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Chemical activation of RARβ induces post-embryonically bilateral limb duplication during *Xenopus* limb regeneration

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The anuran amphibian *Xenopus laevis* can regenerate its limbs for a limited time during the larval stage, while limbs are still developing. Using this regeneration model, we evaluated the proximo-distal blastema cell identity when endogenous retinoids were increased by CYP26 inhibition or when RAR-specific agonists altered RA signaling. Simultaneous proximo-distal and antero-posterior limb duplications were generated, and the RAR-specific agonist can modify blastema identity after amputation, because chemical activation of RARβ produced bilateral hindlimb duplications that resulted in a drastic duplication phenotype of regenerating limbs.

The ability to regenerate lost or damaged body parts is a widespread phenomenon in invertebrates but is exceptional in vertebrates. Only animals capable of asexual reproduction by budding are able to re-establish post-embryonically a new set of axes[1]. Regeneration implies not only the replacement of the lost structures, but also the integration of positional information that specifies the correct spatial patterns. During development, the appendicular skeleton is positioned at specific antero-posterior places with respect to the main body axis and maintains left-right asymmetry with equal size proportion. The mechanisms that establish internal organ asymmetries versus external bilateral symmetry are mostly unknown[2].

Proximo-distal (P-D) and antero-posterior (A-P) limb duplications in regenerating amphibian limbs were first obtained by Niazi and Saxena in 1978 and Maden in 1982 by using exogenously applied retinol (vitamin A) or derived retinoids[3,4]. They revealed for the first time a role of vitamin A derivates in the change of positional information and proximalization of blastema, and showed that the responses are time, stage and concentration-dependent. Retinoic acid (RA) is a metabolic product of retinol that binds and activates nuclear retinoic acid receptors (RARs), which regulate the transcription of specific genes. Retinol dehydrogenases metabolize the retinol obtained from the diet as carotenoids and retinyl esters to retinaldehyde, which is then metabolized by retinaldehyde dehydrogenases to RA. Once RA binds and activates heterodimers formed by RARs and RXRs, it is catabolized in the cytoplasm by CYP26 enzymes yielding polar metabolites that are inactive or less active than RA[5]. Therefore, the balance between its synthesis and its degradation controls intracellular levels of active RA. Studies in *Xenopus laevis* indicate that at-RA and 9-*cis*-RA are not present at detectable levels in embryos[6], while 4-oxo-RA is detected at high levels, binds avidly to and activates RARβ receptor and has strong teratogenic activity[7].

The role of RA on blastema proximalization and regeneration has been studied mainly by exogenous administration of retinoids[3,4]. However, the effect of excess endogenous retinoids on limb regeneration is unknown. Based on this, in the present work CYP26 enzymatic activity was inhibited in amputated limbs by using a pharmacological CYP26 inhibitor. Results show that this inhibitor was able to induce simultaneous P-D and A-P limb duplications during limb regeneration of *Xenopus* larvae, while in developing limbs it promoted apoptosis leading to severe hypomorphic phenotypes. In addition, to determine which retinoic acid receptors (RARs) are involved in this process, specific chemical agonists of RARs were assayed. Using nano-molar concentrations of a RARβ2 agonist induced the most striking A-P and P-D duplications described to date.

Table 1 | Phenotypes obtained after treatment with CYP26 inhibitor and RARβ agonist

Treatment	Normal development	Malformed limbs with no duplications	A-P duplication	Incomplete P-D/ A-P duplication[1]	Complete P-D/ A-P duplication[2]	B-L duplication[3]
DMSO	16/16	0/16	0/16	0/16	0/16	0/16
R115866	0/0	1/43	2/43	21/43	19/43	0/43
AC55649	0/0	0/29	0/29	0/29	1/29	28/29

[1]Duplications of stylopod, zeugopod and autopod elements. No pelvic bone formation.
[2]New pelvic girdle with a well-formed glenoid cavity, duplications of stylopod, zeugopod and autopod elements.
[3]A pair of complete legs emerging from a complete and symmetric new pelvis.

