity. It is also unsurprising that Hox initiation was not temporally collinear. *Xenopus* Hox genes show diverse behaviour in initiation. Some are maternally expressed. Others are not. Some have initially low expression that rises later. Others show swiftly rising expression. See below (*Hoxd* cluster, Fig.1b,) It is also unsurprprising that normalised *Hox* expression maxima showed no temporal collinearity or regularity[3]. Different *Hox* genes have very different expression profiles, no doubt associated with having different functions at various developmental stages[19,20]. In contrast with Kondo et al's apparent assumption, I feel it is very unlikely that *Hox* chromatin opening and initiation of *Hox* transcription are confined to the initiation phase of general *Hox* expression. Successive *Hox* functions in different tissues will determine that these activities are required and occur at many

different times in development. In fact it is already known that *Hox* chromatin opens at least twice during development: in early development and during the initial phase of limb development[9].

4/ The Developmental Importance of *Hox* Temporal Collinearity

Nieuwkoop and collaborators first showed that the Amphibian A-P axis is made in a timed manner. First the forebrain is induced, then progressively more posterior parts all the way back to the tail [21, 22]. These findings showed that the axial neural tissue is first specified as anterior (presumptive forebrain: telencephlon/diencephalon) and then sequentially posteriorised. This transformation involved first a conversion to presumptive mesencephalon, and subsequently to presumptive rhombencephalon, and then to presumptive spinal cord. These findings were confirmed by more recent studies in various vertebrates [7, ,23,24,25,26].

There is evidence that timed A-P axis formation in *Xenopus* is mediated by time space translation (TST) from gastrula stages onwards[7,27] (Fig. 2). *Hox* temporal collinearity acts as a *BMP* dependent timer () in the non-organiser mesoderm (NOM) of the early embryo. Another embryonic region, Spemann's organiser (SO) emits anti-BMP signals. Timed application of either an intact organiser or the organiser anti-*BMP* signal *noggin* to an organniserless embryo at sequential stages blocked the timer at sequential A-P *Hox* values, and thereby fixed timed cell states sequentially, leading to *Hox* spatial collinearity. Either the treatment generated an anteriorly truncated axis with the truncation at sequentially more posterior positions for sequentially later treatments (implanted organisers; continuous anti-*BMP* sources) or it generated one or more A–P/ *Hox* zones; sequentially later treatments gjving sequentially more posterior zones (*noggin*)[7]. In the absence of an organiser signal or *noggin,* temporal collinearity proceeded as normal, in NOM but no spatially collinear *Hox* pattern was ever generated. *Hox* expression then died out. We conclude that sequentially repeated interactions between the two embryonic parts lead to small populations of cells being fixed successively at sequential space-time points/Hox codes. Presumably, specific events including morphogenetic cell movements in the embryo or possibly *Hox* timed mesodermal cell ingression[8], cause these sequentially timed/zoned populations to be arranged in and specify an anterior early to posterior late spatial *Hox* sequence that becomes the initial A-P pattern.. This mechanism was first revealed in *Xenopus* but there is evidence that it operates during gastrulation and later stages in other vertebrates. The conversion of a dynamically changing temporally collinear *Hox* sequence to a stable spatially collinear axial *Hox* pattern

of A-P positional information by timed anti- *BMP* also operates in early chicken and zebrafish embryos[28-30]. It has been shown that *Hox* temporal collinearity in the chicken gastrula determines the order in which primitive streak cells migrate to the node[8,11], that a population of dynamically changing primitive streak cells interacts with the a stable organiser derived cell population to generate the early axial pattern in mouse embryos[31] (, and that *Hox* temporal collinearity during chicken gastrulation generates positional information (e.g., forelimb position) in later development[44]). These parallel and complementary findings in other vertebrate embryos establish that this *Hox* timing mechanism is conserved in evolution. Interestingly, the discoveries above define a believable role for the Spemann organiser, which is well known to be important in A-P patterning. I note that organiser-less (ventralised) *Xenopus* embryos show temporal but not spatial *Hox* collinearity and that reimplantation of an organiser reintroduces and fixes the spatial pattern exactly as predicted above[7].

