The germ theory of regeneration

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Abstract

Amphibians have remarkable regenerative capabilities, but the mechanisms they use to regenerate are largely unknown. Identifying these mechanism would be of great interest for applications in medicine. Reactive oxygen species (ROS) are continually produced and required during tadpole tail regeneration (1). Production of ROS and tadpole tail regeneration are prevented by NADPH oxidase (Nox) inhibitors, suggesting Nox complexes as the source of ROS. However, the role of ROS and the mechanism of their sustained production throughout regeneration, were not known. NF-κB is a rapid-acting transcription factor with the potential to dramatically alter the activity and function of a cell (2). NF-κB is necessary for maintaining the undifferentiated state of human embryonic stem cells (3), human induced pluripotent stem cells (4) and mesenchymal stem cells (5), so may similarly be involved in maintaining the de-differentiated state of regeneration blastema cells. In the absence of an activating signal, NF-κB is sequestered in the cytoplasm by IκB (inhibitor of NF-κB), preventing its nuclear localisation and activity. The IκB kinase (IKK) complex inhibits IκB in response to multiple extracellular stimuli, but ROS can also inhibit IκB (6). Nuclear NF-κB directly activates transcription of several genes encoding Nox proteins (7, 8), so could thereby facilitate ROS production. A positive-feedback loop was hypothesised where ROS inhibit IκB to help maintain continual NF-κB activity and, in turn, facilitate the continual production of ROS by activating the transcription of Nox-encoding genes. Here we demonstrate the involvement of microorganisms in the initiation of tadpole tail regeneration. Microorganisms offer sources of ligands for toll-like receptor (TLR) pathway activation and consequently, IKK complex activity. It was also suggested that sustained NF-κB activity allows the continual expression of the genes encoding Nox4 in blastema cells and Nox2 in professional phagocytes. These findings provide potential targets for the activation of regeneration in non-regenerative animals.

regenerative medicine| *Xenopus laevis*| reactive oxygen species | toll-like receptor| NF-κB

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Main

The ROS concentration greatly increases around the site of injury within 20 minutes after tadpole tail amputation, and is maintained at these high levels throughout the process of regeneration (1). Raising tadpoles in the presence of the Nox inhibitor, DPI, for 3 days following tail amputation prevents regeneration (1). This shows that ROS are important for regeneration, but does not differentiate between the initial burst and the maintenance of ROS production. To see if ROS production was required during the initial hours following amputation, shorter DPI treatment durations were tested. Tadpole tail regeneration was significantly reduced following

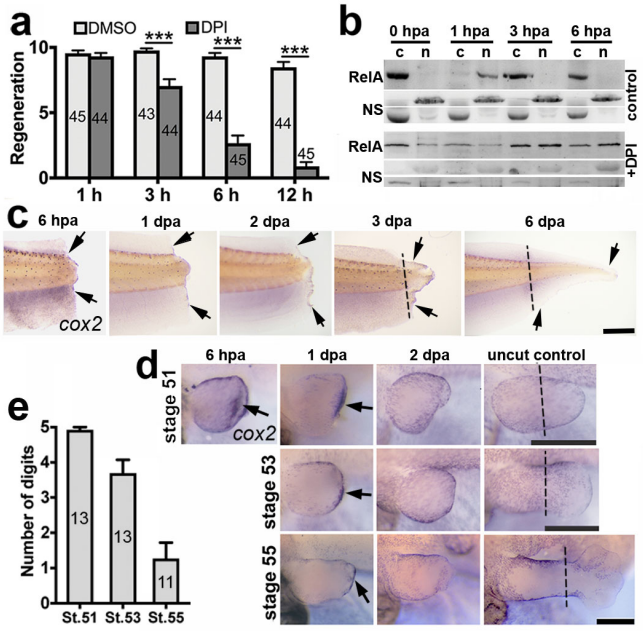

Fig. 1. *Nox* and NF-κB are active within hours after amputation. a, Mean tail regeneration scores + s.e.m of stage 43 tadpoles treated with DPI or DMSO control for 1 h before amputation and the indicated hpa. Sample numbers are indicated inside bars. *p* values are based on ordinal logistic regressions: *** $p < 0.001$. **b**, Western blots showing RelA in cytoplasmic C and nuclear N protein fractions from tail sections at indicated hpa ± DPI. Non-specific NS bands are included as loading controls. **c-d**, *In situ* hybridisations, using a *cox2* probe, of amputated tails (**c**) and hindlimbs (**d**). The stage at amputation and fixation times (hpa/dpa) are labelled. Black arrows indicate staining and dashed lines indicate the amputation planes in unamputated controls. Scale bars: 500 µm. **e**, Mean regeneration scores of hindlimbs amputated at the indicated stages + s.e.m. with sample numbers indicated inside bars.

