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Caractérisation de gènes ostéogéniques chez l'axolotl

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Université de Montréal
Faculté des études supérieures

Ce mémoire intitulé :
Caractérisation de gènes ostéogéniques chez l'axolotl

Présenté par :
Cara Hutchison

À été évalué par un jury composé des personnes suivantes :

Président-rapporteur : Guy Boileau
Directeur de recherche : Stéphane Roy
Membre du jury : Antonio Nanci

Résumé

Les amphibiens urodèles (e.g., axolotl) sont les seuls vertébrés à posséder la capacité exceptionnelle de régénérer parfaitement, tout au long de leur vie, plusieurs parties de leur corps. Parmi les structures complexes, le membre constitue un modèle de choix pour étudier la régénération de l'os. Pour débuter notre étude, nous avons choisi d'étudier l'expression de trois gènes chez l'axolotl impliqués dans l'ostéogénèse; *Cbfa-1, PTHrP* et *Sox-9*. Nos résultats démontrent que ces gènes sont spécifiques pour les éléments squelettiques durant le développement et durant la régénération. De plus, leurs patrons d'expression suggèrent que le mécanisme d'ostéogénèse régulé par *Cbfa-1* et *Sox-9* est conservé durant les stades avancés de ces deux processus. Au contraire, l'expression de *PTHrP* n'était pas conservée, laissant croire qu'il est aussi impliqué dans d'autres processus physiologiques.

Étant capable de régénérer complètement un membre, nous avons questionné si l'axolotl est capable de régénérer une fracture. Nos études démontrent que l'axolotl, comme les autres vertébrés, répare ses fractures jointes, mais est incapable de guérir des fractures non-jointes de dimension critique.

Curieusement, *Sox-9* joue aussi un rôle dans le développement de la crête neurale; nous avons ainsi caractérisé l'expression de *Sox-9* et *Sox-10* (aussi impliqué dans le développement de la crête neurale) durant l'embryogénèse. Notre étude chez l'axolotl confirme que ces protéines sont exprimés et régulés durant le développement embryonnaire et que ces gènes sont exprimés principalement au niveau de la crête neurale et de ses dérivés, tout comme chez les autres vertébrés.

MOTS CLÉS: AXOLOTL, URODÈLE, RÉGÉNÉRATION, OSTÉOGENÈSE, PTHrP, CBFA-1, SOX-9/10, COLLAGÈNE II, FRACTURES, DÉVELOPPEMENT

Abstract

Among vertebrates, urodele amphibians (e.g., axolotl) have the unique ability to perfectly regenerate complex body parts after amputation throughout their life. The limb provides an ideal structure to study skeletogenesis during axolotl regeneration. We report the cloning and characterization of three axolotl genes involved in osteogenesis; *Cbfa-1, PTHrP, Sox-9*. Cartilage staining results confirms that all three gene expression patterns are specific for skeletal elements during limb development and regeneration. Experimental data collected thus far supports the concept that the mechanisms controlling growth and pattern formation during late limb regeneration are a recapitulation of those in developing limbs. Our expression results suggest a possible conserved mechanism in osteogenesis during limb development and late limb regeneration for *Cbfa-1* and *Sox-9* whereas *PTHrP* may have other roles.

With the axolotl's ability to regenerate a perfect limb, we questioned whether the axolotl uses the regeneration process to heal bone fractures. We show that while the axolotl is able to heal a non-stabilized union fracture, like other vertebrates; the axolotl is incapable of healing a bone gap of critical dimension.

Interestingly, *Sox-9* is also involved in neural crest development; we therefore characterized *Sox-9* and *Sox-10* (an important regulator of neural crest cells) during embryogenesis. Western blot results show that *Sox-9* and *Sox-10* proteins are expressed and regulated during embryonic development. Moreover, both genes are expressed mainly in the neural crest and its derivatives, demonstrating comparable expression with other vertebrates.

KEYWORDS: AXOLOTL, URODELE, LIMB REGENERATION, OSTEOGENESIS, PTHrP, CBFA-1, SOX-9/10, COLLAGEN II, FRACTURE HEALING, DEVELOPMENT

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Liste des sigles et abréviations

%	Pourcentage
®	Registered trademark
™	Trade Mark
µ	Micro (10^{-6})
µL	Microlitre
µM	Micromolaire
°C	Degré Celsius
A	Adénine
ADN	Acide désoxyribonucléique
AMPc	Adénosine monophosphate cyclique
ARN	Acide ribonucléique
BSA	Bovine Serum Albumin
C	Cytosine
c-	Carboxy-
CAE	Coiffe Apicale Ectodermique
ADNc	Acide désoxyribonucléique complémentaire
DEPC	Diéthyl pyrocarbonate
dNTP	Déoxynucléotidetriphosphate
EDTA	Éthylènediamine tétra-acétate
EGTA	Ethylen glycol N,N,N',N' tetraacetic acid
EST	Expressed Sequence Tag
et al.	et collaborateurs
EtOH	éthanol
G	Guanine
g	gramme
Hac	Acide acétique glaciale
kDA	KiloDalton
L	Litre
M	Molaire (mole/litre)
MEM	solution saline composée de MOPS, EGTA et $MgSO_4$

MEMFA	fixatif contenant une solution de sel MEM et du formaldéhyde
mg	milligramme
min	minutes
mL	millilitre
mM	millimolaire
pb	paires de bases
PBS	phosphate buffered saline
PCR	réaction de polymérisation en chaîne
RT	reverse transcription
SSC	solution Sodium et Sodium Citrate
sec	seconde
T	Thymine
TAE	tampon Tris-Acide Acétique et EDTA
v	volume
w	poids

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CHAPITRE 1

Introduction

1.1 La régénération : généralités

Le processus de régénération est défini comme étant l'habileté que possède un organisme, pleinement développé ou non, de remplacer ses tissus, organes ou membres par la croissance ou le remodelage de tissu somatique [1]. Ce phénomène a été constaté la première fois dans le dix-huitième siècle par Trembley. Ses travaux sur l'hydre ont démontré que des êtres du règne animal, une fois morcelés, pouvaient reconstruire des organismes complets à partir de simples fragments [2]. C'était Réaumur, qui a continué les recherches de Trembley en découvrant que l'écrevisse possédait aussi une capacité de régénérer ses appendices [3]. En 1768, la recherche en régénération a été révolutionnée lorsque Spallanzani confirme les capacités de régénération de plusieurs espèces tels les vers de terre, les mollusques et les amphibiens [4]. Depuis Spallanzani, de nombreux chercheurs ont concentré leurs études sur la régénération. Leurs découvertes ont permis de comprendre l'étendue véritable des espèces capables de régénérer ainsi que la complexité de ce phénomène.

1.1.1 Régénération morphallaxique versus épimorphique

La régénération morphallaxique et la régénération épimorphique sont les deux modes principaux par lequel un organisme se régénère. La régénération morphallaxique implique la réorganisation des tissus existants afin de reconstituer des parties corporelles manquantes; le tout en absence de prolifération cellulaire [1]. Parmi les espèces qui régénèrent par morphollaxie, l'hydre est un modèle exemplaire. Lorsqu'une hydre est sectionnée transversalement, la partie antérieure reforme un pied et la partie postérieure une tête, donnant deux entités complètes de petite taille. Par la suite, les hydre-filles grandiront jusqu'à ce qu'elles atteignent la taille de l'hydre d'origine [5, 6]. Même après multiples sectionnements transversaux, chacun des tronçons peut redonner un individu normal. De plus, une section longitudinale complète formera deux hémi-hydres qui régénéreront pour

reformer deux hydres complètes. Encore plus remarquable est la capacité de l'hydre à former des animaux complets à partir d'agrégats de cellules [7].

Contrairement à la régénération morphallaxique, la régénération épimorphique implique la division cellulaire. Ce mode de régénération se divise en deux sous-catégories. En ce qui concerne la première catégorie, elle se caractérise par la dédifférenciation cellulaire. Pour reformer la structure manquante, les cellules vont prendre un état dédifférencié prévalant lors du stade embryonnaire. Ces cellules non différenciées vont éventuellement se redifférencier pour former le tissu manquant. La régénération des appendices chez les amphibiens urodèles est un exemple classique de l'épimorphose impliquant la dédifférenciation cellulaire (voir section 1.2.1.1).

Le deuxième mode de régénération épimorphique ne dépend pas de la dédifférenciation des cellules mais plutôt de la migration des cellules-souches de réserve de l'organisme au site d'amputation. Elles vont ultérieurement se différencier pour reformer la structure manquante [1, 6]. C'est le cas des planaires. Lorsqu'il y a perte d'une partie du planaire, les néoblastes (des cellules souches totipotentes réparties dans tout son corps) avoisinants le site d'amputation migrent jusqu'à la blessure et se différencient selon les besoins cellulaires de l'organisme pour remplacer la partie manquante [1, 6, 8-12].

Il est important de préciser qu'il existe un mode de pseudo-régénération dite compensatoire. Elle diffère des deux autres types de régénération puisque lors de la régénération compensatoire hyperplasique, les cellules se divisent tout en conservant leurs fonctions normales. Les cellules en division produisent des cellules similaires à elles-mêmes et ne prennent pas un état dédifférencié. Ce phénomène peut être observé dans le foie chez les mammifères. Lorsqu'un lobe du foie est enlevé, la partie manquante ne repousse pas mais les lobes restants vont croître jusqu'au volume original afin de compenser la perte [13]. Dans ce cas, on dit qu'il y a croissance hyperplasique plutôt que la régénération [14].

1.1.2 Invertébrés versus vertébrés

Le pouvoir de régénération ne dépend pas de la taxonomie et ne suit pas la phylogenèse. Cependant, plusieurs invertébrés ont la capacité de régénérer toutes ou encore certaines parties de leurs corps, tandis que les vertébrés capables de régénérer sont peu nombreux et ont souvent une capacité régénératrice limitée à certains organes ou tissus (sauf quelques exceptions). Parmi les invertébrés capables de régénérer, l'étoile de mer, l'hydre et le vers planaire sont d'intérêt particulier puisqu'ils possèdent la capacité de régénérer leur corps au complet à partir d'un petit fragment [5, 6]. Ce phénomène est généralement associé avec le type de reproduction asexuée et un «turnover» continu des cellules souche indifférenciées qui formeront les principales parties du corps. Un autre aspect essentiel de la capacité régénératrice des invertébrés mentionnés ci-dessus est qu'elle est bidirectionnelle. La même surface de coupure peut régénérer soit une tête, soit une queue. Ni les insectes, ni les vertébrés ne démontrent cette régénération bidirectionnelle. Ils démontrent plutôt une régénération monodirectionnelle. Par exemple, nous pouvons observer la régénération monodirectionnelle lors de la régénération du membre. Une fois l'amputation effectuée, le membre de l'organisme amputé va régénérer mais la partie amputée ne donnera pas un autre organisme complet [15].

Certains insectes qui ne traversent pas un stade pupal (stade de développement où la larve s'entoure d'une enveloppe de cuticule durant sa métamorphose) possèdent aussi la capacité de régénérer [16]. Les insectes appartenant à la famille des hémimétaboles, tels que la sauterelle ou la blatte, se développent graduellement par une série de formes larvaires vers l'âge adulte et ont la capacité de régénérer une patte ou une antenne. Cependant, les holométaboles comme la drosophile, qui présentent un passage abrupt de la larve au stade adulte, ne peuvent régénérer des structures matures [17]. Ces derniers peuvent régénérer au niveau des disques imaginaux (structures qui donnent naissance aux différentes parties de l'insecte telles la patte, l'antenne, les ailes etc.) seulement s'ils sont endommagés avant la métamorphose [18].

La régénération chez les vertébrés est présente principalement (mais non exclusivement) chez les amphibiens. Elle est également présente chez les mammifères mais de manière plus restreinte. Parmi les mammifères ayant la capacité de régénérer, on mentionne le cerf, la souris et l'humain. Le cerf est d'intérêt particulier puisqu'il est le seul modèle mammalien ayant le pouvoir de régénérer un organe au complet. À chaque printemps, le cerf perd ses ramures et les régénère annuellement. La régénération des ces structures osseuses impressionnantes s'effectue via la régénération épimorphique [19, 20]. La repousse des ramures implique la régénération de l'os qui va croître par un processus continu de différenciation cellulaire à partir d'une masse de cellules non différenciées du mésenchyme. Ces cellules vont proliférer et se différencier en chondrocytes et ces derniers vont éventuellement être remplacer par des ostéoblastes pour compléter la repousse de cette structure [21, 22].

Les souris ont aussi une capacité limitée de régénérer les bouts de leurs doigts. La régénération se réalise seulement si l'amputation est distale à la dernière phalange du doigt. Si l'amputation est faite de manière proximale à ce plan d'amputation, il n'y a pas de régénération [23]. En 2003, Han *et al.* ont caractérisé la régénération des bouts des doigts chez la souris et ont trouvé que la capacité de régénérer corrélait avec l'expression du *muscle segment homeobox gene-1 (Msx-1)* [24]. Effectivement, Odelberg *et al.* ont démontré de façon directe l'implication de *Msx-1* dans la dédifférenciation de cellules de mammifères et que ces cellules peuvent être dirigées vers des types cellulaires différents (par exemple : cellules osseuses ou adipocytes) [25]. En fait, il est déjà documenté que les humains peuvent régénérer les bouts de leurs doigts jusqu'à l'adolescence [26]. Comme chez les souris, la capacité régénératrice des doigts de l'humain est limitée à la dernière phalange. De plus, pour qu'il y ait régénération, l'amputation ne peut être traitée de façon chirurgicale; dès que la plaie est refermée par des points de sutures la régénération est inhibée. Notons aussi la capacité de régénération compensatoire, ou plus précisément, la croissance hyperplasique du foie chez l'humain décrite plus haut [14].

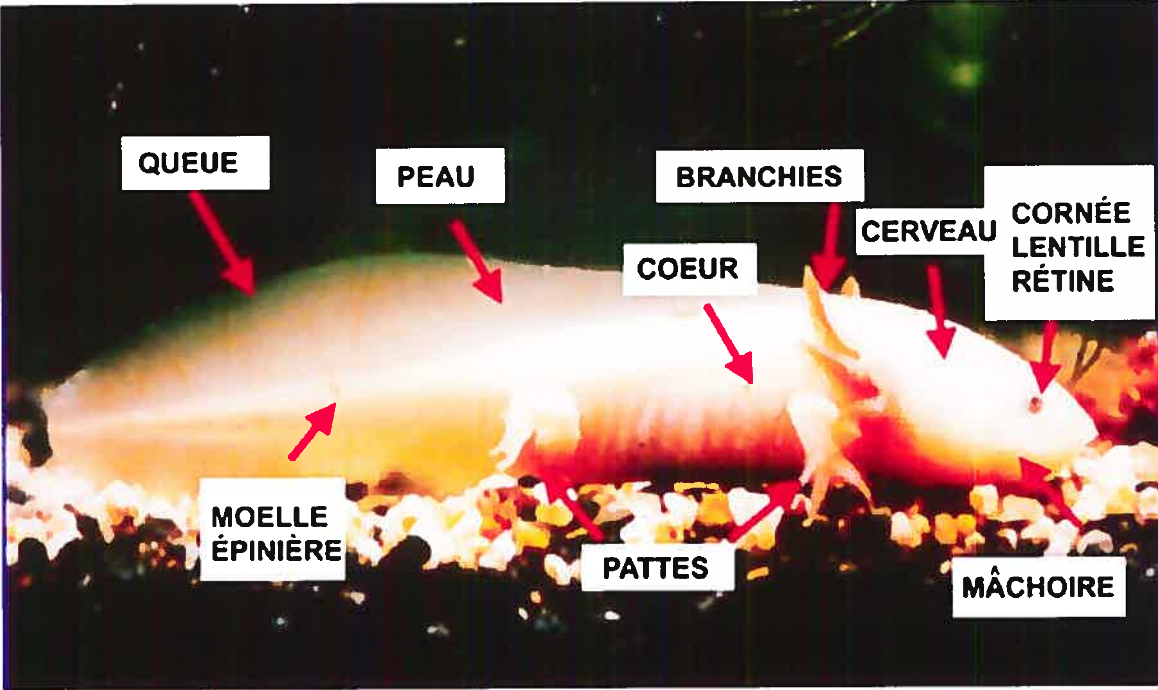
Un autre vertébré ayant la capacité de régénérer est le poisson zèbre (*Danio rerio*). Il peut régénérer le bout de ses nageoires, les muscles cardiaques, les nerfs optiques, la moelle épinière ainsi que les écailles [27-32]. En outre, les amphibiens anoures (grenouilles et crapauds) peuvent régénérer leurs membres et leur queue et tous les tissus qui les composent. Pourtant, tout comme les holométaboles, ils perdent leur capacité régénératrice après la métamorphose [33]. Les champions incontestés de la régénération sont sans doute les amphibiens urodèles (tritons et salamandres). Parmi les urodèles, ont retrouve notre modèle: l'axolotl.

1.2 L'axolotl comme modèle pour étudier la régénération

L'axolotl (*Ambystoma mexicanum*) est un amphibien urodèle originaire du lac Xochimilco au Mexique. Au milieu du dix-neuvième siècle, cette salamandre a été introduite au monde scientifique par l'herpétologiste August Duméril [34]. Actuellement cette espèce est en voie d'extinction dans son habitat naturel. Pour les fins de la recherche, une colonie d'élevage d'axolotls (The Ambystoma Genetic Stock Center) s'est établie à Lexington Kentucky au États-Unis. Le centre maintient un élevage de différentes souches d'axolotls de pigments variés (*wild type, mutant albino, mutant color*). Le mutant albino est particulièrement utile dans le laboratoire puisqu'il n'a pas de pigments noirs dans sa peau. Cette déficience permet de réaliser certaine expérience telle que l'hybridation *in situ* de type «whole mount», où il faut visualiser le marquage des tissus. Aussi, l'entretient des axolotls est facile et ils sont peu coûteux à maintenir.

Les urodèles (e.g. axolotl) sont des modèles exemplaires pour étudier les mécanismes de la régénération puisqu'ils sont les seuls vertébrés à posséder la capacité exceptionnelle de régénérer parfaitement, tout au long de leur vie, leurs membres (pattes et queue) de même que certains de leurs organes (moelle épinière, yeux, apex du cœur etc.) (Figure 1) [15, 35, 36]. Parmi les structures complexes qui régénèrent, le membre est sans doute le plus étudié. De nombreuses études ont permis aux chercheurs d'acquérir une bonne connaissance des mécanismes qui contrôlent la croissance et l'organisation de cette structure durant le développement ainsi que durant la régénération. Ces connaissances offre un aperçu des mécanismes

Figure 1 : Les parties qui régénèrent chez l'axolotl


QUEUE
PEAU
BRANCHIES
CERVEAU
CORNÉE
LENTILLE
RÉTINE
COEUR
MOELLE
ÉPINIÈRE
PATTES
MÂCHOIRE

de régénération chez les vertébrés. L'étude de la régénération du membre présente certains avantages par rapports aux autres structures qui régénèrent. D'abord, le membre possède trois axes définis (antéro-postérieur, proximo-distal et dorso-ventral) et est facile à manipuler. D'ailleurs, le membre est une structure complexe composée de différents tissus incluant l'os, le muscle squelettique, les nerfs et l'épithélium. L'étude de la régénération du membre nécessite une intervention chirurgicale. Les amputations peuvent se faire de façon proximale (au milieu de l'humérus/fémur) ou de façon distale (au milieu des deux os radius/cubitus ou tibia/fibula) par rapport au corps. Peu importe le site d'amputation du membre, la séquence d'évènements menant à la régénération reste la même (Figure 2) [37].

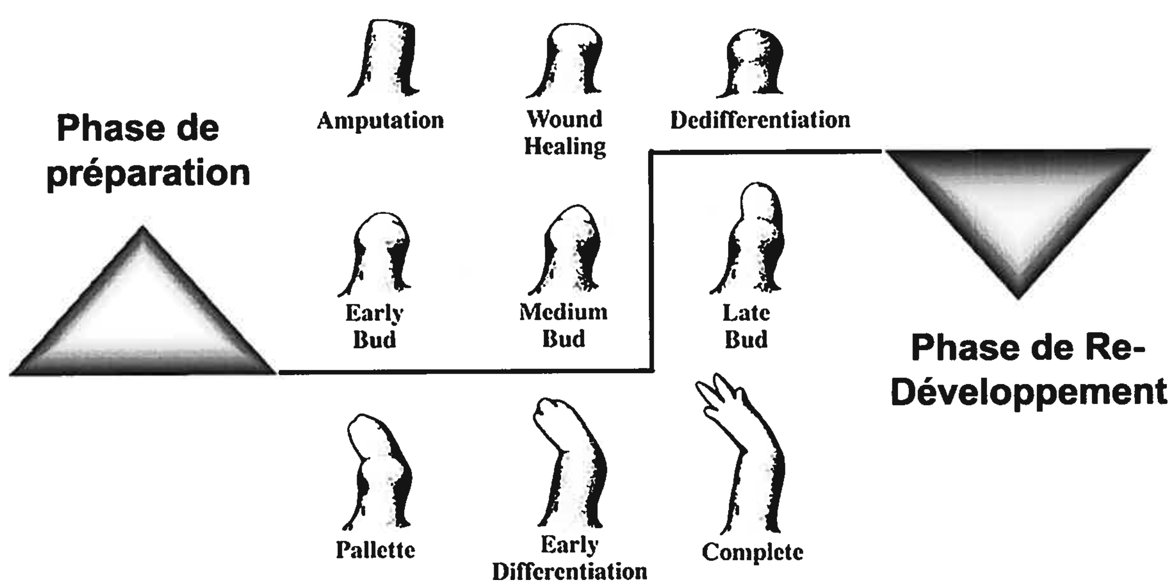
1.2.1 Le processus de la régénération du membre

1.2.1.1 Stades de régénération et les processus cellulaires

Chez les urodèles, le processus de la régénération du membre peut être décrit en tripartie : la phase de guérison, de préparation et la phase de redéveloppement [38]. La première phase du processus de régénération qui est engendrée après l'amputation d'une patte est la guérison de la plaie. En moins d'une heure, les cellules épithéliales de la périphérie du plan d'amputation commencent à migrer pour former une couche mince afin de recouvrir les tissus mésenchymateux exposés [37]. La guérison de la plaie est rapide et, pour des animaux de petite taille, la plaie est fermée dans les 2 heures suivant l'amputation [37, 39]. La phase de guérison est essentielle pour la régénération car si elle est inhibée, la régénération n'aura pas lieu [40, 41]. Après l'établissement de l'épithélium de guérison, celui-ci s'épaissit pour former ce que l'on appelle la coiffe apicale épidermique (CAE). Elle est comparable à la crête apicale ectodermique, impliquée dans la croissance du bourgeon du membre chez le poulet [42-44]. Il semble que la CAE relâche des substances importantes pour la stimulation et la formation du blastème, malgré que leur rôles soient encore mal compris [45].

La prochaine étape dans la régénération du membre implique la dédifférenciation cellulaire. Les cellules provenant des tissus proximaux du plan d'amputation du membre vont se dédifférencier, c'est-à-dire qu'elles vont perdre

Figure 2 : Phénomène biphasique


Phase de préparation
Amputation
Wound Healing
Dedifferentiation
Early Bud
Medium Bud
Late Bud
Phase de Re-Développement
Pallette
Early Differentiation
Complete

leur caractéristiques spécialisées en retournant vers un état pseudo-embryonnaire [37]. Plus précisément, les cellules dérivés des fibroblastes se dédifférencient et vont subséquemment migrer sous l'épiderme de guérison pour former le blastème, une population de cellules prolifératives non-différenciées qui s'accumule au bout distale du moignon amputé [46]. Les fibroblastes jouent un rôle primordial dans la croissance et l'organisation de la patte en régénération [47, 48]. Cette notion a été premièrement testée par Lheureux qui a démontré qu'après avoir greffé un morceau de peau (derme et épiderme) sur une patte irradiée, incapable de régénérer, il y a régénération complète de toutes les structures sauf les muscles [49]. Les expériences suivant celle de Lheureux ont pu démontrer que la peau contient tout ce qui est nécessaire, excluant les cellules capables de former les muscles, pour assurer la régénération parfaite du membre [49-51]. Les derniers stades de la phase de dédifférenciation; les étapes de bourgeon primaire et intermédiaire (*Early bud* EB et *Medium bud* MB) sont aisément identifiées morphologiquement sous un stéréoscope et se sont caractérisées histologiquement par la prolifération et l'accumulation des cellules dédifférenciées du mésenchyme dans le blastème [52].

L'étape finale du processus de la régénération du membre implique le phénomène de redifférenciation cellulaire, c'est-à-dire, les cellules du blastème non-différenciées vont se différencier pour donner tous les tissus nécessaires afin de reformer le membre [53]. Encore plus, il a déjà été démontré que des cellules musculaires qui se sont dédifférenciées peuvent se redifférencier pour donner des cellules cartilagineuses [54], ainsi supportant le concept de transdifférenciation cellulaire dans le blastème. Lorsque la patte continue à régénérer, il y a condensation progressive des cellules mésenchymateuses afin de créer la matrice cartilagineuse qui composera le squelette du membre. Des condensations mésenchymateuses additionnelles se produit de la même manière pour former les doigts à la fin du processus (au stade *early differenciation* ED). Finalement, la patte va croître pour atteindre sa taille normale et rétablir ses fonctions normales [37].

1.2.1.2 Phénomène biphasique

Plusieurs experts dans le domaine de la régénération supportent l'hypothèse que la régénération du membre est un processus biphasique (Figure 2) [38, 46, 55]. La première phase, la phase de préparation, implique les évènements à partir de l'amputation jusqu'à la formation du blastème (bourgeon intermédiaire). La deuxième phase, la phase de redéveloppement, est caractérisée par le contrôle de la croissance et l'organisation axiale du blastème. Les données accumulées jusqu'ici suggèrent que les mécanismes moléculaires impliqués durant la phase de préparation sont uniques au processus de régénération tandis que, durant la phase de redéveloppement, l'organisme reprend les étapes de formation du membre que l'on retrouve normalement lors de la vie embryonnaire [56-63].

Un facteur qui supporte la régénération biphasique de façon importante est la présence des nerfs durant la régénération de la patte. Plusieurs études ont démontré que les nerfs sont requis pour la première phase de régénération; la dénervation du membre durant la phase de préparation inhibe le processus de régénération [37, 48]. De plus, lorsque la patte est dénervée lors de la phase de redéveloppement, la patte devient indépendante de la présence des nerfs et la régénération peut se compléter [64-67].

En plus de la notion de régénération biphasique du membre, il est aussi proposé que les gènes exprimés dans les premiers 24h durant la régénération du membre partagent des mécanismes similaires que celles mis en place pour la guérison d'une plaie chez les mammifères [55, 59]. Cependant, après une amputation, contrairement aux mammifères, l'axolotl va régénérer son membre sans cicatrisation résiduelle entre le moignon et la structure régénérée; ainsi fournissant une patte parfaite. Cette capacité permet l'étude de l'axolotl non seulement pour la régénération mais aussi pour la minimisation des cicatrises chez les mammifères [68]. La caractérisation de plusieurs gènes durant le développement et durant la régénération du membre ont permis davantage de déchiffrer les mécanismes moléculaires impliquées durant la guérison de la plaie et durant la régénération du membre et ainsi, seront traités dans la prochaine section.

1.2.1.3 Les gènes exprimés et leurs rôles

Le premier gène connu à être exprimé lors du processus de la régénération du membre est le *muscle segment homeobox gene-2* (*Msx-2*) qui est détecté aussi tôt qu'une heure suite à l'amputation. Ce gène est également exprimé dans le processus de guérison des plaies, ainsi suggérant que *Msx-2* joue un rôle dans une voie commune à la fois dans la guérison des plaies et dans la régénération [39]. Durant les premières 24 heures de la régénération de la patte, certains gènes de la famille des *Mmp* (e.g. *Mmp-9*) et les gènes *HoxD* (e.g. *Hoxd-10 et Hoxd-8*) sont exprimés. Il est probable que ces gènes ont une fonction dans le rétablissement de l'identité positionnelle du membre amputé, bien que les mécanismes qui régulent ces évènements sont encore inconnus [59, 69, 70]. Les gènes de la famille *HoxA* sont exprimés 24-48h après amputation et ont un rôle dans la spécification de l'axe proximo-distale du membre [57]. Gardiner *et al.* ont décrit l'expression des gènes *HoxA9* et *HoxA13* durant la régénération et durant le développement de la patte. Ils ont démontré que ces gènes sont exprimés de façon conservés durant le développement du membre chez l'axolotl comparé avec d'autres vertébrés; *HoxA9* est exprimé tôt dans l'entier du bourgeon tandis que *HoxA13* est exprimé plus tard dans la région la plus distale du moignon. L'expression proximo-distale de ces gènes durant la régénération est plutôt différent durant la phase de préparation; les deux gènes étant exprimés tôt avec un patron d'expression qui se chevauchent. Lorsque la régénération continue, le patron d'expression pour *HoxA9* et *HoxA13* s'éloignent jusqu'à la phase de redéveloppement où, comme durant le développement, *HoxA9* est exprimé dans l'entièreté du blastème et *HoxA13* est exprimé dans la partie distale du blastème [57]. Curieusement, des études génétiques ont relié l'expression chevauchant de ces gènes avec l'organisation axiale de la région la plus distale du blastème, alors que la région la plus proximale exprimerait *HoxA9* mais non *HoxA13* [71]. Ces résultats sont en accordance avec le model de régénération par intercalation qui stipule que la partie la plus distale du membre est spécifiée en premier et que les sections entre le moignon et le blastème sont intercalées par la suite [72, 73].

