Mechanisms of amphibian macrophage development: characterization of the *Xenopus laevis* colony-stimulating factor-1 receptor

LEON GRAYFER, EVA-STINA EDHOLM and JACQUES ROBERT*

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, USA

ABSTRACT  Macrophage-lineage cells are indispensable to vertebrate homeostasis and immunity. In turn, macrophage development is largely regulated through colony-stimulating factor-1 (CSF1) binding to its cognate receptor (CSF1R). To study amphibian monopoiesis, we identified and characterized the *X. laevis* CSF1R cDNA transcript. Quantitative analysis revealed that CSF1R tissue gene expression increased with *X. laevis* development, with greatest transcript levels detected in the adult lung, spleen and liver tissues. Notably, considerable levels of CSF1R mRNA were also detected in the regressing tails of metamorphosing animals, suggesting macrophage involvement in this process, and in the adult bone marrow; corroborating the roles for this organ in *Xenopus* monopoiesis. Following animal infections with the ranavirus Frog Virus 3 (FV3), both tadpole and adult *X. laevis* exhibited increased kidney CSF1R gene expression. Conversely, while FV3-infected tadpoles increased their spleen and liver CSF1R mRNA levels, the FV3-challenged adults did not. Notably, FV3 induced elevated bone marrow CSF1R expression, and while stimulation of tadpoles with heat-killed *E. coli* had no transcriptional effects, bacterial stimulation of adult frogs resulted in significantly increased spleen, liver and bone marrow CSF1R expression. We produced the *X. laevis* CSF1R in recombinant form (rXlCSF1R) and determined, via *in vitro* cross-linking studies, that two molecules of rXlCSF1R bound the dimeric rXlCSF1. Finally, administration of rXlCSF1R abrogated the rXlCSF1-induced tadpole macrophage recruitment and differentiation as well as bacterial and FV3-elicited peritoneal leukocyte accumulation. This work marks a step towards garnering greater understanding of the unique mechanisms governing amphibian macrophage biology.

KEY WORDS: *Xenopus*, monopoiesis, CSF1, macrophage development, ranavirus

**Introduction**

Macrophage-lineage cells are indispensable to homeostasis and host defenses of all vertebrate species. Monopoiesis, or the development and differentiation of macrophage lineage cells is chiefly mediated by the colony stimulating factor-1 (CSF1; macrophage colony-stimulating factor, MCSF) cytokine and growth factor (Garceau *et al.*, 2010; Hanington *et al.*, 2007; Pixley and Stanley, 2004; Wang *et al.*, 2008). CSF1 functions as a homo-dimer, ligation of the high-affinity tyrosine kinase CSF1 receptor (CSF1R) (Dai *et al.*, 2002), the cell surface expression of which is restricted predominantly to mononuclear phagocytes and their derivative cell populations (Guilbert and Stanley, 1980; Lichanska *et al.*, 1999).

In mammals, the expression levels of the CSF1R increase progressively with macrophage development, from lower levels by myeloid precursors to greater levels by monocytes and further elevated levels by terminally differentiated macrophages (Stanley *et al.*, 1997). Furthermore, the ligation of CSF1R by CSF1 is critical not only to macrophage proliferation, differentiation and survival, but also for effective antimicrobial and anti-tumor host responses orchestrated by these cells (Bober *et al.*, 1995; Karbassi *et al.*, 1987; Munn and Cheung, 1995; Sweet and Hume, 2003). Thus, CSF1R gene expression serves as a reliable marker in the study of monopoiesis during animal development and host immune responses.

Despite the fact that more ancestral vertebrate species possess the CSF1/CSF1R monopoietic systems, the evolutionary origins of these pathways remain poorly understood. CSF1 is present in birds (Garceau *et al.*, 2010), amphibians (Grayfer and Robert, 2013),...
X. laevis CSF1R characterization

