Biochemical and Biophysical Research Communications 423 (2012) 889–894

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Retinoic acid signaling regulates embryonic clock *hairy2* gene expression in the developing chick limb

Caroline J. Sheeba [a,b,c,d], Isabel Palmeirim [c,d,1], Raquel P. Andrade [a,b,1,*]

[a] *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga 4710-057, Portugal*
[b] *ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal*
[c] *Regenerative Medicine Program, Departamento de Ciências Biomédicas e Medicina, Universidade do Algarve, Faro 8005-139, Portugal*
[d] *IBB – Institute for Biotechnology and Bioengineering, Centro de Biomedicina Molecular e Estrutural, Universidade do Algarve, Faro 8005-139, Portugal*

ARTICLE INFO

Article history:
Received 15 June 2012
Available online 21 June 2012

Keywords:
hairy2 expression
Retinoic acid
Limb development
Erk/MAPK
Gli3
BMP4

ABSTRACT

Embryo development proceeds under strict temporal control and an embryonic molecular clock (EC), evidenced by cyclic gene expression, is operating during somite formation and limb development, providing temporal information to precursor cells. In somite precursor cells, EC gene expression and periodicity depends on Retinoic acid (RA) signaling and this morphogen is also essential for limb initiation, outgrowth and patterning. Since the limb EC gene *hairy2* is differentially expressed along the proximal–distal axis as growth proceeds, concomitant with changes in flank-derived RA activity in the mesenchyme, we have interrogated the role of RA signaling on limb *hairy2* expression regulation. We describe RA as a positive regulator of limb *hairy2* expression. Ectopic supplementation of RA induced *hairy2* in a short time period, with simultaneous transient activation of Erk/MAPK, Akt/PI3K and Gli3 intracellular pathways. We further found that FGF8, an inducer of Erk/MAPK, Akt/PI3K pathways, was not sufficient for ectopic *hairy2* induction. However, joint treatment with both RA and FGF8 induced *hairy2*, indicating that RA is creating a permissive condition for p-Erk/p-Akt action on *hairy2*, most likely by enhancing Gli3-A/Gli3-R levels. Finally, we observed an inhibitory action of BMP4 on *hairy2* and propose a model whereby RA shapes limb *hairy2* expression during limb development, by activating its expression and counteracting the inhibitory action of BMP4 on *hairy2*. Overall, our work reports a novel role for RA in the regulation of limb clock *hairy2* gene expression and elucidates the temporal response of multiple intracellular pathways to RA signaling in limb development.

1. Introduction

Spatiotemporal fine-tuned gene expression regulation is a fundamental trait in all biological processes, including embryogenesis. An embryo time-counting mechanism was first reported by describing cyclic expression of *hairy1*, a Hairy/Enhance-of-split (HES) gene, underlying the periodicity of somite formation [1]. Multiple genes belonging to this embryonic molecular clock (EC) have been reported in different species and belonging to the Notch, FGF and WNT signaling pathways [2]. A decade after the somitogenesis clocks discovery, a similar molecular clock was found to operate in the chick distal limb mesenchyme [3]. This tissue presents cyclic expression of another HES family member, *hairy2*, in the chondrogenic precursor cells with a 6 h periodicity [3]. In the following years, oscillations of *HES* expression have also been described in human mesenchymal stem cells [4], mouse neural progenitor cells [5] and in embryonic stem cells [6]. Thus, gene oscillations are a wide-spread mechanism, providing temporal information to multiple systems.

EC genes are regulated by retinoic acid (RA) signaling in the presomitic mesoderm (PSM) [7–9]. Retinoic acid (RA) is a morphogen, derived from vitamin A, which regulates gene expression through its interaction with nuclear receptors [10]. During early embryonic phases, the RA synthesizing enzyme *raldh2* is expressed in the PSM overlapping the *fgf8* expression domain [8]. Over time, the antagonistic action of RA and FGF8 signaling generates opposing anterior-*raldh2* and posterior-*fgf8* gradients, which are absolutely required for proper somitogenesis [7,8,11]. We have further described that RA ensures timely somite formation through modulation of Gli activity [12]. In fact, SHH signaling deprivation in chick PSM delayed both EC periodicity and somite formation rate, which was rescued by RA-mediated Gli activity modulation [12], suggesting the ability of RA to functionally replace SHH signaling.

The developing limb begins as a small bud of homogenous mesenchymal cells under the influence of the opposing proximal–distal RA signaling and the distal–proximal FGF signaling deriving

* Corresponding author. Fax: +351 253604862.
E-mail address: rpandrade@ecsaude.uminho.pt (R.P. Andrade).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.077

from the apical ectodermal ridge (AER) [13]. These two morphogen gradients are essential for limb proximal–distal (PD) outgrowth and patterning [14]. RA also participates in limb anterior–posterior (AP) patterning, since it activates *shh* expression in the zone of polarizing activity (ZPA) [15,16]. While AER-FGFs signal through Erk/MAPK and Akt/PI3K pathway activation [17–19], Gli1–3 proteins function as the intracellular effectors of ZPA-SHH activity [20,21]. As the limb develops, the RA domain is proximalized due to the polarized gene expression distribution of its synthesizing (*Raldh2*) and degrading (*Cyp26*) enzymes, in the embryo flank and distal limb mesenchyme, respectively [22–24]. Importantly, the limb EC *hairy2* gene presents distinct domains of expression during limb development [3], concomitant with the changing influence of RA and FGF signaling in the limb field. In the present study, we have interrogated the role of RA signaling on the regulation of limb EC *hairy2* gene expression. We further assessed the temporal response of the chick embryo forelimb tissue to RA by elucidating the signaling pathways which this morphogen modulates over time.