Results

Inhibition of CYP26 induces P-D and A-P duplications. To explore the effect of increased endogenous retinoids available to RARs we applied R115866 a specific and strong CYP26 enzyme inhibitor[8]. Unilateral amputations were performed leaving the proximal stump spanning the first third of E51-51.5 hindlimbs[9]. After an interval of 8 h for recovery, the epithelized wounds were exposed to 2 μM of R115866 during 72 h. Under this condition a frequency of 49% was obtained of what we named incomplete simultaneous duplications, that is, P-D and A-P duplications within the same regenerate without the formation of any distinguishable new pelvic bone (Table 1). This kind of duplication is basically equal to that reported in regenerating limb buds of *Xenopus laevis* treated with vitamin A[10]. The other 44% were complete simultaneous duplications; those including the formation of a new pelvic girdle with a well-formed glenoid cavity, articulated with the head-bone of the new stylopod. In all cases of simultaneous duplications a mirror-duplicated autopod was formed distally (Fig. 1A–C). The development of the contralateral uncut hindlimbs and both forelimbs were also affected showing hypomorphic malformations. Interestingly, despite displaying severely shortened or even the absence of autopod bones, they developed what seem to be interdigital membranes (Fig. 1D–F).

Based on previous results, the effect of CYP26 enzyme inhibitor was evaluated in another important limb regeneration animal model, the urodele amphibian *Ambystoma mexicanum*. More prolonged treatments with 2 μM of R115866 over 5 days were necessary to induce complete P-D duplications in juvenile axolotls, which included the formation of a secondary shoulder girdle (data not shown). However, although the concentrations of R115866 were either reduced or increased ten-fold or the treatment prolonged, A-P or simultaneous duplications were never obtained. It is important to mention that preliminary assays with the imidazole derivate liarozole, a first generation CYP26 inhibitor, at doses as high as 250 μM, failed to generate duplications in *Xenopus* larvae and juvenile axolotls.

