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Evolution of Developmental Control Mechanisms

Retinoic acid signaling targets *Hox* genes during the amphioxus gastrula stage: Insights into early anterior–posterior patterning of the chordate body plan

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ABSTRACT

Previous studies of vertebrate development have shown that retinoic acid (RA) signaling at the gastrula stage strongly influences anterior–posterior (A–P) patterning of the neurula and later stages. However, much less is known about the more immediate effects of RA signaling on gene transcription and developmental patterning at the gastrula stage. To investigate the targets of RA signaling during the gastrula stage, we used the basal chordate amphioxus, in which gastrulation involves very minimal tissue movements. First, we determined the effect of altered RA signaling on expression of 42 genes (encoding transcription factors and components of major signaling cascades) known to be expressed in restricted domains along the A–P axis during the gastrula and early neurula stage. Of these 42 genes, the expression domains during gastrulation of only four (*Hox1, Hox3, HNF3-1* and *Wnt3*) were spatially altered by exposure of the embryos to excess RA or to the RA antagonist BMS009. Moreover, blocking protein synthesis with puromycin before adding RA or BMS009 showed that only three of these genes (*Hox1, Hox3* and *HNF3-1*) are direct RA targets at the gastrula stage. From these results we conclude that in the amphioxus gastrula RA signaling primarily acts via regulation of *Hox* transcription to establish positional identities along the A–P axis and that *Hox1*, *Hox3*, *HNF3-1* and *Wnt3* constitute a basal module of RA action during chordate gastrulation.

Introduction

Retinoic acid (RA) is a morphogen that acts via heterodimers of the retinoic acid receptor (RAR) and retinoid X receptor (RXR), which bind to retinoic acid response elements (RAREs) in the promoter regions of target genes. The RA signal is mediated by a positive feedback loop involving the direct regulation of *RAR* by RAR/RXR heterodimers (Heyman et al., 1992; Rudert and Gronemeyer, 1993; Blomhoff and Blomhoff, 2006; Campo-Paysaa et al., 2008; Casci, 2008). RA has been extensively studied for its effects on cell cultures, embryonic development, adult growth, regeneration and carcinogenesis in chordates (McCaffery et al., 2003; Mongan and Gudas, 2007; Dann et al., 2008; Niederreither and Dollé, 2008). In embryos of amphioxus and vertebrates, RA has numerous pleiotropic effects. RA signaling is permissive for some tissues–like forelimbs and somites–allowing previously specified structures to complete differentiation, but instructive for other tissues–like hindbrain and foregut–conferring positional information along the anterior–posterior (A–P) axis and specifying tissue identity (Stafford et al., 2006; Duester, 2008).

The earliest known instructive signaling by RA begins at the gastrula stage and mediates patterning of the germ layers along the A–P axis of the embryo (Durston et al., 1989; Sive et al., 1990; Holland and Holland, 1996; Roelen et al., 2002; Grapin-Botton, 2005; White et al., 2007). Even a transitory perturbation of RA signaling in the gastrula can affect A–P patterning during the post-gastrula stages of development. There are few studies on the effects of RA signaling on gene transcription at the gastrula stage, and these are largely limited to *Hox* genes and to vertebrates (Kudoh et al., 2002; Roelen et al., 2002; Li et al., 2008a). Such studies are complicated by the mechanics of gastrulation in vertebrates. The germ layers at the gastrula stage are often more than one cell thick (Delarue et al., 1998; Li et al., 2008b), and the constituent cells undergo complex migrations, either individually or in coherent groups during gastrulation (Schoenwolf and Smith, 2000; Kimura et al., 2006). It is, therefore, difficult to establish the A–P limits of the domains of gene expression in vertebrate gastrulae, raising the possibility that there are early immediate targets of RA signaling at the gastrula stage that act in parallel to *Hox* genes in mediating A–P patterning.

