Mechanisms of Development 153 (2018) 1–9

Contents lists available at ScienceDirect

Mechanisms of Development

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

Exogenous Vitamin D signaling alters skeletal patterning, differentiation, and tissue integration during limb regeneration in the axolotl

Warren A. Vieira, Kaylee M. Wells, Roni Milgrom, Catherine D. McCusker*

Department of Biology, University of Massachusetts Boston, Boston, MA, USA

ARTICLE INFO

Keywords:
Integration
Regeneration
Patterning
Ambystoma mexicanum
Vitamin D signaling
Skeleton

ABSTRACT

Urodele amphibians such as the axolotl regenerate complete limbs as adults, and understanding how the "blueprint", or pattern, of the regenerate is established and manipulated are areas of intense interest. Nutrient signaling plays an important role in pattern formation during regeneration. Retinoic acid signaling is the most characterized pathway during this process. Exogenous retinoic acid (RA) reprograms the pattern information in regenerating cells to a more posterior, ventral, and proximal identity. Vitamin D signaling shares several molecular similarities with RA and has been shown to alter pattern formation during zebrafish pectoral fin regeneration. To determine if exogenous Vitamin D signaling is capable of reprograming pattern in the axolotl limb blastema, we treated regenerating limbs with a potent Vitamin D agonist. Under the studied conditions, exogenous Vitamin D did not act in a manner similar to RA and failed to proximalize the pattern of the resulting regenerates. The Vitamin D treatment did result in several skeletal defects during regeneration, including carpal fusions along the A/P axis; failure to integrate the newly regenerated tissue with the existing tissue, formation of ectopic nodules of cartilage at the site of amputation, and altered bone morphology in uninjured skeletal tissue.

1. Introduction

Nutrient signaling plays key roles in the patterning and differentiation of cells during embryogenesis and regeneration. One of most well-characterized nutrient pathways involved in these processes is retinoic acid (RA) signaling, which has been extensively reviewed elsewhere (Cunningham and Duester, 2015; Rhinn and Dollé, 2012). In the absence of RA, Retinoic Acid Receptors (RAR) form heterodimers with retinoid X receptor (RXR) and together this complex represses transcription of RA-responsive genes. The addition of RA leads to conformational changes in RAR, causing the heterodimer complex to either activate or further repress transcription of its associated genes. RA signaling is critical in the establishment of positional information during primary and secondary field formation in developing vertebrate embryos (as reviewed by (Cunningham and Duester, 2015)), and in regenerating amphibian limbs (Rincón and Scadding, 2002; Nguyen-Yamamoto et al., 2010; Monaghan and Maden, 2013). Additionally, exogenous RA reprograms the positional information within regenerating limb tissue resulting in proximalization and posteriorization of the pattern of the regenerate (Maden, 1996; Mccusker et al., 2014; Bryant and Gardiner, 1992).

The co-receptor to RAR, RXR, binds to other co-receptors that are involved with nutrient signaling as well. One of these receptors is the Vitamin D receptor (VDR), a participant in receptor-mediated Vitamin D signaling (Kliewer et al., 1992). Similar to RAR-associated signaling, VDR is believed to act together with RXR as a transcriptional co-repressor until it is bound by a metabolically active form of Vitamin D. This binding induces a conformational change in the nuclear receptor which results in transcriptional activation (Ohyama et al., 1994; Ohyama et al., 1996) or further suppression (Alroy et al., 1995) of the associated genes.

The involvement of Vitamin D signaling in development and regeneration has not been comprehensively studied. Vitamin D signaling contributes to normal limb formation as components of the vitamin D signaling pathway are expressed in developing limb buds (Liu et al., 2008) and alterations of this pathway negatively effects long bone formation in VDR-knockout mice (Zheng et al., 2004). In the context of regeneration, exogenous Vitamin D, like RA signaling, has been observed to affect pattern formation. The normal anterior/posterior (A/P) pattern in the zebrafish pectoral fin depends on a graded expression of Vitamin D signaling along this axis; however, overexpression of Hand2, an upstream inhibitor of Vitamin D signaling, disrupted pattern formation during pectoral fin regeneration by shortening all the fin rays along the A/P axis (Nachtrab et al., 2013), suggestive of axis posterization. Exogenous Vitamin D treatment, in conjunction with RA signaling, also disrupts A/P patterning in the regenerating axolotl limb