Results

Identification and amino acid alignment analysis of the X. laevis CSF1R

To investigate amphibian macrophage development, we identified the full cDNA transcript encoding the X. laevis principal macrophage growth factor receptor, the colony-stimulating factor-1 receptor (CSF1R) by means of conventional and RACE PCR. The extracellular portion of the putative X. laevis CSF1R exhibits low amino acid sequence identity with the CSF1R molecules of other vertebrate species. However, all of these molecules have in common conserved and structurally important cysteine residues and the presence of five putative immunoglobulin-like domains (D1-D5; Fig. 1). Akin to the other vertebrate CSF1Rs, the putative X. laevis receptor possesses a disrupted intracellular domain composed of an ATP binding motif, a kinase insert domain as well as a major...
tyrosine kinase catalytic domain (Fig. 1). Notably, in contrast to the poor amino acid sequence conservation across the extracellular portions of the vertebrate CSF1Rs (including that of *X. laevis*), the intracellular components of these respective proteins exhibit many stretches of evolutionarily conserved residues, particularly throughout the tyrosine kinase catalytic domains (Fig. 1).

**Phylogenetic analysis of vertebrate CSF1R proteins**

Phylogenetic analysis of the putative vertebrate CSF1R protein sequences indicated a clear evolutionary relationship, wherein the fish CSF1Rs formed a separate clade, ancestral to the amphibian CSF1Rs, which in turn branched independently and ancestral to other vertebrate CSF1Rs (Fig. 2). The orthology of the *Xenopus* sequences is supported by high bootstrap values (Fig. 2). Notably, the avian and reptilian CSF1R molecules branched closer together, in a separate clade from the marsupial and mammalian CSF1R proteins (Fig. 2). The zebrafish KitR, which is structurally related to CSF1R, was used as an outgroup to root the tree (Fig. 2).

**Quantitative analysis of CSF1R gene expression in tissues of tadpoles, metamorphic and adult *X. laevis***

Since CSF1R is primarily expressed by macrophage-lineage cells, we next wanted to examine the quantitative gene expression of the *X. laevis* CSF1R in tadpoles (Stg. 54; (Thors *et al.*, 1982a; Thors *et al.*, 1982b)), metamorphs (Stg. 64) and adults (2 years old) and hence delineate the distribution of macrophage populations during development (Fig. 3). CSF1R gene expression in tadpole and adult muscle was low, whereas CSF1R mRNA levels were significantly greater in regressing tail-muscle tissues of metamorphosing animals (Fig. 3), consistent with the accumulation of active macrophages involved in tail reabsorption (Nishikawa *et al.*, 1998). The spleen, liver and lung CSF1R gene expression increased with development, where the metamorph and adult liver and lung CSF1R transcript levels were significantly greater than those seen in the respective tadpole tissues (Fig. 3). Interestingly, the lung CSF1R mRNA levels were also significantly greater than those seen in tadpoles and metamorphs (Fig. 3). Finally, marginal but detectable levels of CSF1R transcripts were detected in the adult bone marrow (Fig. 3).

**Quantitative analysis of CSF1R gene expression in tissue of *X. laevis* tadpoles and adult frogs immunologically challenged with FV3 and heat-killed *E. coli***

To infer on macrophage distribution upon immunological challenge, we examined the expression of CSF1R following infection with the ranavirus Frog Virus 3 (FV3; *Iridoviridae*) or after injections with heat-killed *E. coli* (Fig. 4). In tadpoles, the kidney, which is the central site of FV3 replication, exhibited a modest but significant increase in the CSF1R gene expression at 3 days post FV3 infection (dpi), followed by a decrease at 6 dpi (Fig. 4A). In contrast, a substantial increase of CSF1R gene expression was observed in tadpole spleens (the primary amphibian immune tissue) at 6 (but not 3) dpi (Fig. 4A). The hematopoietic tadpole liver exhibited significant transcriptional increases in CSF1R at both 3 and 6 dpi (Fig. 4A). Interestingly, challenge of tadpoles with heat-killed *E.
coli had no bearing on CSF1R gene expression (Fig. 4C).

As in tadpoles, FV3 infection of adult frogs resulted in increased kidney CSF1R gene expression at 3 dpi and a subsequent decrease at 6 dpi (Fig. 4B). In contrast to the increased CSF1R gene expression in tadpole spleens following FV3 infection, no significant increase of CSF1R gene expression was observed in adult spleens following ranavalanche challenge (Fig. 4B). Furthermore, the adult liver CSF1R gene expression decreased following FV3 infection, significantly so at 6 dpi (Fig. 4B). The bone marrow CSF1R gene expression was significantly increased at both 3 and 6 dpi (Fig. 4B), supporting our previous findings that the amphibian bone marrow may serve as a site of monopoiesis (Grayfer and Robert, 2013).