L'innervation de la patte est un volet de la régénération qu'attire considérablement d'attention. Les découvertes soutenant le besoin des nerfs pour la régénération du membre ont poussé la recherche pour un facteur neurotrophe responsable pour ce paradigme. Conséquemment, le gène *Dlx3* a été identifié chez l'axolotl [64]. Ce gène fait partie de la famille des gènes *Hox* et est l'homologue du gène *Distalless (Dll)*, un gène reconnu pour sa fonction régulatrice sur la croissance distale des appendices chez la drosophile [74, 75]. *Dlx3* est reconnu pour son implication dans les interactions épithéliaux/mésenchymateux. Chez les vertébrés, ces interactions jouent un rôle dans le développement de plusieurs structures (e.g. dents, appendices, poumons, reins) [76-80]. Une étude fait par Mullen *et al.* a donné un aperçu quant à l'interaction entre les nerfs et l'expression de *Dlx3* durant la régénération chez l'axolotl. Ce groupe a premièrement démontré que pendant la régénération de la patte, les patrons spatio-temporels de *Dlx3* sont consistants avec une fonction dans le contrôle de la croissance distale des appendices. De plus, l'expression de *Dlx3* culmine juste avant la phase de redifférenciation et diminue jusqu'à nul rendue au stade tardifs de la régénération, ainsi coïncidant avec la transition à l'indépendance des nerfs. Ils ont démontré davantage que la dénervation des pattes en régénération des stades jusqu'au bourgeon intermédiaire résulte dans une perte rapide mais éphémère de l'expression de *Dxl3*. Au contraire, la dénervation des stades à partir du bourgeon tardifs n'a aucun effet sur l'expression de ce gène [64].

Il existe aussi des gènes identifiés lors de la phase de redéveloppement de la repousse du membre qui sont exprimés de manière similaire durant le développement. Par exemple, *Sonic hedgehog (Shh)*, un morphogène exprimé dans la région postérieur du bourgeon du membre en développement et qui est important pour l'établissement antéro-postérieur de la patte [81]. Pareillement, ce gène est exprimé dans la région postérieur du blastème de la patte en régénération et semble avoir une fonction conservée durant la régénération de la patte comme dans le développement d'autres vertébrés [47, 82-84]. Semblablement, les études portant sur l'expression des gènes *Hox* distinguent une multitude de gènes appartenant à

cette famille qui, eux aussi, sont exprimés de façon similaire durant le processus du développement et la régénération du membre [56, 57, 85].

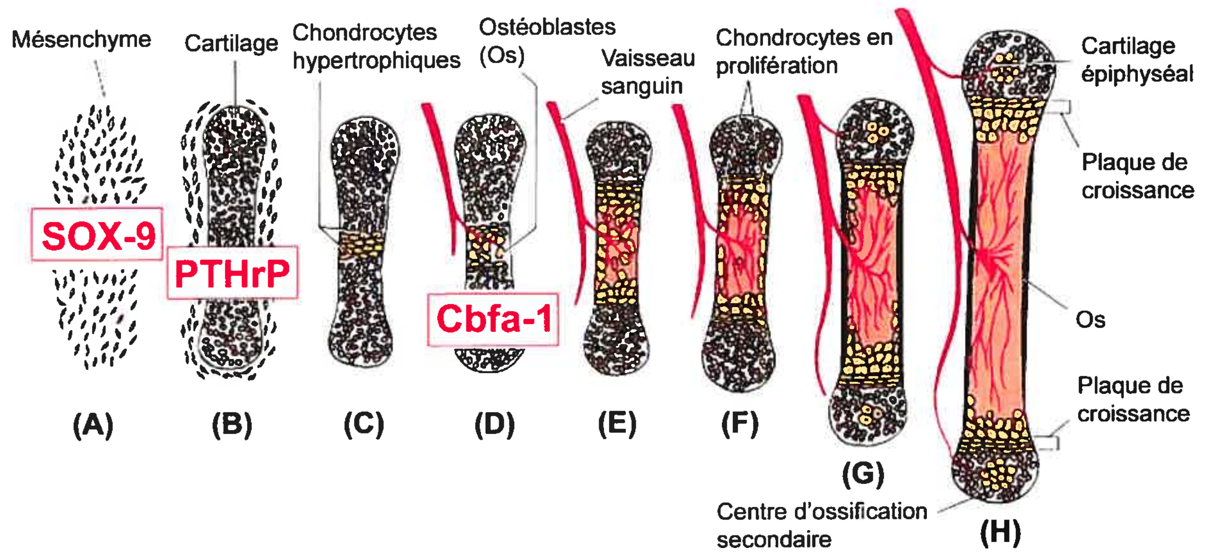
1.3 Étude de gènes exprimés durant le développement et la régénération osseuse

1.3.1 Le développement osseux

Le squelette axial est formé à partir de 3 sources embryonnaires: 1) les somites qui génèrent le squelette axial, 2) la plaque latérale du mésoderme qui génère le squelette des membres et 3) les cellules de la crête neurale (CCN) qui génèrent, entre autre, les arcs branchiaux ainsi que l'os et le cartilage cranio-facial [86]. Quelle que soit la structure squelettique, l'os se formera par une des deux façons suivantes : soit par ossification intramembranaire ou soit par ossification endochondrale [87]. Le processus d'ossification intramembranaire convertit directement les tissus mésenchymateux en os tandis que l'ossification endochondrale passe par plusieurs étapes intermédiaires; c'est-à-dire la différenciation des cellules mésenchymateuses en chondrocytes, la prolifération et l'hypertrophie de ces derniers, suivi par leur remplacement par l'os (le mécanisme détaillé est décrit ci-dessous). Des exemples classiques de structures formées par ossification intramembraneuse sont les os cranio-faciaux. Comme mentionné auparavant, ces dernières structures sont générées à partir des CCN. Les CCN sont formées lors de la fermeture du tube neural durant la neurulation primaire. Les cellules qui composent la crête neurale vont migrer en périphérie et vont progressivement adopter un type cellulaire spécifique afin de contribuer à la formation des différents tissus de l'embryon. Parmi les structures dérivées des CCN, on compte: le tissu conjonctif, les cellules pigmentaires et le ganglion entérique.

Le deuxième mode d'ossification, dit endochondral (Figure 3), est associé à la formation des vertèbres, des côtes et de façon plus importante, des appendices. Le processus d'ossification endochondral peut être décrit en 5 étapes; i) l'engagement des cellules du mésenchyme dans la voie qui mène aux cellules cartilagineuses; ii) la condensation subséquente de ces cellules et leur différentiation en chondrocytes; iii) la prolifération des chondrocytes; iv) l'hypertrophie des chondrocytes; et

Figure 3: Diagramme schématique du processus d'ossification endochondral démontrant les étapes où interviennent les gènes *Sox-9, PTHrP* et *Cbfa-1*. (A, B) Les cellules mésenchymateuses se condensent et différencient en chondrocytes pour former le model cartilagineux de l'os. (C) Les chondrocytes dans le centre de la colonne vont s'hypertrophier et vont subir l'apoptose pendant qu'ils changent et minéralisent leurs matrices extracellulaires. Leurs extinctions permettent aux vaisseaux sanguins d'entrer. (D, E) Les vaisseaux sanguins apportent les ostéoblastes, qui se lient à la matrice cartilagineuse en dégénérescence et vont déposer une matrice de l'os. Concurremment, les ostéoclastes participent à la résorption de la matrice osseuse pour maintenir la solidité de l'os. (F-H) La croissance et la formation de l'os comprennent la prolifération d'un ensemble ordonné des chondrocytes en prolifération, hypertrophie et minéralisation. Les centres secondaires d'ossifications vont aussi former aux extrémités de l'os.



Mésenchyme
Cartilage
Chondrocytes hypertrophiques
Ostéoblastes (Os)
Vaisseau sanguin
Chondrocytes en prolifération
Cartilage épiphyséal
Plaque de croissance
Os
Plaque de croissance
Centre d'ossification secondaire
SOX-9
PTHrP
Cbfa-1
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)

v) l'apoptose des chondrocytes et leur remplacement par des ostéoblastes [88]. Il est donc clair que les os sont des tissus complexes à former. Par conséquent, le processus d'ossification fait intervenir une très grande variété de gènes et molécules. Certains gènes qui jouent des rôles de premier plan lors de la formation du squelette chez l'embryon ont retenu notre attention pour leurs rôles potentiels lors du processus de guérison et de régénération osseuse et seront traités dans les prochaines sections.

1.3.1.1 Les gènes Sox : Sox-9 et Sox-10

Les gènes de la famille *Sry-type* HMG *box* (*Sox*) sont essentiels pour une variété de processus lors du développement embryonnaire. Parmi leurs fonctions, ils jouent un rôle prédominant durant le développement de la crête neurale (résumé dans [89]). Suite à l'induction de cette dernière, il y a quatre gènes *Sox* qui sont exprimés au niveau de la plaque neurale, incluant *Sox-9* et *Sox-10*. Ces deux gènes, avec *Sox-8*, sont regroupés dans la classe *SoxE*, et sont reconnus pour être des régulateurs importants des CCN. Au niveau axial du tube neural, l'expression de *Sox-9* est maintenue généralement dans la région craniale, dans les cellules craniales de la crête neurale, tandis que l'expression de *Sox-10* est située plutôt dans la région du tronc [90, 91]. Des mutations dans le gène *Sox-10* entrainent des défectuosités des dérivés des CCN de la région vagale et du tronc (ganglions entériques, cellules pigmentaires, système nerveux périphérique et arches pharyngiales) tandis que les structures formées à partir des dérivées des cellules craniales de la crête neurale semblent se développer normalement [92]. Des mutations du gène *Sox-10* sont responsables du syndrome de Waardenburg-Shah [92, 93] chez l'humain qui se caractérise, entre autre, par la surdité et des défauts pigmentaires [92, 94].

Quant à *Sox-9*, il est exprimé tôt dans les cellules souches de la crête neurale et est essentiel pour le développement des dérivés squelettiques des cellules craniales de la crête neurale tels les os/cartilages cranio-faciaux. Des expériences «knock-out» pour ce gène chez la souris présentent des embryons chez qui les structures squelettiques endochondrales dérivées des cellules craniales de la crête neurale manquent tandis que les CCN dérivées du tronc semblent normales [91, 95].

Récemment, le groupe de Mori-Akiyama a démontré que la raison pour laquelle les structures squelettiques craniales ne se forment pas est une conséquence de l'inhabilité des cellules craniales de la crête neurale post-migratoires à se différencier en chondrocytes [96]. En plus de son rôle dans le développement des dérivés des cellules craniales de la crête neurale, Sox-9 joue aussi un rôle critique dans le processus de chondrogenèse [83, 97-99]. Il est premièrement exprimé au niveau des cellules précurseurs des chondrocytes et lors de la condensation de ces préchondrocytes. *Sox-9* est également responsable pour la différenciation des cellules mésenchymateuses non-différenciées en chondrocytes et de la maturation et prolifération des cellules chondrocytaires [83, 95]. Des mutations hétérozygotes pour ce gène chez les humains entraînent une maladie qui se nomme la dysplasie campomélique (CD) et se caractérise principalement par de nombreuses malformations squelettiques [99, 100]. Plus récemment, Ng *et al* ont démontré que durant la chondrogenèse chez la souris, Sox-9 est co-exprimé avec le gène collagène type II alpha 1 (*Col2a1)* et qu'il active la transcription de ce dernier. Ceci suggère un rôle régulateur de la protéine *Sox-9* sur *Col2a1* durant la chondrogenèse [83].

1.3.1.2 Collagène type II

Les collagènes sont les glycoprotéines fibreuses les plus abondantes composant la matrice extracellulaire des tissus conjonctifs. Leur présence assure le maintien, la stabilité et l'intégrité structurale de plusieurs tissus et organes [101]. Les divers types de collagènes sont regroupés dans plusieurs familles selon leur structure et organisation supramoléculaire. Par exemple, le collagène type II (*Col2*) est regroupé avec les collagènes de type I, III, V et XI, car ils font tous partie de la famille des collagènes qui forme des fibrilles. Le *Col2* est particulièrement intéressant puisqu'il code pour une composante majeure de la matrice extracellulaire du cartilage [83]. À juste titre, le *Col2* est la composante prédominante dans le cartilage hyalin qui se situe dans les articulations, la cloison nasale, les anneaux des grosses bronches et de la trachée, et à l'extrémité des côtes chez l'humain [101].

L'épissage alternatif de pre-ARNm *Col2* donne 2 formes différentes : *Col2α1*, une forme embryonnaire exprimé dans le mésenchyme pré-chondrocytaire, et *Col2β1*, retrouvé dans le cartilage mature [102, 103]. Durant la chondrogenèse, la transcription de *Col2α1* s'effectue dans les cellules mésenchymateuses, précédant leur différenciation en chondrocytes [104-107]. Des mutations dans *Col2α1* chez l'humain entrainent une variété de chondrodysplasies tels l'achondrogenèse II (Langer-Saldino), qui se caractérise par des malformations graves du cartilage et du squelette, telles un crâne excessivement volumineux, des membres très courts et un tronc large et court; et la maladie de Kniest, qui se caractérise par une dysmorphie faciale, des anomalies rachidiennes et des atteintes articulaires des membres supérieurs [108, 109]. De plus, les études sur des souris transgéniques qui expriment une mutation dans le gène de *Col2* démontrent une formation squelettique anormale [110-113]

1.3.1.3 PTHrP

Parathyroid hormone related peptide (*PTHrP)* est une hormone peptidique premièrement identifiée comme l'agent responsable de l'hypercalcémie humorale maligne [114, 115]. Elle est impliquée dans plusieurs fonctions biologiques et est reconnue pour être, entres autres, un relaxant des muscles lisses, un régulateur du transport calcique trans-épithélial ainsi qu'un régulateur du développement de certains organes et tissus [116]. Durant l'ostéogenèse, le gène *PTHrP* intervient au niveau de la résorption osseuse, de la différenciation et maturation des chondrocytes ainsi que dans la stimulation de la différenciation et prolifération des ostéoblastes [117-119]. Les mutations nulles pour le gène *PTHrP* produisent des anormalités sévères dans le développement endochondral des os; conséquence d'une prolifération réduite, d'hypertrophie et d'apoptose précoce des chondrocytes [120-122].

1.3.1.4 Cbfa1

Core-binding factor α-1 (*Cbfa-1)*, quant à lui, intervient un peu plus tard dans le processus d'ossification. Aussi connu comme *Runt domain 2 (Runx2)*,

Polyomavirus enhancer core binding protein αA (PEBP2αA), *Acute myelocytic leukemia 3 (AML3)*, et *Osteoblast-specific cis-acting element 2 (Osf2)*, *Cbfa-1* est un facteur de transcription qui appartient à la famille des gènes portant des domaines *Runt*. Cette famille comprend trois gènes soit *Cbfa-1*, *Cbfa-2* et *Cbfa-3* [123-125]. Il semble que *Cbfa-1* possède plusieurs rôles durant l'ostéogenèse. En plus d'être connu comme un facteur essentiel pour la différenciation et la maturation des ostéoblastes, *Cbfa-1* est aussi un régulateur de la maturation et de la différenciation des chondrocytes [126]. Récemment, Kim *et al.* ont démontré que *Cbfa-1* semble davantage posséder un rôle dans l'initiation et le maintient de l'état hypertrophique des chondrocytes [127]. Une mutation homozygote pour ce gène chez l'humain entraine la maladie héréditaire de dysplasie cleidocraniale (CCD). Elle se traduit par le nanisme et une altération de la forme, de la taille et du nombre de certains os [128, 129].

1.3.2 La régénération osseuse

On compte beaucoup moins d'études portant sur la régénération/guérison de l'os comparativement à celles portant sur le développement de cette structure. Malgré ce fait, nous pouvons nous fier sur certaines découvertes afin de mieux comprendre le processus d'ostéogenèse durant la régénération. Par exemple, une étude fait par Muneoka *et al.* a démontré qu'après amputation d'une patte d'axolotl, l'os représente environ 50% de la surface amputée, tandis que seulement 2% des cellules en dérivent lors de la régénération [60]. D'autres études portant sur la régénération de la patte suggèrent aussi que les nouvelles structures squelettiques sont générées à partir d'autres cellules [130, 131]. Finalement, plusieurs études supportent la notion que la régénération est en partie une récapitulation des mécanismes moléculaires qui ont lieu durant le développement [56-63]. Ceci étant, nous pouvons nous attendre à ce que certains gènes qui interviennent durant l'ostéogenèse lors du développement interviennent également lors de la régénération, particulièrement *Sox-9, PTHrP* et *Cbfa-1.*

Durant le développement, *Sox-9* agit au niveau des cellules non-différenciées en prolifération qui sont maintenues dans une zone de progression

dans le bourgeon du membre [83, 95]. Ceci est comparable aux stades de dédifférenciation et de prolifération auxquels sont soumises certaines cellules du blastème lors de la régénération du membre d'axolotl [15, 132]. *PTHrP*, lui aussi est un bon candidat pour avoir une fonction dans le processus de la régénération de la patte puisqu'il agit au niveau de la différenciation et de la prolifération des chondrocytes embryonnaires [117]. *PTHrP* pourrait très bien être un élément régulateur dans la différenciation des cellules pluripotentes du blastème durant la régénération afin de les diriger vers un destin chondrocytaire. De plus, il a déjà été démontré que *PTHrP* a un rôle dans la formation du blastème durant la régénération des ramures chez le cerf. Il est exprimé particulièrement dans l'épithélium en régénération ainsi que dans les cellules mésenchymateuses non-différenciées du blastème [20]. Dernièrement, l'implication de *Cbfa-1* dans la différenciation des chondrocytes et ostéoblastes est un processus qu'il faut considérer lors de l'étude de la régénération osseuse du membre [129, 133]. Kim *et al.* ont démontré qu'on retrouve l'expression de *Cbfa-1* au niveau de la zone de croissance des os long [127]. On observe également une zone de croissance osseuse dans le membre en régénération [52], donc nous pourrions spéculer que *Cbfa-1* interviendra d'une manière similaire.

1.3.3 La régénération versus la guérison osseuse

Plusieurs études se concentrent sur la capacité exceptionnelle que possède l'axolotl de régénérer un appendice au complet et ainsi tous les tissus qui le composent, incluant l'os. Par contre chez l'axolotl, peu d'études portent sur le processus de réparation osseuse ou sur la capacité de régénération de l'os suite à une fracture [134]. Les amputations se traduisent par la perte totale d'un bout d'os, mais les fractures ne sont que des blessures ou perte partielle que l'on inflige aux os. Il existe d'abord deux catégories de fractures osseuses : les fractures jointes et les fractures non-jointes de dimension critique («gap» osseux). Pour ce qui concerne les fractures jointes, elles peuvent être de deux types : stabilisées par des interventions de nature médicales (plâtres ou tiges métalliques) ou encore non-stabilisées. Les fractures jointes se guérissent via la formation d'un cale osseux due à une

accumulation cartilagineuse [135]. Concernant les fractures non-jointes, elles sont souvent provoquées par des interventions chirurgicales. Pour ce type de fracture, il est possible de caractériser la dimension critique, c'est-à-dire la longueur maximale que doit avoir l'explant d'os pour que la reformation d'un cale soit possible. Si la longueur critique est dépassée, il est alors impossible d'obtenir une guérison osseuse. La longueur critique d'un explant osseux dépend de la taille de l'animal, quoiqu'elle semble être accrue par certaines molécules telles que *Transforming growth factor β1 (Tgf- β1)* [136].

Chez les vertébrés, le processus de réparation osseuse est bien caractérisé [137-141]. Bien que la plupart des vertébrés soient capables de guérir une fracture osseuse par la formation d'un cale, tous les vertébrés étudiés jusqu'à présent sont incapables de réparer une fracture de dimension critique [142]. Étant donné que l'axolotl adulte possède la capacité exceptionnelle de régénérer complètement un membre amputé, nous sommes particulièrement intéressés aux évènements qui mènent à la réparation et à la guérison de l'os. Est-ce que l'axolotl utilise la régénération pour la réparation/guérison des fractures ou des «gap» osseux? L'étude des mécanismes mis en place lors de la réparation/guérison osseuse va nous permettre de mieux comprendre les mécanismes de régénération des structures squelettiques chez l'axolotl.

1.4 But du projet

Le but principal de cette étude est de caractériser les gènes ostéogéniques exprimés durant la régénération chez l'axolotl afin de mieux comprendre le processus de chondrogenèse et d'ostéogénèse durant ce processus. Des études antérieures suggèrent que les mécanismes qui dirigent la reformation du membre en régénération sont les mêmes que ceux qui dirigent la formation du membre au cours du développement embryonnaire. Nous sommes donc intéressés par l'étude des gênes responsables de la formation de l'os durant la régénération et durant le développement. Parmi les gènes importants impliqués dans ce processus, nous avons étudié particulièrement les gènes *Cbfa-1*, *PTHrP, Sox-9* et *Sox-10.*

Pour atteindre nos objectifs, nous avons, dans un premier temps, cloné les séquences partielles des gènes d'axolotl *Cbfa-1*, *PTHrP, Sox-9* et *Sox-10* en utilisant la technique de RT-PCR avec des amorces dégénérées. Nous avons subséquemment obtenu les séquences complètes de *Cbfa-1* et de *Sox-10* en utilisant la méthode de RACE-PCR. Afin de caractériser l'expression spatio-temporelle et l'expression protéinique des gènes clonés durant le développement et la régénération, nous avons utilisé respectivement la technique d'hybridation *in situ* de type «Whole-mount» et la technique d'immunobuvardage de type «Western».

CHAPITRE 2

Article 1: «The axolotl limb: a model for bone development, regeneration and fracture healing»

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(See Annexe 1)

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2.1 Contribution de l'auteur et déclaration des coauteurs

Contribution de l'auteur

Pour ce premier article, j'ai isolé la séquence complète du gène *Cbfa-1*. J'ai également effectué l'analyse des séquences pour les gènes *Cbfa-1* et *PTHrP*. De plus, j'ai caractérisé l'expression du collagène type II par la technique d'immunohistochimie *in situ* de type «Whole-mount» pour les stades de régénération et développement de la patte. Avec ceci, j'ai rassemblé mes résultats et ceux obtenus antérieurement par Mireille Pilote et j'ai créé les figures appropriées pour me permettre de rédiger et de soumettre l'article ci-dessus. En outre, il y avait plusieurs modifications à faire quant à la re-soumission de ce papier. J'ai refait certaines manipulations pour améliorer la qualité des résultats, par exemple, l'hybridation *in situ* de type «Whole-mount» pour les pattes en développement pour *PTHrP* ainsi que la coloration du cartilage pour plusieurs stades de régénération de pattes. De plus, j'ai aussi re-photographié plusieurs échantillons afin d'améliorer la résolution des images.

1. Mireille Pilote, M.Sc. Biochimie

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Cara Hutchison inclus cet article dans son mémoire de maitrise qui a pour titre «Caractérisation de gènes ostéogéniques chez l'axolotl».

Coauteur Signature Date

2.2 Abstract

Among vertebrates, urodele amphibians (e.g., axolotls) have the unique ability to perfectly regenerate complex body parts after amputation. The limb has been the most widely studied due to the presence of three defined axes and its ease of manipulation. Hence, the limb has been chosen as a model to study the process of skeletogenesis during axolotl development, regeneration and to analyse this animal's ability to heal bone fractures. Extensive studies have allowed researchers to gain some knowledge of the mechanisms controlling growth and pattern formation in regenerating and developing limbs, offering an insight into how vertebrates are able to regenerate tissues. In this study, we report the cloning and characterization of two axolotl genes; *Cbfa-1*, a transcription factor that controls the remodelling of cartilage into bone and *PTHrP*, known for its involvement in the differentiation and maturation of chondrocytes. Whole-mount in situ hybridization and immunohistochemistry results show that *Cbfa-1*, *PTHrP* and type II collagen are expressed during limb development and regeneration. These genes are expressed during specific stages of limb development and regeneration which are consistent with the appearance of skeletal elements. The expression pattern for *Cbfa-1* in late limb development was similar to the expression pattern found in the late stages of limb regeneration (i.e. re-development phase) and it did not overlap with the expression of type II collagen. It has been reported that the molecular mechanisms involved in the re-development phase of limb regeneration are a recapitulation of those used in developing limbs; therefore the detection of *Cbfa-1* expression during regeneration supports this assertion. Conversely, *PTHrP* expression pattern was different during limb development and regeneration, by its intensity and by the localization of the signal. Finally, despite its unsurpassed abilities to regenerate, we tested whether the axolotl was able to regenerate non-union bone fractures. We show that while the axolotl is able to heal a non-stabilized union fracture, like other vertebrates; it is incapable of healing a bone gap of critical dimension. These results suggest that the axolotl does not use the regeneration process to repair bone fractures.

2.3 Introduction

The ability of an adult vertebrate to regenerate lost body parts is very limited; however urodele amphibians such as the axolotl (*Ambystoma mexicanum*) are known to have exceptional abilities to regenerate multiple body parts throughout their life. The axolotl's capacity to regenerate its limbs as an adult offers the opportunity to conduct comparative studies of the genes expressed during development and regeneration in an identical genetic background. Among the complex structures able to regenerate, the limb has been the most widely studied due to the presence of three defined axes and its ease of manipulation. During limb development, the cells that form limb tissues are derived from undifferentiated progenitor cells (50). This process is paralleled in the case of the regenerating limb, with the exception that differentiated cells become undifferentiated before subsequently reforming the missing parts after amputation (7, 50). It has been hypothesized that the process of limb regeneration is biphasic (8, 24, 60). The first phase, known as the preparation phase, involves the events spanning from amputation to blastema formation whereas the second phase, known as the re-development phase, is characterized by the control of growth and pattern formation within the blastema and finally the re-differentiation of cells to reform the missing limb. Experimental data collected to date supports the concept that the molecular mechanisms involved in limb development are recapitulated during the re-development phase of limb regeneration (48, 58, 59, 71). In addition to the aforementioned advantages, the complexity of the limb provides an excellent opportunity for studying many different tissue types including bone and cartilage. Bone represents approximately 50% of the exposed surface following limb amputation, however, as little as 2% of cells in a regenerated limb are derived from bone (48). Previous limb regeneration studies also suggest that new bone is generated from dedifferentiated cells of the blastema and not derived from pre-existing bone (41, 74). Most skeletal bones, including those of the appendage, develop via an endochondral ossification process which involves the replacement of cartilage tissue by bone. This process can be divided into five stages: i) the

commitment of mesenchymal cells to cartilage cells; ii) the subsequent condensation of these cells and their differentiation into chondrocytes; iii) chondrocyte proliferation; iv) chondrocyte hypertrophy; and v) chondrocyte apoptosis and replacement by osteoblasts (30). Among the numerous genes potentially involved in the formation of skeletal elements during limb regeneration, two of them are of particular interest; *Core-binding factor α-1 (Cbfa-1)*, a transcription factor required for mesenchymal condensation, chondrocyte hypertrophy and osteoblast differentiation (34, 53) and *Parathyroid hormone related peptide (PTHrP),* involved in the differentiation and maturation of chondrocytes(37). The *Cbfa-1* gene, also known as Runx2/PEBP2αA/AML3/Osf2, is characterized by a Runt domain that binds DNA and heterodimerizes with *Core-binding factor β (Cbfβ)* (4, 51). Studies have shown *Cbfa-1* to be an essential factor for osteoblast maturation and normal ossification (34). However, additional research has revealed a dual action for *Cbfa-1*: first as an osteoblast differentiation factor and also as a regulator of chondrocyte maturation and differentiation. Homozygous mutations in this gene are responsible for the skeletal malformation syndrome: cleidocranial dysplasia (CCD) (46). This dysplasia is characterized by multiple skeletal malformations and the absence of ossification (46, 53). In fact, without *Cbfa-1*, osteoblasts are unable to differentiate and therefore abolish bone matrix deposition (46). A more recent study by Kim et al. has revealed yet another function for *Cbfa-1* as a regulator of chondrocyte hypertrophy (33). *PTHrP* is another important player to consider when characterizing skeletogenesis during the regeneration process. *PTHrP* is a small peptide first identified as the causative agent for humoral hypercalcemia malignancy (43, 67). This peptide uses the same transmembrane receptor as *parathyroid hormone (PTH)*; consequently they share a well conserved PTH-like domain (54). In addition to causing hypercalcemia, *PTHrP* is a major regulator of chondrocyte maturation, differentiation and proliferation (37). *PTHrP* is known to be expressed in chondrocytes throughout the developing epiphyses, and in osteoblasts in metaphyseal bone (2). *PTHrP*-deficient knockout mice die prematurely due to severe osteochondrodysplasia, characterized by shorter bones due to an early ossification. This can be explained primarily by reduced

proliferation and increased apoptosis of immature chondrocytes as well as the precocious hypertrophy of chondrocytes (3, 32, 36).

