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Retinoic acid signalling is required for specification of pronephric cell fate

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Abstract

The mechanisms by which a subset of mesodermal cells are committed to a nephrogenic fate are largely unknown. In this study, we have investigated the role of retinoic acid (RA) signalling in this process using *Xenopus laevis* as a model system and *Raldh2* knockout mice. Pronephros formation in *Xenopus* embryo is severely impaired when RA signalling is inhibited either through expression of a dominant-negative RA receptor, or by expressing the RA-catabolizing enzyme XCyp26 or through treatment with chemical inhibitors. Conversely, ectopic RA signalling expands the size of the pronephros. Using a transplantation assay that inhibits RA signalling specifically in pronephric precursors, we demonstrate that this signalling is required within this cell population. Timed antagonist treatments show that RA signalling is required during gastrulation for expression of *Xlim-1* and *XPax-8* in pronephric precursors. Moreover, experiments conducted with a protein synthesis inhibitor indicate that RA may directly regulate *Xlim-1*. *Raldh2* knockout mouse embryos fail to initiate the expression of early kidney-specific genes, suggesting that implication of RA signalling in the early steps of kidney formation is evolutionary conserved in vertebrates.

Keywords: Retinoic acid; *Cyp26*; *Raldh2*; *Xenopus*; Pronephros; *Pax-8*; *Lim-1*

Introduction

During early vertebrate development, the fate of initially pluripotent cells becomes progressively restricted to more and more limited developmental choices. The first major events in this process are the allocation of cells to the primary germ layers i.e. ectoderm, mesoderm and endoderm, and the patterning along the embryonic axes. In *Xenopus*, embryonic mesoderm arises from a ring of cells, the marginal zone, located equatorially between the animal and vegetal hemispheres of the blastula. The marginal zone will give rise to a characteristic tissue pattern whose dorso-ventral sequence in the tadpole is notochord, muscle, pronephros and blood (Dale and Slack, 1987; Kessler and Melton, 1994). During gastrulation, molecular signals and morphogenetic movements establish a set of coordinates along the anterior–posterior and dorso-ventral axes. Soluble inducing factors secreted from the Spemann Organizer are proposed to result in the specification of dorsolateral fates such as somites, pronephros and heart (Harland and Gerhart, 1997; Heasman, 1997). Among these factors, inhibitors of the Bone morphogenetic protein (BMP) pathway such as noggin and chordin are crucial for mesoderm patterning (Dale and Jones, 1999). In *Xenopus* and zebrafish, BMP signalling is highest on the ventral side of the embryo and loss of BMP signalling in both species leads to expansion of dorsal structures, such as trunk muscle, at the expense of

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ventral structures (Jones et al., 1996; Kishimoto et al., 1997; Pyati et al., 2005). Most experiments concerning mesodermal patterning are focused on notochord, muscle and blood specification. While several genes have been shown to be crucial for pronephric kidney morphogenesis (Brandli, 1999; Drummond, 2003; Hostetter et al., 2003; Vize et al., 1997), little is known about the signals responsible for the commitment of undifferentiated mesodermal cells to a nephrogenic fate. These signals function during gastrulation and by the early neurula stage pronephric mesoderm can be explanted and form pronephric tubules and glomus in isolation (Brennan et al., 1998; Brennan et al., 1999). In *Xenopus*, *Xlim-1* and *XPax-8* are the earliest identified markers of pronephros specification in the presumptive pronephric field (Carroll and Vize, 1999; Carroll et al., 1999). At late gastrula stage, the expression domains of these two genes overlap in the region that will eventually form the pronephros. Co-expression of XPax-8 and Xlim-1 leads to the formation of enlarged and ectopic pronephroi at high frequency (Carroll and Vize, 1999) and development of the pronephric tubules is inhibited by expression of Xlim-1-Enr, a fusion mutant with an engrailed repressor domain (Chan et al., 2000). Expression of *Lim-1* and *Pax-8* in the intermediate mesoderm fated to give rise to the pronephros is conserved in zebrafish and mouse (Barnes et al., 1994; Bouchard et al., 2002; Fujii et al., 1994; Pfeffer et al., 1998; Plachov et al., 1990; Toyama and Dawid, 1997; Tsang et al., 2000). In these species, in addition to *Pax-8*, *Pax-2* (*Pax-2*.a in zebrafish) marks the intermediate mesoderm during pronephros specification while in *Xenopus* expression of the orthologue *XPax-2* normally commences at tailbud stage, several hours after the pronephros is specified and the *XPax-8* and *Xlim-1* domains are established (Carroll and Vize, 1999). Knockout and transgenic studies identify Lim-1 and Pax-2/8 as critical regulators of nephric lineage specification. Pax-2 and Pax-8 have redundant function in kidney development, but in the absence of both transcription factors the intermediate mesoderm is unable to undergo mesenchymal–epithelial transition necessary for nephric duct formation and fails to express markers of nephric identity (Bouchard et al., 2002; Mansouri et al., 1999). Using microsurgical manipulation of the chick embryo, *Pax-2* expression in the intermediate mesoderm was shown to depend on a BMP-4 signal released by the surface ectoderm and on unidentified signals from the paraxial mesoderm (Mauch et al., 2000; Obara-Ishihara et al., 1999). However, the precise molecular mechanisms regulating *Pax-2/8* and *Lim-1* expression in the intermediate mesoderm remain to be elucidated.

