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Tetrapod axial evolution and developmental constraints; Empirical underpinning by a mouse model

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ABSTRACT

The tetrapod vertebral column has become increasingly complex during evolution as an adaptation to a terrestrial life. At the same time, the evolution of the vertebral formula became subject to developmental constraints acting on the size of the cervical and thoraco-lumbar regions. In the course of our studies concerning the evolution of *Hox* gene regulation, we produced a transgenic mouse model expressing fish *Hox* genes, which displayed a reduced number of thoraco-lumbar vertebrae and concurrent sacral homeotic transformations. Here, we analyze this mutant stock and conclude that the ancestral, pre-tetrapodial *Hox* code already possessed the capacity to induce vertebrae with sacral characteristics. This suggests that alterations in the interpretation of the *Hox* code may have participated to the evolution of this region in tetrapods, along with potential modifications of the HOX proteins themselves. With its reduced vertebral number, this mouse stock violates a previously described developmental constraint, which applies to the thoraco-lumbar region. The resulting offset between motor neuron morphology, vertebral patterning and the relative positioning of hind limbs illustrates that the precise orchestration of the *Hox*-clock in parallel with other ontogenetic pathways places constraints on the evolvability of the body plan.

> 'The whole subject of homology and segmentation is very complex, imperfectly understood, and well worthy of further study'
> [Edwin E. Goodrich, 1913]

1. Introduction

The vertebrate spine is built as a sequence of serial homologous elements, the vertebrae, which develop with different morphologies at different positions along the anterior–posterior axis. Various vertebral formulae reflect both the requirements and the constraints associated with a skeleton that needs to accommodate protective, respiratory and locomotor functions (Romer, 1956; Woltering, 2012). The differentiation of initially similar somites into distinct type of vertebrae (i.e. cervical, thoracic, lumbar, sacral and caudal in mammals) is established early on during embryogenesis, mainly due to a collinear pattern of *Hox* gene expression (Kmita and Duboule, 2003) along the antero-posterior axis (Casaca et al., 2014; Deschamps and van Nes, 2005; Mallo et al., 2010; Wellik, 2009). These coordinated expression patterns indeed generate various combinations of HOX proteins at distinct body levels (or a '*Hox* code' (Kessel and Gruss, 1991)), which genetically instruct somites about their fates in terms of morphology. In addition, the correspondence between particular combinations of HOX proteins and critical morphological transitions are maintained throughout tetrapods, suggesting an instructive role for these proteins in setting up these boundaries (Burke et al., 1995; Gaunt, 1994). However, the fact that various HOX proteins display some functional hierarchies in these processes makes a pure combinatorial system unlikely (see Duboule and Morata, 1994).

The evolution of land vertebrates was paralleled by an increasing complexity of axial regionalization, as an adaptation to a terrestrial lifestyle: the sacrum evolved during the fish–tetrapod transition as a connection between the pelvic girdle and the axial skeleton (Carroll and Holmes, 2007), whereas the lumbar region first appeared in mammals as an adaptation to sagittal flexion during locomotion as well as to accommodate the diaphragm (Carroll, 1988). In mammals, the sacral and lumbar regions are genetically characterized by the transcription of *Hox* genes belonging to paralogy groups 10 and 11 (*Hox10* and *Hox11* genes), evolutionary related to the insect posterior gene *Abd-B* (Izpisua-Belmonte et al., 1991). Functional approaches have revealed that HOX10 proteins suppress rib formation whereas HOX11 proteins can induce sacral

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processes (Carapuco et al., 2005; Wellik and Capecchi, 2003). Interestingly, this collinear distribution of *Hox* transcripts predates the origin of vertebrates and fish for instance already express *Hox10* and *Hox11* genes in paraxial mesoderm, in spite of the absence of both sacral and lumbar regions (Oulion et al., 2011; Prince et al., 1998; van der Hoeven et al., 1996). Therefore, it is likely that these particular region-specific morphologies did not arise through mere changes in *Hox* gene expression domains. Instead, they may have involved concomitant alterations in the activation of downstream target genes, for example via modification in the interpretation of the 'code' either following changes in the *cis*-regulatory modules controlling these targets, or due to changes in the HOX proteins themselves. Altogether, it is currently unknown whether the emergence of these particular body regions involved a simple exaptation of a pre-existing *Hox* pattern, or if it was accompanied by essential structural changes in HOX proteins leading to novel functions.

Regardless of which evolutionary mechanism leads to such critical modifications of the tetrapod spine, its realm of action was likely reduced due to the strong developmental constraints applied to the axial formula. The existence of developmental constraints applied to the organization of segmental patterns in animals was recognized more than a century ago, by the mere observations of natural 'rules' governing the formation of metamerized body plans (see for example the work of Lankester, described in Jeffs and Keynes, 1990). Nowadays, such constraints or limitations in the evolution of otherwise potentially adaptive traits are thought to derive in part from the way the underlying regulatory processes are implemented and shared between various developmental contexts, leading to severe pleiotropic effects at least in vertebrates (Duboule and Wilkins, 1998; Kirschner et al., 2005).