* Corresponding author.
E-mail address: Catherine.McCusker@umb.edu (C.D. McCusker).

https://doi.org/10.1016/j.mod.2018.08.004
Received 17 December 2017; Received in revised form 22 March 2018; Accepted 6 August 2018
Available online 07 August 2018
0925-4773/

(Washabaugh and Tsonis, 1995); although, the effect of exogenous Vitamin D signaling (alone) on pattern formation in this system has not been fully characterized. Thus, a potential role for vitamin D signaling in positional reprogramming during regeneration exists.

Exogenous retinoic acid alters positional identity during limb regeneration in the axolotl in a reproducible manner whereby the regenerating cells, known as blastema cells, assume a more proximalized, posteriorized and ventralized identity (Mccusker et al., 2014). RA signaling is known to slow the cell cycle (Chen and Ross, 2004) and it has been hypothesized that the length of the cell cycle plays a key role in the patterning of embryos and regenerating structures (Bryant and Gardiner, 2016). Similar to RA signaling, Vitamin D regulates transcription by a RXR-associated heterodimer complex and this nutrient molecule also slows the cell cycle (Akutsu et al., 2001). Therefore, we hypothesize that exogenous Vitamin D reprograms positional identity during regeneration in a similar manner as RA.

To test this hypothesis, we sought to better characterize the effect of exogenous Vitamin D signaling on anterior/posterior patterning by treating axolotl with blastemas located on different locations of the limb axis with a potent Vitamin D agonist. Unlike RA, exogenous Vitamin D did not cause the proximalization of the pattern of the regenerates. However, the treatment did result in several skeletal defects during regeneration, including carpal fusions along the A/P axis; the failure to integrate the newly regenerated tissue with the existing tissue; and the formation of ectopic nodules of cartilage. In addition, growth plate morphology and skeletal homeostasis was negatively affected in uninjured, treated tissue.

2. Results

2.1. Exogenous Vitamin D signaling alters the A/P pattern of the carpals

Nachtrab et al. discovered that an A/P gradient of Vitamin D signaling played a role in A/P patterning in regenerating zebrafish fins (Nachtrab et al., 2013). We speculated that the role of Vitamin D signaling in regeneration could be conserved in amphibians. We used RT-PCR to analyze the expression of the *vitamin D receptor* (*VDR*) and downstream transcriptional target of VDR, *cyp24a1* (Pike et al., 2014; Hahn et al., 1994), in differently staged regenerating axolotl limbs - early to mid-bud stage, late bud stage, and tiny limb stage, compared with mature (uninjured limb tissue). We observed that *VDR* is expressed both in regenerating and mature tissues (Fig. 1A), and the expression of *cyp24a1* is significantly increased in early to mid-bud staged regenerating blastema tissue (Fig. 1B). Thus, VDR is present, and apparently activated during normal limb regeneration.

To test whether exogenous Vitamin D signaling alters A/P patterning in the axolotl limb regenerate, we performed a gain-of-function study. Axolotl limbs were amputated either through the humorous (proximal amputation) or between the distal radius and ulna and proximal carpals (distal amputation); allowed to develop mid-bud staged blastema, and then treated with the Vitamin D analogue Doxercalciferol. Blastemas were treated at the mid-bud stage, because we had previously shown that treatment with RA had the greatest effect on the pattern of the regenerate when applied at this stage (Mccusker et al., 2014). The Vitamin D analogue Doxercalciferol has proven biological activity in mammalian systems (Kubodera, 2009; Nguyen-Yamamoto et al., 2010), and here we show that *cyp24a1* expression is significantly increased in treated blastema tissue relative to untreated blastema tissue, indicating that this analogue is also active in amphibian systems (Fig. 1C). We also observed that within two weeks, the treated animals developed what looked like deposits in the skin (Fig. 1D). We suspect that these deposits may be similar to those observed in mammals that are afflicted with Calcinosis cutis, which are mineral deposits in the skin that have been associated with an excess of Vitamin D (Buffenstein et al., 1995). Together these observations indicate that Doxercalciferol treatment activates Vitamin D signaling in the axolotl.

