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Neuregulin-1 signaling is essential for nerve-dependent axolotl limb regeneration

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ABSTRACT

The Mexican axolotl (*Ambystoma mexicanum*) is capable of fully regenerating amputated limbs, but denervation of the limb inhibits the formation of the post-injury proliferative mass called the blastema. The molecular basis behind this phenomenon remains poorly understood, but previous studies have suggested that nerves support regeneration via the secretion of essential growth-promoting factors. An essential nerve-derived factor must be found in the blastema, capable of rescuing regeneration in denervated limbs, and its inhibition must prevent regeneration. Here, we show that the neuronally secreted protein Neuregulin-1 (NRG1) fulfills all these criteria in the axolotl. Immunohistochemistry and *in situ* hybridization of NRG1 and its active receptor ErbB2 revealed that they are expressed in regenerating blastemas but lost upon denervation. NRG1 was localized to the wound epithelium prior to blastema formation and was later strongly expressed in proliferating blastemal cells. Supplementation by implantation of NRG1-soaked beads rescued regeneration to digits in denervated limbs, and pharmacological inhibition of NRG1 signaling reduced cell proliferation, blocked blastema formation and induced aberrant collagen deposition in fully innervated limbs. Taken together, our results show that nerve-dependent NRG1/ErbB2 signaling promotes blastemal proliferation in the regenerating limb and may play an essential role in blastema formation, thus providing insight into the longstanding question of why nerves are required for axolotl limb regeneration.

KEY WORDS: ErbB2, Neuregulin-1, Axolotl, Regeneration, Salamander

INTRODUCTION

Regeneration of the axolotl (*Ambystoma mexicanum*) limb is inhibited by denervation of the limb, but the molecular mechanisms underlying this nerve dependence remain largely unknown. Denervation of the amputated axolotl limb does not inhibit wound healing but blocks the formation of the post-injury proliferative mass called the blastema (Todd, 1823). Nerve dependence is a phenomenon observed during wound healing and regeneration across a wide range of phylogeny (Kumar and Brockes, 2012) and may be due to the secretion of essential growth-promoting factors from peripheral nerves at the wound site (Singer, 1952; Stocum, 2011). Though evidence has been gathered in support of numerous candidate factors, including transferrin (Kiffmeyer et al., 1991; Mescher et al., 1997), fibroblast growth factors (Satoh et al., 2011) and anterior gradient protein (Kumar et al., 2007), no neuronal factor identified thus far has proven capable of rescuing regeneration in the denervated axolotl limb. Here, we examined Neuregulin-1 (NRG1), a neuronally secreted mitogen that promotes proliferation through ErbB2 signaling (Falls, 2003b) and has been found in the newt peripheral nervous system (Brockes and Kintner, 1986) and implicated in newt limb regeneration (Wang et al., 2000). We have shown that NRG1 is found in the axolotl peripheral nervous system and blastema, is capable of rescuing regeneration to the point of digit formation in denervated limbs, and that its inhibition inhibits blastema formation, suggesting that it is a vital upstream proliferative signal during the regenerative process.

RESULTS AND DISCUSSION

NRG1 and ErbB2 are expressed in regenerating limbs

Blastema-specific expression of *nrg1* isoforms and receptors (Fig. 1A-E) was observed by *in situ* hybridization (ISH) in blastemas collected at 16 days post amputation (DPA). Strong expression of the active epidermal growth factor (EGF)-like domain, which is found in all isoforms of NRG1, was observed in the mesenchyme as well as the basal wound epithelium of the blastema. Expression of the immunoglobulin-like domain of *nrg1*, which is common to types I and II NRG1, was strong in the distal mesenchyme and basal wound epithelium and was comparatively absent in cells located proximal to the site of amputation. The cysteine-rich domain of type III *nrg1* was also observed in the blastema, though this expression was found in fewer cells compared with the immunoglobulin-like domain. Type I and type II NRG1 are capable of signaling in a paracrine manner whereas type III NRG1 is limited to juxtacrine signaling (Falls, 2003b), indicating that paracrine NRG1 isoforms in particular are strongly expressed in the blastema during limb regeneration. ISH of *erbb2* revealed that it was expressed in the mesenchymal cells of the distal blastema as well as the basal layer of the wound epithelium, though it was virtually absent from cells that were located proximal to the site of amputation. The ErbB2 co-receptor *erbb3* was similarly expressed in the distal blastema. Taken together, these ISH results suggest that expression of *nrg1* and its receptors is blastema specific during axolotl limb regeneration. RT-PCR of blastemal (21 DPA) and uninjured tissues found that all isoforms of *nrg1* examined were present in the regenerating and uninjured limbs (Fig. 1F). *nrg1* types I and II were upregulated in injured versus uninjured limbs whereas type III *nrg1* was more highly expressed in uninjured limbs. The presence of *nrg1* in intact limbs is consistent with its known roles in Schwann cell and neuromuscular junction maintenance (Falls, 2003a; Sandrock et al., 1997).