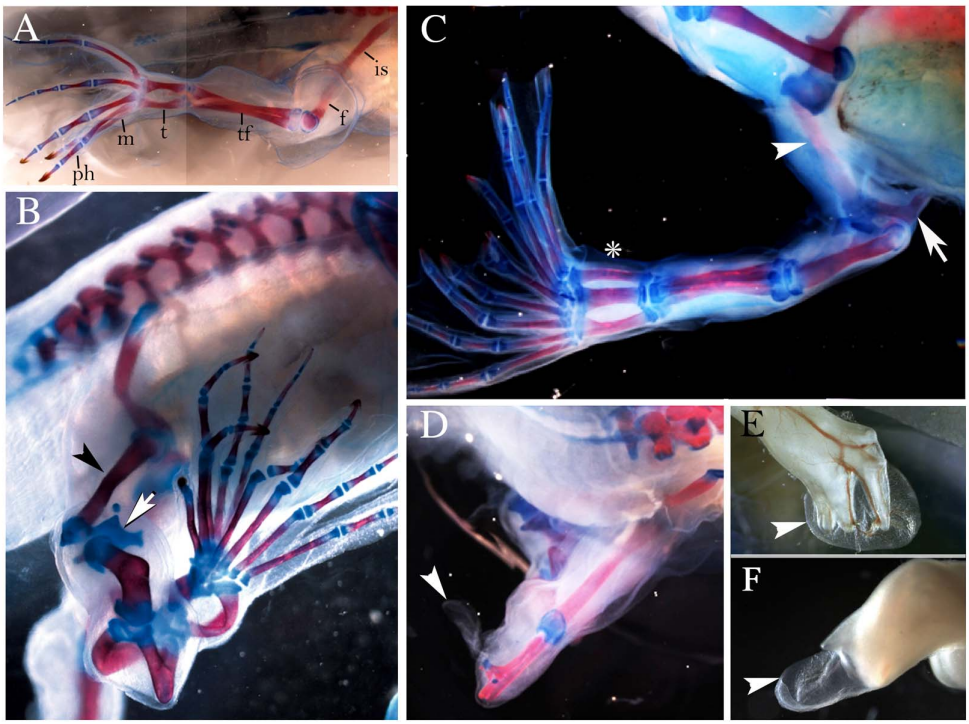

Figure 1 | Duplication of hindlimb regenerates treated with CYP26 inhibitor. (A) Lateral view of Alcian blue and alizarin red-stained control hindlimb 30 days after amputation. Anterior is down. (B) Stage 62 metamorphic larva showing a complete P-D and A-P duplication. White arrow points to the secondary right pelvic bone from which emerges a twisted limb with mirror-image autopod duplication and taumelic long bones. Black arrowhead points to the original femur bone. (C) Other larvae showing complete duplicated limb with an autopod exhibiting A-P duplication included the tarsal bones (asterisk). Arrow points to the iliac shaft of the secondary pelvis and arrowhead points to the original femur bone. (D) Contra-lateral ectromelic hindlimbs showing what seem to be interdigital membranes (white arrowhead). (E, F) Hindlimbs of live froglets showing interdigital membranes. White arrowheads points to the presumptive interdigital membrane attached to distal region of an ectromelic hindlimb. is, iliac shaft., f, femur., tf, tibiofibula., t, tarsals., m, metatarsals., ph, phalanges.