Numerous studies have demonstrated the axolotl's ability to completely regenerate lost appendages in which every tissue including bone is regenerated, yet very few have concentrated on whether the axolotl can regenerate bone in a gap or how well they can heal a fracture (25). The bone healing process has been well characterized in numerous vertebrates (6, 19, 61, 65, 72). Most vertebrates are capable of healing a bone fracture through a callus formation process, however all vertebrates studied to date are unable to heal bone defects of critical dimension (62). These defects are gaps within which bone repair does not take place. The critical dimensions are dependent on the size of the animal. For example, the critical dimension in a rabbit ulna is 15 mm, comparatively; in a rat it is 4-5 mm (11, 29). Under this circumstance, bone formation must be assisted by inductive substances (57). We are particularly interested in the axolotl's abilities to heal and repair bone since they can regenerate entire limbs including the skeletal elements. Does their unique ability to regenerate complex tissues also apply to bone defects or fractures of critical dimension? In this article, we describe the molecular cloning, sequence analysis and expression of *Cbfa-1* as well as *PTHrP* during limb development and regeneration in the axolotl. We also characterized the expression of type II collagen, a cartilage specific protein, during limb regeneration. *Cbfa-1* is an inhibitor of type II collagen and their expression should not overlap (20). Both *Cbfa-1* and *PTHrP* regulate essential aspects of chondrogenesis and osteogenesis in vertebrate development (e.g. mammals & birds). Given that part of the regeneration process is a recapitulation of the developmental process, the regenerating axolotl limb offers an excellent model to analyse whether or not these genes are also required for bone regeneration. Our results show that, *Cbfa-1* and *PTHrP* as well as type II collagen are expressed during limb development and regeneration. Additionally, we found that particular gene expression patterns during axolotl limb development are similar to those during regeneration. Finally, we show that while the axolotl is able to heal a non-stabilized fracture, like other vertebrates; the axolotl is incapable of healing or regenerating a bone gap of critical dimension.

2.4 Materials and methods

Cloning axolotl Cbfa-1 and PTHrP cDNA

Partial axolotl *Cbfa-1* and *PTHrP* cDNA sequences were cloned (5 and 3 clones for each gene respectively) by RT-PCR using degenerate primers (see below). To obtain the full length *Cbfa-1* gene, the SMART RACE cDNA Amplification kit (BD Biosciences, Clontech, USA) was used to construct 5'- and 3'- RACE ready cDNA from total RNA extracted from the axolotl hind limb during the medium bud regeneration stage using Trizol reagent (Invitrogen). Following the manufacturer's instructions, PCR products were obtained using 5'- and 3'- gene specific primers (GSPs) and nested gene specific primers (NGSPs) designed from the partial sequence cloned in the lab. The designs of all primers were as follows:
dFPTHrP: 5'-CAGCT(G/A/C)(A/C)T(G/T/C)CA(C/T)GACAAGGG-3'
dRPTHrP: 5'-GTT(A/T/C/G)GT(C/T)TCCTG(A/G)G(G/T)(C/T)AGGT-3'
dFCbfa-1: 5'-ACAG(C/T)CC(G/C)AACTT(C/T)CT(G/C)TGCT-3'
dRCbfa-1: 5'-GGTA(A/C)G(A/T)CTG(A/G)TCATA(G/C)GAC-3'
5'GSP: 5'-TGGAGGAGATGACTGTGCTT -3'
5'NGSP: 5'-CGTGAAGACAGTTATACTCA -3'
3' GSP: 5'-TGACTATAACTGTCTTCACG-3'
3' NGSP: 5'-AAGCACAGTCATCTCCTCCA-3'

All PCR products were purified by gel extraction, cloned into the pCR II-TOPO sequencing vector (Invitrogen) and sequenced by the McGill University Genome Quebec Sequencing Centre (Montreal, Canada) with M13R and M13F primers. Resulting sequences were assembled using SeqManII (DNASTAR Inc., USA). Full length *Cbfa-1* cDNA was subsequently amplified by PCR using [Axolotl forward CbF1] 5'-ATGGCATCCAACAGCCTGTTCA -3' and [Axolotl reverse CbR1426] 5'-AAAACAGGCACTTTGGCATT-3' primers designed from the sequencing data. The PCR reaction was performed using Taq DNA polymerase (Invitrogen) and the conditions were 35 cycles of 95°C, 30s; 63,5-52,5°C, 20s; 72°C, 1min; followed by 15 min at 72°C. Note that six full-length *Cbfa-1* clones

were isolated from the 5 independent RT-PCR reactions and aligned using MegAlign (DNASTAR Inc., USA).

Animal maintenance and surgery

Axolotl embryos and larvae were purchased from the Ambystoma Genetic Stock Center (Lexington, KY). Animals are kept in a 20% Holtfreter's solution at a room temperature varying from 19-22°C and a photoperiod cycle of 12 hours of light and 12 hours of darkness. For amputations, animals were anaesthetized in 0.1% MS222 solution (ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, St. Louis, MO). Proximal (through humerus or femur) and distal (through radius/ulna or tibia/fibula) amputations were performed with micro-surgical tools (Fine Science Tools, B.C., Canada) on axolotls from 3 to 5 cm in length. For fixation, animals are euthanized and fixed in 1XMEMFA (10X MEM salt (1M MOPS pH 7.4, 20mM EGTA, 10mM $MgSO_4$), 3,7% formaldehyde and DEPC treated H_2O then gradually transferred to a methanol solution and stored at -20°C until needed.

Bone defects of critical dimension were generated by first making an incision in the skin along the longitudinal axe of the distal part of the limb, followed by careful displacement of soft tissues to expose the radius or tibia. The distal bones in axolotl limb (radius/cubitus and tibia/fibula) are nearly identical in size and shape, therefore either one of the two long bones was cut. A section of the radius or tibia was extirpated to generate a gap of 4 mm, this being larger than the critical dimension for this type of bone in the axolotl. The remaining bone was left intact to prevent the limb from collapsing and to preserve the distance between the disengaged bone extremities. It also served as an internal control for intact bone. In order to generate bone fractures, surgeries were performed identically except the radius or tibia was simply cut with surgical scissors. Following surgical intervention, limbs were fixed at different periods and the double staining technique was used to verify new cartilage and bone formation. All animal care and experiments were done in accordance with the Université de Montréal animal care committee's guidelines.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described in Gardiner et al. with a few modifications (22). Digoxigenin (DIG) –labeled antisens RNA probes were created using T7 RNA polymerase (Invitrogen) and DIG RNA labelling mix (Roche Diagnostics, Laval, Quebec, Canada). Sense probes were generated with SP6 RNA polymerase (Invitrogen). For probe synthesis, a 400 bp *Cbfa-1* and a 230 bp *PTHrP* fragment were cloned into the pCR II-TOPO plasmid which was then linearized using the appropriate restriction enzymes. For tissue permeabilization, developing embryos and amputated limbs were incubated with 20μg/ml and 30 μg/ml proteinase K respectively for 15 min on ice and then at 37°C for 5 min or for 1h on ice then 1h at 37°C. Probe hybridization was 24h for embryos and 72h for limbs. Prehybridization and hybridization temperatures were done between 55°C and 65°C. Finally, BM purple (Roche) was used as the enzyme substrate for the colorimetric reaction for the alkaline phosphatase reaction.

Type II Collagen whole-mount in situ immunohistochemistry

Samples were rehydrated from methanol and rinsed three times in a phosphate-buffered saline solution with 0.1% Tween-20 (PTW). Limbs were then bathed in 2.5% trypsin for either 15 min (developing limbs) or 40 min (regenerating limbs) followed by three 5-min rinses in water. After 10 min in -20°C acetone, specimens were rinsed in water for 10 min and then washed three times for 5 min with PTW. Samples were then transferred to a blocking solution (phosphate-buffer solution (0.7 X PBS) + 1% DMSO + 5% sheep serum + 0.1% Tween) for 1 hour and incubated in a mouse monoclonal antibody against Type II collagen (II-II6B3, Lab Vision Corporation, Fremont, CA) diluted 1:100 in blocking solution overnight at 4°C. The next day, limbs were rinsed eight times for 15 min in a PTW + 1% DMSO solution before being placed in an anti-mouse horseradish peroxidase labelled secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted 1:500 in blocking solution overnight at 4°C. The following morning, samples were rinsed eight times for 30 min with PTW and subsequently bathed in a diaminobenzidine

(DAB) solution (Invitrogen). The reaction was stopped by two 5-min washes with PTW and fixed in a formalin solution (3.7% formaldehyde in 0.7X PBS). Specimens were cleared through a graded series of ethanol/glycerol and stored in 100% glycerol. As a control for specificity, the same protocol was followed except the primary antibody was omitted (Fig. 4 F).

Skeletal staining of embryos and limbs

Developing and regenerating limbs were stained using the Victoria blue method to verify new cartilage formation (9). Limbs were fixed in alcoholic Bouin's solution for 24 hours before being rinsed several times with 70% ethanol. Specimens were rinsed regularly in 3.5% NH_4OH for 2 days and subsequently treated with Acid alcohol for 2 hours. Specimens were stained with 1% Victoria Blue for 2 hours and then rinsed with 70% ethanol. Limbs were gradually dehydrated to 100% ethanol, then cleared and stored in methyl salicylate.

A double staining technique of Alizarin Red and Alcian Blue was used to stain cartilage blue and calcified matrix red for bone fracture experiments (12). Fixed specimens were rinsed regularly with distilled water for 1 h. Specimens were placed in an alcian blue staining solution (20 mg of alcian blue powder and a 7:3 EtOH/glacial acetic acid solution (EtOH/Hac)) for one day, and then were transferred to an EtOH/Hac solution for one hour. After being placed in 100% EtOH for 24 h, the sample was rinsed with tap water frequently for 2 days before being transferred to a 1% trypsin solution diluted in a 30% saturated sodium borate solution. The specimen is trypsinized for a period of about 24 h, depending on the size, and then transferred for 24 h to an alizarin red solution (saturated alizarin red in water added to a 0.5% KOH solution until it takes on a dark purple color). This step was followed by several changes of a 0.5% KOH solution within a 24 h period before specimens were finally treated with 0.5%KOH/glycerol solutions: 2:1, 1:1, 1:2 and preserved in 100% glycerol.

2.5 Results

Isolation and sequence analysis of axolotl Cbfa-1 and PTHrP cDNA

A partial axolotl *Cbfa-1* sequence was obtained by first isolating a 400 bp cDNA fragment from axolotl larvae total RNA by RT-PCR using degenerate primers. After using the RACE-nested PCR strategy (21), a 1371 bp contig was analyzed using the NCBI BLAST database. Results show a 975 bp ORF (Fig. 1A) which encodes a 325 aa protein containing a partial Runt domain of 40 aa (Fig. 1B). *Cbfa-1* is known to belong to the Runt domain gene family which are characterized by a conserved Runt domain (51). Axolotl protein analysis using BLAST showed a 94% sequence homology with chicken (39), 93% with human (51) and 86% with mouse (56) (Fig. 1C). A phylogenetic tree was created by comparing full length *Cbfa-1* cDNA sequences from different species and placed the axolotl sequence closer to that of birds than to humans which is consistent with the phylogeny of this specie (Fig. 1D).

The same strategy was used to isolate a 225 bp fragment of axolotl *PTHrP* cDNA. The results show a corresponding 75 aa partial *PTHrP* sequence located towards the 5' of the gene (Fig. 2A). Protein alignment analysis demonstrate that our partial sequence shares a 77%, 65% and 64% homology with chicken (52), human (26) and mouse (66) *PTHrP* respectively (Fig. 2B). High homology is expected between species since the cloned sequence contains a partial Parathyroid domain characteristic to the parathyroid hormone family. Integrated in this sequence, is a partial PTH-like domain, unique to the parathyroid hormone, this sequence is required for ligand-receptor interactions (63). Protein alignment of the partial PTH-like domain shows even higher conservation having 90% chicken and 69% mouse and human homology (Fig. 2B). Analysis of the cloned 225 nt sequence with *PTHrP* cDNA from other species was performed using MegAlign (DNASTAR Inc., USA). The resulting phylogenetic tree is consistent with the sequence homology data and evolutionary relationships are conserved (Fig. 2C). This result shows a similar evolutionary relationship for *PTHrP* as that of *Cbfa-1*, where the axolotl sequences are closest to the chicken.

Figure 1. Analysis of the axolotl *Cbfa-1* sequence. (A) cDNA sequence. (B) Amino acid sequence. Red characters indicate start and stop codons. Blue characters indicate conserved partial Runt domain. Green characters indicate Runx conserved carboxyterminal pentapeptide. (C) Amino acid alignment of *Cbfa-1* from multiple model organisms used for developmental and biomedical research (mice, human, chicken and axolotl). Blue box contains the runt domain and the green box indicates Runx conserved carboxyterminal pentapeptide. (D) Phylogenetic tree. Species evolutionary comparison of *Cbfa-1* ORF sequences. [GenBank accession number DQ885240]

A)

TGTACTACCTTCCTGGCTTTTTGCATGTTTCCAGCGCAATACAATCTTTGGACCAAAAAGG
GAGTAGGCACGCAGTAGAACTTCTGAGCGAATAAGTTGAGAATCTAAAACCAGACATGGTC
TGTACTTACAGATTAAGGATTGCAGGAGTGTTTACACCGTAAGGTGCAAGACCTGACTGGG
AAAGACAACAAGAGAACAGGAACCATGGCATCCAACAGCCTGTTCAGCTCAGTGGCACCTT
GTCAGCAAAACTTCTTTTGGGGCAAGAGTTTTACCCTGACTATAACTGTCTTCACGAACCC
GCCCCAAGTAGCCACCTACCACAGAGCTATTAAAGTCACGGTGGATGGACCACGGGAGCCC
AGGAGGCACAGACAGAAGCTTGATGACTCTAAACCTAGTTTGTTCTCTGAACGCCTCAGTG
ATTTAGGGCGTATTCCTCATCCCAGTATGAGAGTGGGTGTCCCAACTCAGAGTCCTCGGCC
TTCCCTAAACTCTGCACCAAGTCCTTTTAATCCACAAGGACAGAGTCAGACTGCAGATCCT
AGACAAGCACAGTCATCTCCTCCATGGTCCTATGACCAGTCCTACCCAGCATACTTGAGCC
AGATGACCTCGCCGTCCATTCACTCCACAACACCCCTTTCATCCTCCCGAGGTACAGGACT
TCCTGCTATCACTGATGTGCCCAGACGTCTCTCAGGTGCTTCAGAACTTGGTCCATTTTCT
GATCCCAGGCAGTTTACAAGCATTTCGTCCCTCACCGAAAGTCGTTTTTCCAATCAACGAA
TGCACTATCCAACCACATTTACTTATACACCGCCTGTCACTTCTGCCATGTCTCTGGGAAT
GTCAGCGMCCACCCATTACCACACCTATTTACCACCACCCTATCCTGGATCTTCACAAAAC
CAAAGTGGACACTTCCAAACAAGCAGCACCCCGTACCTATACTATGGTACATCATCAGGAT
CATATCAGTTCCCCATGGTGCCCGGCGGAGACCGTTCTCCCTCTAGGATGCTACCACCATG
CACCACCACATCAAACGGAGGCACGCTGATCAACCCAAACCTGTCAAACCAAAATGATGGC
GTGGATGCCGATGGAAGCCATAGCAGCTCCCCGACAATTCTGAATTCAAGTGGCAGAATGG
GTGAGTCGGTTTGGAGACCTTATTGAAATATTTATTCGGTGGCCTAACTTCGTTTGGCCCA
ACCTGATCAATGAATGCCAAAGTGCCTGTTTTCATAACGTTTACTGTTAATATACTGAATA
TATGCAAAATCAGATGGCTGTATGGCCATAAGCAGACAATTGCATTATGTTTTTAAAGCMA
ACGCTGACACGTTTATGCTGTTTACTTTG

B)

MASNSLFSSVAPCQQNFFWGKSFTLTITVFTNPPQVATYHRAIKVTVDGPREPRRHRQKLDDS
KPSLFSERLSDLGRIPHPSMRVGVPTQSPRPSLNSAPSPFNPQGQSQTADPRQAQSSPPWSYD
QSYPAYLSQMTSPSIHSTTPLSSSRGTGLPAITDVPRRLSGASELGPFSDPRQFTSISSLTES
RFSNQRMHYPTTFTYTPPVTSAMSLGMSAXTHYHTYLPPPYPGSSQNQSGHFQTSSTPYLYYG
TSSGSYQFPMVPGGDRSPSRMLPPCTTTSNGGTLINPNLSNQNDGVDADGSHSSSPTILNSSG
RMGESVWRPY

C)

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1    MASNSLFSSVAPCQQNFFW-----------------------------------------    Ambystoma mexicanum
1    MASNSLFSTVTPCQQNFFWDPSTSRRFSPPSSSLQPGKMSDVSPVVAAQQQQQQQQQQQQ    Homo sapens
1    MASNSLFSSVTPCQQNFFWDPSTSRRFSPPSSSLQPGKMSEVSPVVVAQQQQQQQQQQQQ    Galus galus
1    MASNSLFSAVTPCQQSFFWDPSTSRRFSPPSSSLQPGKMSDVSPVVAAQQQQQQQQQQQQ    Mus musculus

20   ------------------------------------------------------------    Ambystoma mexicanum
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61   E---------------------------------AAVPRLR-PHDNRTMVEIIADHPAEL    Galus galus
61   QQQQQQQQQQQQQQQQEAAAAAAAAAAAAAAAAAAAVPRLRPPHDNRTMVEIIADHPAEL    Mus musculus

20   ------------------------------------------------------------    Ambystoma mexicanum
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87   VRTDSPNFLCSVLPSHWRCNKTLPVAFKVVALGEVPDGTVVTVMAGNDENYSAELRNASA    Galus galus
121  VRTDSPNFLCSVLPSHWRCNKTLPVAFKVVALGEVPDGTVVTVMAGNDENYSAELRNASA    Mus musculus

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147  VMKNQVARFNDLRFVGRSGRGKSFTLTITVLTNPPQVATYHRAIKVTVDGPREPRRHRQK    Galus galus
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234  LDDSKPSLFSDRLSDLGRIPHPSMRVGVPPQNPRPSLNSAPSPFNPQGQSQITDPRQAQS    Homo sapens
207  LDDSKPSLFPERLSDLGRIPHPSMRVGVPTQSPRPSLNSAPSPFNPQGQSQITDPRQAQS    Galus galus
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267  SPPWSYDQSYPSYLSQMTSPSIHSTTPLSSTRGTGLPAITDVPRRLS-------------    Galus galus
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314  ---------GASELGPFSDPRQFTSISSLTESRFSNPRMHYPATFTYTPPVTSGMSLGMS    Galus galus
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218  AXTHYHTYLPPPYPGSSQNQSGHFQTSSTPYLYYGTSSGSYQFPMVPGGDRSPSRMLPPC    Ambystoma mexicanum
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365  ATTHYHTYLPPPYPGSSQNQSGPFQTSSTPYLYYGTSSGSYQFPMVPGGDRSPSRMLPPC    Galus galus
421  ATTHYHTYLPPPYPGSSQSQSGPFQTSSTPYLYYGTSSASYQFPMVPGGDRSPSRMVPPC    Mus musculus

278  TTTSNGGTLINPNLSNQNDGVDADGSHSSSPTILNSSGRMGESVWRPY    Ambystoma mexicanum
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425  TTTSNGSTLLNPNLPNQSDGVEADGSHSSSPTVLNSSGRMDESVWRPY    Galus galus
481  TTTSNGSTLLNPNLPNQNDGVDADGSHSSSPTVLNSSGRMDESVWRPY    Mus musculus
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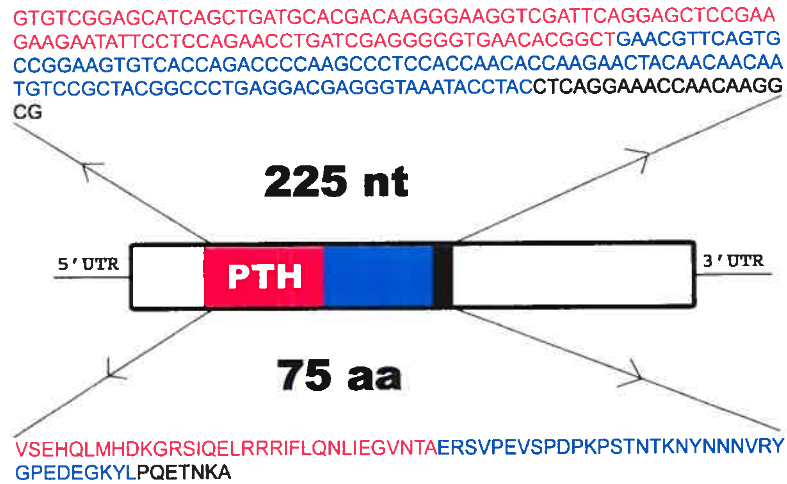
D)

Mus musculus
Rattus norvegicus
Bos taurus
Homo sapiens
Gallus gallus
Ambystoma mexicanum
Danio rerio
Takifugu rubripes
Xenopus

Figure 2. Analysis of the axolotl *PTHrP* sequence. (A) cDNA and corresponding amino acid sequence with respect to the full length sequence. Red characters indicate partial PTH-like domain. Amalgamation of blue and red characters indicate partial Parathyroid domain. (B) Amino acid alignment of *PTHrP* from multiple model organisms used for developmental and biomedical research (mice, human, chicken and axolotl). Red box shows the PTH-like domain and the combined red and blue boxes the Parathyroid domain. (C) Phylogenetic tree. Partial axolotl cDNA was compared to *PTHrP* ORF sequences from other species. [GenBank accession number DQ885239]

A)



B)

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1   MMFTKLFQQWSFAVFLLSYSVPSYGRSVEGISRRLKRAVSEHQLLHDKGKSIQDLRRRIF  Gallus gallus
1   -MLRRLVQQWSVLVFLLSYSVPSRGRSVEGLGRRLKRAVSEHQLLHDKGKSIQDLRRRFF  Mus musculus

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61  LQNLIEGVNTAEIRATSEVSPNPKPATNTKNYP--VRFGSEDEGRYLTQETNKSQTYKEQ  Gallus gallus
60  LHHLIAEIHTAEIRATSEVSPNSKPAPNTKNHP--VRFGSDDEGRYLTQETNKVETYKEQ  Mus musculus

75                                                                Ambystoma mexicanum
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119 PLKVSGKKKKAKPGKRKEQEKKKRRARSAWLNSGMYGSNVTESPVLDNSVTTHNHILR    Gallus gallus
118 PLKTPGKKKKGKPGKRREQEKKKRRTRSAWPST--AASGLLEDPLPHTSRTSLEPSLRTH  Mus musculus
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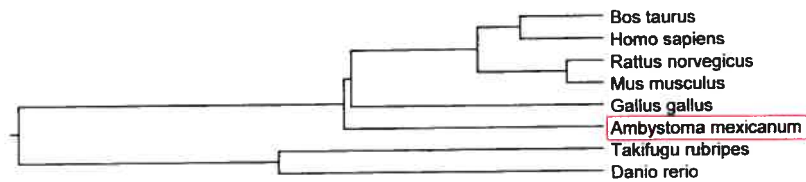
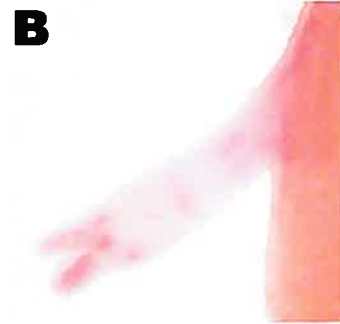
C)



Whole-mount in situ analysis of Cbfa-1 and PTHrP expression during limb development

Whole-mount in situ hybridization was used to determine the expression pattern of *Cbfa-1* and *PTHrP* at different developmental stages of the limb in axolotl embryos using RNA anti-sense probes (Fig. 3). Victoria blue was used to stain skeletal elements at different developmental stages of the limb in order to compare *Cbfa-1* and *PTHrP* expression with the appearance of skeletal elements. Type II collagen protein expression was determined by whole-mount in situ immunohistochemistry to further demarcate the skeleton in developing limbs. Cells in permanent cartilage express type II collagen in prehypertropic chondrocytes (40, 44) but do not express *Cbfa-1* (10, 17, 31, 33, 35, 69). The type II collagen results provide confirmation that the *Cbfa-1* expression observed is specific as they do not overlap. *Cbfa-1* expression is observed throughout the developmental process of the axolotl limb (Fig. 3A-C). Forelimb development in the axolotl begins at stages 44-45 (49) where it is possible to distinguish prominent budding containing some *Cbfa-1* expression (data not shown). At stage 48, weak expression is observed at the base of the limb and in the mid region of the extremity. In the distal region, two strong expression points are noticeable (Fig. 3A). Comparatively, it is possible to distinguish a completely formed humerus and a budding radius at the tip of the same stage (Fig. 3G). With the extension of the radius and the ulna in place, by stage 50, carpal skeletal structures within the hand and the metacarpals of the first two digits are in formation (Fig. 3H). Two *Cbfa-1* expression regions corresponding to the earliest forming digits can be observed as well as expression points in the carpal region and weak expression in the mid limb region (Fig 3B). Reaching stage 52, *Cbfa-1* expression is restricted solely within the diaphysis of the phalanges showing a particularly strong signal in the digit still in formation (Fig. 3C). At the same time, cartilage staining shows that by this stage, the first two digits are complete and there is evidence for the formation of digit III (Fig. 3I). Also, type II collagen staining in the later stages of limb development displays differential expression to *Cbfa-1*, further substantiating our whole-mount results (Fig. 3D-F). *PTHrP* expression was

Figure 3. Whole-mount in situ hybridization, immunohistochemistry and cartilage staining in developing limbs for *Cbfa-1*, type II collagen, cartilage and *PTHrP*. (A-C) *Cbfa-1* expression, (D-F) Type II collagen protein expression, (G-I) Cartilage staining with Victoria blue, and (J-L) *PTHrP* expression. Developing limbs at stage 48 (A, D, G), Stage 50 (B, E, H, K) and Stage 52 (C, F, I, L). *PTHrP* control limb using a sense probe (J). Black arrow indicates humerus. Note that axolotl embryos were photographed anterior to the top and posterior to the bottom.

A

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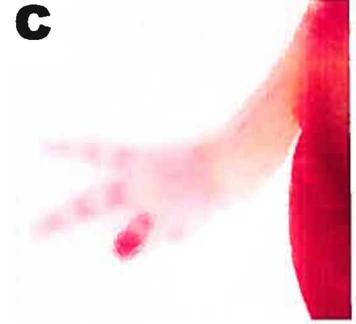
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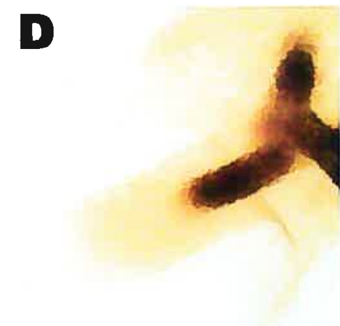
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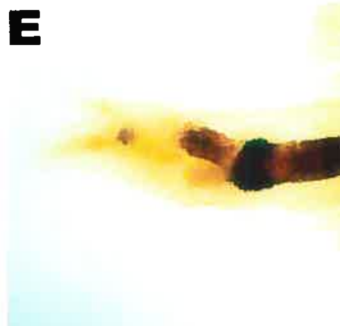
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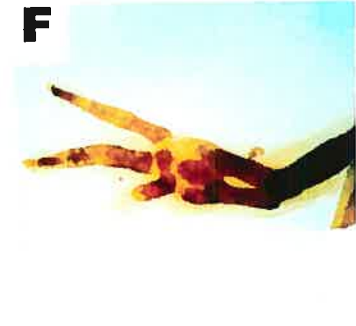
F

G

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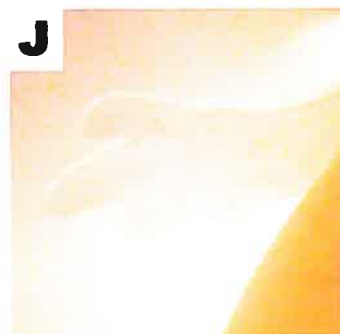
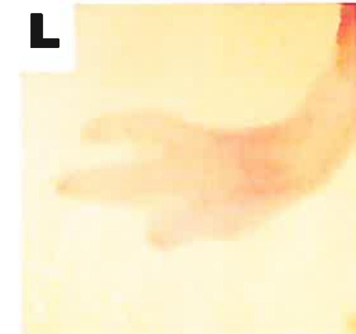
I

J

K

L

also verified during limb development (Fig. 3J-L). Expression remained undetected until stage 50, where expression is observed in the tips of the more developed digits and in the carpal region (Fig. 3K). Stage 52 shows a similar expression pattern, though a little more pronounced (Fig. 3L). Since the *PTHrP* expression detection during these stages is weak, a sense probe was used as a control (Fig. 3J) to confirm that the signal detected was specific.