To test this hypothesis, we used the invertebrate chordate amphioxus. Amphioxus resembles vertebrates in using RA signaling for axial patterning (Holland and Holland, 1996; Escriva et al., 2002; Marlétaz et al., 2006), but has the advantage of an early development

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that is morphologically uncomplicated: the spherical blastula has a single layer of cells surrounding a hollow blastocoel. Gastrulation begins with invagination from the posterior pole of the embryo. The resulting gastrula has an outer ectoderm and an inner mesendoderm, both only one cell thick. The invaginated mesendoderm largely obliterates the blastocoel and creates a new space, the archenteron, which opens to the exterior via a blastopore. Cell movements during invagination of the mesendoderm are minimal: the cells at the blastoporal lip do not converge towards the dorsal midline, and there is little involution over the lip of the blastopore (Zhang et al., 1997; Holland and Holland, 2007). Amphioxus is also more amenable for such a study than tunicates, where RA signaling of the A–P axis is either highly modified (ascidians) or absent (appendicularians) (Ishibashi et al., 2003; Nagatomo and Fujiwara, 2003; Fujiwara, 2005; Cañestro and Postlethwait, 2007; Imai et al., 2009).

For vertebrates, except for the study of Kudoh et al. (2002), the effects of altering RA signaling during the gastrula stage have not been tallied until the neurula and larval stages (reviewed by Duester, 2008). Similarly, for amphioxus, previous studies altering RA signaling during the gastrula stage only looked at the effects of this treatment at later stages. Therefore, although these studies documented effects of RA on expression of a number of genes (i.e. several *Hox* genes, *Cdx*, *Hedgehog*, *HNF3-1*, *Nodal*, *Notch*, *Otx*, *Pitx*, *Wnt3* and *Wnt5*) at the neurula and larval stages (Holland and Holland, 1996; Schubert et al., 2004, 2005, Schubert et al., 2006; Osborne et al., 2009), they could not differentiate direct targets of RA signaling from indirect ones.

In the present study, we administered RA or an RA antagonist (BMS009) continuously to cultures of developing amphioxus from the beginning of the gastrula stage and assayed for effects later in the gastrula stage on expression of 42 genes known to be transcribed in restricted anterior–posterior patterns at the gastrula stage (listed in Supplementary Table S1). In addition, we inhibited protein synthesis with puromycin to distinguish between direct and indirect targets of RA signaling. Our results show that only 3 of these 42 genes (*Hox1*, *Hox3* and *HNF3-1*) are direct targets of RA at the gastrula stage, while only one (*Wnt3*) is an indirect target. Moreover, we present evidence suggesting that, in the amphioxus early neurula, RA may also directly regulate *Hox4* and *Hox6*. Since, in amphioxus, the control regions of at least *Hox1* and *Hox3* include functional RAREs (Manzanares et al., 2000; Wada et al., 2006), we conclude that RA acts and acted primarily through *Hox* genes as direct targets in patterning the A–P body axis at the gastrula stage not only in amphioxus, but probably also in the last invertebrate chordate ancestor of vertebrates.

Materials and methods

Sexually mature males and females of the Florida amphioxus (*Branchiostoma floridae*) were collected in Tampa Bay, Florida, USA, during the summer breeding season. The animals were stimulated to spawn electrically (Holland and Holland, 1993). After fertilization, the embryos were raised in filtered seawater at 28 °C and staged according to Holland and Yu (2004) as very early, early, mid and late gastrulae (respectively, 3 h, 4 h, 5 h and 6 h after fertilization) or early neurulae (9 h after fertilization).

In a first series of experiments, RA or the RA antagonist BMS009, each dissolved in DMSO (for a final concentration of 1×10^{-6} M), were added to cultures of very early gastrulae. DMSO at a 1:1000 dilution alone (Holland and Holland, 1996; Escriva et al., 2002) had no detectable effect. Samples of gastrulae at 4 h, 5 h or 6 h of development were fixed in 4% paraformaldehyde in MOPS buffer (0.1 M MOPS, 0.5 M NaCl, 2 mM MgSO4, 1 mM EGTA, pH 7.4) (Holland et al., 1996). This fixation solution is referred to hereafter simply as PFA. After fixation overnight at 4 °C, the specimens were transferred to 70% ethanol and stored at −20 °C until subjected to *in situ* hybridization. For each gene tested, antisense riboprobes were synthesized according to Holland et al. (1996) from the originally described clones or matching EST clones.