The comparative analysis of vertebral columns provides many instances of such canalized processes (Asher et al., 2011), as for example the well-known constraint that fixes the number of cervical vertebrae to seven in all mammals but manatees and sloths (see e.g. Galis, 1999; Galis and Metz, 2007; Varela-Lasheras et al., 2011), even though natural selection favored in some instances either the increase or the decrease in neck length, as in giraffes and whales, respectively. In such cases however, variations occurred through changes in the sizes of vertebrae rather than in their number. It was suggested that this constraint was generated by a potential interference with the migration of the diaphragm muscles, thus leading to an impaired respiration (Buchholtz et al., 2012; Hirasawa and Kuratani, 2013). Likewise, in the thoraco-lumbar region, a constraint seems to restrict the overall number of vertebrae to 19 or 20 in most mammals (Narita and Kuratani, 2005), perhaps associated with the proper implementation of locomotor mechanisms (Buchholtz, 2014; Galis et al., 2014).

Unfortunately, even though the mechanisms underlying both the time-sequenced production of somites (Pourquie, 2003) and the concurrent progressive activation of *Hox* genes (Noordermeer et al., 2014) start to be understood, evolutionary scenarios accounting for the macroevolution of the axial skeleton remain complex to address experimentally and lack empirical support. In this study, we investigate the phenotypic abnormalities in a transgenic mouse containing a *HoxAa* BAC from the pufferfish (*Tetraodon nigroviridis*) genome. This transgenic line was produced in the aim of studying interspecies regulatory controls (Woltering et al., 2014) and expresses fish *HoxAa* genes at slightly ectopic positions during the developing mouse body axis. The resulting morphological transformations indicate that pre-tetrapodial HOX proteins can induce tetrapod-specific anatomical features. Also, the number of thoracic and lumbar vertebrae obtained scores below the developmental constraint identified by Narita and Kuratani (2005). The paraplegia observed in these mice suggests that the origin of the constraint on the evolutionary bias in TL vertebral number may lie in the mechanistic independence between axial patterning by *Hox* genes, on the one hand, and hind limb positioning, on the other hand.

2. Results

2.1. A mouse stock with a reduced number of TL vertebrae

In the course of our studies of *Hox* gene regulation during the fin to limb transition, a transgenic mouse stock was generated by using a Pufferfish (*T. nigroviridis*) BAC covering part of the posterior *HoxAa* cluster and containing from *HoxA9a* through *HoxA13a* (Woltering et al., 2014). Three F0 males were obtained, which all showed locomotory incapacitation of the hind limbs (paraplegia), as well as a trunk shortened along the anterior to posterior axis (Fig. 1A, Supplementary movies 1–2). One male died shortly after weaning and the cadaver was lost; a second male died without any apparent pathological cause, at approximately two years of age, but never reproduced; the third male proved capable of reproducing and was used to establish a line through natural mating. As further paternal transmission of this transgenic condition was never achieved in this line, it was maintained through hemizygous maternal crosses. In addition to the male reproductive defects, the problems in hind limb coordination with an abnormal gate caused by (partial) hind-limb paralysis persisted in this line. Adult animals improved in this respect after 5 months of age.

To try and understand the etiology of these various phenotypes, we initially analyzed these mice for potential skeletal and/or neural abnormalities. Alizarin red-alcian blue staining in newborns and adults revealed major homeotic transformations in the posterior trunk, including a large anterior shift of the sacrum leading to a reduction of the lumbar region from usually six (sometimes five) lumbar vertebrae in wild-type mice, to only three lumbar vertebrae in the transgenic condition. In addition, the second and third lumbar vertebrae (L2 and L3) showed partial sacral transformation, as shown by clearly broadened lateral processes (Fig. 1B, C, Supplementary Fig. 1). This phenotype was also scored in the skeleton of the second F0 male, for which no line could be established (Fig. 1B [tni *HoxAa#2*]). The early innervation pattern of the hind limbs was investigated in 12.5 days old fetuses (E12.5), using immune-staining of neurofilaments (Fig. 1D). In transgenic mice, an abnormal truncation of the peroneal nerve, which innervates the dorsal aspect of the hind limbs, was observed consistent with the locomotory abnormalities detected in these mice. Both the neuronal and reproductive phenotypes proved very similar to those observed for the loss of function of *Hox10* group genes. These latter mutants indeed display a misspecification of the sciatic part of the lumbosacral plexus, which normally innervates the hind limbs (Carpenter et al., 1997; Tarchini et al., 2005; Wu et al., 2008).

2.2. Gain of function of Tetraodon Hoxa11a

In order to associate the phenotypic abnormalities observed in the transgenic line with the potential expression of the transgenic *Tetraodon Hox* genes present in the BAC, we performed in situ hybridization for the *Tetraodon Hoxa9a* to *Hoxa13a* genes (Fig. 2). Interestingly, these genes were expressed during mouse development with the expected spatial collinear pattern along the main body axis, with *Hoxa9a* being expressed most anteriorly and *Hoxa13a* being confined to the posterior tail region. Comparison with the expression of the endogenous mouse *Hoxa* genes however, indicated the presence of transgenic *Hoxa11a* transcripts at a too anterior position, i.e. about three to four somites more anterior than the corresponding pattern for the mouse *Hoxa11* gene. Such an anteriorized transcriptional pattern associated with a *Hox11* gene was previously reported, associated with the replacement in vivo of an endogenous *Hoxd11* enhancer by its teleost counterpart. Mice carrying this fish enhancer at the correct endogenous position expressed their own *Hoxd11* gene too anteriorly (Gerard et al., 1997). Therefore, an anterior gain of function of the *Tetraodon Hoxa11a* gene as reported here may reflect a specific *cis*-regulatory difference between fish and mammals, as somewhat supported by the high divergence in non-coding DNA sequences between the fish and tetrapods posterior

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