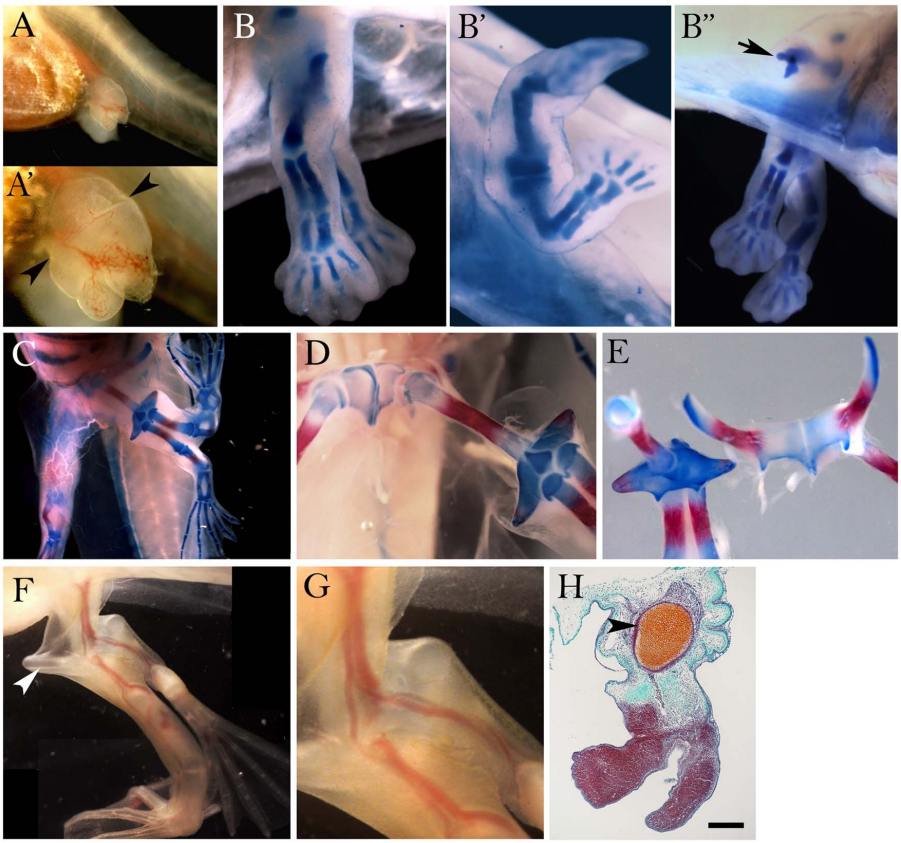

Figure 2 | Bilateral limb duplication in regenerates treated with the RARβ agonist AC55649. (A) Ventro-lateral view of left hindlimb nine days post amputation treated AC55649. (A′) Closer view of the same hindlimb showed in (A). Black arrowheads point to the amputation plane. (B, B′, B″) Lateral, ventral and contralateral view respectively of B-L duplicated at 16 days post-amputation and stained with Alcian blue. Black arrow points to the hypomorphic contralateral hindlimb. (C) Ventro-lateral view of B-L duplicated hindlimb of stage 62 Alcian blue and alizarin red-stained larva. (D) Closer view of the same duplicate partially dissected. (E) Dorsal view of dissected duplicate; compare the primary pelvis (right) and secondary pelvis (left) dorsally attached to the original femur bone. (F) Posterior view of limbs showing the bifurcated femoral blood vessel. Arrowhead points to the well-developed secondary iliac shafts. (G) Closer view of the bifurcated blood vessel. (H) Histology of duplicates at twelve days after amputation stained with safranine-O. Arrowhead points to the femur bone from which duplicated limbs emerge. No notochord cells are observed. Scale bar 200 μm.

Over-activation of RARβ induces bilateral hindlimb duplication. The effects of the specific commercially available RAR agonists such as adapalene, AC55649, AM-580 and CD437 were then explored. The most consistent and striking effects were obtained with AC55649, a strong RARβ2 receptor agonist that displays 100-fold selectivity over other retinoid receptors[11]. Under the same experimental conditions as described above we determined that exposing regenerates for 18 h to just 100 nM of AC-55649 was sufficient to induce what we named bilateral limb (B-L) duplications. With this concentration, approximately 65% (n = 45) of larvae survived the treatment and practically all began to form a pair of bilaterally symmetric limb buds (Table 1; Fig. 2A, A′). Skeletal staining showed a pair of legs emerging from a complete and symmetric new pelvis, which consistently maintained the same position regarding the A-P, P-D and dorso-ventral (D-P) axes of the femur bone from which it emerged (Fig. 2B, B′, B″). The pelvic bones were well formed, symmetric and emerged dorsally attached to the distal stump of the original femur (Fig. 2C, D, E). The original femoral blood vessel that runs down the ventral side of the thigh divided and continued toward the ventral side of both new hindlimbs (Fig. 2F, G). In *Xenopus* the opening of the cloaca is located between the thighs under the pelvis and in front of the ventral fin fold; in the present experiments, no formation of a cloaca or fin-fold tissue was observed in B-L duplication. Furthermore, it has been reported that near of the midline, notochord cells are sources of molecules that are involved in tissue patterning, and that amputated tails of frogs treated with retinoids develop an overgrowth of notochord cells at the base of the limb duplicates[12]; such overgrowth of notochord cells was not observed in this study (Fig. 2H). Contralateral developing hindlimbs and forelimbs also showed severe hypomorphic malformations (data not shown).

Interestingly, treatments with R115866 and AC-55649 provoked death of larvae at the onset of metamorphosis at around stage 62, a stage when the levels of T3 and T4 thyroid hormones peak, meanwhile all control larvae were metamorphosed.

Discussion

The role of endogenous RA in limb regeneration has been studied in conditions of RA deficiency using alcohol and aldehyde dehydrogenase enzyme inhibitors; these treatments impair limb regeneration[13,14]. In contrast, in the present study we uncovered the effects of excess endogenous retinoids by using a CYP26 enzyme inhibitor, which consists in simultaneous P-D and A-P limb duplications and complete simultaneous duplications at higher frequency than in experiments applying exogenous vitamin A (see Table 1)[10,15]. With this inhibitor, an excess of endogenous retinoids was possibly generated in tissues with active synthesis-degradation of RA other than limbs. In this case, a systemic increase of endogenous retinoids may affect the blastema. However, it is known that blastema produces RA[16], up-regulates the protein CRABP2, expresses *Cyp26b* and *Raldh2* genes[17] and RA signaling is active in the apical wound epithelium[18]. Moreover, the expression and up-regulation of *Rar*β2 was recently

reported in the regenerating limb and tail of *N. viridescens,* and their function in limb regeneration was demonstrated by using the specific RARβ2 inhibitor LE135[19].