Immunohistochemical staining of 16 DPA blastemas further confirmed the nerve-dependent presence of NRG1 and its active receptor in the regenerating limb. At 16 DPA, NRG1-positive cells

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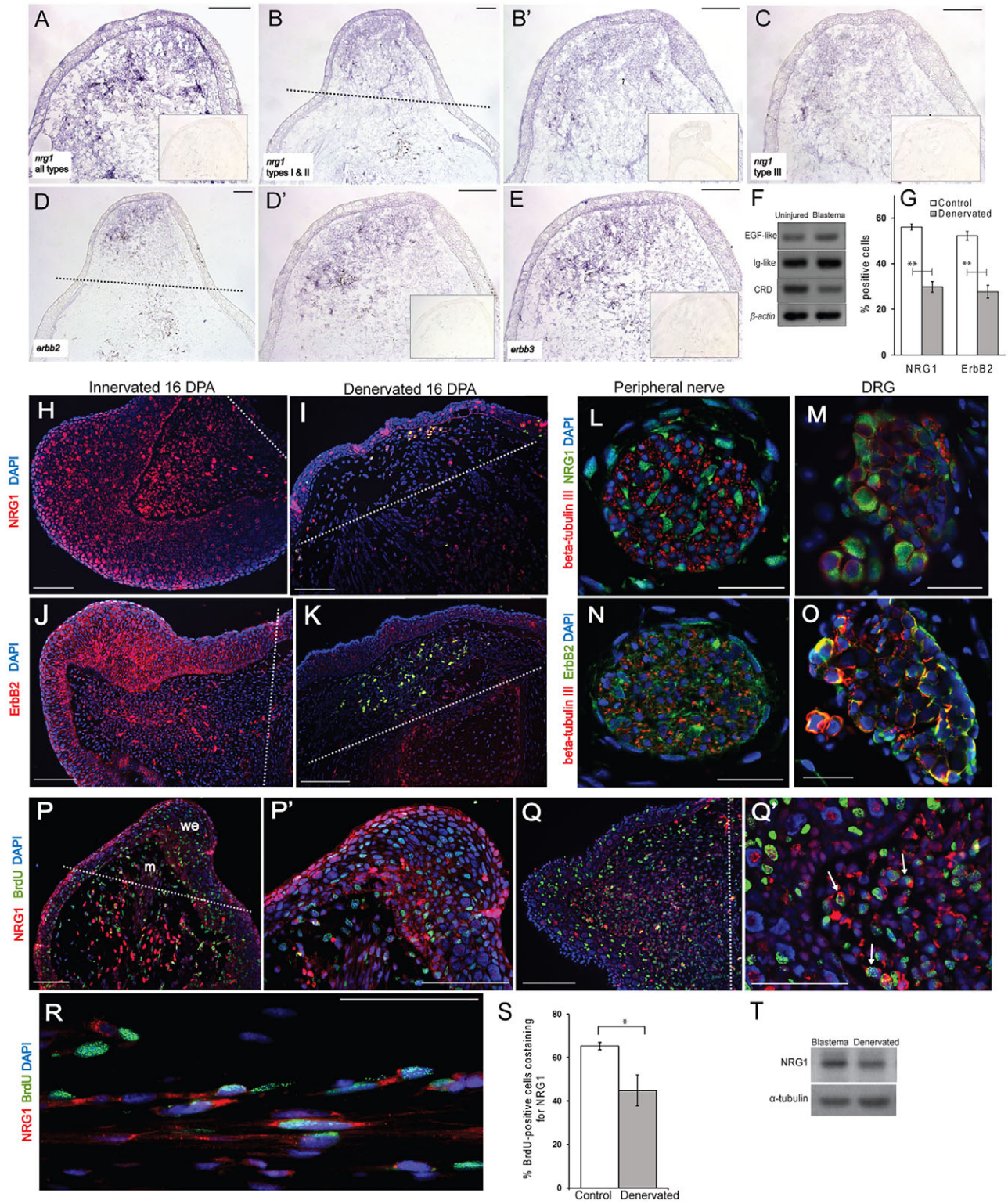