Intriguingly and again in contrast to the tadpole expression patterns; six days following heat-killed E. coli stimulation of adult frogs, these animals possessed significantly elevated spleen, liver and bone marrow CSF1R gene expression, where the magnitude of the bone marrow upregulation of this gene far exceeded any other CSF1R transcriptional changes reported here (Fig. 4C).

**In vitro rXlCSF1 and rXlCSF1R binding studies**

To confirm that the *Xenopus* CSF1R is the cognate receptor for the monopoietic CSF1 ligand, we produced recombinant rXlCSF1R and rXlCSF1 molecules using an insect expression system, and assessed ligand-receptor binding in vitro using the disuccinimidyl suberate (DSS, 2.5 mM final concentration) crosslinker to stabilize protein interactions and western blot analysis against the V5 tags on these recombinant proteins (Fig. 5). Since the mammalian CSF1 interacts with the D2 and D3 domains of its cognate CSF1R (Chen et al., 2008), we engineered the (rX)CSF1R to comprise of the D2 and D3 extracellular domains of the *X. laevis* receptor. Western blot analysis of the crosslinked 25 kDa rXlCSF1 ligand revealed a band shift to 50 kDa; indicative of rXlCSF1imerization (Fig. 5). Following DSS crosslinking, rXlCSF1R could no longer be detected by western blot, which is typical of non-interacting proteins (Fig. 5). Notably, when the rXlCSF1 and rXlCSF1R were coincubated and crosslinked with DSS, a band shift to 130 kDa was observed, indicating that the dimerized rXlCSF1 interacted with two 40 kDa molecules of rXlCSF1R in solution (Fig. 5). The crosslinking of rXlCSF1R in the presence of bovine serum albumin (BSA) did not yield a banding pattern, indicating lack of non-specific interactions (Fig. 5).

**rXlCSF1R abrogates the rXlCSF1-mediated recruitment and differentiation of tadpole peritoneal macrophages**

To confirm the biological roles of rXlCSF1 and rXlCSF1R, we examined the ability of the recombinant ligand to elicit and differentiate tadpole peritoneal macrophages, and the capacity of the CSF1R D2-D3 extracellular domain-containing rXlCSF1R to abrogate this process by antagonizing the effects induced by rXlCSF1 (Fig. 6). Twenty-four hours following intraperitoneal injection of tadpoles with the rXlCSF1 resulted in significant accumulation of peritoneal macrophages (Fig. 6A). In contrast, administration of rXlCSF1R induced a reduction in the numbers of tadpole resident peritoneal phagocyte populations (Fig. 6A). Furthermore, a four-
fold excess of rXCSF1R co-injected with rXCSF1 abrogated the rXCSF1-mediated macrophage elicitation (Fig. 6A).

Microscopy analysis of cytospin preparations stained by Giemsa revealed that peritoneal leukocytes obtained from tadpoles injected with vector control were comprised primarily of smaller mononuclear phagocytes, whereas rXCSF1 treatment resulted in the accumulation/differentiation of considerably larger, ruffled and highly vacuolated macrophages (Fig. 6B). On average, cells from vector control administered animals consisted of only 14.9% large, ruffled macrophages, whereas these larger cells comprised 69.6% of the rXCSF1R-derived macrophages (Fig. 6C). Notably, peritoneal phagocytes isolated from tadpoles administered rXCSF1R alone had significantly lower numbers of large, ruffled macrophages (6.4%) than even those seen in the vector control cultures (Fig. 6C), suggesting that resident peritoneal macrophages also rely on native CSF1 for survival.

Given that the administration of rXCSF1R significantly affected the differentiation and abundance of resident tadpole peritoneal macrophages (see Fig. 6), we next assessed how rXCSF1R treatment would impact the tadpoles' capacity to recruit leukocytes into the peritoneum following heat-killed *E. coli* or FV3 challenge (Fig. 7A). Notably, the numbers of peritoneal leukocytes elicited one day after stimulation with heat-killed *E. coli* were dramatically decreased by co-injection with rXCSF1R (Fig. 7A). Similarly, macrophage infiltration induced one day after FV3 infection was significantly decreased by co-injecting rXCSF1R with the FV3 (Fig. 7A). These findings suggest that the macrophage-suppressive effects conferred by rXCSF1R administration (Fig. 6) culminate in decreased capacities of resident mononuclear phagocytes to recruit additional leukocyte populations in response to immune stimuli.