Whole-mount in situ analysis of Cbfa-1 and PTHrP expression during limb regeneration

Limbs were amputated proximally (mid-humerus) and whole-mount in situ hybridization was used to detect *Cbfa-1* and *PTHrP* expression at different stages of regeneration. We used the Victoria blue staining technique for cartilage and type II collagen immunohistochemistry to characterize the developing skeleton during limb regeneration in order to compare this process with *Cbfa-1* and *PTHrP* expression. There was no visible *Cbfa-1* signal in the first stages of regeneration, specifically 6h, 24h, and 48h after amputation (data not shown). The absence of expression is not surprising since at these stages the process of chondrogenesis has not yet begun (70). At the early bud and medium bud stages, Victoria blue staining shows no sign of cartilaginous structures in the blastema, only the skeletal element in the stump is detected (Fig. 4K, L). The first appearance of *Cbfa-1* expression in the blastema occurs at medium bud (Fig. 4B). At the late bud stage, new cartilage is formed at the end of the amputated bone (proximal portion of the blastema) as resolved by both Victoria blue staining and type II collagen expression (Fig. 4H, M) and a *Cbfa*-1 signal is detected in the distal region of the blastema (Fig. 4C). Accordingly, this is the stage when cartilage differentiation begins in the periosteum of the amputated bone (70). Kim et al. have previously shown that *Cbfa-1* is localized in the growth plates of long bones during development (33). Interestingly, a growth plate is also formed during regeneration (70). As the limb continues to regenerate, the *Cbfa-1* signal becomes more restricted to the distal part of the regenerating blastema at the palette stage (Fig. 4D) and is concentrated in areas of future developing digits

Figure 4. Whole-mount in situ hybridization, immunohistochemistry and cartilage staining of regenerating limbs for *Cbfa-1*, type II collagen, cartilage and *PTHrP*. (A-E) *Cbfa-1* expression, (F-J) type II collagen staining, (K-O) cartilage staining with Victoria blue and (P-T) *PTHrP* expression in proximal amputations during forelimb regeneration. Regeneration stages are: (A, K, P) Early bud stage, (B, G, L, Q) Medium bud stage, (C, H, M, R) Late bud stage, (D, I, N, S) Palette stage and (E, J, O, T) Early differentiation stage. A negative control limb for type II collagen expression is illustrated in (F). Note that axolotl regeneration stages are characterized according to Tank et al. (70).

A

B

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D

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O

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Q

R

S

T

similar to the expression pattern observed during limb development (Fig. 3A-C). Type II collagen expression at this stage shows a differential expression pattern to that of *Cbfa-1* (Fig. 4I). The palette stage designates the beginning of cartilage differentiation in the zeugopod of the limb and in bones of the forelimb (70). This is illustrated in figure 4N where cartilage staining allows us to see a rough outline of the radius and ulna. At the early differentiation stage, the first digits have completely formed and the third digit already has two metacarpals (Fig 4O). At this stage, the *Cbfa-1* signal shows specific expression points in the diaphysis of the phalanges and a particularly strong signal in the digit still in formation (Fig. 4E). As was observed in development, type II collagen expression at the early differentiation stage of regeneration corresponded to the appearance of cartilage (Fig. 4O) and did not overlap with the domains of *Cbfa-1* expression (Fig. 4E, J).

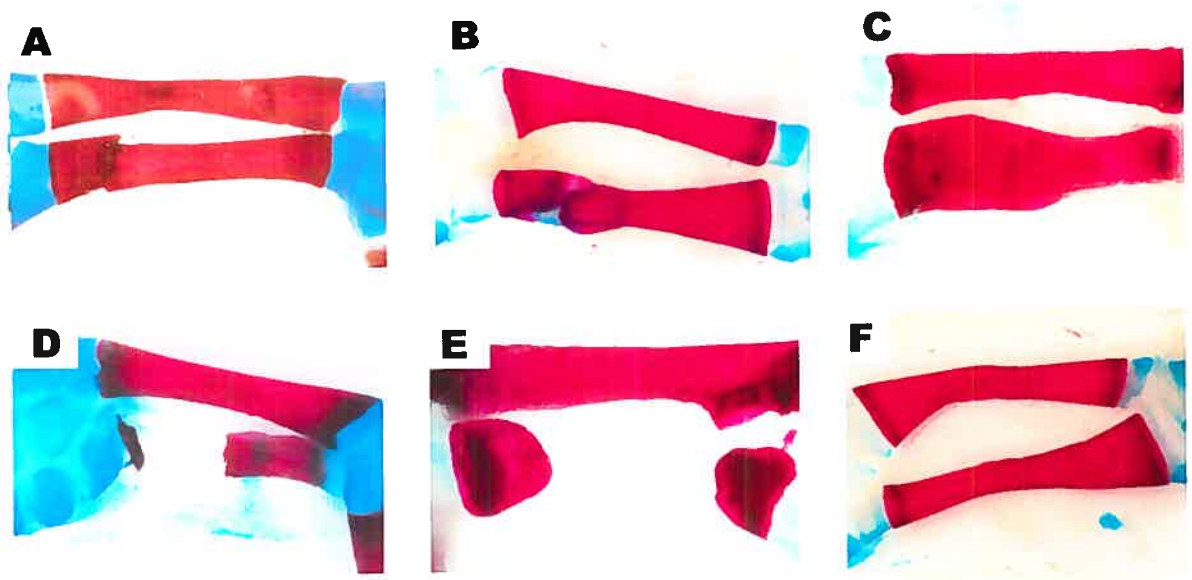
PTHrP expression was also characterized during axolotl limb regeneration (Fig. 4P-T). In the early stages of regeneration (6h, 24h, and 48h after amputation), *PTHrP* showed no visible signal of expression (data not shown). However, from the early bud to the late bud stage, strong expression is detected in the regenerating blastema (Fig. 4P-R). At the palette stage, expression is localized to the digit forming region (Fig. 4S) corresponding to formation of the first two digits. Cartilage staining at the early differentiation stage is characterized by the differentiation of the digits (Fig. 4O) where *PTHrP* expression can be detected around the contours of the future digits of the limb (Fig. 4T).

Bone fracture and critical gap healing in axolotl limbs

As previously mentioned, most vertebrates are able to heal bone fractures (6, 19, 61, 65, 72) yet all vertebrates studied to date are incapable of healing bone defects of critical dimension (62). Therefore, since axolotls are capable of regenerating entire limb structures including the skeletal elements without any resulting scar or demarcation lines between the stump and the regenerated tissues, we questioned whether the axolotl is also able to heal bone defects of critical dimension. The results for the non-stabilized union and non-union fractures of

critical dimension are shown in figure 5. A clear-cut union fracture is shown for the limb 15 days after fracturing (Fig. 5A) with no visible signs of healing. At 45 days post-fracture, the two extremities of the fractured bone are unhinged and a blue halo around the fracture site is noticeable (Fig. 5B). This indicates that cartilaginous cells have migrated to the fracture site and that the healing process has been initiated. After two months, the extremities of the fractured bones are joined together by a cartilaginous matrix which will eventually form a soft callus around the fracture site (results not shown). Five months post-fracture, the soft callus is completely replaced by an envelope of calcified matrix that permanently joins the two bone extremities, otherwise known as a hard callus (Figure 5C shows a completely healed bone fracture after seven months). Figure 5D shows a non-union fracture 15 days after fracturing. Unlike the non-stabilized union fractures, staining results in the following months show no sign of cartilage at the fracture site (results not shown). Furthermore, during this period there is no evidence that a healing process via a cartilaginous phase has been triggered at the fracture site. Finally, seven months post-surgery, the bones extremities are slightly rounded and there are still no signs of a healing process or bone regeneration (Fig. 5E).

Figure 5. Double staining of non-stabilized union fractures. (A-C) and non-stabilized non-union fractures (D, E) on axolotl forelimbs. (A, D) 15 days after surgery, (B) 1½ months post-fracture, (C, E) 7 months post-surgery and (F) sham operated forelimb.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2.5 Discussion

Cbfa-1 and PTHrP expression during limb development and regeneration

In this study, we describe for the first time the cloning, analysis and expression of axolotl *Cbfa-1* and *PTHrP* genes during limb development and regeneration. We also describe the expression of the type II collagen during limb regeneration. Sequence analyses of both *Cbfa-1* and *PTHrP* show high homologies between the axolotl and other species. *Cbfa-1* is known to be expressed during mesenchymal cell condensation and to participate in the remodelling of cartilage and bone precursor cells (13, 53, 68), which could explain its expression during the early stages of limb development. In addition to its role in osteoblast differentiation, a function in limb patterning has been proposed to explain early *Cbfa-1* expression during development (14, 45, 53). In fact, null-mutant mice for *Cbfa-1* show structural defects of the skeleton and, in some cases, lacked scapulae and clavicle bones (46). It has been shown that *Cbfa-1* is expressed in hypertrophic chondrocytes and mature osteoblasts to regulate their maturation. These cells are mostly concentrated in the diaphysis of the bone (33) which correlates to *Cbfa-1* expression. To corroborate the *Cbfa-1* expression detected, type II collagen protein expression was characterized in the developing limb. Our results were comparable to those reported by Franssen et al. where they demonstrated that type II collagen expression is exclusive to condensing chondrocytes (20) and, more importantly for the present study, is absent in regions expressing *Cbfa-1* which confirms the expression data.

Unlike *Cbfa-1*, *PTHrP* expression was not detected until reaching the more advanced stages of limb development. It was not until stages 50 and 52 that a weak *PTHrP* signal was observed at the distal end of the developing digits and in the carpal region. Similar results were found in developing rat fetuses, the presence of detectable *PTHrP* mRNA being attributed to the proliferative activity of chondrocytes required for proper carpal and metacarpal formation (37, 54).

Cbfa-1 and *PTHrP* are also expressed during axolotl limb regeneration. During the first two-days following amputation, there is no detectable *Cbfa-1* or *PTHrP* signal. The lacking expression may be due to the absence of cell segregation and differentiation in the blastema during these stages (70). The first noticeable *Cbfa-1* expression is near the amputation site of the blastema at the medium stage. Interestingly, *Cbfa-1* action is not required until the replacement of chondrocytes by osteoblasts (46), events that do not occur until the late bud stage (70). Tank et al. described the differentiation of the cuff of periosteal cartilage at the amputation site around the time the medium bud stage occurs; these events take place before cartilage differentiation occurs within the blastema and could explain the expression results for of *Cbfa-1* at this stage of regeneration (70). Tank et al. also reported that during the palette stage of regeneration, there is overt differentiation of chondrocytes at the digital primordial; these findings correlate with the expression of *Cbfa-1* as a regulator of chondrocyte replacement by osteblasts (14).

Despite the weak expression of *PTHrP* found in developing axolotl limbs, expression during limb regeneration was strong. During regeneration, the blastema is subject to intense mitotic activity and total RNA quantities become significantly more important (73) thereby possibly explaining increased *PTHrP* levels. However, there are several alternative reasons to explain the increased *PTHrP* expression at the early bud stage. First, the perichondrium at the bone amputation site produces chondrocytes that accumulate around the existing bone, and *PTHrP* is known to play a role in chondrocyte proliferation (1, 36). Second, it has been proposed that during this stage, the blastema contains exclusively undifferentiated cells (70), interestingly, *PTHrP* expression has been associated with non-differentiated mesenchymal cells destined to become chondrocytes (37, 54). Third, *PTHrP* has been proposed to regulate *Ihh*, one of the molecules responsible for the differentiation of mesenchymal cells into chondrocytes (42, 72). Stark et al. reported that the newt *banded hedgehog (bhh)* (homologue of mammalian and avian *Ihh* (16)) was uniformly expressed in the blastema from the initial stages of regeneration. Interestingly, *N-bhh* expression was also dramatically reduced at the palette and early differentiation stages of regeneration (64) as is *PTHrP*. Apart from

the absence of *PTHrP* expression prior to blastema formation, *N-bhh* expression pattern correlates with that of *PTHrP* observed during axolotl limb regeneration (64). Studies on the regenerating deer antler have also shown *PTHrP* expression in the regenerating blastema, particularly in the dermis of the skin, perichondrium, underlying proliferating undifferentiated mesenchymal cells as well as in recently differentiated, non proliferating chondrocytes (18), all of which support the expanded expression found throughout the axolotl blastema.

In general, both *Cbfa-1* and *PTHrP* exhibited different expression patterns during limb development and regeneration which correlated with their own functions in other species. *Cbfa-1* expression pattern in late limb development is conserved with the late regeneration stages, supporting the hypothesis of a biphasic process consisting of an initial preparation phase that is followed by a re-development phase (23). It was also noted that *Cbfa-1* expression during limb regeneration is weaker than during limb development despite having a conserved expression pattern between both processes. The presence of more mature tissues and extracellular matrix could be an explanation for the difference in the intensity of *Cbfa-1* signal. On the other hand, the expression pattern of *PTHrP* was different between limb development and regeneration, by its intensity but also by the localization of the signal, thus suggesting a possible dual function of this gene during these processes (23). Overall, the data for these 2 genes supports the hypothesis that limb regeneration is a biphasic process. Recent research has shown that there are corresponding sets of early and late genes associated with each phase (23). Of particular interest, events during the preparation phase of regeneration differ significantly from what is observed during limb development (23, 47, 48, 60) whereas the mechanisms for controlling growth and pattern formation in the blastema are recapitulated during development (23, 48, 58, 59, 71). These findings are supported by the expression patterns observed for *Cbfa-1* during regeneration and development, being differentially expressed throughout the early stages and having nearly identical expression patterns in the later stages. The different *PTHrP* expression observed during the early stages of development and regeneration can also be rationalized according to Muneoka's findings (48); however to support the

notion of biphasic process, similar expression patterns in the later stages would be expected, as observed for *Cbfa-1*. The different *PTHrP* expression between development and regeneration can be attributed to its many physiological roles. *PTHrP* has been reported to regulate keratinocyte growth and differentiation (5, 28) suggesting that it could be an important mediator for skin growth and wound healing. In fact, the wound healing process shares numerous mechanisms with the preparation phase of regeneration (60). *PTHrP* has also been shown to participate in blastema formation during deer antler regeneration, particularly in the regenerating epithelium and in the mesenchymal cells of the blastema (55). These observations provide a plausible explanation for the pronounced expression of *PTHrP* during the preparation phase of regeneration and the differing expression patterns observed during the re-development phase of regeneration and the late stages of limb development.

Fracture healing of non-stabilized union and non-union fracture in the axolotl limb

Finally, we verified the axolotl's ability to heal union and non-union bone fractures in order to establish whether these animals can specifically or directly regenerate their bones. The results from this study demonstrate that the axolotl heals union fractures like any other vertebrates (72). Surprisingly, based on the regenerative capacity of these animals, no healing in the axolotls subjected to non-union fractures of critical dimension was observed, the gap length had not changed 7 months after injury (Fig. 5E). This leads us to conclude that the axolotl does not use the regeneration process to heal fractures. Previous experiments had shown that the adult axolotl is incapable of replacing a missing bone when this one was completely extirpated which is quite different than fracture healing (73). Studies pertaining to the healing mechanisms used by the axolotl to heal bone fractures are rare; though numerous hypotheses can be formulated to explain the reason this organism is unable to regenerate bone fractures. First, after fracturing, unlike limb amputation, there is no open wound, thereby inhibiting important epithial-mesenchymal interactions essential for the regeneration process (7, 23, 73).

Although this is a plausible explanation, it is not likely since the axolotl is able to regenerate missing parts of the heart after ablation (7). Another important event, essential for limb regeneration, is the large trauma to surrounding tissues that occurs after amputation. Goss (1969) demonstrated that if an axolotl limb is amputated directly through the plane where bone has previously been extirpated, the redeveloped section will contain half of the extracted bone, without the base of the bone being present (73). This experiment not only reveals the importance of a trauma factor, but also that cells composing the skeletal structures already in place in the amputated limb seem to have little implication in the regeneration process (48). This notion was further tested by Lheureux who demonstrated that after grafting a piece of skin (dermis and epidermis) on an irradiated limb, incapable of regeneration, there is complete regeneration of all structures aside muscle (38). These experiments demonstrate that the skin contains all the necessary components, excluding cells capable of forming muscles, to assure perfect limb regeneration (15, 38). Another important factor to consider when comparing bone healing and bone regeneration is the presence of dedifferentiated cells during the regeneration process. Cellular dedifferentiation is crucial to the regeneration process, distinguishing regenerating limbs from nonregenerating limbs (27), therefore the presence of dedifferentiated cells may be a determining factor in order to regenerate a bone fracture of critical dimension. Further studies will be needed for a better understanding of the genes involved in bone formation and fracture healing during axolotl limb regeneration in order to establish their requirements during this process.

Acknowledgements

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2.7 References

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CHAPITRE 3

Article 2: « Cloning and developmental expression of Sox genes in the axolotl and their regulation during limb regeneration»

Article in Review, Developmental Dynamics

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3.1 Contribution de l'auteur et déclaration des coauteurs

Contribution de l'auteur

Pour cet article, j'ai effectué toute les expériences pour le gène *SOX-10*, à l'exception de son clonage (RACE-PCR) qui a été effectué par Mathieu Lévesque. J'ai d'abord caractérisé son expression spatio-temporelle pour chaque stade embryonnaire du développement et pour chaque stade du membre en régénération par la technique d'hybridation *in situ* de type «Whole-mount». J'ai également effectué des immunobuvardages de type «Western» avec des anticorps dirigés contre *SOX-9* et *SOX-10* (stades du développement et de la régénération). Mireille Pilote a accompli le restant des résultats portant sur le gène *Sox-9*. Encore une fois, j'ai fait les analyses de séquences et j'ai rassemblé tous les résultats pour les gènes *SOX* afin de créer les figures et rédiger l'article.

Déclaration des coauteurs

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À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Cara Hutchison inclus cet article dans son mémoire de maitrise qui a pour titre «**Cloning and developmental expression of Sox genes in the axolotl and their regulation during limb regeneration**».

Mathieu Lévesque		8/11/06
Coauteur	Signature	Date
Mireille Pilote		8/11/06
Coauteur	Signature	Date

3.2 Abstract

In this paper, we report the cloning of axolotl *Sox-9* and *Sox-10* genes and the characterization of their expression during embryogenesis by whole mount *in situ* hybridization and western blot analysis. Cloning results show that both genes have significant homologies with other species, in addition to containing conserved HMG domains common to the Sox gene family. Both genes demonstrated regulated protein expression consistent with their respective RNA expression. Furthermore, these genes are expressed mainly in the neural crest and its derivatives, demonstrating comparable expression with other vertebrates. Results reveal that *Sox-9* and *Sox-10* have similar expression patterns during early development, though demonstrating differential expression as the embryo develops. In early embryogenesis, similar expression is observed particularly in the neural folds, prospective cephalic and trunk regions, otic placodes and somites. In later embryonic stages, *Sox-10* expression is restricted to the otic placodes, while *Sox-9* expression persists during skeletal formation in the cranial region and in developing limbs. In order to better understand the process of chondrogenesis during limb regeneration, we verified *Sox-9* expression pattern during limb development and regeneration. Previous limb regeneration studies suggest that new bone is generated from the dedifferentiated cells of the blastema and is not derived from pre-existing bone. We therefore used the Victoria blue staining technique and whole-mount *in situ* immunohistochemistry for the type II collagen protein to directly correlate *Sox-9* expression with chondrogenic events during these processes. Results show that *Sox-9* is expressed at the onset of chondrogenesis during limb development and during limb regeneration.

3.3 Introduction

Urodele amphibians, such as the axolotl (*Ambystoma mexicanum*), are the only vertebrates known to have the exceptional ability to regenerate complete body parts following amputation throughout their life. The limb has been the most widely studied structure due to the presence of three defined axes and its ease of manipulation. Once amputated, the process of urodele limb regeneration goes through several phases including a dedifferentiation phase in which the mesenchymal cells proximal to the amputation site dedifferentiate to form a blastema [1, 2]. These cells will subsequently proliferate and eventually redifferentiate to form the lost parts of the appendage[3]. Limb regeneration is often considered to be a biphasic process, consisting of a preparation phase and a re-development phase. The preparation phase is unique to the regeneration process and is characterised by several events such as wound healing, cellular dedifferentiation and nerve dependency. The re-development phase is characterised by growth and pattern formation of the blastema [4-7]. Experimental data collected thus far supports the concept that during the re-development phase, the mechanisms controlling growth and pattern formation of limb regeneration are a recapitulation of those in developing limbs [8-11]. During limb development, cells that form limb tissues are derived from undifferentiated progenitor cells [12] whereas in the case of the regenerating limb, the process is similar except that differentiated cells become undifferentiated and then reform the missing parts following amputation [1, 12].

The complexity of the axolotl limb provides a unique model for regeneration research since it implicates various cellular types such as epithelial, muscle, connective tissue and bone. Bone represents approximately 50% of the exposed surface following limb amputation, however, as little as 2% of cells in a regenerated limb are derived from bone [8]. Previous limb regeneration studies also suggest that new bone is generated from dedifferentiated cells of the blastema and is not derived from pre-existing bone [13, 14]. Most skeletal bones, including those of the appendage, develop via an endochondral ossification process which involves

the replacement of cartilage tissue by bone. This process is initiated by the commitment of mesenchymal cells to cartilage cells which subsequently condense and differentiate into chondrocytes. Afterwards, the chondrocytes will proliferate, hypertrophy and finally be replaced by osteoblasts [15]. In order to understand the mechanisms underlying skeletal regeneration, it is important to characterise the expression of genes known for their role in the formation of skeletal elements during bone development. The *Sox-9* gene is an ideal candidate since it is a key regulator of chondrogenesis. It acts not only during the initial stages of chondrogenesis by stimulating mesenchymal condensations, but also acts to regulate the differentiation of chondrocytes to osteoblasts [16].

Sox-9 belongs to the *Sox* (*Sry-type* HMG *box*) family, a group of transcription factors that play diverse roles in many important developmental processes. These proteins contain a highly conserved DNA-binding domain known as the HMG (high mobility group) domain, comprising 79 amino acids [17] first identified in the mammalian *sex determination-related to Y chromosome* (*Sry*) protein [18-20]. Outside the HMG domain, *Sox* sequences are variable though having several other conserved domains which categorize these proteins into 10 subgroups (A-J). The *SoxE* genes (*Sox-8*, *Sox-9* and *Sox-10*) are known as important regulators of neural crest cells, thereby playing a central role in the development of the vertebrate body plan. However, in addition to having a role in early neural crest specification, *Sox-9* is also identified as a major regulator of chondrogenesis [21-24]. In humans, Sox-9 heterozygous mutations in one of the gene alleles are responsible of campomelic dysplasia (CD), a skeletal dysplasia that is characterized by sex-reversal and many skeletal malformations, particularly in the craniofacial region [24, 25]. Additionally, it has been demonstrated that *Sox-9* is co-expressed with *Col2a1*, the gene encoding for the major extracellular matrix component: type IIa collagen [23]. During development, *Sox-9* was shown to be expressed in early neural crest progenitors and is required for development of skeletogenic derivatives of the mammalian cranial neural crest [26, 27]. *Sox-9* knockdown experiments have shown that embryos lack endochondral skeletal elements derived from the cranial neural crest while neural crest cells in the trunk

appear to develop normally. This is due to an inability of the post-migrating cranial neural crest cells to differentiate into chondrocytes [28].

All of the SoxE genes are known to be expressed in neural crest progenitors though having differential expression depending on the species [29]. Generally, *Sox-9* expression is maintained in the migrating cranial neural crest cells and down regulated in the pre-migratroy trunk neural crest cells, while *Sox-10* expression persists in the trunk and diminishes in the branchial arches [26, 30]. *Sox-10* is recognised as a neural crest cell regulator. The first evidence for *Sox-10* in neural crest development was discovered from the neural crest phenotype observed in Dominant megacolon (*Dom*) mice, in which the *Sox-10* gene carries a frameshift mutant rendering the protein functionally inactive [31, 32]. These mutations have since been found in humans (Waardenburg-Shah disease), and zebrafish [31-34] who suffer from defects in the vagal/trunk neural crest derivatives (enteric ganglia, pigment cells and dorsal root ganglia) but not in cranial (craniofacial cartilages and bones) or cardiac neural crest derivatives [34]. Studies performed in the *Dom* mouse model have shown that mutants appear to have normal neural crest formation and that neural crest-derived embryonic structures are present, thus suggesting that *Sox-10* intervenes after the initial stages of neural crest specification [31, 32, 35, 36]. In fact, *Sox-10* is believed to have a role as a survival factor since in its absence, post-migratory neural crest cells that form and migrate undergo apoptosis before they are able to differentiate [32, 33, 37, 38].

In this article, we describe the molecular cloning, sequence analysis and expression of Sox-*9* and *Sox-10* in the axolotl during embryonic development. Expression patterns for both genes correlated with their respective functions and were similar to the expression patterns found in other species [26, 27, 30, 33, 39-42]. Recently, Tanaka's group have described the expression of *Sox-9* during tail regeneration [43]. We are therefore interested in the expression of *Sox-9* in the developing and regenerating limb. Since part of the regeneration process is a recapitulation of development, the regenerating axolotl limb offers an excellent model to study bone formation/regeneration. We used a whole-mount in situ immunohistochemistry technique to characterise the expression of type II collagen

(a cartilage specific protein) and the Victoria blue cartilage staining method in order to demarcate the developing skeleton during limb development and regeneration. Results reveal that the expression of *Sox-9* correlates with skeletal events during axolotl limb development and regeneration, thus suggesting a role for this gene in chondrogenesis during both processes.

3.4 Materials and methods

Cloning and sequence analysis of axolotl Sox-9 and Sox-10 cDNA

Partial axolotl *Sox-9* and *Sox-10 cDNA* sequences were cloned by RT-PCR using degenerate primers (see below). To obtain the full length *Sox-10* gene, the SMART RACE cDNA Amplification kit (BD Biosciences, Clontech, USA) was used to construct 5'- and 3'- RACE ready cDNA from total RNA extracted from the axolotl hind limb during the medium bud regeneration stage using Trizol reagent (Invitrogen). Following the manufacturer's instructions, PCR products were obtained using 5'- and 3'- gene specific primers (GSPs) and nested gene specific primers (NGSPs) designed from the partial sequence cloned in the lab. The designs of all primers were as follows:
dFSox: 5'-GA(C/T)CAGTA(C/T)CC(G/C)CA(C/T)CTGCA-3'
dRSox: 5'-TGCT(G/C)AG(C/T)TC(A/T/C/G)CC(A/G)ATGTCC-3'
5'GSP: 5'-GTGTGTGTTCTTCTAGTGTC-3'
5'NGSP: 5'-TCGGCCTCCTCTATAAAGG-3'
3' GSP: 5'-ACCGGTCACCCATGTCTGAT-3'
3' NGSP: 5'-AGCAGCAAACCACCCATTGA-3'
All PCR products were purified by gel extraction, cloned into the pCR II-TOPO sequencing vector and sequenced by the McGill Genome Sequencing Centre with M13R and M13F primers. Resulting sequences were assembled using SeqManII (DNASTAR Inc., USA). Full length *Sox-10* cDNA was subsequently amplified by PCR using [Axolotl forward SOX10 258] 5'-AGAGACAAAATGGCGGATGA-3' and [Axolotl reverse SOX10 1646] 5'-GCTCTCCGTCTTTCCCTCAG-3' primers designed from the sequencing data. The PCR reaction was performed using Taq DNA polymerase (Invitrogen) and the conditions were 30 cycles of 95°C, 30s; 64°C, 20s; 72°C, 45s; followed by 15 min at 72°C. All sequences were analysed on the NCBI BLAST database and phylogenetic trees were created using MegAlign (DNASTAR Inc., USA).