Fig. 1. NRG1 and ErbB2 are expressed in the PNS and the regenerating blastema. (A-E) ISH showing that *nrg1* isoforms and receptors are expressed in the blastema at 14 DPA. Insets show sense controls. (F) RT-PCR analysis showing upregulation of type I and II *nrg1* isoforms in regenerating versus uninjured limbs. (G-K) NRG1 and ErbB2 are expressed in the mesenchyme of regenerating blastemas but lost upon denervation (n=4 biological replicates each). Green and orange fluorescence is due to autofluorescent cellular debris. (L-O) NRG1 and ErbB2 are expressed in dorsal root ganglia and peripheral nerves. (P,P′) NRG1 is expressed in the wound epithelium (we) and mesenchyme (m) of 6 DPA limbs along with proliferating BrdU-positive cells. (Q,Q′) Extensive NRG1 expression and colocalization with BrdU in a 16 DPA blastema. Arrows indicate co-labeled cells. (R) NRG1 and BrdU colocalization along peripheral nerves in a regenerating limb at 16 DPA. (S) Denervation significantly decreases the percentage of BrdU/NRG1 colocalization in 16 DPA limbs (n=4 biological replicates). (T) Western blot of NRG1 at 16 DPA showing a band at the expected size of 47 kDa and greater band intensity in blastemal tissue relative to denervated tissue. Data are represented as mean±s.e.m.; statistical analysis performed by Student's t-test, $^{**}P<0.01$, $^{*}P<0.05$. Dotted lines indicate the plane of amputation. Scale bars: 100 µm.

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were found both in the wound epithelium and in 56.18% of mesenchymal blastemal cells (Fig. 1G,H). By contrast, the percentage of mesenchymal NRG1-positive cells was significantly reduced to 29.87% in denervated limbs (Fig. 1G,I). These findings indicate that NRG1 protein is found in the blastema and reduced upon denervation, suggesting that nerves support a positive-feedback loop that sustains NRG1 and ErbB2 expression. NRG1 antibody specificity was tested by western blot analysis, which showed a band at the expected size of 47 kDa and demonstrated stronger band intensity in blastemal tissue compared with denervated tissue at 16 DPA (Fig. 1T). Immunohistochemical staining for the receptor ErbB2 was consistent with these findings, as ErbB2 was strongly expressed in both the mesenchyme and wound epithelium of blastemas at 16 DPA (Fig. 1G,J) but reduced upon denervation (Fig. 1G,K). Overall, these results show that RNA and protein of both NRG1 and ErbB2 are highly expressed in the blastema during axolotl limb regeneration.

Dorsal root ganglia, which are capable of rescuing regeneration if grafted into a denervated limb (Goldhamer et al., 1992; Kamrin and Singer, 1959; Tomlinson and Tassava, 1987), showed extensive NRG1 and ErbB2 staining (Fig. 1M,O). NRG1 (Fig. 1L) and ErbB2 (Fig. 1N) were further observed in cross-sectioned peripheral nerves. NRG1 staining in the basal wound epithelium was observed before blastema formation and as early as 6 DPA (Fig. 1P). Co-staining with bromodeoxyuridine (BrdU) showed that at 6 DPA NRG1 was also present in a subpopulation of proliferating mesenchymal cells located just underneath the wound epithelium. Though the mechanism behind blastema formation remains poorly understood, previous studies have shown that signals from the basal wound epithelium may work in conjunction with nerves to induce the accumulation of de-differentiated mesenchymal cells at the site of amputation (Goss, 1956a,b; Loyd and Tassava, 1980; Tassava and Garling, 1979). The pre-blastemal presence of NRG1 in the wound epithelium as well as the proliferating mesenchyme thus indicates that it may play an important role in blastema induction.

NRG1 and BrdU co-staining was further observed in 16 DPA blastemas (Fig. 1Q). BrdU-positive mesenchymal cells co-stained with NRG1 in 65.89% of cells in control limbs and 46.55% of cells in denervated limbs (Fig. 1S), indicating a pro-proliferative function that is consistent with its known roles in cell proliferation and survival (Canoll et al., 1996; Flores et al., 2000; Garratt et al., 2000). Furthermore, cells that co-stained with NRG1 and BrdU were observed along peripheral nerves near the site of amputation (Fig. 1R), suggesting that Schwann cells may also be secreting NRG1. Taken together, these immunohistochemical results show that NRG1 and its active receptor are localized in peripheral nerves and the proliferating blastema and thus may promote nerve-dependent blastemal formation and proliferation.