Since we have shown that *X. laevis* macrophages derived by the recombinant ligand rXCSF1 increases tadpoles susceptibility to FV3 (Grayfer and Robert, 2014) and that converging evidence
indicates that ranaviruses rely on macrophages to disseminate inside their hosts (reviewed in Grayfer et al., 2012), we postulated that ablation of tadpole resident peritoneal macrophages by rXCSF1R should also reduce FV3 dissemination into tadpole organs. Accordingly, tadpoles were injected with FV3 alone, or in combination with rXCSF1R and virus loads in various tissues were assessed the following day (Fig. 7B). In support of our previous findings, the rXCSF1R administration significantly reduced FV3 loads in kidney and spleen, but not liver (Fig. 7B). This suggests that peritoneal CSF1-dependent phagocytes are critical for the dissemination of this virus.

Discussion

This manuscript represents the first characterization of an amphibian CSF1R. Despite the poor conservation of the overall amino acid sequence among vertebrate CSF1R molecules, all CSFR1 gene products, including the X. laevis, share hallmark features including 5 putative immunoglobulin domains, structurally conserved cysteine residues as well as a disrupted tyrosine kinase domain. The extracellular portions of CSF1R molecules exhibit more divergence, possibly reflecting evolutionary drift to facilitate the binding of respective cognate CSF1 ligands, which also display low amino acid sequence conservation (Grayfer and Robert, 2013). By contrast, the intracellular catalytic tyrosine kinase domains of these respective CSF1R proteins are remarkably well conserved, perhaps marking the evolutionary functional necessity for retaining these protein sequences. The evolutionarily diverged CSF1R amino acid sequences of distinct vertebrate classes are also reflected in their phylogenetic relationships, wherein the fish, amphibian, avian, reptilian and mammalian receptors all branched in respective separate clades. Indeed the CSF1R catalytic domains have been evolutionarily conserved across vertebrates, suggesting conservation in downstream CSF1R cell signaling and presumably the resulting biological outcomes. However, many other aspects of lower vertebrate macrophage biology appear to be distinct from what has been documented in mammals. This includes varying teleost CSF1 ligand and receptor gene copy numbers (Hanington et al., 2007; Wang et al., 2008; Wang et al., 2014; Williams et al., 2002) and unique physiological localization of amphibian macrophages (Grayfer and Robert, 2013). It remains to be determined whether these differences arise from and/or are dependent on CSF1-CSF1R functions which are different to those of mammals. Thus, it is possible that the monopoietic roles conferred by CSF1-CSF1R of distinct lower vertebrate species are at least partially unique to those described in mammals.

Whereas birds and mammals possess a single CSF1 gene expressing alternatively spliced transcripts, fish possess at least two distinct CSF1 genes that do not appear to produce alternatively spliced products (Hanington et al., 2007; Wang et al., 2008; Wang et al., 2014). Presently, it is unknown whether the functions of the different fish CSF1 genes correspond to those mediated by the respective alternatively spliced mammalian transcript products. Furthermore, at least some fish species, including Fugu, possess 2 distinct CSF1R genes (Williams et al., 2002). Therefore, it is quite possible that similar to the fish type II IFN system (Aggad et al., 2012; Grayfer and Belosevic, 2009; Grayfer et al., 2010; Shibasaki et al., 2013; Yabu et al., 2011), the multiple teleost CSF1 receptor and ligand gene products may exhibit complex interactions, distinct from the single ligand, single receptor strategy of higher vertebrates. In our recent study of the X. tropicalis and X. laevis CSF1 genes (Grayfer and Robert, 2013), we did not identify additional CSF1 gene copies in X. laevis and X. tropicalis genomes, nor did we detect additional CSF1R genes during the studies described here. It is noteworthy that despite our best efforts, using both conventional and RACE PCR approaches, we were unable to identify alternatively spliced X. laevis CSF1 transcripts (data not shown). Although more investigation are needed, it appears that alternatively spliced CSF1 and/or CSF1R genes are absent or at least of minor importance in Xenopus. It is possibly that the unique Xenopus monopoietic strategy requires a single non-alternatively spliced Xenopus CSF1 and a single CSF1R.