Animal maintenance and surgery

Axolotl embryos and larvae were purchased from the Ambystoma Genetic Stock Center (Lexington, KY). Animals are kept in a 20% Holtfreter's solution at a room temperature varying from 19-22°C and a photoperiod cycle of 12 hours of light and 12 hours of darkness. For amputations, animals were anaesthetized in 0.1% MS222 solution (ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, St. Louis, MO). Proximal (through humerus or femur) and distal (through radius/ulna or tibia/fibula) amputations were performed with micro-surgical tools (Fine Science Tools, B.C., Canada) on axolotls from 3 to 5 cm in length. For fixation, animals are euthanized and fixed in 1XMEMFA (10X MEM salt (1M MOPS pH 7.4, 20mM EGTA, 10mM $MgSO_4$), 3,7% formaldehyde and DEPC treated H_2O then gradually transferred to a methanol solution and stored at -20°C until needed. All animal care and experiments were done in accordance with the Université de Montréal animal care committee's guidelines.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described in Gardiner *et al.* with a few modifications [44]. Digoxigenin (DIG) –labeled antisens RNA probes were created using T7 RNA polymerase (Invitrogen) and DIG RNA labelling mix (Roche Diagnostics, Laval, Quebec, Canada). For probe synthesis, a 458 bp *Sox-9* and a 334 bp *Sox-10* fragment were cloned into the pCR II-TOPO plasmid which was then linearized using the appropriate restriction enzymes. For tissue permeabilization, developing embryos and amputated limbs were incubated with 20μg/ml and 30 μg/ml proteinase K respectively for 15 min on ice and then at 37°C for 5 min or for 1h on ice then 1h at 37°C. Probe hybridization was 24h for embryos and 72h for limbs. Prehybridization and hybridization temperatures were done between 55°C and 65°C. Finally, BM purple (Roche) was used as the enzyme substrate for the colorimetric reaction for the alkaline phosphatase reaction.

Type II Collagen whole-mount in situ immunohistochemistry

Samples were rehydrated from methanol and rinsed three times in a phosphate-buffered saline solution with 0.1% Tween-20 (PTW). Limbs were then bathed in 2.5% trypsin for either 15 min (developing limbs) or 40 min (regenerating limbs) followed by three 5-min rinses in water. After 10 min in -20°C acetone, specimens were rinsed in water for 10 min and then washed three times for 5 min with PTW. Samples were then transferred to a blocking solution (phosphate-buffer solution (0.7 X PBS) + 1% DMSO + 5% sheep serum + 0.1% Tween) for 1 hour and incubated in a mouse monoclonal antibody against Type II collagen (II-II6B3, Lab Vision Corporation, Fremont, CA) diluted 1:100 in blocking solution overnight at 4°C. The next day, limbs were rinsed eight times for 15 min in a PTW + 1% DMSO solution before being placed in an anti-mouse horseradish peroxidase labelled secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted 1:500 in blocking solution overnight at 4°C. The following morning, samples were rinsed eight times for 30 min with PTW and subsequently bathed in a diaminobenzidine (DAB) solution (Invitrogen). The reaction was stopped by two 5-min washes with PTW and fixed in a formalin solution (3.7% formaldehyde in 0.7X PBS). Specimens were cleared through a graded series of ethanol/glycerol and stored in 100% glycerol. As a control for specificity, the same protocol was followed except the primary antibody was omitted (Fig. 7 F).

Skeletal staining of embryos and limbs

Staining was performed using the Victoria blue method to verify new cartilage formation [45]. Limbs were fixed in alcoholic Bouin's solution for 24 hours before being rinsed several times with 70% ethanol. Specimens were rinsed regularly in 3.5% NH_4OH for 2 days and subsequently treated with Acid alcohol for 2 hours. Specimens were stained with 1% Victoria Blue for 2 hours and then rinsed with 70% ethanol. Limbs were gradually dehydrated to 100% ethanol, then cleared and stored in methyl salicylate.

Western Blot

Total proteins of axolotl blastemas and embryos were extracted by sonification in sodium dodecyl sulphate (SDS) sample buffer. Proteins were denatured in boiling water for 5 min and 50 μg per lane was loaded on 12% polyacrylamide-SDS gels following the Laemmli method [46]. Transfer of proteins onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) was done by electrophoresis. Anti-Sox9 and Anti-Sox10 rabbit affinity isolated antibodies (Sigma-Aldrich), which specifically recognize their respectful proteins, were used to detect axolotl *Sox-9* and *Sox-10.* Immunodetection of primary antibodies was visualized using the ECL Western blotting kit (Amersham Biosciences, Piscataway, NJ) following manufacturer's guidelines. Coomasie blue staining of the proteins on the membrane was used for a loading control.

3.5 Results

Isolation and sequence analysis of axolotl Sox-9 and Sox-10 cDNA

A partial axolotl *Sox-9* sequence was obtained by isolating a 458 bp cDNA fragment from axolotl larvae total RNA by RT-PCR using degenerate primers. Using the NCBI BLAST database for sequence analysis, the isolated fragment was shown to contain a 171 bp sequence corresponding to around 75% of the conserved HMG domain (Fig. 1A). Moreover, results reveal that the isolated sequence is located towards the 5' of the gene and contains the endmost region of the HMG domain (Fig. 1B). Nucleotide alignment with other species showed that the partial axolotl *Sox-9* sequence shared 82% homology with chicken [47] and 81% with human [48], monkey [49] and frog [50]. A phylogenetic tree was created in order to compare the evolutionary relationships of the *Sox-9* gene between different species (Fig. 1C). Results indicate that the axolotl sequence is conserved with other species and is consistent with specie hierarchy.

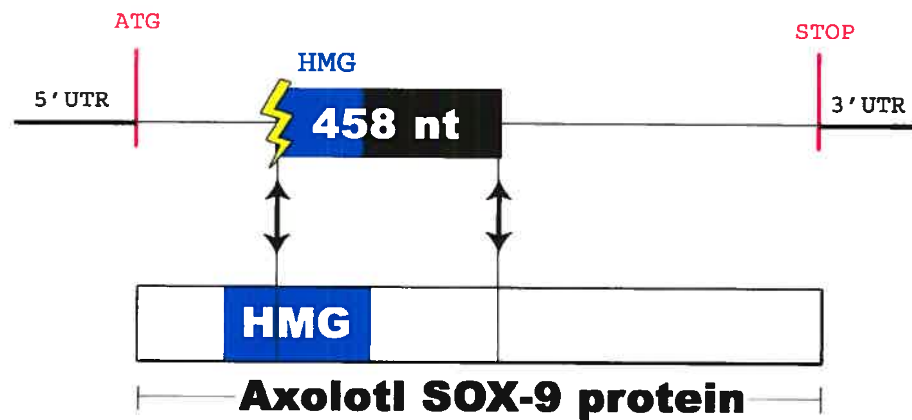
A 1725 bp contig was also analyzed using the NCBI BLAST database revealing the isolation of the axolotl *Sox-10* gene. Results show a 1341 bp ORF (Fig. 2A) which encodes a 447 aa protein containing an HMG domain of 79 aa (Fig. 2B). Analysis of the axolotl protein sequence indicates a high homology with other species. Axolotl *Sox-10* was shown to have a sequence of homology of 72% with chicken [51], 70% with human and xenopus [30, 34] and 69% with mouse [48]. The HMG region has a 94% homology with the HMG region of compared species. A phylogenetic tree is illustrated in figure 2C comparing the *Sox-10* protein between different species. The axolotl displays the closest relationships with chicken and xenopus, further substantiating the high homologies obtained for these species when compared to the axolotl *Sox-10* protein sequence.

Fig. 1. Sequence analysis of a partial axolotl *Sox-9* gene. (A) cDNA sequence. Blue characters represent conserved HMG domain. Character legend is as follows: W=A+T, Y=C+T, M=A+C, K=G+T (B) Schematic representation of the cloned sequence. (C) Phylogenetic tree. Partial axolotl cDNA was compared to the *Sox-9* complete cds sequences from other species. [GenBank accession number DQ901548]

A)

GAGCAGTATCCCCACCTGCACAWYGCCGTTCTCAGCAAGACCCTGGGAAAGCTGTGGAGG
CTTCTGAATGAGAACGACAAGCGCCCCTTTATAGAGGAGGCCGAGCGCCTSCGTACTCGC
CCACAAGAAGGACCACCCCGATTAMAAGTACCAGCCGCGCAGGCGCAAGAATGGCAAGGC
CACGACAGGAGAGGGAGAGGTGCCGGGCGAAGGCGACACGGTGCAGGGACACTACAAGAA
CACACACCTGGACCTGCGGCGTGCCGGCGACCGGTCACCCATGTCTGATGGGGCGTCAGA
ACACGGGGCAGGTCAGAGTCACGGGCCCCCTACGCCACCCACCACGCCCAAGACGGAGCT
GCAAAACGGCAAGGACCCCAAACGCGATGGCCGCYTACTKTTMGASAGCAGCAAACCACC
CATTGACTTCGGCAACGTGGACATCGGTGAGCTCAGCA

B)



C)

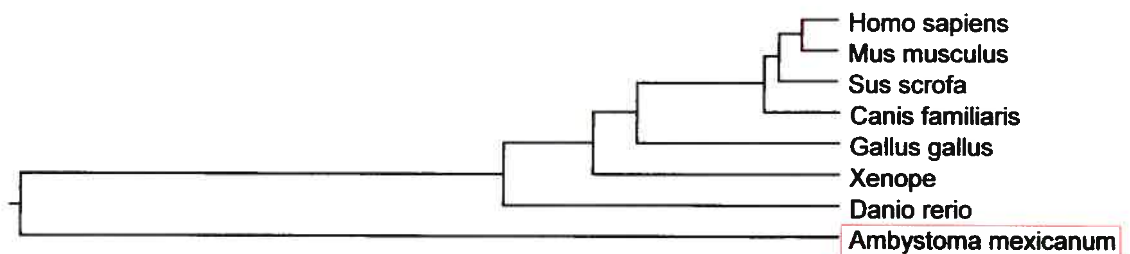


Fig. 2. Sequence analysis of the axolotl *Sox-10* gene. (A) cDNA sequence containing a 1341 bp ORF. Red characters indicate start and stop codons. Blue characters indicate conserved HMG domain. (B) Protein sequence of 447 aa containing a HMG domain of 79 aa. (C) Phylogenetic tree showing specie comparison of Sox-*10* protein sequence. [GenBank accession number DQ901549]

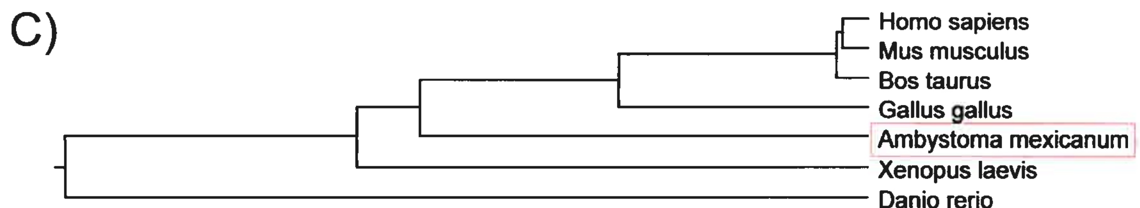
A)

GGAGGACCCCCTGTGCCCACACCCCTGACGCGGTTAGCCGAGAGAGAGAGGGGGACCGGTCAGTCGAAT
TAGAAGTGGGGCAGTGCAAGCAAGACCAGCTGTGAGGGGCAGGCCGTGTGCCCACCAGCAAGATCCAGG
AGGGGTCCCTGTGCCCACGCCGGAGCGAGCTGTCACAGGAGGGAGCCGATGAGAGCCAAGGCTGCATTC
CAATAGAAAGTGAACCAGACCACAACACTTTCAAGAAGACCACAGAGACAAAATGGCGGATGAGCAGAG
CCTTTCTGAGGTCGAGATGAGCCCGACGGGGTCCGAGGAGGAGCAGTGCCTGTCGCCAGGCACTTCAAC
GGCCTCGGATGGCCTGCGGCCTGGCAGCCCGAGTGACAATGACATGGCCAAGGTGAAGAAGGAGCTGGA
CTCAGAGACTGAGGACGAGAAGTTCCCCATTTGCATCCGCGAGGCCGTCAGCCAGGTGCTGAGTGGCTA
TGACTGGACGCTGGTGCCCATGCCCGTCCGCGTCAACGGCAGCAGCAAGTCCAAACCACACGTGAAGCG
GCCCATGAATGCCTTCATGGTCTGGGCGCAGGCGGCGCGCCGAAAACTGGCTGACCAGTACCCGCACCT
GCACAACGCCGAGCTCAGCAAGACCCTGGGAAAGCTGTGGAGGCTTCTGAATGAGAACGACAAGCGCCC
CTTTATAGAGGAGGCCGAGCGCCTGCGTATGCAGCACAAGAAGGACCACCCCGATTACAAGTACCAGCC
GCGCAGGCGCAAGAATGGCAAGGCCACGACAGGAGAGGGAGAGGTGCCGGGCGAAGGCGACACGGTGCA
GGGACACTACAAGAACACACACCTGGACCTGCGGCGTGCCGGCGACCGGTCACCCATGTCTGATGGGGC
GTCAGAACACGGGGCAGGTCAGAGTCACGGGCCCCCTACGCCACCCACCACGCCCAAGACGGAGCTGCA
AAACGGCAAGGACCCCAAACGCGATGGCCGCCCACTGGGAGAGAGCAGCAAACCACCCATTGACTTCGG
CAACGTGGACATCGGTGAGATCAGCCATGATGTCATGTCCAACATGGAGCCCTTTGATGTCAACGAGTT
CGACCAGTACCTGCCACCTAATGGCCACGGGGGCCATGCCAGCCACGTAGCTGGGAGCTACACCGGCGG
ATATGGCCTAGGCAGTGCCCTGGCAGCTGCCGCCAGCAGTGGAAGTCACTCTGCTTGGATTTCCAAGCC
GCATGGCGCTGCCTCACTAGCTGAATCAAAGGCCCAGGTTAAGACGGAGAGCTCCGGTGCCGGCCATTA
CCCGGAACAGTCTTCAGCCTCTCAGATCACCTATGCCTCACTTGGCCTGCCACATTATGGCTCAGCCTT
TCCTGCCATTCCCCGGCCACAGTTTGACTACTCAGATCATCAGCCATCTGGATCCTACTATAGCCACGG
CGGTCAGACCTCTGGCCTCTATTCTGCGTTCTCCTACATGGGACCATCCCAGCGCCCACTTTATACAGC
CCTCACTGAATCCAGTAGTGTGGCCCAGTCCCACAGCCCTACCCACTGGGAACAGCCTGTCTACACAAC
TCTGTCTAGGCCATGAAGGTGATGGGGAGCTGAGGGAAAGACGGAGAGCAATAGCCGTCTGAAGACTAA
GGAGTCTTCTTTCTGCGTAGACAATGGACATTGCGCGCTCCATCTAAACTCTACCACGTCTTCCTGTTC

B)

MADEQSLSEVEMSPTGSEEEQCLSPGTSTASDGLRPGSPSDNDMAKVKKELDSETEDEKFPICIREAVSQ
VLSGYDWTLVPMPVRVNGSSKSKPHVKRPMNAFMVWAQAARRKLADQYPHLHNAELSKTLGKLWRLLNEN
DKRPFIEEAERLRMQHKKDHPDYKYQPRRRKNGKATTGEGEVPGEGDTVQGHYKNTHLDLRRAGDRSPMS
DGASEHGAGQSHGPPTPPTTPKTELQNGKDPKRDGRPLGESSKPPIDFGNVDIGEISHDVMSNMEPFDVN
EFDQYLPPNGHGGHASHVAGSYTGGYGLGSALAAAASSGSHSAWISKPHGAASLAESKAQVKTESSGAGH
YPEQSSASQITYASLGLPHYGSAFPAIPRPQFDYSDHQPSGSYYSHGGQTSGLYSAFSYMGPSQRPLYTA
LTESSSVAQSHSPTHWEQPVYTTLSRP

C)



Analysis of Sox-9 and Sox-10 expression during embryonic development

Whole mount *in situ* hybridization was used to characterise the gene expression of *Sox-9* and *Sox-10* in the axolotl during embryonic development using RNA anti-sense probes (Figs. 3 and 4). Results indicate that *Sox-9* expression is absent in the most precocious stages of embryogenesis, showing no expression during the gastrula stage (Fig. 3A). The signal was first detected at mid-neurulation on the neural folds of the neural crest forming region (Fig. 3B). This signal intensified with neural crest formation showing expression along the neural tube and in the anterior region of the embryo, corresponding to the prospective cephalic and trunk regions (Fig. 3C). At stage 26, *Sox-9* expression is maintained in the anterior region of the embryo (Fig. 3D), corresponding to the areas in which neural crest cells migrate in order to participate in the formation of various structures such as the otic placodes, branchial arches and craniofacial skeletal element [26, 28, 52]. Effectively, as the embryo develops, expression is detected in these structures. Figure 3E shows a stage 33 embryo with specific expression in the otic placodes. By stage 36, additional expression is observed along the spinal cord in regions corresponding to somites, in the orbital cavities, branchial arches and the developing mandible (Fig. 3F,G). At later stages of embryogenesis, *Sox-9* expression is concentrated to skeletal elements, showing a particularly strong signal in the cranial bone ridge and in the lower jaw (Fig. 3H). Finally, besides the weak expression remaining in the cranial region at stage 46 (Fig. 3I), expression is also detected in the limb, where skeletal formation is just beginning.

Sox-10 expression during embryogenesis exhibited a similar pattern to *Sox-9* with a few differences (Fig 4). *Sox-10* expression is detected during the mid-neurulation stage, having a signal confined to neural folds of the neural crest forming region (Fig. 4A). This expression is analogous to that observed for *Sox-9* at this stage, though the *Sox-10* signal seems to be more widespread. At the late neurula stage, expression is observed in the prospective trunk and cephalic regions, again showing similar expression to *Sox-9* (Fig 4B,C). By stage 25, specific expression is present in the otic placodes and weak expression along the spinal cord

Fig. 3. Whole-mount in situ hybridization of *Sox-9* expression during embryonic development. (A) Stage 11 embryo. (B) Dorsal view of a stage 16 embryo. (C) Dorsal view of a stage 22 embryo. (D) Lateral view of a stage 26 embryo. (E) Lateral view of a stage 33 embryo. Lateral (F) and lateral rostral (G) view of a stage 39 embryo. (H) Stage 43 embryo. (I) Dorsal view of a stage 46 embryo. Stages were characterised according to Armstrong *et al*. [53].

A

B

C

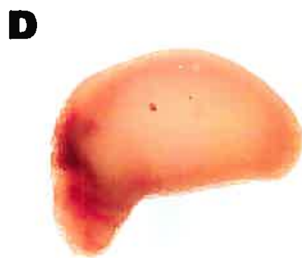

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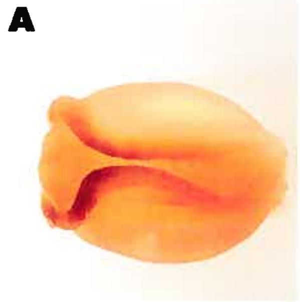
Fig. 4. Whole-mount in situ hybridization of *Sox-10* expression during embryonic development. (A) Dorsal view of a stage 16 embryo. Lateral (B) and dorsal (C) view of an embryo at stage 22. Lateral (D) and dorsal (E) view of a stage 25 embryo. (F) Dorsal view of a stage 31 embryo. Lateral view of a stage 36 embryo (G) and a lateral rostral view at stage 41 (H). Stage 47 embryo (I). Note that axolotl embryos were photographed anterior to the left and posterior to the right. Stages were characterised according to Armstrong *et al*. [53].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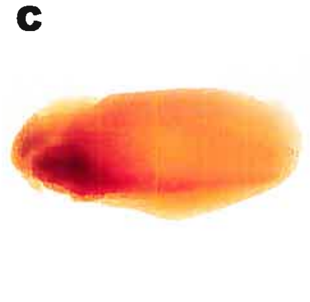
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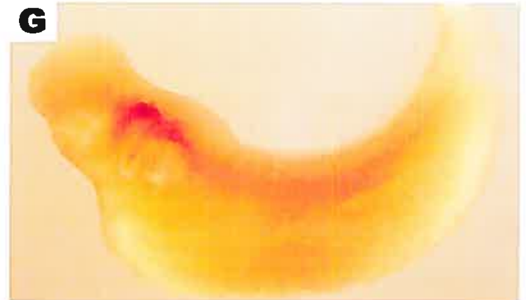
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can be observed (Fig 4D,E). At stage 31, expression is maintained in the otic placodes in addition to having expression in the somites along the neural tube (Fig. 4F). Contrary to *Sox-9*, *Sox-10* signal diminished significantly in the later stages of development becoming restricted to the otic placodes (Fig 4G,H). By stage 49, there was no *Sox-10* signal detected (Fig. 4I).

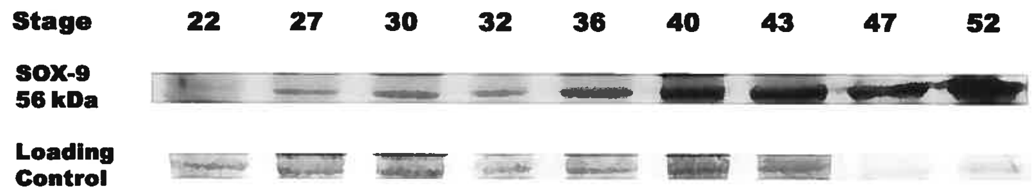
To further corroborate our results, we verified *Sox-9* and *Sox-10* protein expression in the axolotl during embryonic development. Using antibodies raised against the human *Sox-9* and *Sox-10* proteins, we were able to detect a band of approximately 56 kDa and 50 kDa respectively (Fig. 5). *Sox-9* protein expression increased throughout development (Fig 5A). *Sox-10* protein expression was strong during early embryogenesis and decreased gradually in the final stages until no expression was observed (Fig. 5B).

Analysis of Sox-9 expression during limb development and regeneration

In the final stages of the embryogenesis, we also looked at limb development. Whole mount *in situ* hybridization was used to characterise the expression of *Sox-9* RNA in the developing limb (Fig. 6A-C). In order to directly correlate the expression of *Sox-9* with chondrogenesis, type II collagen protein expression was determined by whole-mount in situ immunohistochemistry to demarcate the skeleton in developing limbs (Fig. 6D-F). A Victoria blue staining technique was also used to stain cartilage at different developmental stages of the limb (Fig. G-I). Results demonstrate that at stage 44, when the limb bud is only beginning to appear, there was no expression of *Sox-9* (data not shown). By stage 46, a strong signal in the whole limb bud can be observed and it is possible to distinguish initial cartilage formation in the limb bud (data not shown). At stage 47, the limb starts to take shape and flattens out in the distal region where *Sox-9* expression is particularly intense (Fig. 6A). Cartilage staining reveals a budding radius at the tip of a completely formed humerus (Fig. 6G) while type II collagen protein expression is restricted to the humerus (Fig. 6D). *Sox-9* expression correlates with skeletal elements, having a stronger expression in the regions still in

Fig. 5. Western blot analysis of *Sox-9* and *Sox-10* expression during embryonic development. Western blot analysis from embryonic stages 22 to 52 for *Sox-9* (A) *Sox-10* (B) protein expression. Coomasie blue staining of total axolotl proteins on the membrane was used for a loading control.

A)



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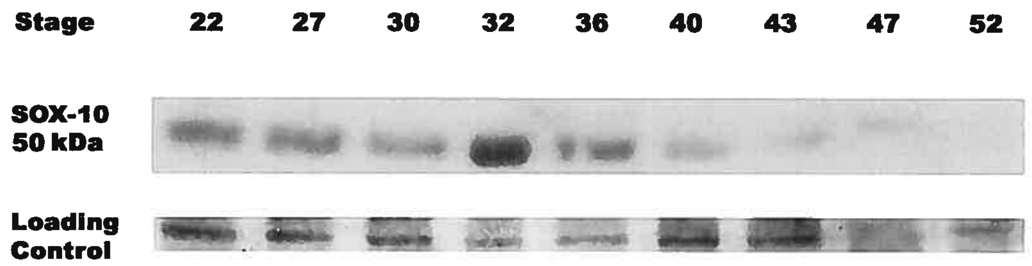
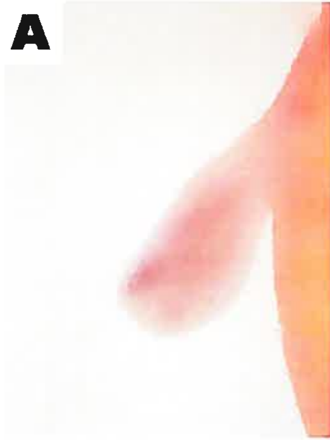


Fig. 6. Whole-mount in situ hybridization, immunohistochemistry and cartilage staining in developing limbs for *Sox-9*, type II collagen and cartilage. *Sox-9* expression (A-C) type II collagen protein expression (D-F) and cartilage staining with Victoria blue (G-I) in developing limbs at stage 47 (A,D,G), stage 50 (B,E,H) and stage 53 (C,F,I). Black arrow indicates humerus. Note that axolotl embryos were photographed anterior to the top and posterior to the bottom. Stages were characterised according to Nye *et al*. [2].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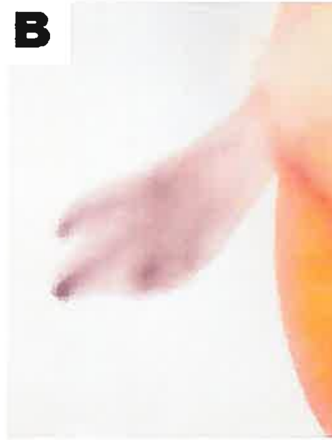
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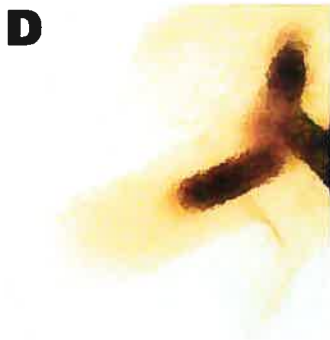
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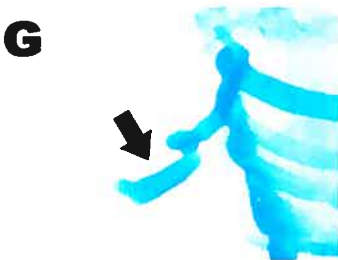
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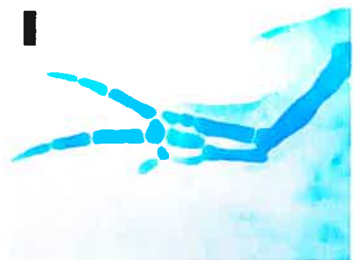
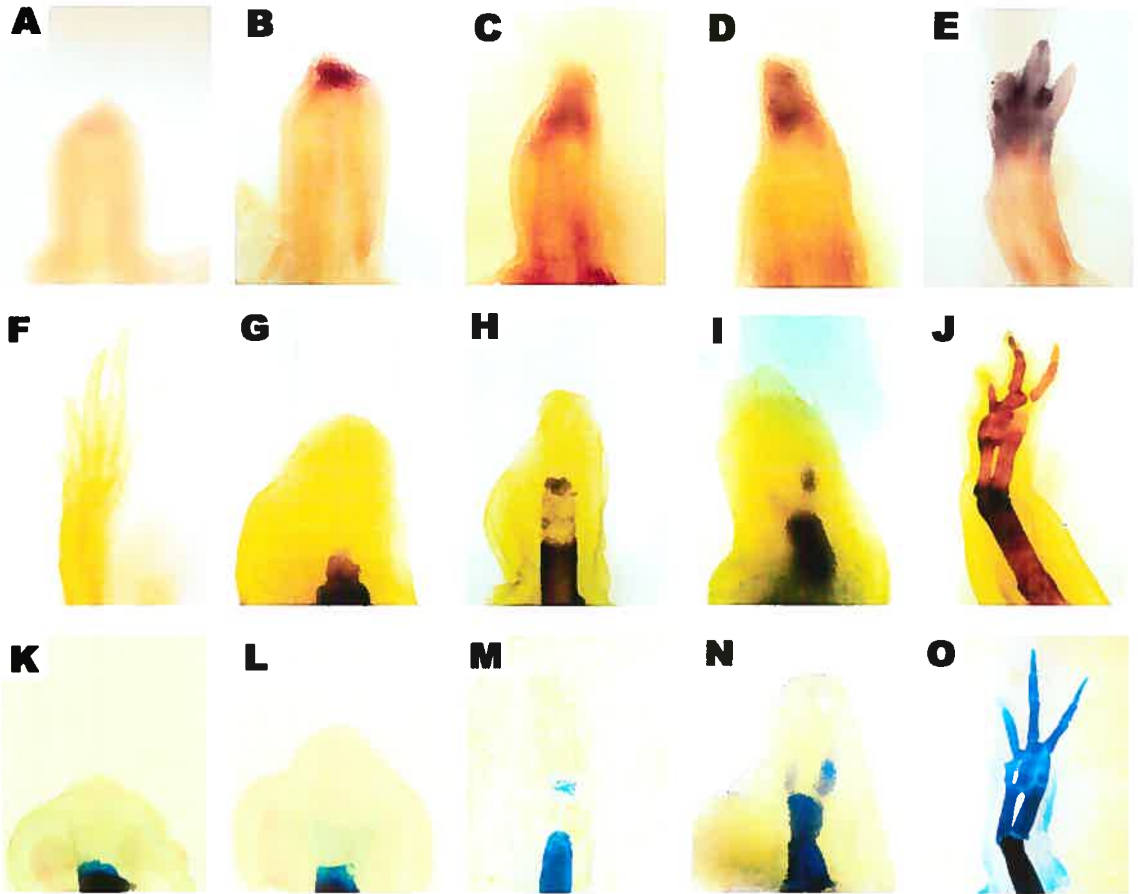

I

formation. With the extension of the radius and ulna, by stage 50, carpal skeletal structures within the hand and the phalanges of the first two digits are in formation (Fig. 6E,H). Again, the signal is more intense in the distal end of the limb, corresponding to the digit forming area (Fig. 6B). Nearing the final stages of limb development, *Sox-9* is expressed in the tip of the developing digits and in the metacarpal region (Fig 6C). Type II collagen expression and cartilage staining shows that by this stage, the first two digits are complete and there is evidence for the formation of digit III (Fig. 6F,I). As expected, the more developed digits (I and II) have a weaker signal than the one in development (III). At this stage the phalangial region is still in formation, thereby substantiating the *Sox-9* signal in this area.