NRG1 supplementation rescues regeneration in denervated limbs

To determine whether NRG1 supplementation is sufficient to rescue regeneration in denervated limbs, NRG1β-1 peptide-soaked beads were implanted underneath the wound epithelium of limbs at 7 DPA (Fig. 2A). Supplementation with NRG1 induced blastema formation in six of seven denervated limbs (Fig. 2B-D), which regenerated significantly more tissue than PBS-treated denervated limbs but not innervated limbs across a span of 2 weeks (Fig. 2E). Blastema formation was not the result of nerve survival or regeneration, as demonstrated by the fact that immunohistochemical staining for nerves was deficient in denervated and NRG1-treated limbs at 21 DPA (Fig. 2F-H).

Because axolotl limbs cannot be reliably denervated for longer than ∼20 days, in a separate experiment we denervated blastemas at 19 DPA and supplemented them with NRG1-soaked beads every 4 days in order to determine whether this treatment was sufficient to rescue limb regeneration all the way to digit formation. By 36 DPA, four of five NRG1-treated limbs had regenerated to the point of digit formation, whereas three of three controls and zero of three denervated limbs developed digits (Fig. 2I-L; Fig. S1). NRG1-supplemented limbs regenerated significantly more tissue than did PBS-treated limbs, although they did not regenerate to the same degree as the fully innervated controls (Fig. 2M), suggesting that greater NRG1 supplementation or the inclusion of additional factors may be necessary to achieve total rescue. Alcian Blue staining demonstrated the presence of chondrogenesis in the new digits in control and NRG1-treated limbs (Fig. 2N,O), and beta-tubulin III immunohistochemistry further confirmed the lack of nerves in both denervated conditions (Fig. 2P-R).

NRG1 supplementation thus appears to be capable of bypassing the nerve requirement for blastema induction and limb regeneration, a finding with considerable implications for explaining the longstanding question of nerve-dependent regeneration. Although it has been found that application of Gdf5 and Fgfs can induce limb formation in an accessory limb model of axolotl regeneration (Satoh et al., 2011), this is the first example to our knowledge of a single protein rescuing regeneration in the denervated axolotl limb. These results suggest that NRG1 acts as an essential link between nerves and the blastema, as it promotes blastemal growth and proliferation throughout the entire process of limb regeneration, from early blastemal growth to later digit formation.

Inhibition of NRG1/ErbB2 signaling blocks regeneration

NRG1 signaling was inhibited with the specific (Nagasawa et al., 2006; Ufkin et al., 2014) ErbB2 inhibitor mubritinib. Submersion in 500 nM mubritinib did not impair wound healing but completely inhibited blastema formation in fully innervated limbs, rendering them outwardly identical to denervated limbs (Fig. 3A-C). Limbs treated with mubritinib regenerated significantly less tissue than control but not denervated limbs (Fig. 3M), lacked cellular accumulation at the wound site, and morphologically resembled denervated limbs (Fig. 3G,H). BrdU cell counts of 12 DPA blastemas found that cellular proliferation was significantly decreased and in fact virtually abolished in drug-treated limbs (Fig. 3I,J,N) despite the presence of healthy nerves, suggesting that the observed lack of proliferation was the direct result of ErbB2 inhibition rather than any inadvertent loss of innervation.

To examine the similarities between denervated and mubritinib-treated limbs further, animals were bathed in 500 nM mubritinib starting at 16 DPA, well after blastema formation. Previous studies have found that denervation after blastema formation substantially reduces cell cycling and proliferation (Goldhamer and Tassava, 1987; Maden, 1978; Tassava et al., 1974) and results in the formation of a miniature limb (Powell, 1969; Schotté and Butler, 1944; Singer and Craven, 1948), suggesting that nerves are required for blastemal proliferation but not limb differentiation and morphogenesis. We found that mubritinib inhibited blastemal growth but not limb patterning over a span of 12 days, inducing the formation of miniature limbs that were phenotypically similar to those formed as a result of late denervation (Fig. 3D-F). Limbs treated with mubritinib regenerated significantly less tissue than control limbs but not denervated limbs (Fig. 3O), underlining the similarity between denervated and ErbB2-inhibited limbs. Our