The highest level of CSF1R gene expression was observed in the lung and liver of X. laevis adults. Presumably these expression patterns reflect the presence of alveolar macrophages (Lin et al., 1989) and Kupffer cells (Amemiya et al., 2011), wherein the mammalian counterparts of both of these macrophage-lineage populations express high CSF1R levels. Notably, CSF1R gene expression increased with X. laevis development in the majority of tissues examined, including kidney, spleen, liver, lung and skin. This may represent the accumulation of resident macrophages, reflecting complex growing biological necessities for these cells with frog development and maturation. Alternatively, it is possible that the level of CSF-1R gene expression per cell increases during development. In addition, the CSF1R gene expression increased in the muscle tissue of regressing tails from metamorphosing animal. Indeed, macrophages have been previously demonstrated to play crucial roles in tail and body muscle remodeling during metamorphosis (Nishikawa et al., 1998), where our present observations corroborate with these earlier findings. It will be interesting to examine the differences in CSF1 responsiveness of macrophage precursors in tadpoles and adult X. laevis, since they are both CSF1-responsive (Grayfer and Robert, 2013; Grayfer and Robert, 2014), whereas macrophage development in adult involves the bone marrow that is absent in tadpoles (Grayfer and Robert, 2013).

Following FV3 infection, both tadpoles and adults exhibited increased kidney CSF1R gene expression, which is interesting considering that the kidney is the primary site of FV3 replication (Gantress et al., 2003; Robert et al., 2007). In addition, macrophages are intimately involved in both immunity and the infection strategy of FV3 (Morales et al., 2010; Grayfer et al., 2012). Therefore, it seems reasonable to speculate that CSFR gene expression increases due to the infiltration of kidney tissues by macrophage-lineage cells. Intriguingly, the spleen and liver CSF1R gene expression patterns induced by FV3 infection were markedly different between tadpoles and adults, possibly underlining their distinct monopoietic strategies. In light of our recent findings that the X. laevis bone marrow serves as the prime source of macrophage precursors (Grayfer and Robert, 2013), together with our present observation that FV3 and heat-killed E. coli both elicit increased bone marrow CSF1R expression may indicate that in response to immunological challenges, adult Xenopus increase monopoietic activity at the level of the bone marrow. By contrast, tadpoles do not possess bone marrow and thus, presumably orchestrate monopoiesis in the hematopoietic liver. This would explain the increased CSF1R gene expression in FV3-infected tadpole, but not adult liver tissues. In comparison to adults, the tadpole spleen may likewise be more prominently involved in macrophage development and immunity as
reflected in substantially more upregulated CSF1R gene expression within this tadpole tissue following FV3 challenge. It is interesting that heat-killed *E. coli* did not elicit significant CSF1R expression changes in any of the tadpole tissues examined, whereas following this inflammatory stimulus adults exhibited substantially more robust CSF1R gene expression increases in the spleen, liver and bone marrow than observed following FV3 infection. These disparities may reflect differences in pathogen pattern recognition receptor expression between tadpoles and adults, and a physiological necessity for more prominent adult monopoietic responses to inflammatory, rather than viral challenges. A greater understanding of *Xenopus* macrophage development and immune responses will no doubt shed light on this present enigma. We emphasize that in our present work *E. coli* was used as an inflammatory stimulus rather than a direct point of comparison with the more relevant FV3 viral challenge. Future *X. laevis* infection studies using relevant bacterial pathogens will lend to our understanding of the differences in macrophage involvement during different host responses.