Using the same techniques described for limb development, we characterised *Sox-9* expression and chondrogenesis during limb regeneration. Results show that there is no *Sox-9* expression detected during the first few days following amputation, specifically 6h, 24h, and 48h after amputation (data not shown). The absence of expression at these stages is not surprising since the process of chondrogenesis has not yet begun [3]. At the early bud stage, there is still no signal (Fig. 7A). At this stage, the humerus is stained blue and denotes the initial amputation site underlying the blastema (Fig. 7K). It is not until reaching the medium bud stage, approximately 12 days after the amputation, that we detect a strong *Sox-9* signal in the regenerating blastema (Fig. 7B). Both type II collagen expression and cartilage staining still show no signs of cartilaginous structures in the blastema (Fig. 7G,L), however the number of osteoclasts undergo a definite decline and the deposition of periosteal cartilage is initiated [3]. By the end of this stage, occasional small areas of cells alongside the bone have begun to produce a distinct cartilage matrix [3], thus providing a rationale for the *Sox-9* signal. The same expression pattern for *Sox-9* was present at the late bud stage which is characterised by the lengthening of the blastema (Fig. 7C). It is at the late bud stage that we are able to observe new cartilage formation at the distal tip of the amputated bone (Fig. 7H,M). Accordingly, this is the stage when cartilage differentiation begins in the periosteum of the amputated bone [3]. As the limb continues to

Fig. 7. Whole-mount in situ hybridization, immunohistochemistry and cartilage staining of regenerating limbs for *Sox-9*, type II collagen and cartilage. *Sox-9* expression (A-E) type II collagen protein expression (F-J) and cartilage staining with Victoria blue (K-O) at different stages of the regenerating forelimb after proximal amputation. Regeneration stages are: (A,K) Early bud stage. (B,G,L) Medium bud stage. (C,H,M) Late bud stage. (D,I,N) Palette stage. (E,J,O) Early differentiation stage. A negative control limb for type II collagen expression is illustrated in panel F. Note that axolotl regeneration stages are characterised according to Tank *et al.* [3]. Similar expression was observed in distal amputations for these stages.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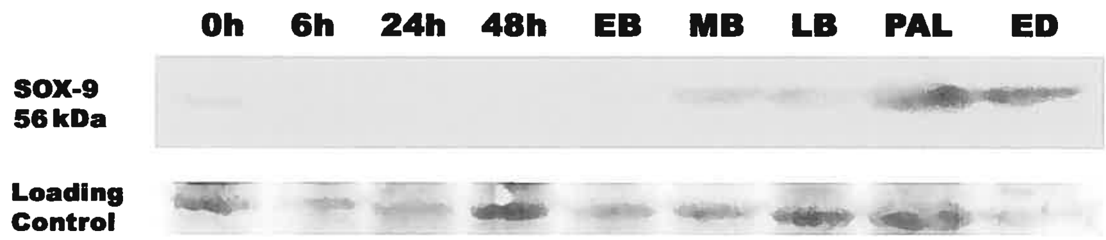
regenerate, a different pattern in *Sox-9* expression was observed at the palette stage (Fig 7D). The palette stage designates the beginning of cartilage differentiation in the zeugopod of the limb and in bones of the forelimb [3]. This is illustrated in figures 7I&N where type II collagen expression and cartilage staining reveals a rough outline of the radius, ulna and some metacarpals. Interestingly, while maintaining expression throughout the entire blastema, the *Sox*-9 signal is more intense at the outer extremities of the blastema, corresponding to areas of developing digits and elements of zeugopodial nature (Fig. 7D). The last stage of regeneration studied was the digit differentiation stage in which the first digits have formed and the third digit already has two phalanges formed (Fig 7J,O). Comparatively, at this stage the expression of *Sox-9* is restricted to the ridge of the growing digits and more intensely at the bottom of the interdigital space (Fig. 7E).

To further characterise the expression of *Sox-9* during limb regeneration, we verified protein expression by Western blot analysis. Figure 8 shows the detection of a 56 kDa protein corresponding to *Sox-9*. Results show that in the initial stages of regeneration, *Sox-9* protein expression is comparable to that of a mature limb (0h) whereas in final stages of regeneration the signal intensifies considerably. This is in accordance with RNA expression pattern observed during these stages of regeneration.

Fig. 8. Western blot analysis of *Sox-9* expression during limb regeneration. Western blot analysis showing the temporal expression and regulation of *Sox-9* protein during forelimb regeneration. The 0h represents mature non regenerating limb tissue. Coomasie blue staining of total axolotl proteins on the membrane was used for a loading control.


0h
6h
24h
48h
EB
MB
LB
PAL
ED
SOX-9
56 kDa
Loading
Control

2.5 Discussion

Cloning of Sox genes and their expression during embryogenesis

In this study, we describe the cloning, analysis and expression of axolotl *Sox-9* and *Sox-10* genes and their regulation during embryogenesis. Sequence analyses of the cloned sequences show; i) conserved HMG domains; and ii) high homologies with other species, thus validating their identities. Whole-mount *in situ* hybridization results reveal that during the early stages of embryogenesis, *Sox-9* is expressed in the anterior region of the neural folds and in the spinal chord. This is in accordance with the expression patterns detected by whole mount *in situ* hybridization performed in Xenopus [26, 54] and chick [52]. Furthermore, previous studies in other species have demonstrated that *Sox-9* is in fact required for neural crest development and differentiation [26, 40, 52, 55]. Eventually, Sox-9 expression becomes more localized to specific regions such as the otic placodes. Saint-Germain *et al.* have proposed that *Sox-9* is one of the key regulators of inner ear specification, demonstrating that *Sox-9* function is required for otic placode specification [54]. In the later stages of development, *Sox-9* expression is detected in cartilaginous structures in the head such as the mandible and other cranial bones (occipital, synotic). This expression is expected in cranial skeletal formation since *Sox-9* is primarily known for its extensive role throughout chondrogenesis, particularly during mesenchymal condensation and the regulation of chondrocyte differentiation [56-59]. Moreover, *Sox-9* null embryos have been shown to display multiple skeletal abnormalities including cranial defects, consistent with an important role in normal cranial development [26-28, 39].

The *Sox-10* expression detected during the early stages of embryogenesis is similar to that observed for *Sox-9* and is comparable with the expression patterns observed in Xenopus, chick and zebrafish [30, 33, 40, 41]. Expression detected in the neural and spinal regions is not surprising since: i) *Sox-10* has been previously shown to play a role in late neural crest development [32, 37, 38, 40] and ii) *Sox-10* mutant models suffer from defects in the vagal and trunk neural crest derivatives,

suggesting a strict requirement for this gene in the vagal and trunk neural crest [31-34]. In more advanced stages of development, *Sox-10* expression diminishes and is restricted to the otic placodes. *Sox-10* has been shown to be expressed in the otic placodes of multiple species [40, 42, 52, 60, 61] and has been proposed to play a particularly important role in inner ear development [61]. All together, the results observed for axolotl *Sox-9* and *Sox-10* gene expression during embryogenesis are similar to the expression found in other species and are in accordance with their respective roles during embryonic development.

Western blot analysis was performed to characterize *Sox-9* and *Sox-10* protein expression during embryogenesis. Results show that *Sox-9* protein expression increases throughout development. The augmented protein expression seen in the later stages of axolotl development can be attributed to the beginning of skeletal formation. The *Sox-9* RNA signal detected by whole-mount *in situ* hybridization is increasingly strong up until embryonic stage 43 (Fig. 3). Interestingly, forelimb development in the axolotl begins at stages 44-45 and is concurrent with initial cartilage formation of the limb [2]. As the limb continues to develop, new cartilage is formed (Fig. 4E-H) thus justifying the observed increase in *Sox-9* protein expression through to stage 52. Contrary to *Sox-9*, *Sox-10* protein expression is more pronounced early in embryogenesis. These results are consistent with the RNA expression detected during the same stages by whole-mount *in situ* hybridization. These results demonstrate that *Sox-9* and *Sox-10* proteins are expressed and regulated during axolotl embryonic development. Additionally, the protein expression detected for both genes during this process correlate with their respective RNA expressions.

Sox-9 expression during limb development and regeneration

Whole-mount *in situ* hybridization results confirmed that *Sox-9* is expressed during limb development and limb regeneration in the axolotl. During early limb development, expression is dispersed throughout the entire limb despite the fact that cartilage staining reveals the appearance of only a small bud of cartilage within

the limb. This expression pattern suggests that *Sox-9* is expressed in prechondrogenic mesoderm [62] and supports the role for this gene in the differentiation and aggregation of prechondrogenic precursors [58, 62]. Later during limb development, a stronger signal is detected in the distal region of the limb although the signal remains present in the whole limb bud. Skeletal staining results confirm that the more pronounced signal corresponds with the skeletal setting of limb bone (humerus, ulna/radius) [2]. Finally, towards the end of development, the *Sox-9* signal is present in regions corresponding to developing digits. A recent study by Franssen *et al.* investigated the expression of type II collagen protein and chondrogenesis during axolotl limb development. Results were similar to our own, revealing that type II collagen is expressed concomitantly with cartilage formation throughout limb development. Given that during chondrogenesis *Sox-9* is co-expressed with *Col2a1*, the gene encoding for type II collagen [23], our results together with the results obtained by Franssen et al. further supports the idea that *Sox-9* is in fact a precursor of cartilage formation in the developing axolotl limb.

Unlike embryonic and limb development, the regeneration process is not as well-studied; little is known regarding gene expression and function. Here, we report the expression pattern of *Sox-9* during axolotl limb regeneration. Results show that during the early phases of the regeneration process, there is no *Sox-9* expression detected. The lack of expression during these first days following amputation may be justified by the fact that *Sox-9* has no known function in the inflammatory or healing processes triggered following amputation [20]. *Sox-9* expression first appears at the medium bud stage. Tank *et al.* have reported this stage demarks beginning of cartilage deposition around the amputated bone which may explain the *Sox-9* expression detected. Accordingly, *Sox-9* has previously been shown to be essential for chondrocyte proliferation and maintenance during the entire chondrogenesis process in mice [56, 63]. At the late bud stage, a strong signal detected in the anterior region of the blastema can be explained by intense chondrocyte proliferation that occurs around the amputated bone [3]. During the palette stage, precursor cells differentiate into chondrocytes and it is likely that *Sox-*

9 is responsible for the differentiation and proliferation of prechondrocytic cells [58, 62, 63]. An interesting expression pattern for *Sox-9* is observed during the digit differentiation stage, particularly in the first interdigital space and at the tips of the developing digits. The same expression pattern is also observed in the developing limb of mouse and chick embryos [27, 56, 57, 62]. In these species, *Sox-9* expression at the end of the digits corresponds to zones where chondrogenesis is still active, an event required for the formation of normal-sized digits [57]. The *Sox-9* expression detected in the interdigital spaces may be due to *Bone morphogenetic protein*-2 (*BMP-2*) signaling. *BMP-2* belongs to the *TGF-β* superfamily and is widely recognized for its ability to stimulate bone formation and osteoblast differentiation [64]. Furthermore, *Sox-9* has been shown to be an important downstream mediator of the *BMP-2* signaling pathway in osteogenic cells [65]. Finally, it can be noted that the *Sox-9* expression is significantly less intense in the more mature digit. This may indicate that chondrogenesis is finished and that the chondrocytes in this digit have hypertrophied. Ng *et al.* have demonstrated that hypertrophic chondrocytes no longer express *Sox-9* [23]. Our Western blot confirms that *Sox-9* protein expression is weak in normal limbs and increases in the later stages of regeneration.

It is worth mentioning that we did not detect any *Sox-10* expression during limb development or during limb regeneration. This is not surprising since *Sox-10* is not recognized to have a role in skeletal formation or limb patterning. Current literature supports the concept that *Sox-10* expression is detected primarily in the peripheral nervous system and neural crest derived cells [30, 32, 33, 41, 42, 60] and shows no expression in developing limbs.

All together, our results suggest that *Sox-9* expression precedes the appearance of skeletal structures during axolotl limb development and regeneration. During limb regeneration, *Sox-9* is expressed in the last stage of the preparation phase, a process unique to the regeneration process that includes events such as wound healing and cellular dedifferentiation. Interestingly, *Sox-9* expression is predominantly detected in the late stages of limb regeneration (i.e. re-development phase). It has been reported that the molecular mechanisms of this phase are a

recapitulation of those used in developing limbs. Thus, this suggests a possible conserved mechanism for this gene in osteogenesis during limb development and late limb regeneration. Further studies will be needed for the characterization of the genes involved in bone regeneration during axolotl limb regeneration in order to establish their requirements during this process.

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CHAPITRE 4

Discussion

L'étude du phénomène de la régénération a fleuri considérablement pendant les deux derniers siècles. Présentement, la recherche sur la régénération tissulaire se fait dans toute une gamme d'espèces, du protozoaire à l'humain, parmi lesquelles on retrouve l'urodèle. Chez ce dernier, le membre est la structure la plus étudiée et en conséquence, les mécanismes de la mise en place de différents tissus et cellules lors du processus de développement et de la repousse du membre sont bien caractérisés. Parmi ces tissus, nous nous intéressons particulièrement au développement des structures squelettiques. L'étude des signaux qui régulent leur développement peut donner un aperçu aux mécanismes moléculaires qui pourront potentiellement aussi être impliqués dans la régénération des tissus squelettiques. Dans cette étude, nous avons réussi à cloner et à caractériser les gènes *Sox-9, Sox-10, PTHrP* et *Cbfa-1* de l'axolotl. Ces gènes sont tous reconnus pour leurs fonctions importantes lors du développement, particulièrement au niveau de la formation du squelette, chez les mammifères.

4.1 Expression de Sox-9 et Sox-10 durant l'embryogénèse

Les gènes *Sox-9* et *Sox-10* sont importants dans plusieurs processus développementaux incluant le développement de la crête neurale et la régulation des NCC chez plusieurs espèces. Pour notre étude, nous avons caractérisé l'expression de *Sox-9* et *Sox-10* à plusieurs stades embryonnaire en utilisant la technique d'hybridation *in situ* de type «whole-mount» et d'immunobuvardage de type ««western». Nos résultats ont révélé que *Sox-9* et *Sox-10* ont un patron d'expression similaire durant les stades précoces du développement tandis que lors de l'embryogénèse, leurs patrons diffèrent. De manière générale, les patrons d'expression de *Sox-9* et *Sox-10* chez l'axolotl sont comparables à ceux démontrés parmi les autres espèces et sont en accordance avec leurs rôles respectifs durant l'embryogenèse. De plus, l'expression protéinique durant le développement de l'embryon démontre une régulation qui corrèle avec l'expression génique observée.

4.2 Expression de Sox-9, Cbfa-1 et PTHrP durant le développement et la régénération du membre

4.2.1 Sox-9

Durant le développement, *Sox-9* est exprimé partout dans le membre lors des stades précoces puis se concentre plus tard dans les régions qui correspondent aux doigts en développement. Ce patron d'expression coïncide dans un premier temps avec son rôle dans la différenciation et l'agrégation des précurseurs préchondrocytaires ainsi qu'avec son rôle plus tardif dans la maturation et prolifération des chondrocytes [95, 143-148]. Durant la régénération du membre, *Sox-9* est exprimé exclusivement à partir des stades tardifs, étant premièrement détecté au stade du bourgeon intermédiaire, qui démarque le début de la déposition du cartilage autour de l'os amputé [52]. Ultérieurement, l'expression de *Sox-9* est maintenue durant la totalité de la phase de redéveloppement, ce qui correspond avec la mise en place de plusieurs structures squelettiques du membre. Nos résultats ont démontré que l'expression de *Sox-9* durant le développement et la régénération précède la formation du cartilage. Encore plus, *Sox-9* présente un patron d'expression durant les stades avancés de développement du membre similaire à celui observé durant la phase de redéveloppement de la régénération. Ces résultats supportent le phénomène de régénération biphasique ainsi qu'un mécanisme ostéogénique conservé pour ce gène lors des deux processus.

4.2.2 PTHrP

Pour ce qui concerne l'expression de *PTHrP*, elle n'est que faiblement détectée aux derniers stades du développement du membre, particulièrement dans la région carpienne et dans les doigts en développement. Cette expression peut être attribuée à ses rôles régulateurs pour le maintient de la prolifération des chondrocytes requise pour la formation des structures carpiennes et métacarpiennes [116, 119]. Quant à l'expression de *PTHrP* durant la régénération du membre, il est exprimé aussitôt que le blastème est visible, à l'étape du bourgeon primaire, et continue d'être fortement exprimé jusqu'aux derniers stades de régénération. L'intensité du signal observé pour *PTHrP* durant la régénération pourrait être

justifiée par : i) son rôle dans la prolifération des chondrocytes [122] ii) l'association de son expression avec les cellules mésenchymateuses non-différenciées destinées à se transformer en chondrocytes [116, 119] et iii) son rôle régulateur sur *Ihh*, une molécule responsable pour la différenciation des cellules mésenchymateuses en chondrocytes [149, 150].

L'expression de *PTHrP* est différente lors du développement et de la régénération du membre, par la localisation du signal et aussi par l'intensité. Ceci suggère qu'il aurait plus qu'une fonction lors de ces processus. L'expression différentielle pourrait être attribuée aux divers rôles physiologiques de *PTHrP* en plus de son rôle durant l'ostéogénèse. Par exemple, diverses études ont déjà démontré que *PTHrP* est impliqué dans la régulation de la croissance et de la différenciation des kératinocytes [151, 152]. *PHTrP* est aussi réputé d'avoir un rôle dans l'établissement et l'accumulation des tissus musculaires [116] et de favoriser la dédifférenciation des cellules de Schwann dans la régénération des nerfs [153]. D'ailleurs, il a déjà été démontré que *PTHrP* participe à la formation du blastème durant la régénération des ramures du cerf [20]. Donc, il est plausible que l'expression de *PTHrP* détectée durant le développement et la régénération du membre correspond aux autres fonctions connues de ce gène.

4.2.3 Cbfa-1

Nous avons également vérifié l'expression de *Cbfa-1* au cours du développement du membre. Il est connu que *Cbfa-1* est exprimé durant la condensation mésenchymateuse et qu'il participe à la différenciation des cellules précurseurs de l'os et du cartilage. Il n'est donc pas surprenant qu'on détecte son expression tôt durant le développement [129, 154, 155]. *Cbfa-1* est aussi réputé pour être exprimé dans les chondrocytes hypertrophiques et les ostéoblastes pour réguler leur maturation [127]. Ces cellules se retrouvent dans les diaphyses de l'os ainsi corroborant l'expression détectée dans ces régions vers la fin du développement du membre. Durant la régénération, *Cbfa-1* n'est que faiblement exprimé à l'étape de bourgeon tardif et lors des derniers stades de régénération du membre, son expression est limitée aux régions de la formation des doigts, particulièrement dans

les diaphyses. Son patron d'expression durant ces stades coïncide avec des régions squelettiques en formation, notamment à l'étape de différenciation chondrocytaires [52], confirmant nos résultats avec l'implication de *Cbfa-1* dans la différenciation des chondrocytes.

De manière générale, le patron d'expression de ce gène corrèle avec la formation des structures squelettiques du membre durant le développement et la régénération. Les résultats obtenues pour *Cbfa-1* supportant la régénération biphasique du membre nous laissent croire que ces gènes sont autant impliqués durant la régénération osseuse que durant le développement.

4.3 Guérison des fractures osseuses

Finalement, nous avons vérifié la capacité de l'axolotl de guérir des fractures non-stabilisées jointes et non-jointes afin d'établir les mécanismes de réparation mis en place lors d'une blessure de ce type dans un organisme capable de régénérer des membres en entier. Nos résultats démontrent que l'axolotl, tout comme les autres vertébrés, guérit ses fractures non-stabilisées jointes par le processus de formation d'un cale de cartilage qui sera ensuite remplacé par de la matrice osseuse [140]. Pour ce qui est des fractures non-jointes de dimension critique, on a remarqué que l'axolotl est incapable de réparer l'espace laissé entre les deux bouts d'os et ce, même après sept mois. L'axolotl n'utilise donc pas la régénération pour réparer ses fractures. Étant donné que l'axolotl adulte possède la capacité exceptionnelle de régénérer des membres complets, la raison pour laquelle il n'utilise pas la régénération pour réparer ses fractures reste incomprise. Cependant, plusieurs hypothèses peuvent être formulées afin de tenter d'expliquer cette énigme. Tout d'abord, à la différence d'une amputation, après une fracture il y a absence d'une plaie ouverte, ainsi inhibant les interactions épithélio-mésenchymateuses importantes pour le processus de régénération [15, 37, 59]. Quoique ceci pourrait être une explication plausible, cela est peu probable car l'axolotl est capable de régénérer des parties manquantes du cœur et de la moelle épinière après résection en l'absence de ces interactions [15]. Un autre évènement essentiel à la régénération du membre est le trauma qui est infligé aux tissus avoisinants après amputation. Goss a

démontré que si un membre d'axolotl est amputé directement à travers une section où l'os aurait été préalablement extirpé, la partie qui va régénérer va contenir la moitié de l'os correspondant à l'os extirpé. Par contre, il n'y aura pas de régénération osseuse dans le moignon [35]. Cette expérience démontre non seulement l'importance du facteur de trauma, mais aussi que les cellules qui composent les structures squelettiques déjà mise en place dans le membre amputé semblent avoir peu d'implication dans le processus de régénération. Cette notion a été testé davantage par Lheureux qui a démontré qu'après avoir greffé un morceau de peau (derme et épiderme) sur une patte irradiée, incapable de régénérer, il y a régénération complète de toutes les structures sauf les muscles [49]. Ensemble, ces expériences ont démontré que les matériaux présents dans le moignon ne sont pas nécessaires pour la régénération des nouvelles structures squelettiques du membre [49-51]. Un autre facteur auquel il faut penser lorsqu'on compare le processus de guérison et de régénération est la présence de cellules dédifférenciées. La dédifférenciation cellulaire est critique au processus de régénération, distinguant les membres qui régénèrent de ceux qui ne régénèrent pas [156]. Ceci étant, la présence des cellules dédifférenciées pourrait être un facteur déterminant pour la régénération d'une fracture non-jointe de dimension critique.

Nos résultats obtenues pour les fractures osseuses (Chapitre 2, figure 5) sont consistants avec le modèle de régénération par intercalation, ce qui requiert un blastème en contact avec le moignon pour que la régénération épimorphique ait lieu [72, 73]. Il a été démontré que si une main mature est greffée sur un moignon proximal, il n'y a rien qui se passe, c'est-à-dire pas d'intercalation. Au contraire, si une blastème distale est greffée sur un moignon proximal, un membre normal va régénérer [37]. Un «gap» osseux de dimension critique ressemble à la greffe de la main mature sur le moignon où il y aurait absence de régénération par intercalation. Naturellement, ceci pourrait être attribué au fait que comme dans le cas d'une fracture osseuse, la main mature ne contient pas une CAE, ce qui empêchera l'établissement des interactions épithélio-mésenchymateuses.

Malgré qu'il existe une ressemblance en termes d'expression génique et de changements cellulaires entre les processus de la guérison des plaies et les étapes

précoces de la régénération, basée sur nos résultats et ceux discutés antérieurement, l'idée que la régénération soit simplement une guérison amélioré est peu probable. Une étude réalisée par Mescher *et al.* présente une analyse intéressante sur la réponse immunitaire par rapport à la régénération épimorphique. L'étude révèle une réponse immunologique diminuée, ou plutôt, une réponse d'immunotolérance chez les amphibiens à la suite d'une amputation [157]. Cette expérience supporte l'idée que la régénération n'est pas une guérison améliorée mais plutôt une réponse différente des urodèles aux blessures, reliée à l'immunotolérance, qui pourrait être en partie responsable de permettre ou de diriger les cellules situées au plan d'amputation vers le processus de régénération.

Bien que les études ci-dessus nous apportent quelques réponses sur les mécanismes de guérison et de régénération des traumatismes osseux, il faut impérativement continuer à étudier l'expression des gènes ostéogéniques qui interviennent durant la formation, la régénération et la guérison osseuse afin de tenter de mettre en évidence les différences moléculaires entre ces processus.

CHAPITRE 5

Conclusions et perspectives

L'exploitation de l'axolotl pour étudier les mécanismes cellulaires et moléculaires responsables de la régénération chez les vertébrés a contribué à l'avancement des connaissances dans le domaine de la régénération. Ayant maintenant les outils nécessaires, il est possible d'identifier les divers gènes qui interviennent lors du processus de régénération. Actuellement, des projets de séquençage EST [158, 159] ont réussi à cloner plus de 60 000 EST, ce qui a contribuer à la réalisation des puces à ADN [160]. Avec cette technologie, il serait possible d'identifier à grande échelle les gènes impliqués dans plusieurs processus essentiels à la régénération, telle que la dédifférenciation cellulaire et la dépendance des nerfs, afin de mieux comprendre leurs rôles durant la régénération. À l'instant, nous nous fions sur les gènes essentiels lors des processus développementaux puisqu'ils démontrent une conservation parmi un grand éventail d'espèces.

Pour notre étude, nous avons choisi d'étudier l'expression de *Sox-9, Sox-10, PTHrP* et *Cbfa-1*, ces gènes étant déjà caractérisés durant le développement chez plusieurs vertébrés. La caractérisation de ces gènes chez l'axolotl durant le développement et la régénération du membre nous a permis d'éclaircir leurs implications durant ces processus. Pour ce qui concerne *Sox-9* et *Sox-10*, comme démontré chez d'autres espèces, leurs patrons d'expression durant l'embryogenèse ont confirmé leur présence durant le développement de la crête neurale et dans les structures qui en dérivent. *Sox-9* joue aussi un rôle dans la chondrogenèse durant le développement et la régénération de la patte, ayant une expression omniprésente qui précède la formation de cartilage lors des deux processus. De l'autre côté, l'expression différentielle du gène *PTHrP* lors du développement et de la régénération peut être attribuée au fait que *PTHrP* est impliqué dans plusieurs processus cellulaires. Quant à *Cbfa-1*, le patron d'expression durant la régénération ressemble beaucoup à celui obtenu lors du développement de la patte et correspond avec la mise en place du cartilage. On peut alors penser que *Cbfa-1* possède un rôle ostéogénique conservé durant les deux processus ainsi supportant l'hypothèse de la

régénération biphasique du membre. Finalement, l'autre volet du projet visait à caractériser le processus de guérison des fractures osseuses chez l'axolotl. Nos expériences ont confirmé que l'axolotl n'utilise pas la régénération pour réparer des fractures osseuses, soit jointes ou non-jointes.

La prochaine étape dans cette étude serait de caractériser la fonction de *Sox-9, PTHrP* et *Cbfa-1*, à l'aide des vecteurs viraux fonctionnels chez l'axolotl, afin de mieux comprendre leurs rôles durant le développement et la régénération de l'os. Étant donné que les gènes et leurs fonctions sont conservés parmi différentes espèces, il n'est pas déraisonnable de s'attendre à ce que les gènes impliqués dans la régénération du membre chez les amphibiens urodèles soient conservés chez l'humain. Une meilleure compréhension des mécanismes de régénération des structures squelettiques chez l'axolotl pourrait bénéficier au développement de nouveaux traitements des fractures et des maladies dégénératives et inflammatoires de l'os comme l'arthrite et l'ostéoporose chez l'humain.