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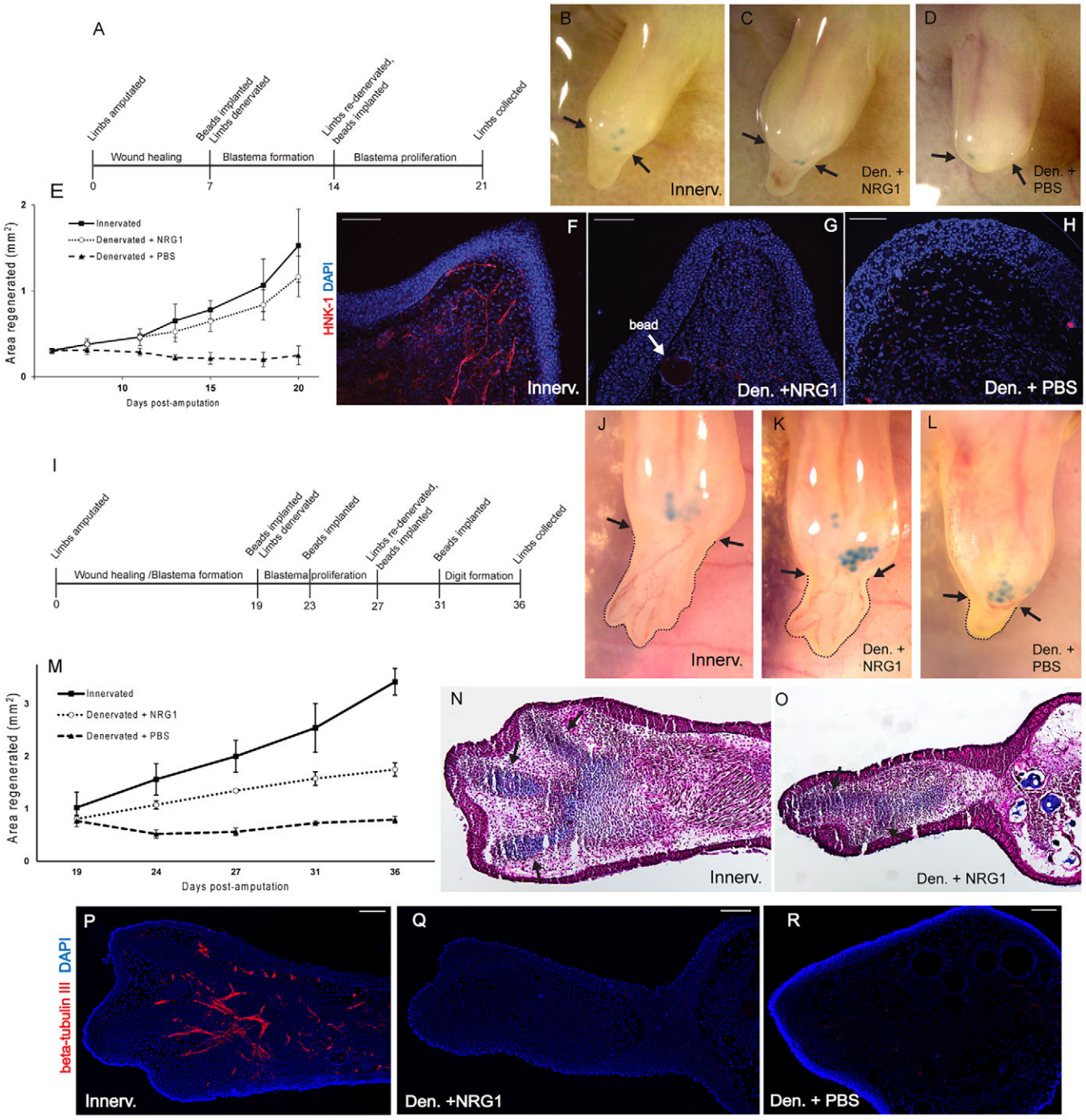

Fig. 2. Supplementation with NRG1 rescues regeneration in denervated limbs. (A) Timeline of early NRG1 supplementation experiment. (B-D) Supplementation with NRG1 rescues regeneration in denervated limbs at 20 DPA. Arrows indicate the plane of amputation. (E) From 6 to 20 DPA, NRG1-supplemented (n=7) limbs regenerated significantly more tissue than denervated (P<0.05, n=7), but not control limbs (P>0.05, n=3). (F-H) NRG1-supplemented limbs regenerated in the absence of hyperinnervation. (I) Timeline of late NRG1 supplementation experiment. (J-L) Implantation of NRG1-soaked beads into denervated limbs rescues regeneration to the point of digit formation at 36 DPA. Arrows indicate the plane of amputation and dotted lines outline the regenerating tissue. (M) From 19 to 36 DPA, NRG1-supplemented (n=5) limbs regenerated significantly more tissue than denervated (P<0.05, n=3) and significantly less tissue than innervated (P<0.01, n=3) limbs. (N,O) Alcian Blue staining showing digit formation in control and NRG1-treated limbs. (P-R) NRG1 induced growth and digit formation in fully denervated limbs. Data are represented as mean±s.e.m.; statistical analysis performed by one-way ANOVA with Tukey's post-hoc test. Scale bars: 100 µm.

inhibition experiments thus indicate that ErbB2 signaling is necessary for promoting blastema formation and maintaining blastemal proliferation during the early tissue growth and late tissue patterning phases of regeneration.