During these stages, function of the *Hox* genes themselves is clearly a part of the timing and time space translation mechanisms. The timing mechanism operates and generating the spatial *Hox* pattern involves autoregulation and collinear cross-regulation among the *Hox* genes and Hox associated miRNA's, leading to a sequence of collinear interactions among them. This is thus at least partly a *Hox* cascade mechanism. The fact that *Hox* cascades are involved in specifying the axis is clearly demonstrated by the cascade of phenotypes generated by *Hox* gain and loss of function experiments in *Xenopus* and other systems[32-39]. We have called this aspect of collinearity: macrocollinearity[40].

Hox Temporal Collinearity in a Cell Line

In an important early paper that received too little attention[32] Faiella et al. demonstrated that NT2/D1 cells show *Hox* temporal collinearity (though they do not make a spatial *Hox* pattern). NT2/D1 was not just any old cell line: these are human pluripotent embryonal carcinoma (EC) cells. They have properties in common with the types of cells that display temporal collinearity in embryos (NOM mesoderm and primitive streak). The fact that these E.C. cells can not generate stable *Hox* expression or a *Hox* pattern presumably reflects the absence of any organiser function in this cell line.   This study was the first to reveal the *Hox-Hox* interaction that underlies temporal collinearity (posterior induction (PI)). Interestingly, the cascade LOF phenotypes generated in temporal collinearity in this cell line are mimicked by some of the Hox LOF phenotypes in spatial collinearity generated in the Xenopus embryo[33-36].

This is as expected and is a further endorsement of the proven connection between temporal and spatial collinearity.

Conclusion

In conclusion, while accepting the new findings by Kondo et al., I doubt their contention that "experimental evidence for the temporal collinearity hypothesis is not strong". Hox temporal collinearity appears to occur in a population of pluripotent stem cells in the early vertebrate embryo, and is part of the embryo-building program.

Acknowledgement

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Figure Legends

Fig. 1 **Temporal collinearity and deviations from it in X. tropicalis**

1A Temporal order r of Hox expression in the D,A,C,B clusters. Hox genes that re out oif the temporally collinear sequence are marked in red.

1B Hoxd cluster: detailed expression profiles. Note that Hoxd1, Hoxd8 and Hoxd9 start their expression much too early. Their temporally

collinear positions are regained at a slightly later stage due to a prolonged period of low level jnitial expression.

Fig. 2. **Timing, Axial patterning, and Time Space Translation**

Above: The domain structure of the vertebrate A-P axis Domains with significant Hox genes and other markers. An unexpected element is introduced by the newly characterised EAD: extreme anterior domain, which makes the face. This is shown as the most anterior part of the straight axis. Actually. the anterior end of the dorsal A-P axis bends around to the ventral side of the embryo to face posteriorly- like the handle of a walking stick (not shown). A Head: anterior head (corresponding to telencephalon,diencephalon, mesencephalon). P Head: posterior head (corresponding to anterior rhombencephalon, occipital somites). Neck: cervical somites, posterior rhombencephalon, Thorax: thoracic vertebrae, anterior spinal cord. Abdomen: Lumbar and sacral regions, spinal cord. Tail: caudal vertebrae, spinal cord.

Above and below: Time space translation. A biological timer, represented by the clockface below, proceeds from 1-12 (red numbers). The timer starts with information needed for making the EAD, proceeds to the anterior head, then to posterior head, then to neck, then to thororax , then abdomen, then tail. The timer needs *BMP* to function so is in tissues like NOM with high *BMP* (yellow/orange). Anti –*BMP* (blue) (produced by the organiser) interacts with the timer sequentially to freeze the identities of an early/A-late/P sequence of axial zones. In the axial sequence, th   e *Hox* genes are each both a component of the timer at their appropriate times and are sequentially involved in setting up the A–P sequence of axial zones. The genes involved in time space translation in the EAD-head zones are unknown. The heavy red dashed arrows represent transport of cells from the high BMP environment (yellow) to the BMP inhibited environment (blue). The continuous dark red arrows connecting yellow to blue for the head and EAD I indicate that the details he   re are unknown.   The head and tail of the A-P timer are close together because of their representation on a clockface. No statement about molecular identities is intended.