2. Materials and methods

2.1. Eggs and embryos

Fertilized Gallus gallus eggs were incubated at 37.8 °C in a 49% humidified atmosphere and staged according to Hamburger and Hamilton (HH) classification [25]. All the experiments were performed in stage HH22–24 forelimb buds.

2.2. Microsurgical ablation of ZPA tissue

A window was cut in the shell of incubated eggs and the vitelline membrane was carefully removed. The ZPA was microsurgically ablated from the right wing bud of embryos using a tungsten needle. As a control, ZPA extirpated embryos were randomly selected for direct fixation and hybridization with *shh*. Operated embryos were re-incubated for 6–7 h, either collected in PBS and fixed for in situ hybridization or subjected to additional manipulations.

2.3. Bead implantation experiments

AG1-X2 beads (Bio-Rad) were soaked for 20 min at room temperature in *all-trans* RA (5 μg/μl; Sigma) in DMSO. The beads were implanted in ovo into the mesoderm of right chick wing buds at the desired position and for the desired time. Beads soaked in DMSO served as control and they did not show any effect on gene expression.

2.4. In situ hybridization and imaging

In situ hybridization was performed as previously described [26], using antisense digoxigenin-labeled RNA probes: *shh* [27] and *hairy2* [28]. Limbs processed for in situ hybridization were photographed using an Olympus DP71 digital camera coupled to an Olympus SZX16 stereomicroscope.

2.5. Immunoblot analysis

RA-beads were implanted in the forelimb AND and incubated either for 1 h or 6 h time period. Experimental and contralateral control limbs were surgically ablated and divided along the proximal–distal axis and the untreated halves were discarded. Portions from at least twelve different limbs were collected and total protein was extracted from each limb pool. 10 μg and 50 μg of protein extracts were loaded per well on a 12% and 7% SDS–PAGE minigel, respectively, for Erk/MAPK, Akt/PI3K and Gli3 western-blots. Blots were probed with p44/42 MAPK, phosphor-p44/42 MAPK, Akt, phosphor-Akt (Cell signaling) and Gli3 polyclonal [21] primary antibodies. β-tubulin (Abcam) antibody was used as loading control. Blots were incubated with anti-rabbit secondary antibody (Abcam), developed with Super Signal West Femto Substrate (Pierce Biotechnology, Inc., Rockford, IL) and exposed in Chemidoc (Bio-Rad). Bands were quantified using Quantity one (Bio-Rad) and normalized with loading control.

3. Results and discussion

The chick distal limb mesenchyme displays distinct *hairy2* expression patterns between stages HH20–28, cyclically recapitulated in the sub-ridge mesenchyme (DCD, distal cyclic domain) every 6 h [3,29]. *hairy2* transcripts are always detectable in the central mesenchymal limb domain containing the muscle precursors (CPD, central positive domain) and in the posterior limb encompassing the ZPA (PPD, posterior positive domain) (Fig. 1A). In contrast, *hairy2* expression is absent in both the anterior and posterior limb mesenchyme (AND, anterior negative domain; PND, posterior negative domain) (Fig. 1A). Understanding the regulatory mechanisms of limb *hairy2* expression is very important since it is proposed to provide temporal information to chondrogenic precursor cells before they enter their differentiation program [3]. Taking into account the previous knowledge of RA regulatory effects on EC gene expression during somitogenesis

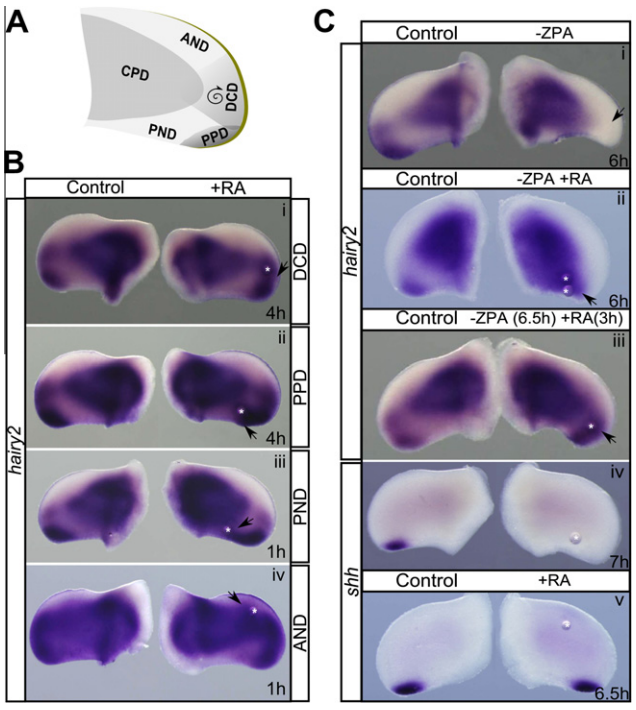

Fig. 1. RA signaling induces limb *hairy2* expression in a SHH-independent manner. (A) Schematic representation of *hairy2* expression domains in HH24 chick forelimb. *hairy2* is always observed in the central positive domain (CPD) and in the posterior positive domain (PPD). *hairy2* is absent from both anterior negative (AND) and posterior negative (PND) domains and is cyclically expressed in the distal cyclic domain (DCD), with a 6 h periodicity. (B) *hairy2* is upregulated by implantation of RA-beads in all limb domains. (C) Distal *hairy2* expression is lost upon ZPA ablation (i) and rescued by RA-bead implantation, either immediately (ii) or after 6.5 h of tissue manipulation (iii). RA-induced *hairy2* expression is not mediated by *shh* induction (iv, v). Dorsal view; anterior to the top. *RA-Beads. Arrows indicate altered *hairy2* expression.