Long-term (23 DPA) exposure to 10 µM mubritinib induced contraction of the wound epidermis, similar to that observed in mice after injury (Dunn et al., 2013), in contrast to the minimal wound contraction observed in control limbs (Fig. 3K,L). Axolotl tissue regeneration is a typically scar-free process that occurs with minimal collagen deposition (Levesque et al., 2010; Seifert et al., 2012), but extensive and aberrant collagen deposition was observed in the mesenchyme of mubritinib-treated limbs. The phenotype observed

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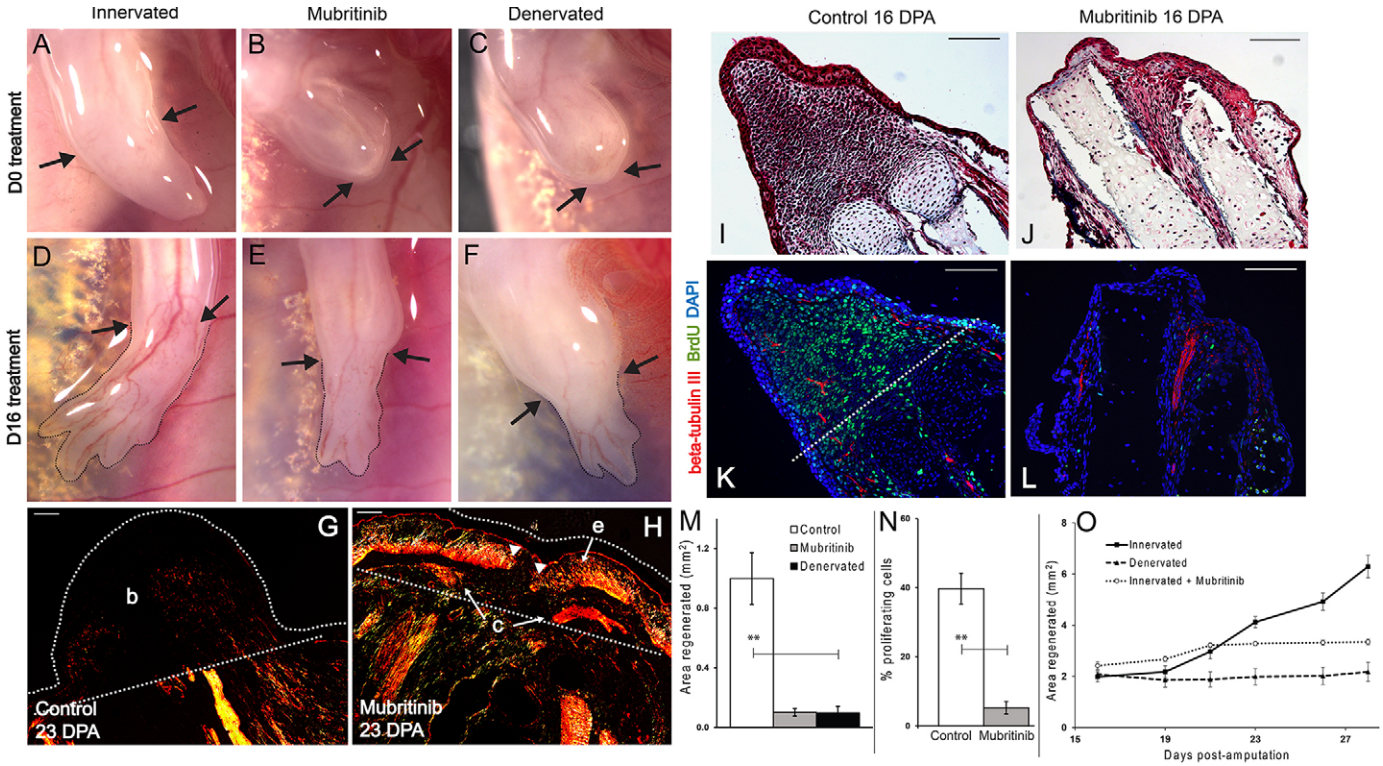