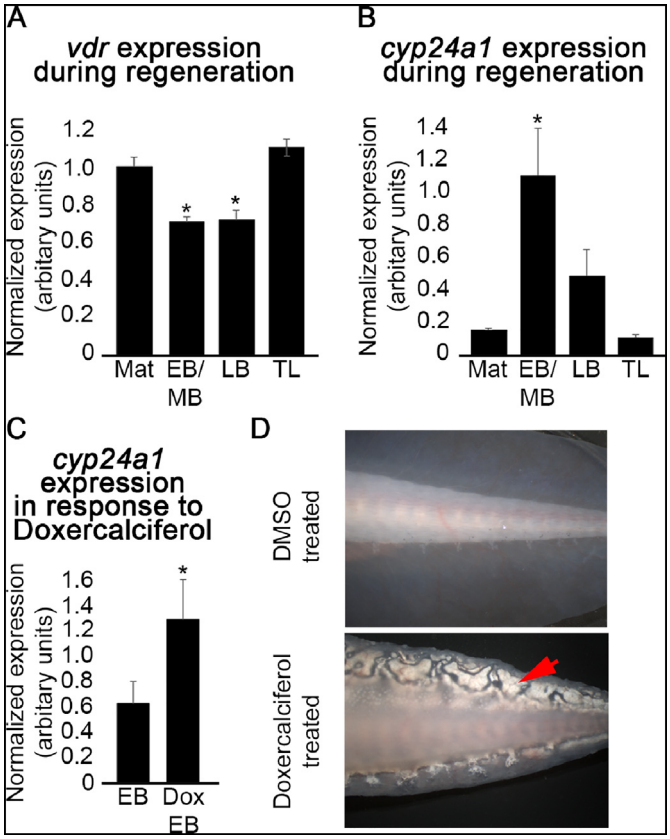

Fig. 1. Vitamin D signaling is activated during regeneration and Doxercalciferol further enhances this signaling in the axolotl. (A) Semi-quantitative analysis by RT-PCR of (A) *vdr* and (B) *cyp24a1* gene expression during the course of regeneration, relative to the expression of the house keeping gene, *ef1α*. Mat - mature, uninjured limb tissue; EB/MB - early to mid-bud stage blastema tissue; LB – late bud stage blastema tissue; TL – tiny limb tissue; $p < 0.05$ (one way Anova; Tukey post hoc test relative to mature, uninjured tissue; $N = 3$). (C) Semi-quantitative analysis by RT-PCR of *cyp24a1* revealed a significant increase in the expression of this gene in early bud stage blastema tissue in response to Doxercalciferol treatment (10 mg/kg; Dox EB); $p < 0.05$ (*t*-test, $N = 3$). Error bars represent standard error of the mean. (D) Representative images of vehicle (top) and Doxercalciferol treated (10 mg/kg, bottom) axolotl tail flaps. Doxcercaliferol treatment resulted in aberrant structures (red arrow) in the tail flap. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We observed several different carpal patterning phenotypes within the treated regenerates (Fig. 2). Limbs were scored by the location of the fusion along the A/P axis (Fig. 2, Table 1). We observed that the frequency and type of carpal fusions greatly depended on whether the treated blastema was located in a proximal or distal limb location (0–75% for proximal amputations versus 70–100% in distal amputations). Carpal fusions have previously been found to occur at high frequency during normal (untreated) regeneration in response to distal amputation (Rincón and Scadding, 2002). In the current study, fusions were more regularly observed in distally located regenerates regardless of Doxercalciferol treatment, as expected. However, more fusions were observed in the regenerates with the highest dosage of Doxercalciferol (Fig. 2B). Additionally, both low and high doses of Doxercalciferol resulted in an increase in the number of fusions in regenerates from proximal located amputations (Fig. 2C). Interestingly, the highest dose of Doxercalciferol resulted in anterior-located fusions in over 50% of the regenerates.

In zebrafish fins, the over expression of Hand2 (a posterior marker) increases the expression of Cyp24a1, a Vitamin D inactivating enzyme,