Fig. 3. ***Hox* sequences for axial cascade phenotypes** Above: Wild type *Hox* sequence. second and third down: blue diagrams: Loss of function affecting temporal collinearity in ES cells. *Hox 1* and *Hox3* LOF each cut off the *Hox* temporal collinearity sequence from their paralogue
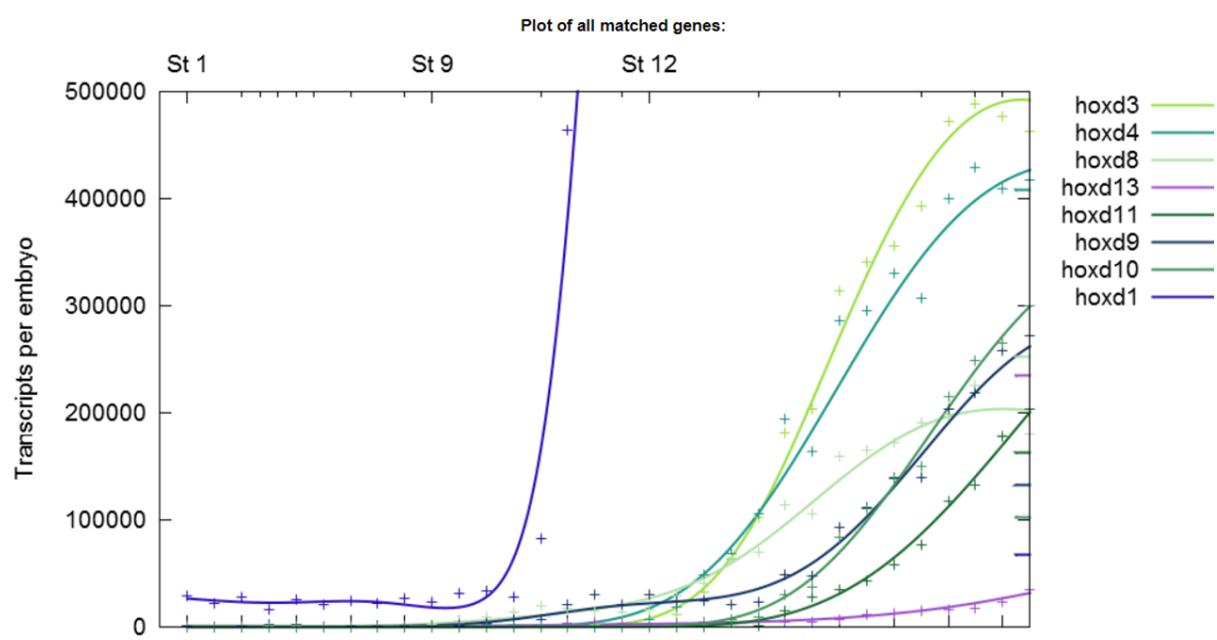
position. Fourth and fifth down: Xenopus spatial collinearity, loss of function. Fourth: *Hox1* loss of function (LOF) (all 3 *Hox1* genes knocked down by morpholinos (MOs)). The axis from *Hox1* backward is compromised. The dotted line indicates there is still reduced residual expression for some posterior Hox genes. Fifth down:*Hoxc6* LOF (MO) The axis from *Hoxc6* backward is compromised/deleted. Sixth down: *Hoxb4* gain of function (GOF): ectopic expression of *Hoxb4* in *Hox* free dorsalised embryoss. A partial posterior axis is generated, starting with *Hoxb4*. Seventh down: *Hoxb9* gain of function. Details similarly as above for *Hoxb4* gain of function. Please note that: In *Hox1* loss of function, the immediately more anterior marker (*Gbx2)* has enhanced expression, presumably reflecting absence of posterior dominance exerted by *Hox1* genes. Similarly, in *Hoxc6* loss of function, *Hox4* and *Hox5* are hyperinduced, presumably reflecting absence of *Hoxc6* posterior dominance.

Figures

Fig.1
A

Cluster				Para	Expr	Seq						
Hoxd	d1	d3	d4	d8	d9	d10	d11	d13				
Hoxa	a1	a2	a7	a9	a3	a10	a5	a4		a6	a11	a13
Hoxc	c6	c4	c9	c8	c5	c10	c3	c11	c12=c13			
Hoxb	b1	b3	b4	b8	b9	b7	b6	b2	b5			

B

Fig. 2

Fig. 3