Fig. 3. Inhibition of ErbB2 blocks regeneration, inhibits proliferation, and induces aberrant collagen deposition. (A-C) Inhibition of ErbB2 with 500 nM mubritinib blocks blastema formation at 13 DPA. (D-F) Mubritinib application after 16 DPA blocks limb proliferation but not patterning and appears phenotypically similar to day 16 denervation. Dotted lines outline the regenerating tissue. (G,H) Picrosirius staining showing that 23 days of submersion in 10 µM mubritinib results in contraction of the epidermis (e) and aberrant collagen deposition (c) in the mesenchyme, in contrast to the minimal fibrotic deposition seen in control blastemas (b). Arrowheads indicate the contracted wound margin. Dotted lines delineate the boundary of the epidermis and the plane of amputation. (I,J) Masson's trichrome staining of control and mubritinib-treated limbs at 12 DPA show a lack of blastemal accumulation in the drug-treated limbs. (K,L,N) Treatment with mubritinib does not reduce innervation but significantly decreases the proliferative index of amputated limbs (n=5). Dotted line indicates the plane of amputation. (M) At 14 DPA, mubritinib-treated limbs (n=5) had regenerated significantly less area than control (n=8) but not denervated (n=8) limbs. (O) Limbs that were either denervated (n=8) or treated with mubritinib (n=8) at 16 DPA regenerated significantly less tissue than control limbs (P<0.001, n=7). Arrows indicate the planes of amputation. Data are represented as mean±s.e.m.; statistical analysis performed by Student's t-test, **P<0.01. Scale bars: 100 µm.

here after long-term ErbB2 inhibition indicates a disruption of these scar-preventing programs and resembles the phenotype observed in amputated limbs after total macrophage ablation (Godwin et al., 2013).

As ErbB2 can also heterodimerize with epidermal growth factor receptor (EGFR), we pharmacologically inhibited EGFR in order to ensure that the effects of mubritinib were due to NRG1 and not to EGF signaling inhibition. Animals bathed in the specific (Goishi et al., 2003; Han et al., 1996) EGFR inhibitor AG1478 for 6 days post-amputation exhibited a markedly different phenotype from mubritinib-treated animals, as EGFR inhibition resulted in improper wound healing and eventual tissue regression (Fig. 4D-G). Strikingly, these animals also developed excessive numbers of iridophores after just 10 days of treatment (Fig. 4H). Furthermore, EGFR inhibition significantly reduced epidermal but not mesenchymal proliferation at 5-6 DPA, whereas ErbB2 inhibition significantly reduced mesenchymal but not epidermal proliferation (Fig. 4A-C,I,J). These results suggest that inhibition via mubritinib primarily blocks NRG1/ErbB2 signaling rather than EGF/ErbB2 signaling, which instead appears to play a crucial role in wound healing and epidermal proliferation after amputation. Overall, our data show that NRG1/ErbB2 signaling is essential for limb regeneration and may play a vital role in preventing scar formation during this process as well.

Conclusions

We have shown that a single nerve-derived protein, Neuregulin-1, is capable of supporting blastemal growth and tissue regeneration up to the point of digit formation in the denervated axolotl limb. We propose that nerve-dependent NRG1/ErbB2 signaling is crucial for blastemal proliferation and may also be an essential component of blastema formation and scar-prevention programs. Although NRG1 is the first protein to our knowledge that has been shown to be capable of rescuing regeneration to digits in the axolotl limb, these findings do not rule out the possibility of other factors playing a crucial role in this process. Newt anterior gradient protein has been shown to rescue regeneration in denervated newt limbs (Kumar et al., 2007), and despite some prominent species differences between axolotls and newts, which demonstrate a different recovery response to denervation (Liversage and McLaughlin, 1983) as well as a phylogenetically unique method of regenerating muscular tissues (Sandoval-Guzman et al., 2014; Tanaka et al., 2016), further exploration of the relationship between these two signaling pathways is necessary in order to characterize fully the underlying cause of nerve dependency in the axolotl limb. Given the conserved role of NRG1/ErbB2 signaling in the peripheral nerves as well as the burgeoning evidence of its necessity in other animal models of cardiac (Bersell et al., 2009; D'Uva et al., 2015; Gemberling et al., 2015) and peripheral nerve (Fricker et al., 2011; Ronchi et al., 2013, 2015) regeneration, elucidating the function and mechanism of this