DPI treatment for 3, 6 and 12 hours post-amputation (hpa) (Fig 1a). Longer treatments resulted in greater reductions in regeneration, with post-amputation treatments of 6 and 12 hours reducing regeneration scores to less than half of their respective controls. These data suggest, on average, a requirement for Nox within the first 6 hours, but not within the first 3 hours, following amputation.

Inactive NF-κB is sequestered in the cytoplasm and translocates to the nucleus upon activation. A Western blot of nuclear and cytoplasmic protein extracts from tadpole tail sections showed RelA (p65 subunit of NF-κB) predominantly in the cytoplasm of uninjured tails and in the nucleus at 1 hpa (Fig 1b). By 3 hpa, it was once again located predominantly in the cytoplasm. This translocation was greatly delayed in the presence of DPI (Fig 1b), with RelA predominantly in the cytoplasm at 1 hpa, suggesting the involvement of ROS produced by Nox. The initial surge of ROS, where ROS were observed far beyond the amputation

plane (1), would account for the NF-κB nuclear translocation. The initial, widespread NF-κB translocation may not be essential as DPI did not prevent regeneration within the timeframe of this event. The Western blots were performed with protein extracts from large tail sections and do not have the sensitivity to detect NF-κB activity immediately adjacent to the amputation plane.

The direct NF-κB target and marker of inflammation (9), *cox2*, is expressed along the amputation plane at 6 hpa and along the edge of tadpole tail regenerates throughout regeneration (Fig 1c). Expression of *cox2* was strong in stage 51 (regenerative) hindlimbs at 6 hpa and 1 day post-amputation (dpa) in the tissue beneath the wound epithelium, but was not observed at 2 dpa (Fig 1d). *Xenopus* limbs regenerate well early in development, but this capacity becomes lost as development progresses (10), allowing regenerative competency and incompetency to be studied in the same appendage (Fig 1e). Expression along the amputation plane at 1 dpa was reduced with decreasing regenerative capacity (Fig 1d, e). These data suggest that NF-κB is active along the amputation plane in regenerating tails and limbs and its activity in limbs is correlated with regenerative success.

NF-κB also directly activates *nox1*, *nox2* and *nox4* (7, 8), which encode the catalytic subunit of different Nox complexes (11). Since Nox is required for tail regeneration (1) and the NF-κB target, *cox2*, is expressed in regenerating tails and hindlimbs, it was hypothesised that at least one of these NF-κB-targeted *nox* genes would also be expressed during regeneration. Indeed, both *nox2* and *nox4* were expressed in regenerating stage 51 hindlimbs at all timepoints tested (Fig 2, which span the timeframe of blastema formation (12). Expression of *nox2* was punctate and comparable to the distribution of neutrophils and macrophages in regenerating hindlimbs (13), consistent with its expression being confined to these cells. Expression of *nox4* was observed around the injury and throughout the regenerate. No obvious expression of *nox1* was observed at any timepoints. These results suggest Nox2 and Nox4 as drivers of ROS production during regeneration. Preventing inflammatory cell recruitment to the injury does not significantly alter ROS production in regenerating tadpole tails (1), suggesting that Nox2 does not substantially contribute to overall ROS production during tail regeneration.

Xenopus laevis tadpoles can regenerate their tails up to metamorphosis, but temporarily lose this ability during the 'refractory period' from stages 45 to 47 (14). It was hypothesised that this refractory period could be due to a reduction in ROS signalling. To see if acute exposure to exogenous ROS during the refractory period improved regeneration, tadpole tails were dipped in different concentrations of hydrogen peroxide (H_2O_2) after amputation at stage 46. Unexpectedly, the H_2O_2 dip reduced, rather than enhanced, regeneration at concentrations of 3% and 0.3% (data not shown). Interestingly, the control tadpoles had good regeneration, which