CHAPITRE 6

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Annexe 1: Article in press

«The axolotl limb: a model for bone development, regeneration and fracture healing»

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The axolotl limb: A model for bone development, regeneration and fracture healing

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Abstract

Among vertebrates, urodele amphibians (e.g., axolotls) have the unique ability to perfectly regenerate complex body parts after amputation. The limb has been the most widely studied due to the presence of three defined axes and its ease of manipulation. Hence, the limb has been chosen as a model to study the process of skeletogenesis during axolotl development, regeneration and to analyze this animal's ability to heal bone fractures. Extensive studies have allowed researchers to gain some knowledge of the mechanisms controlling growth and pattern formation in regenerating and developing limbs, offering an insight into how vertebrates are able to regenerate tissues. In this study, we report the cloning and characterization of two axolotl genes; *Cbfa-1*, a transcription factor that controls the remodeling of cartilage into bone and *PTHrP*, known for its involvement in the differentiation and maturation of chondrocytes. Whole-mount in situ hybridization and immunohistochemistry results show that *Cbfa-1*, *PTHrP* and type II collagen are expressed during limb development and regeneration. These genes are expressed during specific stages of limb development and regeneration which are consistent with the appearance of skeletal elements. The expression pattern for *Cbfa-1* in late limb development was similar to the expression pattern found in the late stages of limb regeneration (i.e. re-development phase) and it did not overlap with the expression of type II collagen. It has been reported that the molecular mechanisms involved in the re-development phase of limb regeneration are a recapitulation of those used in developing limbs; therefore the detection of *Cbfa-1* expression during regeneration supports this assertion. Conversely, *PTHrP* expression pattern was different during limb development and regeneration, by its intensity and by the localization of the signal. Finally, despite its unsurpassed abilities to regenerate, we tested whether the axolotl was able to regenerate non-union bone fractures. We show that while the axolotl is able to heal a non-stabilized union fracture, like other vertebrates, it is incapable of healing a bone gap of critical dimension. These results suggest that the axolotl does not use the regeneration process to repair bone fractures.
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Keywords: Urodele/axolotl; Fracture healing; Limb regeneration; Osteogenesis; *Cbfa-1*/*PTHrP*/collagen

Introduction

The ability of an adult vertebrate to regenerate lost body parts is very limited; however, urodele amphibians such as the axolotl (*Ambystoma mexicanum*) are known to have exceptional abilities to regenerate multiple body parts throughout their life. The axolotl's capacity to regenerate its limbs as an adult offers the opportunity to conduct comparative studies of the genes expressed during development and regeneration in an identical genetic background. Among the complex structures able to regenerate, the limb has been the most widely studied due to the presence of three defined axes and its ease of manipulation. During limb development, the cells that form limb tissues are derived from undifferentiated progenitor cells [50]. This process is paralleled in the case of the regenerating limb, with the exception that differentiated cells become undifferentiated before subsequently reforming the missing parts after amputation [7,50]. It has been hypothesized that the process of limb regeneration is biphasic [8,24,60]. The first phase, known as the preparation phase, involves the events

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spanning from amputation to blastema formation whereas the second phase, known as the re-development phase, is characterized by the control of growth and pattern formation within the blastema and finally the re-differentiation of cells to reform the missing limb. Experimental data collected to date support the concept that the molecular mechanisms involved in limb development are recapitulated during the re-development phase of limb regeneration [48,58,59,71]. In addition to the aforementioned advantages, the complexity of the limb provides an excellent opportunity for studying many different tissue types including bone and cartilage. Bone represents approximately 50% of the exposed surface following limb amputation; however, as little as 2% of cells in a regenerated limb are derived from bone [48]. Previous limb regeneration studies also suggest that new bone is generated from dedifferentiated cells of the blastema and not derived from pre-existing bone [41,74]. Most skeletal bones, including those of the appendage, develop via an endochondral ossification process which involves the replacement of cartilage tissue by bone. This process can be divided into five stages: (i) the commitment of mesenchymal cells to cartilage cells; (ii) the subsequent condensation of these cells and their differentiation into chondrocytes; (iii) chondrocyte proliferation; (iv) chondrocyte hypertrophy and (v) chondrocyte apoptosis and replacement by osteoblasts [30]. Among the numerous genes potentially involved in the formation of skeletal elements during limb regeneration, two of them are of particular interest; *Core-binding factor α-1* (*Cbfa-1*), a transcription factor required for mesenchymal condensation, chondrocyte hypertrophy and osteoblast differentiation [34,53] and *Parathyroid hormone related peptide* (*PTHrP*), involved in the differentiation and maturation of chondrocytes [37]. The *Cbfa-1* gene, also known as Runx2/PEBP2αA/AML3/Osf2, is characterized by a Runt domain that binds DNA and heterodimerizes with Core-binding factor β (Cbfβ) [4,51]. Studies have shown *Cbfa-1* to be an essential factor for osteoblast maturation and normal ossification [34]. However, additional research has revealed a dual action for *Cbfa-1*: first as an osteoblast differentiation factor and also as a regulator of chondrocyte maturation and differentiation. Homozygous mutations in this gene are responsible for the skeletal malformation syndrome: cleidocranial dysplasia (CCD) [46]. This dysplasia is characterized by multiple skeletal malformations and the absence of ossification [46,53]. In fact, without *Cbfa-1*, osteoblasts are unable to differentiate and therefore abolish bone matrix deposition [46]. A more recent study by Kim et al. has revealed yet another function for *Cbfa-1* as a regulator of chondrocyte hypertrophy [33]. *PTHrP* is another important player to consider when characterizing skeletogenesis during the regeneration process. *PTHrP* is a small peptide first identified as the causative agent for humoral hypercalcemia malignancy [43,67]. This peptide uses the same transmembrane receptor as *parathyroid hormone* (*PTH*); consequently they share a well conserved PTH-like domain [54]. In addition to causing hypercalcemia, *PTHrP* is a major regulator of chondrocyte maturation, differentiation and proliferation [37]. *PTHrP* is known to be expressed in chondrocytes throughout the developing epiphyses, and in osteoblasts in metaphyseal bone [2]. *PTHrP*-deficient knockout mice die prematurely due to severe osteochondrodysplasia, characterized by shorter bones due to an early ossification. This can be explained primarily by reduced proliferation and increased apoptosis of immature chondrocytes as well as the precocious hypertrophy of chondrocytes [3,32,36].

Numerous studies have demonstrated the axolotl's ability to completely regenerate lost appendages in which every tissue including bone is regenerated, yet very few have concentrated on whether the axolotl can regenerate bone in a gap or how well they can heal a fracture [25]. The bone healing process has been well characterized in numerous vertebrates [6,19,61,65,72]. Most vertebrates are capable of healing a bone fracture through a callus formation process; however, all vertebrates studied to date are unable to heal bone defects of critical dimension [62]. These defects are gaps within which bone repair does not take place. The critical dimensions are dependent on the size of the animal. For example, the critical dimension in a rabbit ulna is 15 mm, comparatively; in a rat it is 4–5 mm [11,29]. Under this circumstance, bone formation must be assisted by inductive substances [57]. We are particularly interested in the axolotl's abilities to heal and repair bone since they can regenerate entire limbs including the skeletal elements. Does their unique ability to regenerate complex tissues also apply to bone defects or fractures of critical dimension? In this article, we describe the molecular cloning, sequence analysis and expression of *Cbfa-1* as well as *PTHrP* during limb development and regeneration in the axolotl. We also characterized the expression of type II collagen, a cartilage specific protein, during limb regeneration. *Cbfa-1* is an inhibitor of type II collagen and their expression should not overlap [20]. Both *Cbfa-1* and *PTHrP* regulate essential aspects of chondrogenesis and osteogenesis in vertebrate development (e.g. mammals and birds). Given that part of the regeneration process is a recapitulation of the developmental process, the regenerating axolotl limb offers an excellent model to analyze whether or not these genes are also required for bone regeneration. Our results show that *Cbfa-1* and *PTHrP* as well as type II collagen are expressed during limb development and regeneration. Additionally, we found that particular gene expression patterns during axolotl limb development are similar to those during regeneration. Finally, we show that while the axolotl is able to heal a non-stabilized fracture, like other vertebrates, the axolotl is incapable of healing or regenerating a bone gap of critical dimension.

Materials and methods

Cloning axolotl Cbfa-1 and PTHrP cDNA

Partial axolotl *Cbfa-1* and *PTHrP* cDNA sequences were cloned (5 and 3 clones for each gene, respectively) by RT-PCR using degenerate primers (see below). To obtain the full-length *Cbfa-1* gene, the SMART RACE cDNA Amplification kit (BD Biosciences, Clontech, USA) was used to construct 5′- and 3′-RACE ready cDNA from total RNA extracted from the axolotl hind limb during the medium bud regeneration stage using Trizol reagent (Invitrogen). Following the manufacturer's instructions, PCR products were obtained using 5′- and 3′-gene specific primers (GSPs) and nested gene specific primers (NGSPs)

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designed from the partial sequence cloned in the lab. The designs of all primers were as follows:

dFPTHrP:
5′-CAGCT(G/A/C)(A/C)T(G/T/C)CA(C/T)GACAAGGG-3′
dRPTHrP: 5′-GTT(A/T/C/G)GT(C/T)TCCTG(A/G)G(G/T)(C/T)AGGT-3′
dFCbfa-1: 5′-ACAG(C/T)CC(G/C)AACTT(C/T)CT(G/C)TGCT-3′
dRCbfa-1: 5′-GGTA(A/C)G(A/T)CTG(A/G)TCATA(G/C)GAC-3′
5′GSP: 5′-TGGAGGAGATGACTGTGCTT -3′
5′NGSP: 5′-CGTGAAGACAGTTATACTCA -3′
3′GSP: 5′-TGACTATAACTGTCTTCACG-3′
3′NGSP: 5′-AAGCACAGTCATCTCCTCCA-3′.

All PCR products were purified by gel extraction, cloned into the pCR II-TOPO sequencing vector (Invitrogen) and sequenced by the McGill University Genome Quebec Sequencing Centre (Montreal, Canada) with M13R and M13F primers. Resulting sequences were assembled using SeqManII (DNASTAR Inc., USA). Full-length *Cbfa-1* cDNA was subsequently amplified by PCR using [Axolotl forward CbF1] 5′-ATGGCATCCAACAGCCTGTTCA -3′ and [Axolotl reverse CbR1426] 5′-AAAACAGGCACTTTGGCATT-3′ primers designed from the sequencing data. The PCR reaction was performed using Taq DNA polymerase (Invitrogen) and the conditions were 35 cycles of 95°C, 30 s; 63.5–52.5°C, 20 s; 72°C, 1 min; followed by 15 min at 72°C. Note that six full-length *Cbfa-1* clones were isolated from the 5 independent RT-PCR reactions and aligned using MegAlign (DNASTAR Inc., USA).

Animal maintenance and surgery

Axolotl embryos and larvae were purchased from the Ambystoma Genetic Stock Center (Lexington, KY). Animals are kept in a 20% Holtfreter's solution at a room temperature varying from 19–22°C and a photoperiod cycle of 12 h of light and 12 h of darkness. For amputations, animals were anaesthetized in 0.1% MS222 solution (ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, St. Louis, MO). Proximal (through humerus or femur) and distal (through radius/ulna or tibia/fibula) amputations were performed with micro-surgical tools (Fine Science Tools, B.C., Canada) on axolotls from 3 to 5 cm in length. For fixation, animals are euthanized and fixed in 1XMEMFA (10× MEM salt (1 M MOPS pH 7.4, 20 mM EGTA, 10 mM $MgSO_4$), 3.7% formaldehyde and DPEC treated H_2O then gradually transferred to a methanol solution and stored at −20°C until needed.

Bone defects of critical dimension were generated by first making an incision in the skin along the longitudinal axe of the distal part of the limb, followed by careful displacement of soft tissues to expose the radius or tibia. The distal bones in axolotl limb (radius/cubitus and tibia/fibula) are nearly identical in size and shape, therefore either one of the two long bones was cut. A section of the radius or tibia was extirpated to generate a gap of 4 mm, this being larger than the critical dimension for this type of bone in the axolotl. The remaining bone was left intact to prevent the limb from collapsing and to preserve the distance between the disengaged bone extremities. It also served as an internal control for intact bone. In order to generate bone fractures, surgeries were performed identically except the radius or tibia was simply cut with surgical scissors. Following surgical intervention, limbs were fixed at different periods and the double staining technique was used to verify new cartilage and bone formation. All animal care and experiments were done in accordance with the Université de Montréal animal care committee's guidelines.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described in Gardiner et al. with a few modifications [22]. Digoxigenin (DIG)-labeled antisense RNA probes were created using T7 RNA polymerase (Invitrogen) and DIG RNA labeling mix (Roche Diagnostics, Laval, Quebec, Canada). Sense probes were generated with SP6 RNA polymerase (Invitrogen). For probe synthesis, a 400 bp *Cbfa-1* and a 230 bp *PTHrP* fragment were cloned into the pCR II-TOPO plasmid which was then linearized using the appropriate restriction enzymes. For tissue permeabilization, developing embryos and amputated limbs were incubated with 20 μg/ml and 30 μg/ml proteinase K respectively for 15 min on ice and then at 37°C for 5 min or for 1 h on ice then 1 h at 37°C. Probe hybridization was 24 h for embryos and 72 h for limbs. Prehybridization and hybridization temperatures were done between 55°C and 65°C. Finally, BM purple (Roche) was used as the enzyme substrate for the colorimetric reaction for the alkaline phosphatase reaction.

Type II collagen whole-mount in situ immunohistochemistry

Samples were rehydrated from methanol and rinsed three times in a phosphate-buffered saline solution with 0.1% Tween-20 (PTW). Limbs were then bathed in 2.5% trypsin for either 15 min (developing limbs) or 40 min (regenerating limbs) followed by three 5-min rinses in water. After 10 min in −20°C acetone, specimens were rinsed in water for 10 min and then washed three times for 5 min with PTW. Samples were then transferred to a blocking solution (phosphate-buffer solution (0.7×PBS)+1% DMSO+5% sheep serum+0.1% Tween) for 1 h and incubated in a mouse monoclonal antibody against Type II collagen (II-II6B3, Lab Vision Corporation, Fremont, CA) diluted 1:100 in blocking solution overnight at 4°C. The next day, limbs were rinsed eight times for 15 min in a PTW+1% DMSO solution before being placed in an anti-mouse horseradish peroxidase labeled secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted 1:500 in blocking solution overnight at 4°C. The following morning, samples were rinsed eight times for 30 min with PTW and subsequently bathed in a diaminobenzidine (DAB) solution (Invitrogen). The reaction was stopped by two 5-min washes with PTW and fixed in a formalin solution (3.7% formaldehyde in 0.7× PBS). Specimens were cleared through a graded series of ethanol/glycerol and stored

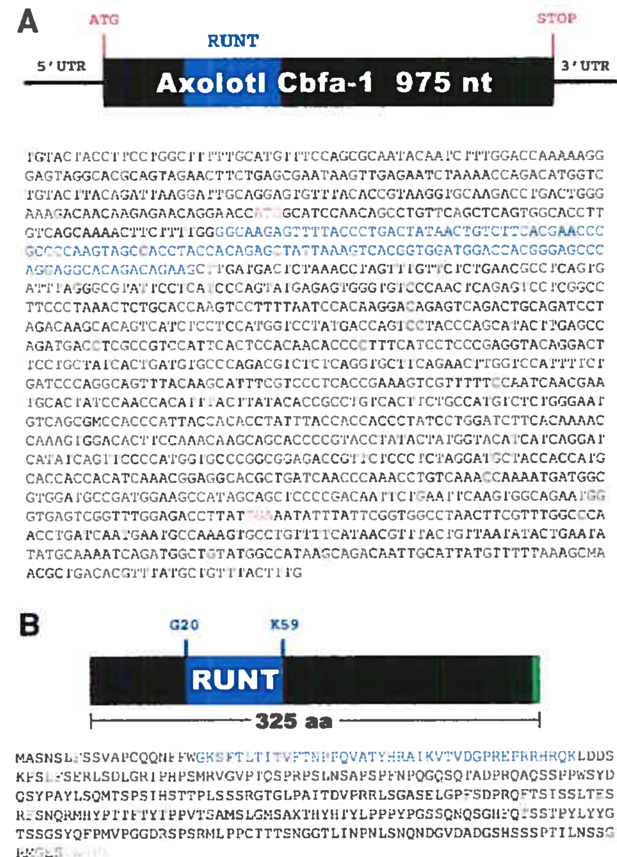

Fig 1. Analysis of the axolotl *Cbfa-1* sequence. (A) cDNA sequence. (B) Amino acid sequence. Red characters indicate start and stop codons. Blue characters indicate conserved partial Runt domain. Green characters indicate Runx conserved carboxyterminal pentapeptide. (C) Amino acid alignment of *Cbfa-1* from multiple model organisms used for developmental and biomedical research (mice, human, chicken and axolotl). Blue box contains the runt domain and the green box indicates Runx conserved carboxyterminal pentapeptide. (D) Phylogenetic tree. Species evolutionary comparison of *Cbfa-1* ORF sequences. GenBank accession number 828471. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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C

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1    MASNSLFSSVAPCQQNFFW-----------------------------------------  Ambystoma mexicanum
1    MASNSLFSTVTPCQQNFFWDPSTSRRFSPPSSSLQPGKMSDVSPVVAAQQQQQQQQQQQQ  Homo sapiens
1    MASNSLFSSVTPCQQNFFWDPSTSRRFSPPSSSLQPGKMSEVSPVVVAQQQQQQQQQQQQ  Gallus gallus
1    MASNSLFSAVTPCQQSFFWDPSTSRRFSPPSSSLQPGKMSDVSPVVAAQQQQQQQQQQQQ  Mus musculus

20   ------------------------------------------------------------  Ambystoma mexicanum
61   QQQQQQQQQQQE-------AAAAAAAAAAAAAAAAAVPRLRPPHDNRTMVEIIADHPAEL  Homo sapiens
61   E---------------------------------AAVPRLR-PHDNRTMVEIIADHPAEL  Gallus gallus
61   QQQQQQQQQQQQQQQQQEAAAAAAAAAAAAAAAAAAVPRLRPPHDNRTMVEIIADHPAEL  Mus musculus

20   ------------------------------------------------------------  Ambystoma mexicanum
114  VRTDSPNFLCSVLPSHWRCNKTLPVAFKVVALGEVPDGTVVTVMAGNDENYSAELRNASA  Homo sapiens
87   VRTDSPNFLCSVLPSHWRCNKTLPVAFKVVALGEVPDGTVVTVMAGNDENYSAELRNASA  Gallus gallus
121  VRTDSPNFLCSVLPSHWRCNKTLPVAFKVVALGEVPDGTVVTVMAGNDENYSAELRNASA  Mus musculus

20   --------------------GKSFTLTITVFTNPPQVATYHRAIKVTVDGPREPRRHRQK  Ambystoma mexicanum
174  VMKNQVARFNDLRFVGRSGRGKSFTLTITVFTNPPQVATYHRAIKVTVDGPREPRRHRQK  Homo sapiens
147  VMKNQVARFNDLRFVGRSGRGKSFTLTITVLTNPPQVATYHRAIKVTVDGPREPRRHRQK  Gallus gallus
181  VMKNQVARFNDLRFVGRSGRGKSFTLTITVFTNPPQVATYHRAIKVTVDGPREPRRHRQK  Mus musculus

60   LDDSKPSLFSERLSDLGRIPHPSMRVGVPTQSPRPSLNSAPSPFNPQGQSQTADPRQAQS  Ambystoma mexicanum
234  LDDSKPSLFSDRLSDLGRIPHPSMRVGVPPQNPRPSLNSAPSPFNPQGQSQITDPRQAQS  Homo sapiens
207  LDDSKPSLFPERLSDLGRIPHPSMRVGVPTQSPRPSLNSAPSPFNPQGQSQITDPRQAQS  Gallus gallus
241  LDDSKPSLFSDRLSDLGRIPHPSMRVGVPPQNPRPSLNSAPSPFNPQGQSQITDPRQAQS  Mus musculus

120  SPPWSYDQSYPAYLSQMTSPSIHSTTPLSSSRGTGLPAITDVPRRLS-------------  Ambystoma mexicanum
294  SPPWSYDQSYPSYLSQMTSPSIHSTTPLSSTRGTGLPAITDVPRRISDDDTATSDFCLWP  Homo sapiens
267  SPPWSYDQSYPSYLSQMTSPSIHSTTPLSSTRGTGLPAITDVPRRLS-------------  Gallus gallus
301  SPPWSYDQSYPSYLSQMTSPSIHSTTPLSSTRGTGLPAITDVPRRISDDDTATSDFCLWP  Mus musculus

167  ---------GASELGPFSDPRQFTSISSLTESRFSNQRMHYPTTFTYTPPVTSAMSLGMS  Ambystoma mexicanum
354  STLSKKSQAGASELGPFSDPRQFPSISSLTESRFSNPRMHYPATFTYTPPVTSGMSLGMS  Homo sapiens
314  ---------GASELGPFSDPRQFTSISSLTESRFSNPRMHYPATFTYTPPVTSGMSLGMS  Gallus gallus
361  SSLSKKSQAGASELGPFSDPRQFPSISSLTESRFSNPRMHYPATFTYTPPVTSGMSLGMS  Mus musculus

218  AXTHYHTYLPPPYPGSSQNQSGHFQTSSTPYLYYGTSSGSYQFPMVPGGDRSPSRMLPPC  Ambystoma mexicanum
414  ATTHYHTYLPPPYPGSSQSQSGPFQTSSTPYLYYGTSSGSYQFPMVPGGDRSPSRMLPPC  Homo sapiens
385  ATTHYHTYLPPPYPGSSQNQSGPFQTSSTPYLYYGTSSGSYQFPMVPGGDRSPSRMLPPC  Gallus gallus
421  ATTHYHTYLPPPYPGSSQSQSGPFQTSSTPYLYYGTSSASYQFPMVPGGDRSPSRMVPPC  Mus musculus

278  TTTSNGGTLINPNLSNQNDGVDADGSHSSSPTILNSSGRMGESVWRPY              Ambystoma mexicanum
474  TTTSNGSTLLNPNLPNQNDGVDADGSHSSSPTVLNSSGRMDESVWRPY              Homo sapiens
425  TTTSNGSTLLNPNLPNQSDGVEADGSHSSSPTVLNSSGRMDESVWRPY              Gallus gallus
481  TTTSNGSTLLNPNLPNQNDGVDADGSHSSSPTVLNSSGRMDESVWRPY              Mus musculus
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D

Mus musculus
Rattus norvegicus
Bos taurus
Homo sapiens
Gallus gallus
Ambystoma mexicanum
Danio rerio
Takifugu rubripes
Xenopus

Fig. 1 (*continued*).

in 100% glycerol. As a control for specificity, the same protocol was followed except the primary antibody was omitted (Fig. 4F).

Skeletal staining of embryos and limbs

Developing and regenerating limbs were stained using the Victoria blue method to verify new cartilage formation [9]. Limbs were fixed in alcoholic Bouin's solution for 24 h before being rinsed several times with 70% ethanol. Specimens were rinsed regularly in 3.5% NH_4OH for 2 days and subsequently treated with Acid alcohol for 2 h. Specimens were stained with 1% Victoria Blue for 2 h and then rinsed with 70% ethanol. Limbs were gradually dehydrated to 100% ethanol, then cleared and stored in methyl salicylate.

A double staining technique of Alizarin Red and Alcian Blue was used to stain cartilage blue and calcified matrix red for bone fracture experiments [12]. Fixed specimens were rinsed regularly with distilled water for 1 h. Specimens were placed in an alcian blue staining solution (20 mg of alcian blue powder and a 7:3 EtOH/glacial acetic acid solution (EtOH/Hac)) for 1 day, and then were transferred to an EtOH/Hac solution for 1 h. After being placed in 100% EtOH for 24 h, the sample was rinsed with tap water frequently for 2 days before being transferred to a 1% trypsin solution diluted in a 30% saturated sodium borate solution. The specimen is trypsinized for a period of about 24 h, depending on the size, and then transferred for 24 h to an alizarin red solution (saturated alizarin red in water added to a 0.5% KOH solution until it takes on a dark purple color). This step was followed by several changes of a 0.5% KOH solution within a 24 h period before specimens were finally treated with 0.5% KOH/glycerol solutions: 2:1, 1:1, 1:2 and preserved in 100% glycerol.

Results

Isolation and sequence analysis of axolotl Cbfa-1 and PTHrP cDNA

A partial axolotl *Cbfa-1* sequence was obtained by first isolating a 400 bp cDNA fragment from axolotl larvae total RNA by RT-PCR using degenerate primers. After using the

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RACE-nested PCR strategy [21], a 1371 bp contig was analyzed using the NCBI BLAST database. Results show a 975 bp ORF (Fig. 1A) which encodes a 325 aa protein containing a partial Runt domain of 40 aa (Fig. 1B). *Cbfa-1* is known to belong to the Runt domain gene family which is characterized by a conserved Runt domain [51]. Axolotl protein analysis using BLAST showed a 94% sequence homology with chicken [39], 93% with human [51] and 86% with mouse [56] (Fig. 1C). A phylogenetic tree was created by comparing full-length *Cbfa-1* cDNA sequences from different species and placed the axolotl sequence closer to that of birds than to humans which is consistent with the phylogeny of this specie (Fig. 1D).

The same strategy was used to isolate a 225 bp fragment of axolotl *PTHrP* cDNA. The results show a corresponding 75 aa partial *PTHrP* sequence located towards the 5′ of the gene (Fig. 2A). Protein alignment analysis demonstrates that our partial sequence shares a 77%, 65% and 64% homology with chicken [52], human [26] and mouse [66] *PTHrP*, respectively (Fig. 2B). High homology is expected between species since the cloned sequence contains a partial Parathyroid domain characteristic to the parathyroid hormone family. Integrated in this sequence, is a partial PTH-like domain, unique to the parathyroid hormone, this sequence is required for ligand–receptor interactions [63]. Protein alignment of the partial PTH-like domain shows even higher conservation having 90% chicken and 69% mouse and human homology (Fig. 2B). Analysis of the cloned 225 nt sequence with *PTHrP* cDNA from other species was performed using MegAlign (DNASTAR Inc., USA). The resulting phylogenetic tree is consistent with the sequence homology data and evolutionary relationships are conserved (Fig. 2C). This result shows a similar evolutionary relationship for *PTHrP* as that of *Cbfa-1*, where the axolotl sequences are closest to the chicken.