Because the loss of function of CYP26 by R115866 preferentially avoids at-RA catabolism[8], it is reasonable to speculate that levels of free at-RA may increase within the blastema. On this basis, at-RA may be an endogenous ligand during *Xenopus* limb regeneration. However, though *in vitro* experiments show that 4-oxo-RA is one of the catabolic products of CYP26[20], *in vivo* it is detected at high levels in the *Xenopus* embryo[6]. It also activates RARβ and is known to be as potent as RA[7]. Therefore, 4 oxo-RA should not be excluded as the retinoid that may activate RARβ2 *in vivo*, particularly considering the existence of several CYP26 and P450 enzymes. In addition, the possibility that RA could be transformed into other as yet unexplored active retinoids should not be ruled out.

Based on the literature and on present results, the main role of CYP26 during limb development could be to protect against the teratogenic effect of RA[21,22], but in the regeneration process it would be to protect the blastema from re-specification and change of positional information. On the basis of gene expression pattern of *Raldh2, Cyp26b* and *Crabp2* during *Xenopus* limb regeneration, it has been suggested that a dynamic and concentric RA gradient is formed during this process[17]. *Cyp26b* is expressed in the early blastema at 3 days of regeneration, in principle a time window sensitive to CYP26 enzyme inhibitors. We suggest that in our experiments the treatment with R115866 would be able to break that RA gradient, giving rise to re-specification of E51-51.5 blastema during its formation. Thus it would be possible to consider that low doses of the inhibitor, or treatment at later stages may result in mild proximalized phenotypes.

A remarkable result of the present work were the B-L duplications obtained after chemical activation with RARβ, no previously reported with any retinoid assayed, representing a *bona fide* homeotic transformation: one secondary pelvic girdle developed bearing two symmetric legs that maintained a constant position with regard to the stump from which they emerged (see Fig. 3). Several reports have shown the effects of different regimes of exogenously applied vitamin A and derived retinoids in regenerating limbs and tails in developing embryos[10,23,24]. From our point of view, the two most striking phenotypes obtained are the homeotic transformation of amputated tails into limbs in two anuran species[12,25], and the RA homeotic transformation induced in mouse embryos resulting in multiple ectopic hindlimbs and lower-body duplication[26]. The experimental design proposed here suggests that blastema must change its coordinate system and re-specify the axes at a very early stage post-amputation, within a narrow time window of less than 24 h. This early specification[27] implies that the instructive/permissive action of RA must occur before a distinguishable blastema forms. Fate maps and transplantation experiments have shown that the blastema specifies its P-D axis and is already divided into growth zones as early as 3 days post amputation[28]. Also, the blastema is more sensitive to retinoids during early blastema formation, when cells actively proliferate[29].

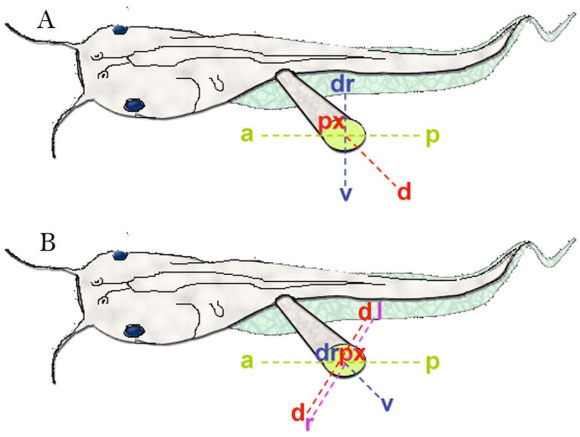

Figure 3 | Schematized comparative of axes in normal regeneration and B-L duplication. (A) Normally the blastema places the proximo-distal axis accordingly with the level of amputation (red dotted line), maintaining the antero-posterior and dorso-ventral axes unaltered. (B) However, the blastema is competent to form bilateral limbs by reestablishing the most proximal position of hindlimbs (*i.e.* the dorsal part of a new pelvis). Hence in this blastema a new dorso-ventral axis forms (blue dotted line) and the left-right axis (cyan dotted line) emerges from the crossroad with the antero-posterior axis (green dotted line). a, anterior; p, posterior; dr, dorsal; v, ventral; px, proximal; d, distal; l, left; r, right.