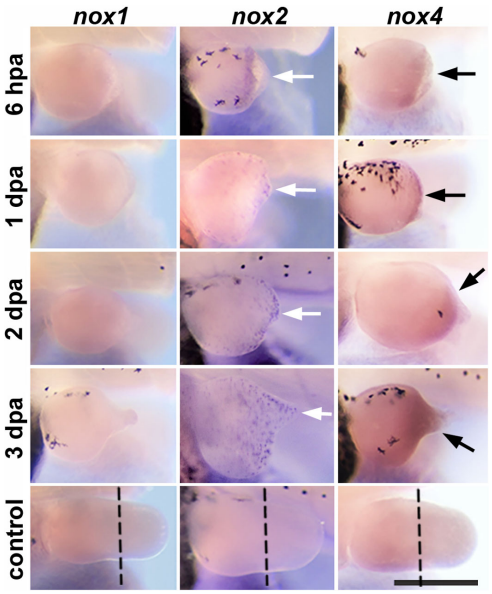

Fig. 2. *nox2* and *nox4* are expressed in regenerating hindlimbs. *In situ* hybridisations, using indicated probes, of amputated stage 51 tadpole hindlimbs. Fixation times (hpa/dpa) are labelled. White arrows indicate punctate staining, black arrows indicated continuous staining and dashed lines indicate the amputation planes in unamputated controls. Scale bar: 500 µm.

is not consistent with the refractory period. This prompted a re-validation of the refractory period under current culture protocols. Despite repeated attempts using different batches of tadpoles, a clear refractory period was not observed.

The unexpected reduction in regeneration following H_2O_2 treatment prompted a search for an explanation. The NF-κB activator, IKK, can be activated by the binding of ligands from microorganisms to toll-like receptors (TLR) (15). Lipopolysaccharides (LPS) are present on the surface of Gram-negative bacteria and bind to TLR4, eliciting activation of NF-κB via IKK (15). LPS which have been treated with H_2O_2 no longer function as TLR4 ligands (16). It was hypothesised that dipping tails in H_2O_2 was inhibiting regeneration by disabling TLR ligands of bacteria around the wound, preventing TLR activation. In the absence of TLR activation, IKK may not be activated on amputation, potentially preventing NF-κB activity and the ROS burst being sustained.

To test this, heat-killed (HK) *Escherichia coli* (Gram-negative bacteria with surface LPS (17)) were added following tail amputation and H_2O_2 treatment (Fig 3a). Regeneration was greatly improved following addition of HK *E. coli*. Strikingly, tadpoles treated with 0.3% H_2O_2 and HK *E. coli* had significantly better regeneration than the untreated controls. To see if H_2O_2 treatment of tadpole skin, and not the wound itself, was sufficient to inhibit regeneration, stage 46 tadpoles were briefly immersed in H_2O_2 prior to amputation (Fig 3b). Regeneration was significantly reduced with H_2O_2 pre-treatment, consistent with the hypothesis that naturally occurring bacterial ligands on the skin surface have an important role in initiating regeneration. The reduction in regeneration with H_2O_2 treatment varied

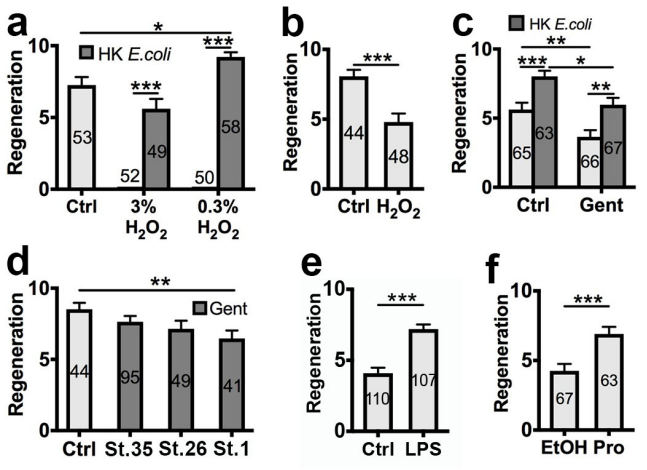

Fig. 3. Regeneration is activated by bacterial ligands. Mean regeneration scores + s.e.m. of stage 46 tadpoles: **a**, with amputated tails dipped in the indicated concentration of H_2O_2 ± HK *E. coli* and untreated control; **b**, immersed in 0.3% H_2O_2 prior to tail amputation and untreated control; **c**, raised with or without gentamicin prior to tail amputation ± HK *E. coli*; **d**, raised with gentamicin beginning at the indicated developmental stage before tail amputation + HK *E. coli*; **e**, with tails amputated ± LPS; **f**, with tails amputated + prostratin or ethanol control. Sample numbers are indicated inside bars. *p* values are based on ordinal logistic regressions: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

between batches of tadpoles (data not shown), possibly due to varying bacterial loads.