Whole-mount in situ analysis of Cbfa-1 and PTHrP expression during limb development

Whole-mount in situ hybridization was used to determine the expression pattern of *Cbfa-1* and *PTHrP* at different developmental stages of the limb in axolotl embryos using RNA

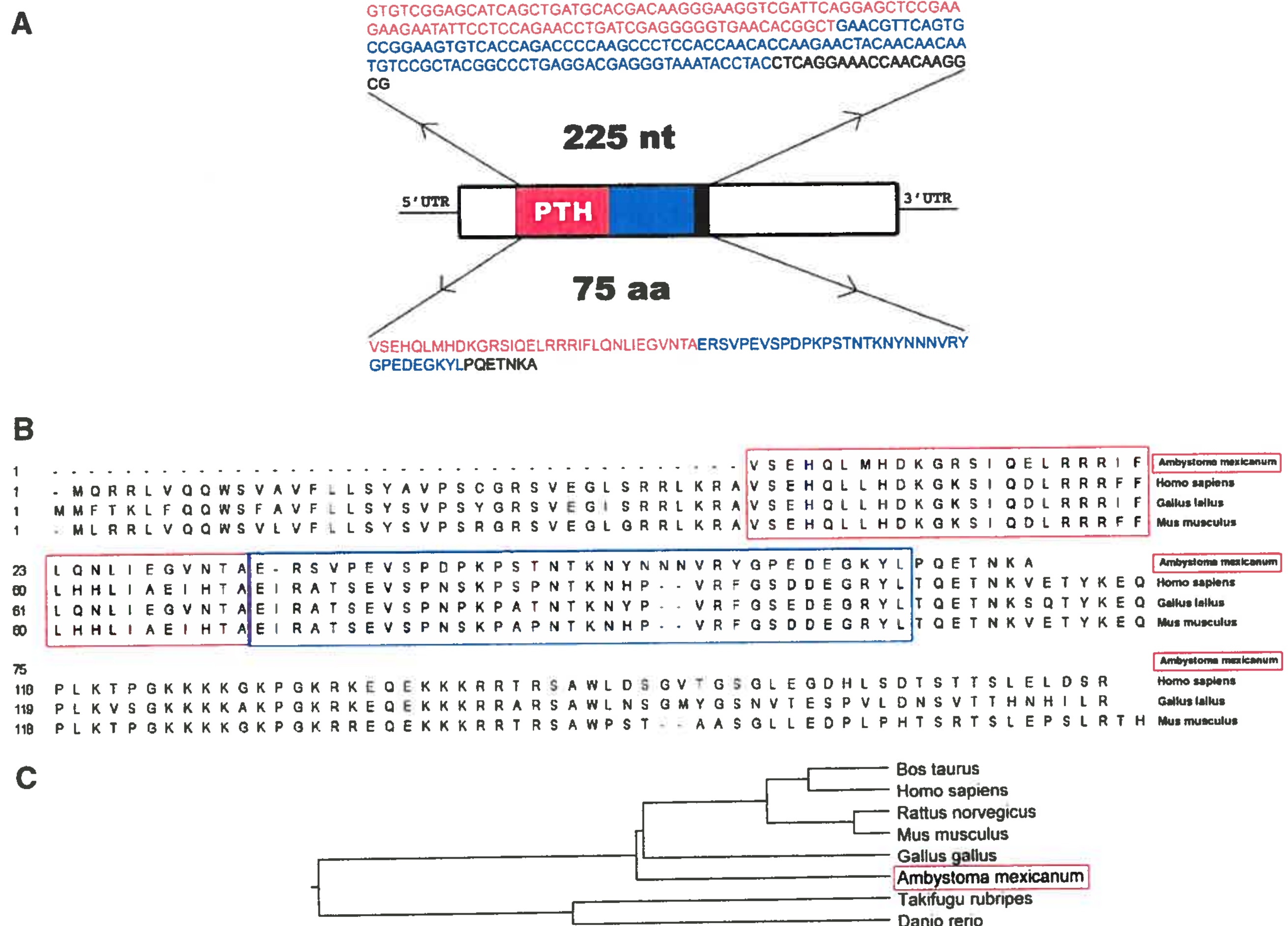

Fig 2. Analysis of the axolotl *PTHrP* sequence. (A) cDNA and corresponding amino acid sequence with respect to the full-length sequence. Red characters indicate partial PTH-like domain. Amalgamation of blue and red characters indicates partial Parathyroid domain. (B) Amino acid alignment of *PTHrP* from multiple model organisms used for developmental and biomedical research (mice, human, chicken and axolotl). Red box shows the PTH-like domain and the combined red and blue boxes the Parathyroid domain. (C) Phylogenetic tree. Partial axolotl cDNA was compared to *PTHrP* ORF sequences from other species. GenBank accession number 835466. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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anti-sense probes (Fig. 3). Victoria blue was used to stain skeletal elements at different developmental stages of the limb in order to compare *Cbfa-1* and *PTHrP* expression with the appearance of skeletal elements. Type II collagen protein expression was determined by whole-mount in situ immunohistochemistry to further demarcate the skeleton in developing limbs. Cells in permanent cartilage express type II collagen in prehypertropic chondrocytes [40,44] but do not express *Cbfa-1* [10,17,31,33, 35,69]. The type II collagen results provide confirmation that the *Cbfa-1* expression observed is specific as they do not overlap. *Cbfa-1* expression is observed throughout the developmental process of the axolotl limb (Figs. 3A–C). Forelimb development in the axolotl begins at stages 44–45 [49] where it is possible to distinguish prominent budding containing some *Cbfa-1* expression (data not shown). At stage 48, weak expression is observed at the base of the limb and in the mid region of the extremity. In the distal region, two strong expression points are noticeable (Fig. 3A). Comparatively, it is possible to distinguish a completely formed humerus and a budding radius at the tip of the same stage (Fig. 3G). With the extension of the radius and the ulna in place, by stage 50, carpal skeletal structures within the hand and the metacarpals of the first two digits are in formation (Fig. 3H). Two *Cbfa-1* expression regions corresponding to the earliest forming digits can be observed as well as expression points in the carpal region and weak expression in the mid limb region (Fig. 3B). Reaching stage 52, *Cbfa-1* expression is restricted solely within the diaphysis of the phalanges showing a particularly

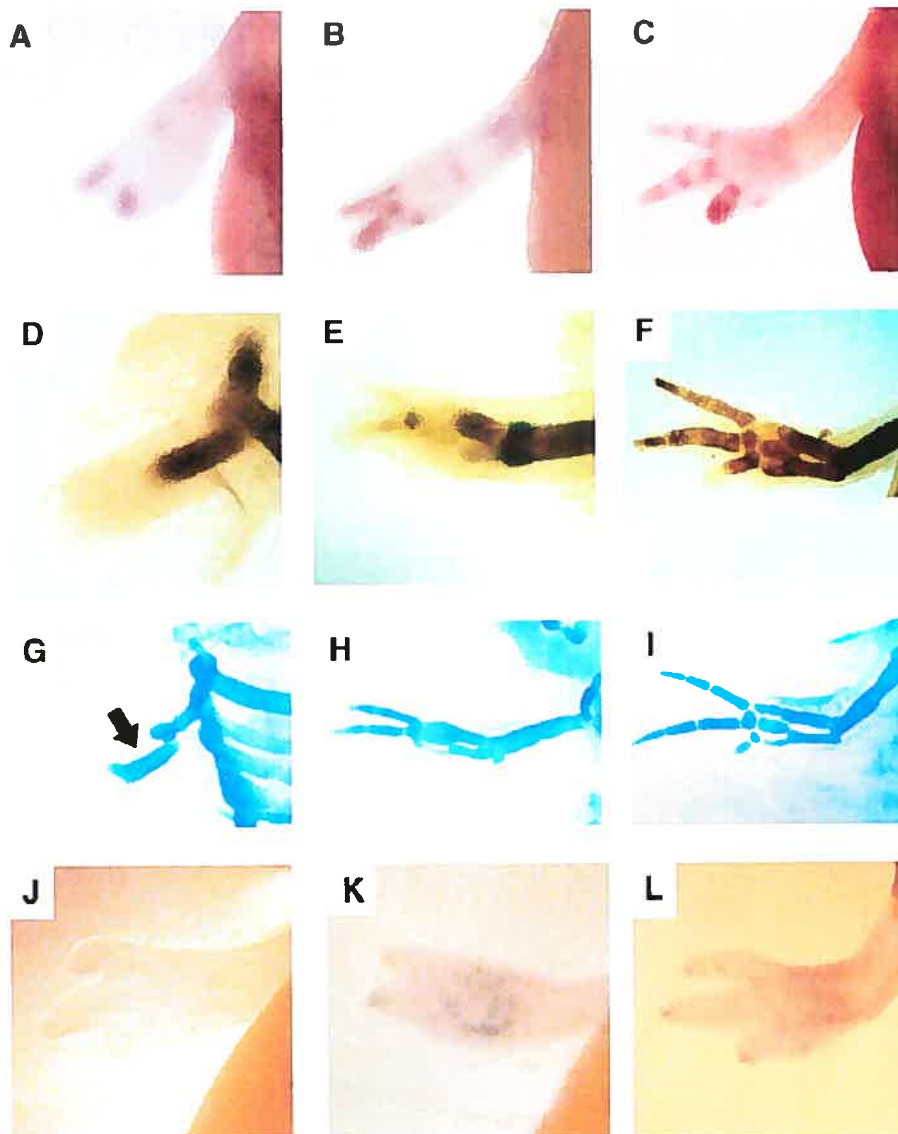

Fig 3. Whole-mount in situ hybridization, immunohistochemistry and cartilage staining in developing limbs for *Cbfa-1*, type II collagen, cartilage and *PTHrP*. (A–C) *Cbfa-1* expression, (D–F) Type II collagen protein expression, (G–I) Cartilage staining with Victoria blue and (J–L) *PTHrP* expression. Developing limbs at stage 48 (A, D, G), Stage 50 (B, E, H, K) and Stage 52 (C, F, I, L). *PTHrP* control limb using a sense probe (J). Black arrow indicates humerus. Note that axolotl embryos were photographed anterior to the top and posterior to the bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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strong signal in the digit still in formation (Fig. 3C). At the same time, cartilage staining shows that by this stage, the first two digits are complete and there is evidence for the formation of digit III (Fig. 3I). Also, type II collagen staining in the later stages of limb development displays differential expression to *Cbfa-1*, further substantiating our whole-mount results (Figs. 3D–F). *PTHrP* expression was also verified during limb development (Figs. 3J–L). Expression remained undetected until stage 50, where expression is observed in the tips of the more developed digits and in the carpal region (Fig. 3K). Stage 52 shows a similar expression pattern, though a little more pronounced (Fig. 3L). Since the *PTHrP* expression detection during these stages is weak, a sense probe was used as a control (Fig. 3J) to confirm that the signal detected was specific.

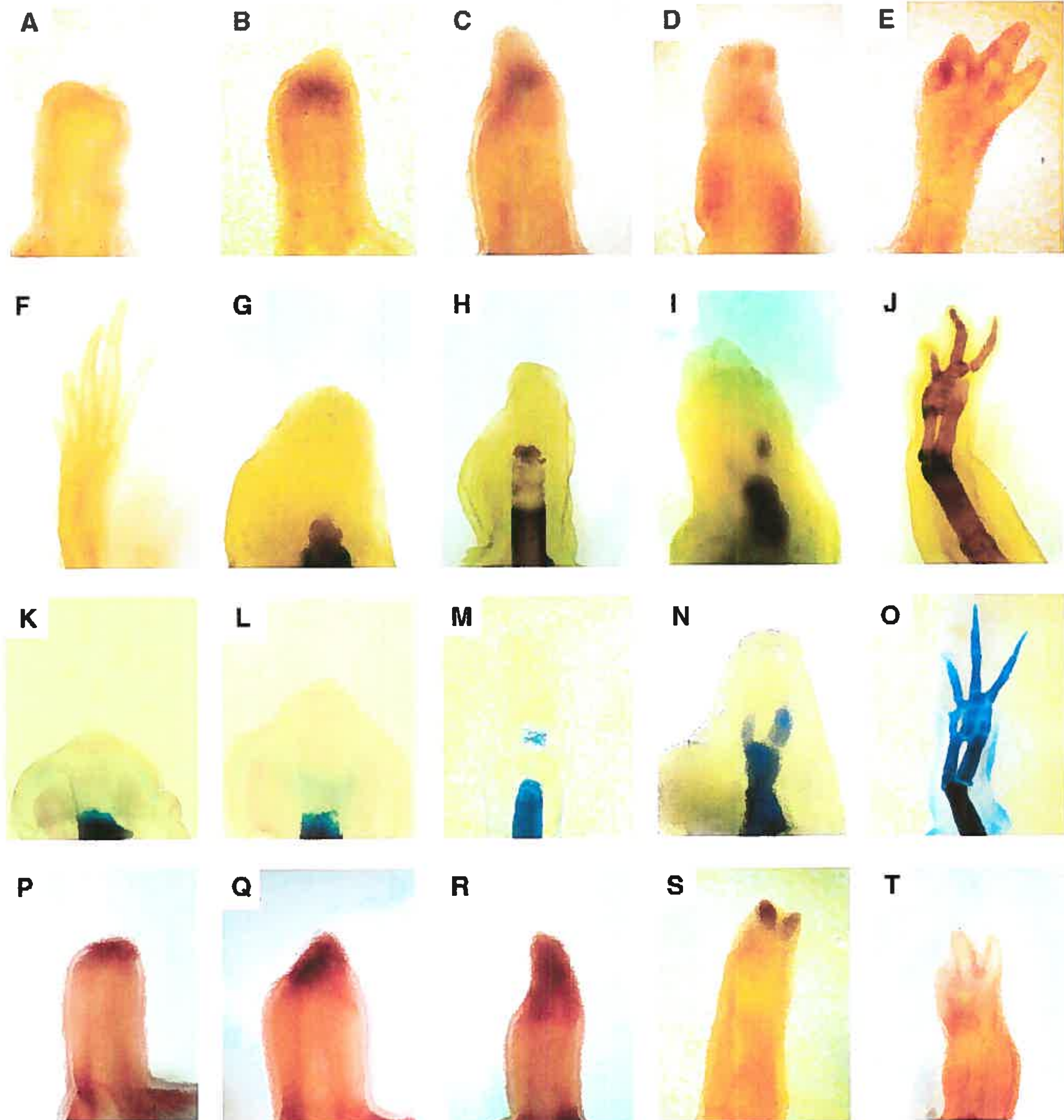

Fig 4. Whole-mount in situ hybridization, immunohistochemistry and cartilage staining of regenerating limbs for *Cbfa-1*, type II collagen, cartilage and *PTHrP*. (A–E) *Cbfa-1* expression, (F–J) type II collagen staining, (K–O) cartilage staining with Victoria blue and (P–T) *PTHrP* expression in proximal amputations during forelimb regeneration. Regeneration stages are: (A, K, P) Early bud stage, (B, G, L, Q) Medium bud stage, (C, H, M, R) Late bud stage, (D, I, N, S) Palette stage and (E, J, O, T) Early differentiation stage. A negative control limb for type II collagen expression is illustrated in panel F. Note that axolotl regeneration stages are characterized according to Tank et al. [70]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Whole-mount in situ analysis of Cbfa-1 and PTHrP expression during limb regeneration

Limbs were amputated proximally (mid-humerus) and whole-mount in situ hybridization was used to detect *Cbfa-1* and *PTHrP* expression at different stages of regeneration. We used the Victoria blue staining technique for cartilage and type II collagen immunohistochemistry to characterize the developing skeleton during limb regeneration in order to compare this process with *Cbfa-1* and *PTHrP* expression. There was no visible *Cbfa-1* signal in the first stages of regeneration, specifically 6 h, 24 h and 48 h after amputation (data not shown). The absence of expression is not surprising since at these stages the process of chondrogenesis has not yet begun [70]. At the early bud and medium bud stages, Victoria blue staining shows no sign of cartilaginous structures in the blastema, only the skeletal element in the stump is detected (Figs. 4K, L). The first appearance of *Cbfa-1* expression in the blastema occurs at medium bud (Fig. 4B). At the late bud stage, new cartilage is formed at the end of the amputated bone (proximal portion of the blastema) as resolved by both Victoria blue staining and type II collagen expression (Figs. 4H, M) and a *Cbfa-1* signal is detected in the distal region of the blastema (Fig. 4C). Accordingly, this is the stage when cartilage differentiation begins in the periosteum of the amputated bone [70]. Kim et al. have previously shown that *Cbfa-1* is localized in the growth plates of long bones during development [33]. Interestingly, a growth plate is also formed during regeneration [70]. As the limb continues to regenerate, the *Cbfa-1* signal becomes more restricted to the distal part of the regenerating blastema at the palette stage (Fig. 4D) and is concentrated in areas of future developing digits similar to the expression pattern observed during limb development (Figs. 3A–C). Type II collagen expression at this stage shows a differential expression pattern to that of *Cbfa-1* (Fig. 4I). The palette stage designates the beginning of cartilage differentiation in the zeugopod of the limb and in bones of the forelimb [70]. This is illustrated in Fig. 4N where cartilage staining allows us to see a rough outline of the radius and ulna. At the early differentiation stage, the first digits have completely formed and the third digit already has two metacarpals (Fig. 4O). At this stage, the *Cbfa-1* signal shows specific expression points in the diaphysis of the phalanges and a particularly strong signal in the digit still in formation (Fig. 4E). As was observed in development, type II collagen expression at the early differentiation stage of regeneration corresponded to the appearance of cartilage (Fig. 4O) and did not overlap with the domains of *Cbfa-1* expression (Figs. 4E, J).

PTHrP expression was also characterized during axolotl limb regeneration (Figs. 4P–T). In the early stages of regeneration (6 h, 24 h and 48 h after amputation), *PTHrP* showed no visible signal of expression (data not shown). However, from the early bud to the late bud stage, strong expression is detected in the regenerating blastema (Figs. 4P–R). At the palette stage, expression is localized to the digit forming region (Fig. 4S) corresponding to formation of the first two digits. Cartilage staining at the early differentiation stage is characterized by the differentiation of the digits (Fig. 4O) where *PTHrP* expression can be detected around the contours of the future digits of the limb (Fig. 4T).

Bone fracture and critical gap healing in axolotl limbs

As previously mentioned, most vertebrates are able to heal bone fractures [6,19,61,65,72] yet all vertebrates studied to date are incapable of healing bone defects of critical dimension [62]. Therefore, since axolotls are capable of regenerating entire limb

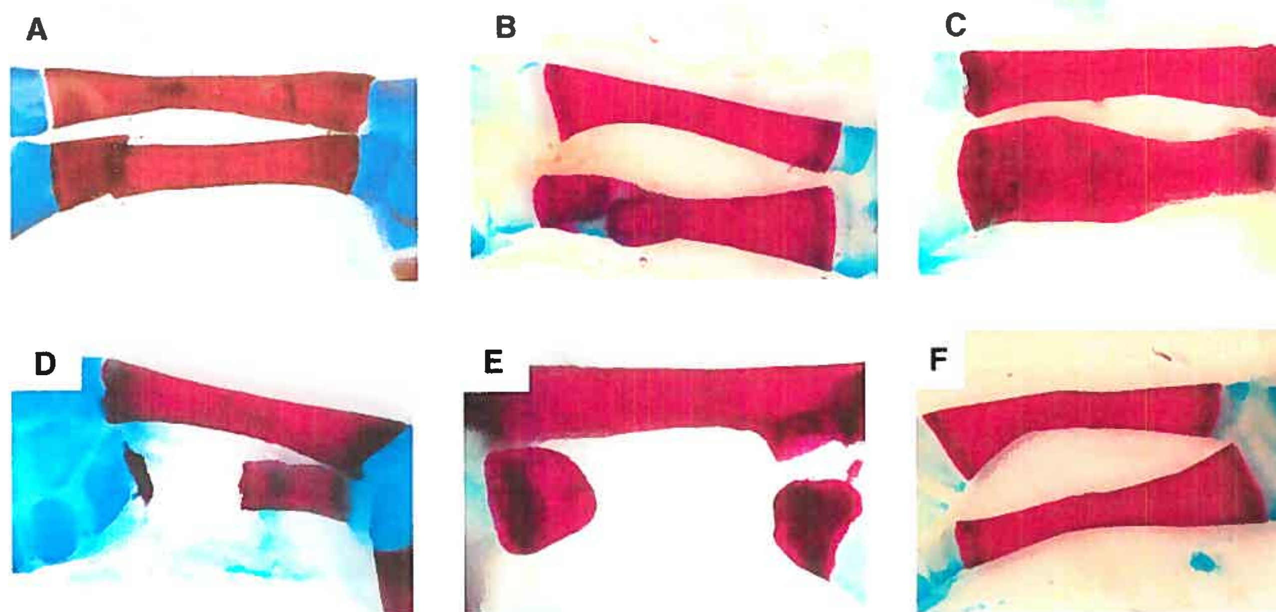

Fig 5. Double staining of non-stabilized union fractures (A–C) and non-stabilized non-union fractures (D, E) on axolotl forelimbs. (A, D) 15 days after surgery, (B) 1 1/2 months post-fracture, (C, E) 7 months post-surgery and (F) sham operated forelimb.

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structures including the skeletal elements without any resulting scar or demarcation lines between the stump and the regenerated tissues, we questioned whether the axolotl is also able to heal bone defects of critical dimension. The results for the non-stabilized union and non-union fractures of critical dimension are shown in Fig. 5. A clear-cut union fracture is shown for the limb 15 days after fracturing (Fig. 5A) with no visible signs of healing. At 45 days post-fracture, the two extremities of the fractured bone are unhinged and a blue halo around the fracture site is noticeable (Fig. 5B). This indicates that cartilaginous cells have migrated to the fracture site and that the healing process has been initiated. After 2 months, the extremities of the fractured bones are joined together by a cartilaginous matrix which will eventually form a soft callus around the fracture site (results not shown). Five months post-fracture, the soft callus is completely replaced by an envelope of calcified matrix that permanently joins the two bone extremities, otherwise known as a hard callus (Fig. 5C shows a completely healed bone fracture after seven months). Fig. 5D shows a non-union fracture 15 days after fracturing. Unlike the non-stabilized union fractures, staining results in the following months show no sign of cartilage at the fracture site (results not shown). Furthermore, during this period there is no evidence that a healing process via a cartilaginous phase has been triggered at the fracture site. Finally, 7 months post-surgery, the bone extremities are slightly rounded and there are still no signs of a healing process or bone regeneration (Fig. 5E).

Discussion

Cbfa-1 and PTHrP expression during limb development and regeneration

In this study, we describe for the first time the cloning, analysis and expression of axolotl *Cbfa-1* and *PTHrP* genes during limb development and regeneration. We also describe the expression of the type II collagen during limb regeneration. Sequence analyses of both *Cbfa-1* and *PTHrP* show high homologies between the axolotl and other species. *Cbfa-1* is known to be expressed during mesenchymal cell condensation and to participate in the remodeling of cartilage and bone precursor cells [13,53,68], which could explain its expression during the early stages of limb development. In addition to its role in osteoblast differentiation, a function in limb patterning has been proposed to explain early *Cbfa-1* expression during development [14,45,53]. In fact, null-mutant mice for *Cbfa-1* show structural defects of the skeleton and, in some cases, lacked scapulae and clavicle bones [46]. It has been shown that *Cbfa-1* is expressed in hypertrophic chondrocytes and mature osteoblasts to regulate their maturation. These cells are mostly concentrated in the diaphysis of the bone [33] which correlates to *Cbfa-1* expression. To corroborate the *Cbfa-1* expression detected, type II collagen protein expression was characterized in the developing limb. Our results were comparable to those reported by Franssen et al. where they demonstrated that type II collagen expression is exclusive to condensing chondrocytes [20] and, more importantly for the present study, is absent in regions expressing *Cbfa-1* which confirms the expression data.

Unlike *Cbfa-1*, *PTHrP* expression was not detected until reaching the more advanced stages of limb development. It was not until stages 50 and 52 that a weak *PTHrP* signal was observed at the distal end of the developing digits and in the carpal region. Similar results were found in developing rat fetuses, the presence of detectable *PTHrP* mRNA being attributed to the proliferative activity of chondrocytes required for proper carpal and metacarpal formation [37,54].

Cbfa-1 and *PTHrP* are also expressed during axolotl limb regeneration. During the first 2-days following amputation, there is no detectable *Cbfa-1* or *PTHrP* signal. The lacking expression may be due to the absence of cell segregation and differentiation in the blastema during these stages [70]. The first noticeable *Cbfa-1* expression is near the amputation site of the blastema at the medium stage. Interestingly, *Cbfa-1* action is not required until the replacement of chondrocytes by osteoblasts [46], events that do not occur until the late bud stage [70]. Tank et al. described the differentiation of the cuff of periosteal cartilage at the amputation site around the time the medium bud stage occurs; these events take place before cartilage differentiation occurs within the blastema and could explain the expression results for of *Cbfa-1* at this stage of regeneration [70]. Tank et al. also reported that during the palette stage of regeneration, there is overt differentiation of chondrocytes at the digital primordial; these findings correlate with the expression of *Cbfa-1* as a regulator of chondrocyte replacement by osteblasts [14].

Despite the weak expression of *PTHrP* found in developing axolotl limbs, expression during limb regeneration was strong. During regeneration, the blastema is subject to intense mitotic activity and total RNA quantities become significantly more important [73] thereby possibly explaining increased *PTHrP* levels. However, there are several alternative reasons to explain the increased *PTHrP* expression at the early bud stage. First, the perichondrium at the bone amputation site produces chondrocytes that accumulate around the existing bone, and *PTHrP* is known to play a role in chondrocyte proliferation [1,36]. Second, it has been proposed that during this stage, the blastema contains exclusively undifferentiated cells [70], interestingly, *PTHrP* expression has been associated with non-differentiated mesenchymal cells destined to become chondrocytes [37,54]. Third, *PTHrP* has been proposed to regulate *Ihh*, one of the molecules responsible for the differentiation of mesenchymal cells into chondrocytes [42,72]. Stark et al. reported that the newt *banded hedgehog* (*bhh*) (homologue of mammalian and avian *Ihh* [16]) was uniformly expressed in the blastema from the initial stages of regeneration. Interestingly, *N-bhh* expression was also dramatically reduced at the palette and early differentiation stages of regeneration [64] as is *PTHrP*. Apart from the absence of *PTHrP* expression prior to blastema formation, *N-bhh* expression pattern correlates with that of *PTHrP* observed during axolotl limb regeneration [64]. Studies on the regenerating deer antler have also shown *PTHrP* expression in the regenerating blastema, particularly in the dermis of the skin, perichondrium, underlying proliferating undifferentiated mesenchymal cells as well as in recently differentiated, non-proliferating chondrocytes [18], all of which

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support the expanded expression found throughout the axolotl blastema.

In general, both *Cbfa-1* and *PTHrP* exhibited different expression patterns during limb development and regeneration which correlated with their own functions in other species. *Cbfa-1* expression pattern in late limb development is conserved with the late regeneration stages, supporting the hypothesis of a biphasic process consisting of an initial preparation phase that is followed by a re-development phase [23]. It was also noted that *Cbfa-1* expression during limb regeneration is weaker than during limb development despite having a conserved expression pattern between both processes. The presence of more mature tissues and extracellular matrix could be an explanation for the difference in the intensity of *Cbfa-1* signal. On the other hand, the expression pattern of *PTHrP* was different between limb development and regeneration, by its intensity but also by the localization of the signal, thus suggesting a possible dual function of this gene during these processes [23]. Overall, the data for these 2 genes support the hypothesis that limb regeneration is a biphasic process. Recent research has shown that there are corresponding sets of early and late genes associated with each phase [23]. Of particular interest, events during the preparation phase of regeneration differ significantly from what is observed during limb development [23,47,48,60] whereas the mechanisms for controlling growth and pattern formation in the blastema are recapitulated during development [23,48,58,59,71]. These findings are supported by the expression patterns observed for *Cbfa-1* during regeneration and development, being differentially expressed throughout the early stages and having nearly identical expression patterns in the later stages. The different *PTHrP* expression observed during the early stages of development and regeneration can also be rationalized according to Muneoka's findings [48]; however, to support the notion of biphasic process, similar expression patterns in the later stages would be expected, as observed for *Cbfa-1*. The different *PTHrP* expression between development and regeneration can be attributed to its many physiological roles. *PTHrP* has been reported to regulate keratinocyte growth and differentiation [5,28] suggesting that it could be an important mediator for skin growth and wound healing. In fact, the wound healing process shares numerous mechanisms with the preparation phase of regeneration [60]. *PTHrP* has also been shown to participate in blastema formation during deer antler regeneration, particularly in the regenerating epithelium and in the mesenchymal cells of the blastema [55]. These observations provide a plausible explanation for the pronounced expression of *PTHrP* during the preparation phase of regeneration and the differing expression patterns observed during the re-development phase of regeneration and the late stages of limb development.

Fracture healing of non-stabilized union and non-union fracture in the axolotl limb

Finally, we verified the axolotl's ability to heal union and non-union bone fractures in order to establish whether these animals can specifically or directly regenerate their bones. The results from this study demonstrate that the axolotl heals union fractures like any other vertebrates [72]. Surprisingly, based on the regenerative capacity of these animals, no healing in the axolotls subjected to non-union fractures of critical dimension was observed, the gap length had not changed 7 months after injury (Fig. 5E). This leads us to conclude that the axolotl does not use the regeneration process to heal fractures. Previous experiments had shown that the adult axolotl is incapable of replacing a missing bone when this one was completely extirpated which is quite different than fracture healing [73]. Studies pertaining to the healing mechanisms used by the axolotl to heal bone fractures are rare; though numerous hypotheses can be formulated to explain the reason why this organism is unable to regenerate bone fractures. First, after fracturing, unlike limb amputation, there is no open wound, thereby inhibiting important epithial–mesenchymal interactions essential for the regeneration process [7,23,73]. Although this is a plausible explanation, it is not likely since the axolotl is able to regenerate missing parts of the heart after ablation [7]. Another important event, essential for limb regeneration, is the large trauma to surrounding tissues that occurs after amputation. Goss (1969) demonstrated that if an axolotl limb is amputated directly through the plane where bone has previously been extirpated, the re-developed section will contain half of the extracted bone, without the base of the bone being present [73]. This experiment not only reveals the importance of a trauma factor, but also that cells composing the skeletal structures already in place in the amputated limb seem to have little implication in the regeneration process [48]. This notion was further tested by Lheureux who demonstrated that after grafting a piece of skin (dermis and epidermis) on an irradiated limb, incapable of regeneration, there is complete regeneration of all structures aside muscle [38]. These experiments demonstrate that the skin contains all the necessary components, excluding cells capable of forming muscles, to assure perfect limb regeneration [15,38]. Another important factor to consider when comparing bone healing and bone regeneration is the presence of dedifferentiated cells during the regeneration process. Cellular dedifferentiation is crucial to the regeneration process, distinguishing regenerating limbs from nonregenerating limbs [27], therefore the presence of dedifferentiated cells may be a determining factor in order to regenerate a bone fracture of critical dimension. Further studies will be needed for a better understanding of the genes involved in bone formation and fracture healing during axolotl limb regeneration in order to establish their requirements during this process.

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