We speculate that B-L limb duplication observed in this study arises because a new dorso-ventral axis in the blastema forms and as a consequence a new left-right axis emerges in the regenerating limb (Fig. 2E, Fig. 3). Importantly, this dorso-ventral axis maintains a constant orientation with respect the main body axis as shown in Fig. 3. This constant position is not observed in the case of incomplete duplicates that show a heterogeneous orientation with respect the main body axis. It is known that RARβ is expressed and up regulated in blastema after amputation of the limb in adult newt[19]. It is possible that over activation of RARβ may intensify the phenomenon of proximalization by RA on limb regeneration[4].

RA has been proposed to act as a morphogen in development and regeneration, specifically as an instructive molecule. It has also been stated that RA may act as a permissive molecule[21,30]. Regardless of the mechanism of action, RA has been demonstrated as a necessary signaling molecule in the generation and maintenance of blastema in regenerating zebrafish fins, and as the diffusive signal arising from the trunk that together with FGF establishes the identity of P-D axis in the developing limb[31,32]. Investigating the dynamics between RA and FGFs signaling[33,34] with the use of transcriptome strategies could contribute to our understanding of regulatory hierarchies and elusive unidentified molecules controlling axial patterning. Also of interest is whether the activation of RARβ in blastema regulates a mechanism similar to that occurring in bilateral symmetry establishment during somitogenesis by RA[35]. Based on the B-L duplication, we propose that the mirror-limb duplications generated in several animal models may also be interpreted as a partial bilateral duplication that will be evident as more and more proximal bones become duplicated.

Methods

Larvae hindlimb amputation. *Xenopus laevis* were injected with chorionic gonadotropin to induce spawning. Larvae were fed with powdered staple fish food. Stage 51–51.5 larvae were anesthetized by immersion in cool water and hindlimbs were unilaterally amputated using tungsten needles and leaving intact the proximal third. Contralateral limbs were left as developing internal control. Five cm-long juvenile axolotls were maintained in aquaria and fed daily with *Artemia salina.* Forelimb amputations were performed at the mid-stylopod with nail clippers and treated with R115866 as described below. This research protocol was reviewed and approved by the Institutional Review Board for the Care and Use of Laboratory Animals of Instituto de Investigaciones Biomedicas, UNAM.

CYP-26 inhibitor and RARβ agonist treatment. Amputated larvae were left to recover for 8 h. They were then transferred in groups of 3 to glass containers with 100 ml of aquarium water and treated with R115866 (obtained from Janssen Research Foundation) from a 50 mM stock solution in DMSO, or with AC-55649 (BIOMOL) from a 50 mM stock solution in DMSO, or added with vehicle control (v/v DMSO). DMSO treated larvae regenerate and develop normal limbs in all cases assessed (16/16). Containers were covered with aluminum foil to protect them from light. Treatment with the CYP26 inhibitor lasted for 72 h, while with the RARβ2 agonist treatment it lasted only for 18 h. Following treatment, regeneration and development was allowed to continue until early metamorphosis, around stage 62[9], when all treated larvae died.

Skeletal staining, histology and cell death detection. Pre-metamorphic larvae were euthanized with an overdose of tricaine, then fixed and dehydrated overnight (O/N) in 100% ethanol. Thereafter they were incubated in acetone for 24 h and stained for 3 h at 37°C and then O/N at room temperature in Alcian Blue and Alizarin red in 70%

ethanol with 5% acetic acid. Finally, they were rinsed in tap water before clearing in 1% KOH and 20% glycerol for 24 h and then placed in graded glycerol.

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Author contributions

Study conception and design: R.C., J.C.-M. Acquisition of data: R.C. Analysis and interpretation of data: R.C., J.C.-M. Wrote the paper: R.C., J.C.-M.

Additional information

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