To further test this hypothesis, tadpoles were raised with the antibiotic, gentamicin, to reduce the bacterial load on the skin (Fig 3c). Tadpoles raised with gentamicin had low regeneration scores after amputation at stage 46, which were significantly increased by addition of HK *E. coli*. Interestingly, with added HK *E. coli*, the gentamicin treated tadpoles had significantly lower regeneration scores than their siblings raised without gentamicin. Gentamicin reduced the regenerative potential of tadpole tails, even with addition of HK *E. coli*, and this reduction was greater the earlier gentamicin treatment began (Fig 3d). Purified LPS also significantly improved regeneration of stage 46 tadpole tails (Fig 3e), as did the protein kinase C activator, prostratin (Fig 3f), which activates IKK intracellularly, independent of TLRs (18). These results reveal the involvement of ligands binding to TLRs in the activation of regeneration, possibly through the IKK pathway.

Gentamicin is commonly used by researches raising *Xenopus* embryos to reduce early deaths, but we have found untreated tadpoles do better as adults. In order to maintain a healthy breeding colony, we have not been routinely raising our embryos with antibiotics. The refractory period may exist as a product of a sterile laboratory environment. The refractory period ends at stage 48. In order to progress from stage 47 to 48, tadpoles need to be fed and would therefore no longer be in a sterile environment and regain regenerative capacity through TLR pathway activation.

TLR4 is limited to only a few cell types, including macrophages (19), so TLR4 would not be sufficient to activate NF-κB-mediated regeneration in other cell types. TLR4 pathway activation in macrophages leads to the production and release of pro-inflammatory cytokines including TNFα and IL-1β (20) which have receptors on most cell types (21–23). Binding of these cytokines to their respective cell-surface receptors leads to the activation of IKK (24) and could consequently spread the activation signal to many cell types. TNFα is important for skeletal muscle regeneration (25, 26) and liver regeneration (27, 28) and essential for zebrafish fin regeneration (29). The recruitment of macrophages may thereby be important for TLR-dependent regeneration. Interestingly, macrophage removal prevents regeneration of salamander limbs (30). The regenerative competency of pre-refractory tadpole tails, raised in a sterile environment (14), indicates that they must have an alternative, TLR-independent, and perhaps also IKK-independent, mechanism of regeneration. Reducing inflammatory cell migration does not prevent tail regeneration in stage 43 (pre-refractory-equivalent) *Xenopus tropicalis* tadpoles (1).

This study suggests a role for NF-κB in regeneration and a mechanism for its continual activity in blastemal cells through a positive-feedback loop involving Nox4 and ROS. A method to initiate this positive-feedback loop in human cells may have the potential to improve our regenerative capabilities.

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Author contributions. TB and CB conceived the study and the project outline. TB designed and carried out the experiments and interpreted results. CB supervised the study and collated figures. TB wrote the manuscript. TB and CB contributed to revision of, and approved, the final manuscript.

Author information. The Authors declare no competing interests.

Methods

Animal ethics. All animal experiments were approved by the University of Otago Animal Ethics Committee under protocol AEC56/12.

Tail regeneration. Tadpoles were raised in petri dishes (40-60 per dish) in 0.1× Mark's modified Ringer's (MMR) at 24°C. They were divided evenly between treatment groups either before (when using pre-amputation treatment) or after amputation. A sharp scalpel blade was used to amputate 30% of the tail under MS222 (1/4000 w/v) anaesthesia (>stage 44) or no anaesthesia (<stage 45). Following any post-amputation treatment, each group of tadpoles was divided into at least three petri dishes. Tadpoles were kept at 24 °C for 7 days with daily feedings of spirulina and 50% 0.1x MMR changes. Regeneration was scored as follows: 0=no regeneration, 5=partial regeneration and 10=complete regeneration.

Hindlimb regeneration. Stage 51-55 tadpoles were anaesthetised with MS222 (Sigma, 1/4000 w/v) and placed on a moistened paper towel. One hindlimb was amputated using Vannas iridectomy scissors at the approximate level of the knee. Tadpoles were transferred to dechlorinated water with air bubbled through an aeration stone. Tadpoles were kept in the aquarium at 25°C for the stated time (or for 1 month when regeneration was scored). Regeneration scores were assigned as follows: 0=stump, 0.5=spike, 1-5=the number of regenerated digits.

Heat killed *E. coli.* TOP10 *Escherichia coli* were grown in 10ml of Luria broth to stationary phase before centrifugation at 13,000 rpm and resuspension in 1x PBS three times. They were then heated to 70 °C for 1 h, centrifuged and resuspended in 2 mL of 0.1x MMR. As indicated, the solution was added at a dilution of 1:100 in 0.1x MMR for 1 h following amputation.