Retinoic acid (RA), a metabolite of vitamin A, has proven to be necessary for proper development of the vertebrate embryo. Vitamin A deficiency (VAD) results in a spectrum of developmental malformations including patterning defects in the central nervous system, circulatory and hematopoietic systems, limbs and trunk (Clagett-Dame and DeLuca, 2002; Maden, 2000). Conversely, when RA is available to embryos in the wrong places or the wrong times it is a potent teratogen. RA binds to a family of nuclear receptors, the RA receptors (RARs-α, -β, -γ and their isoforms), which form heterodimers with retinoid X receptors (RXRs -α, -β, -γ) to control the expression of target genes containing RA response elements (RAREs) (Bastien and Rochette-Egly, 2004; Chambon, 1996). RA signalling depends on the coordinated expression of specific synthesizing enzymes, the RA-synthesizing retinaldehyde dehydrogenases (RALDHs) and the CYP26 catabolizing enzymes. As RA can diffuse freely across solid tissues, the local expression sites of the metabolic enzymes represent potential spatial patterning tools: the RALDHs form the origins of RA diffusion gradients and the CYP26s generate abrupt steps in RA levels (Sirbu et al., 2005). In situ hybridization (Niederreither et al., 1997) and enzymatic studies (McCaffery and Drager, 1995) have shown that Raldh2 is the prominent RALDH activity during early embryogenesis. Knockout of the *Raldh2* gene results in embryonic lethality at embryonic day E9.5–10.5, caused by severe trunk, hindbrain and heart defects (Niederreither et al., 1999; Niederreither et al., 2001; Niederreither et al., 2000). Using a RARE-LacZ reporter transgene, *Raldh2*$^{-/-}$ null embryos were shown to lack any detectable RA activity, except in the eye field which contains other RALDH enzymes (Mic et al., 2002). *Raldh2* and *Cyp26a1* knockout and expression studies indicate that the tightly regulated synthesis and distribution of RA is crucial during late stages of gastrulation (Abu-Abed et al., 2001; Fujii et al., 1997; Niederreither et al., 1997; Sakai et al., 2001). As there is good evidence that RA signalling is essential for mesodermal patterning (Maden, 1999; Ruiz i Altaba and Jessell, 1991), we hypothesized that it might be essential for pronephros development. This hypothesis is based on two observations. First, the expression characteristics of the synthesizing and catabolizing enzymes in *Xenopus* suggest that RA signalling is active in the mesoderm during gastrulation and early neurulation when the pronephric precursors are being specified. *XRaldh2* and *XCyp26* show non-overlapping, complementary expression domains in the gastrula marginal zone. *XRaldh2* expressing cells are located in the internal involuting mesoderm, whereas *XCyp26* mRNA appears to be restricted to the epithelial layer of the involuting domain (Chen et al., 2001; Hollemann et al., 1998). Second, treatment of *Xenopus* blastula animal caps with activin A plus RA results in the formation of pronephric cells. Although RA are used at non physiological doses in this in vitro assay, the induced cells express differentiation markers characteristic of the correct developmental stage and in the correct developmental sequence (Brennan et al., 1998; Moriya et al., 1993; Osafune et al., 2002).

Yet, involvement of endogenous RA signalling in pronephros specification has not been demonstrated. In order to test this hypothesis, we have analysed pronephros development in *Xenopus* and mouse embryos in which RA signalling has been manipulated. Our results show that defective RA signalling greatly impairs pronephros development. We further demonstrate that RA signalling is required during gastrulation for the onset of *Xlim-1* and *XPax-8* expression within the pronephric precursors.