The mammalian dimeric CSF1 binds exclusively to the D2 and D3 domains of the CSF1R, dimerizing the receptor (Chen et al., 2008). Our *in vitro* binding studies indicate that the D2 and D3 domains of a recombinant amphibian CSF1R are also sufficient to bind the recombinant homodimerized CSF1 ligand. More detailed studies will be needed to elucidate the modalities and stoichiometry of these interactions. Notably, the rXlCSF1R very effectively inhibited rXlCSF1-mediated tadpole elicitation and development of macrophages into cells morphologically resembling mature, differentiated populations. Furthermore, rXlCSF1R administration also ablated the recruitment of peritoneal leukocytes elicited by heat-killed *E. coli* and the FV3. Since tadpoles administered with rXlCSF1R exhibited diminishing numbers of mature resident peritoneal macrophages, the compromised capacities of rXlCSF1R-treated tadpoles to recruit leukocytes into the peritoneum upon ip immune challenges likely reflects a functional impairment of peritoneal macrophages, which would normally chemo-attract additional immune populations in response to immune stimuli. Notably, teleost fish appear to have evolved an additional regulatory mechanism of monopoiesis and inflammation whereby they produce an alternatively spliced, soluble CSF1R (Barreda et al., 2005; Rieger et al., 2014a; Rieger et al., 2014b). Intriguingly, this moity only possesses the D1 and D2 domains of the membrane-bound CSF1R and yet this molecule is highly biologically active and inhibits fish macrophage proliferation and inflammatory responses (Barreda et al., 2005; Rieger et al., 2014a; Rieger et al., 2014b). It will be interesting to learn which specific domains are involved in the interactions of the respective teleost CSF1 ligand(s), soluble and membrane bound CSF1Rs and whether a similar system is present in amphibian species.

Converging evidence indicate that macrophage-lineage cells are utilized by ranaviruses as a means of dissemination within their hosts (Grayfer et al., 2012). In addition, CSF1-derived macrophages render tadpoles more susceptible to FV3 (Grayfer and Robert, 2014). Here, we show that intraperitoneal administration of rXlCSF1R reduces resident phagocyte numbers and impairs some of their functions such as leukocyte recruitment. The treatment with rXlCSF1R also results in decreased dissemination of FV3 from the inoculation site of the peritoneum to the kidney (central site of ranavirus replication) and the spleen (central immune organ), whereas it does not affect low viral dissemination into the liver. These observations underline the complexity of the roles of mononuclear phagocytes as both mediators of anti-RV immune responses and culprits in the progression of these infections.

The CSF1-CSF1R axis represents the focal point of vertebrate monopoiesis and it has become apparent that evolutionarily more ancestral species such as teleosts and amphibians may possess varying strategies for CSF1-mediated macrophages development, distinct from those described in mammalian species. Further research into CSF1 and CSF1R macrophage biology of lower vertebrates will yield new insights into the evolutionary basis of monopoiesis and permit the development of more effective preventative measures against pathogens that infiltrate macrophage-lineage cells of more ancient species.

**Materials and Methods**

**Animals**

Outbred pre-metamorphic (stage 54, (Thors et al., 1982a; Thors et al., 1982b) tadpoles, metamorphic (stage 64) and adult (2 years old) frogs were obtained from our *X. laevis* research resource for immunology at the University of Rochester (http://www.urmc.rochester.edu/mbi/resources/Xenopus-laevis/). All animals were handled under strict laboratory and University Committee on Animal Research regulations (Approval number 100577/2003-151).

**Identification and analysis of X. laevis colony-stimulating factor-1 receptor**

The identification of the *X. laevis* CSF1 was described previously (Grayfer and Robert, 2013). A fragment of the *X. laevis* CSF1R cDNA transcript was identified using primers against the predicted *X. tropicalis* CSF1R sequence (Acc. no.: BC082504). Subsequently, RACE PCR was performed in accordance with manufacturers’ directions (Clonetech) to identify the complete *X. laevis* CSF1R cDNA transcript (Acc. No.: KM400585). All primer sequences are available upon request.

Protein sequence alignments were performed using the Clustal W software (http://www.ebi.ac.uk/clustalw/). Signal peptide regions were identified using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP). Protein motif and domain predictions were achieved using the ELM (http://elm.eu.org/) and SMART (http://smart.embl-heidelberg.de/) servers. Phylogenetic analysis was performed by Clustal X software using the neighbor joining method and bootstrapped 10,000 times, with values expressed as percentages.