H_2O_2 dip. Stage 46 tadpoles were anaesthetised with MS222 and tails were amputated using a sharp scalpel blade. Tadpoles were placed in 0.1× MMR (MS222 and H_2O_2 form a toxic product, so tadpoles must be removed from MS222 before H_2O_2 dip) and individually sucked headfirst into an unmodified 3 mL plastic pipette with tails projecting out the end. Tails were dipped in a H_2O_2 solution for 3 s and the tadpoles were placed in fresh 0.1× MMR three times before being transferred to their post-H_2O_2 treatments.

H_2O_2 immersion. Stage 46 tadpoles were immersed in 0.3% H_2O_2 in 0.1× MMR for 2 m and transferred to fresh 0.1× MMR and anesthetised with MS222 prior to tail amputation.

DPI treatment. Stage 43 tadpoles were treated with 2 µM diphenyleneiodonium chloride (DPI; Sigma; 2 mM in DMSO stock) or 0.1% DMSO (in 0.1× MMR) for 1 h before and 1, 3, 6 or 12 h after tail amputation.

LPS and prostratin treatments. Stage 46 tadpoles (raised at a low density of approx. 40 per dish) were anaesthetised with MS222 and tails were amputated using a sharp scalpel blade. Tadpoles were treated with 50 $\mu g.mL^{-1}$ lipopolysaccharides (LPS) from *E coli* 0111:B4 purified by phenol extraction (Sigma; 5 $mg.L^{-1}$ in water stock) with untreated control for 1 h, or 10 µM prostratin (Sigma; 4 $mg.mL^{-1}$ in ethanol stock) with 0.1% ethanol control, for 40 m.

Gentamicin treatment. Tadpoles used for gentamicin experiments were raised at 17°C and placed in petri dishes (40-60 per dish) containing 100 $\mu g.L^{-1}$ gentamicin in 0.1× MMR from the stage indicated, or if no stage is indicated from just after the embryos were sorted at stage 2-6. Embryos were moved to new petri dishes containing fresh gentamicin every two days. Following amputation, tadpoles were incubated at 24°C and remained in gentamicin for a further day.

Cytoplasmic and nuclear protein extractions and Western blots. Stage 55 tadpole tails (four per timepoint) were amputated (approx. 40% of tail length) with a sharp scalpel blade under MS222 anaesthesia. After 0, 1, 3 or 6 h, tadpoles were re-anaesthetised and a further 3 mm (20% of the remaining tail) was amputated. For DPI treatment, tadpoles were incubated with 2 µM DP1 (0.1% DMSO) for 1 h prior to the first amputation until the second amputation. The four 3 mm sections for each timepoint and treatment were homogenised in a bead beater with CER I (NE-PER) reagent, Halt protease inhibitor cocktail (Thermo Fisher) and EDTA. Cytoplasmic and nuclear protein fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher) following the manufacturer's protocol. Nuclear extracts were diluted 1:1 with PBS. Samples were prepared with sample loading buffer and heated to 100°C for 3 m. The samples (10 µL) were run on 12.5% SDS-PAGE gels along with a Precision Plus Protein dual colour standard (Bio-Rad) and transferred to PVDF membranes. The membranes were blocked with 2% BSA in PBS/tween (25 m) and incubated with 1:200 rabbit-anti NF-κB p65 polyclonal antibody (Pierce) (2 h) and 1:5000 Goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad) (1 h). Reactivity was detected with Clarity Western ECL Substrate (Bio-Rad).

Whole mount *in situ* hybridisation. *Coding regions of cox2 (primers: 5' gctctagaatgatcgtactacccgc, 3' cggtaccttaaagttcggatgtgtgc),* nox1 (primers: 5' tcctgttttccagggcagtg, 3' agattggacgcccatagctg), *nox2* (primers: 5' ctatgacgagggcgaagcat, 3' tcatcccagccagtgaggta) and *nox4* (primers: 5' taggcaggaatccagtgatgg, 3' cactcccgcaacagaagtga) were amplified from stage 12 *X. laevis* embryo cDNA using Pwo DNA Polymerase (Roche) and ligated into the TOPO Cloning site of the Pcr4-TOPO plasmid (Invitrogen Life Technologies). Insertions were verified by DNA sequencing. Plasmids were linearised with NotI and digoxigenin-labelled RNA probes were transcribed using T3 polymerase with a DIG-NTP mix (Roche). Templates were removed using DNase I (Roche) and the probes were precipitated with 2.5 M LiCl. Whole-mount in situ hybridisations were performed as previously described in Pearl et al (12).

Data availability. Data are available on request from the corresponding author.

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