The effects of RA or BMS009 were studied for the following genes expressed at the gastrula stage as well as for two *Hox* genes expressed very shortly thereafter (the normal expression of all of these genes was already known from previous studies, as listed in Supplementary Table S1): *FoxD* (AF512537), *FoxQ2* (AY163864), *HNF3-2* (Y09236), *HNF3-1* (X96519), *Pax3/7* (AF165886), *Hex* (EU296398), *Pitx* (AJ438768), *Otx* (AF043740), *Cdx* (AF052465), *EvxA* (AF374191), *Gbx* (DQ416766), *Lim1/5* (DQ399521), *Six1/2* (EF195742), *Six3/6* (EF195743), *Six4/5* (EF195741), *Sox1/2/3* (AF271787), *Blimp1* (EU708968), *Neurogenin* (AF271788), *Brachyury* (X91903), *Eya* (EF195740), *Delta* (BW899056), *Notch* (Y12539), *Nodal* (AY083838), *Lefty* (EST clone bfne107n04), *Fgf8/17/18* (FJ266460), *Hedgehog* (Y13858), *Wnt1* (AF061974), *Wnt3* (AF361013), *Wnt4* (AF061973), *Wnt5* (AF361014), *Wnt6* (AF361015), *Wnt7* (AF061975), *Wnt8* (AF190470), *Wnt11* (AF187553), *Dkk1/2/4* (EST clone bfga017h15), *Dkk3* (EST clone bflv049h10), *sFRP2-like* (EST clone bfga018e02), *sFRP3/4* (EST clone bfad036d02), *Hox1* (AB028206), *Hox3* (X68045), *Hox4* (AB028208), *Hox6* (Z35146). The last two genes in this list, although not conspicuously transcribed at the gastrula stage, have expression domains known to be influenced by RA signaling at the early neurula stage (Schubert et al., 2004, 2005, 2006).

For blocking protein synthesis, puromycin (Sigma-Aldrich, Saint Louis, MO, USA) was added to gastrula cultures to a final concentration of 200 μg/ml at 3 h, 4 h and 5 h of development. After 5 min, RA or BMS009 (1×10^{-6}M) or DMSO was added and after 1 h embryos were fixed for *in situ* hybridization. The effectiveness of the puromycin concentration was verified by its ability to block synthesis of endogenous alkaline phosphatase in the amphioxus gut endoderm (Supplementary Fig. S1) (Holland et al., 1996).

Results

Altered RA signaling affects gene expression domains in amphioxus gastrulae

To identify potential direct targets of RA signaling during the gastrula stage in amphioxus, we first determined the effect of altered RA signaling on 40 genes with limited domains of expression along the A–P axis of the gastrula (Fig. 1; Supplementary Table S1) plus *Hox4* and *Hox6*, although expression of the last two only begins at the very end of (*Hox4*) or shortly after (*Hox6*) gastrulation (Schubert et al., 2004, 2005, 2006). Expression of *Hox2* at the gastrula stage was too weak to allow proper interpretation and was therefore excluded from our analysis. The other sampled genes fall into several categories: Notch, Nodal, Wnt and FGF signaling, forkhead and homeobox genes as well as transcription factors (*Neurogenin*, *Sox1/2/3*). About half have a single expression domain in a single tissue layer (either the outer ectoderm or the inner mesendoderm), and about half have domains in both tissue layers and/or two domains in a given tissue layer. Expression of thirteen of these 42 genes (*Cdx*, *Hedgehog*, *HNF3-1*, *Hox1*, *Hox3*, *Hox4*, *Hox6*, *Nodal*, *Notch*, *Otx*, *Pitx*, *Wnt3* and *Wnt5*) at the neurula and later stages was already known to be affected by RA applied during the gastrula stage, but it was not known if their expression in the gastrula itself was also affected (Holland and Holland, 1996; Schubert et al., 2004, 2005, 2006; Osborne et al., 2009). Therefore, it was not known whether any or all are direct targets of RA signaling. Administration of RA or the RA antagonist BMS009 from the onset of gastrulation did not alter the expression patterns of *Pitx*, *Otx*, *Cdx*, *Notch*, *Nodal*, *Hedgehog* and *Wnt5* during the gastrula stage (Figs. 1I–K, X, Y, B′, F′), even though their expression was affected by altered RA signaling in later embryos and larvae (Schubert et al., 2005, 2006; Osborne et al., 2009). *Nodal*, *Hedgehog* and *Wnt5* encode secreted signaling proteins involved in axial patterning in early development, and these results suggest that they are acting in parallel to RA signaling at the gastrula stage, with any crosstalk occurring only later in development. Moreover, none of