**Production of rXlCSF1 and rXlCSF1R**

The production of the rXlCSF1 has been described previously (Grayfer and Robert, 2013). The rXlCSF1R was produced by transfecting Sf9 insect cells (cellfectin II, Invitrogen) with the pMIB/V5 HisA insect expression vector (Invitrogen) containing the *X. laevis* CSF1R sequence corresponding to the IG2 and IG3 domains of the extracellular portion of the protein. Transfected Sf9 supernatants were confirmed for rXlCSF1R expression, positive transfectants were scaled up to 500 mL in liquid cultures and grown for 6 days in blastocidin (10 μg/mL)-containing medium. Resulting supernatants were dialyzed overnight at 4/C against 150 mM sodium phosphate, concentrated against polyethylene glycol flakes (8 kDa), dialyzed overnight at 4/C against 150 mM sodium phosphate and passed through Ni-NTA agarose columns (Qiagen). Columns were washed with 2x10 volumes of high stringency wash buffer (0.5% Tween 20; 50 mM Sodium Phosphate; 500 mM Sodium Chloride; 100 mM Imidazole) and 5x with low stringency wash buffer (as above, but with 40 mM Imidazole). The rXlCSF1R was eluted in fractions using 400 mM Imidazole. The purity of the eluted fractions was confirmed by silver-stain and the presence of rXlCSF1R was assessed by western blot against the V5 epitope on the recombinant protein. Fractions containing the rXlCSF1R were pooled and the protein concentration determined by the Bradford Protein Assay (BioRad). A protease inhibitor cocktail (Roche) was added to the purified protein, which was then aliquoted.
In vitro rXICSF1 and rXICSF1R cross-linking studies

One microgram each of rXICSF1, rXICSF1R, rXICSF1 + rXICSF1R and BSA + rXICSF1R were incubated in APBS (100μl final volume) for 30 min, cross-linked for 30 min using 2.5 mM disuccinimidyl suberate (DSS, final concentration, Thermo Scientific). Cross-linking was terminated for 15 min by the addition of 50 mM Tris (final concentration). The reactions were then resolved and visualized using SDS-PAGE and western blot against the V5 epitopes on the recombinant proteins and developed using ECL (Pierce) on X-ray film (Eastman Kodak Co).

**Statistical analysis**

Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey’s post hoc test, using Vassar Stat (http://faculty.vassar.edu/lowry/anova1u.html) statistical program. Probability level of P<0.05 was considered significant.

**Acknowledgements**

This work was supported by National Institute of Health (R24-AI-059830) and National Science Foundation (IOB-074271) grants to J.R. L.G. was supported by a National Sciences and Engineering Research Council of Canada Postdoctoral Fellowship and a Life Sciences Research Fellowship from the Howard Hughes Medical Institute. We thank Tina Martin for animal husbandry. This manuscript was improved by the insightful suggestions of one anonymous reviewer.

**References**


Further Related Reading, published previously in the *Int. J. Dev. Biol.*

**Sexual dimorphism of AMH, DMRT1 and RSPO1 localization in the developing gonads of six anuran species**  
Rafal P. Piprek, Anna Pecio, Katarzyna Laskowska-Kaszub, Jacek Z. Kubiak and Jacek M. Szymura  
*Int. J. Dev. Biol.* (2013) 57: 891-895

**Dual embryonic origin of the hyobranchial apparatus in the Mexican axolotl (Ambystoma mexicanum)**  
Asya Davidian and Yegor Malashichev  
*Int. J. Dev. Biol.* (2013) 57: 821-828

**Clonal analyses in the anterior pre-placodal region: implications for the early lineage bias of placodal progenitors**  
Sujata Bhattacharya and Marianne E. Bronner  
*Int. J. Dev. Biol.* (2013) 57: 753-757

**Amphibian interorder nuclear transfer embryos reveal conserved embryonic gene transcription, but deficient DNA replication or chromosome segregation**  
Patrick Narbonne and John B. Gurdon  
*Int. J. Dev. Biol.* (2012) 56: 975-986

**Origins of Cdx1 regulatory elements suggest roles in vertebrate evolution**  
Stephen J. Gaunt and Yu-Lee Paul  
*Int. J. Dev. Biol.* (2011) 55: 93-98

**Reptile scale paradigm: Evo-Devo, pattern formation and regeneration**  
*Int. J. Dev. Biol.* (2009) 53: 813-826

**Proteomics analysis of regenerating amphibian limbs: changes during the onset of regeneration**  
Michael W. King, Anton W. Neff and Anthony L. Mescher  
*Int. J. Dev. Biol.* (2009) 53: 955-969

5 yr ISI Impact Factor (2013) = 2.879