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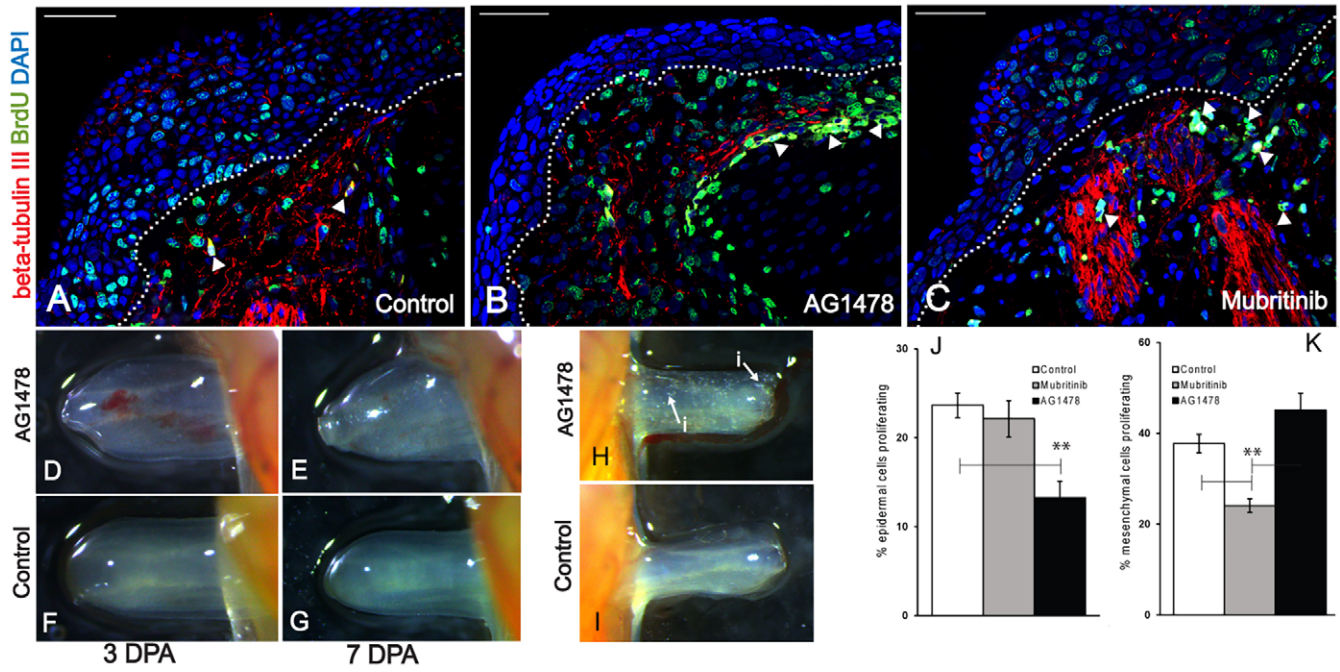

Fig. 4. EGFR inhibition inhibits wound closure and is phenotypically distinct from ErbB2 inhibition. (A-C) Proliferating cells are localized to the mesenchyme in AG1478-treated limbs and the epidermis in mubritinib-treated limbs at 6 DPA. Arrowheads indicate autofluorescent cellular debris and red blood cells; dotted lines indicate the boundary between the wound epidermis and mesenchyme. (D-G) AG1478-treated limb showing aberrant wound closure over time compared with control limb at 3 DPA and 7 DPA. (H) Limb treated with AG1478 for 10 days demonstrating aberrant development of iridophores (i). (I) Control limb treated with DMSO for 10 days showing lack of iridophores. (J,K) Percentage of proliferating epidermal and mesenchymal cells in control, mubritinib-treated and AG1478-treated limbs at 6 DPA (n=5 biological replicates each). Data are represented as mean±s.e.m.; statistical analysis performed by one-way ANOVA with Tukey's post-hoc test, $^{**}P$<0.01. Scale bars: 100 µm.

signaling pathway in the axolotl may have far-reaching impacts on the field of regenerative medicine.

MATERIALS AND METHODS

Surgical procedures

Leucistic axolotls (*Ambystoma mexicanum*) were bred and raised at Northeastern University according to the methods of Farkas and Monaghan (2015). Animals were anesthetized in 0.01% benzocaine and amputation was performed just proximal to the elbow joint. Recombinant human NRG1β-1 peptide (0.5 mg/ml in PBS; PeproTech) was incubated overnight with Affi-gel 50-100 mesh agarose beads (Bio-Rad) according to Niswander (2008). An incision was made 1-2 mm above the site of amputation, then two beads were probed with forceps through the incision until they rested underneath the wound epithelium. Animals were denervated 1 h later. Two more beads were implanted, limbs were re-denervated at 14 DPA, and blastemas were imaged three times a week. Area regenerated was assessed blind to the experimental condition by the tracing of blastemas in ImageJ. For the digit rescue experiment, three beads were implanted into the base of blastemas at 19 DPA, and denervations were performed 1 h post-implantation. Three more beads were added every 4 days, limbs were re-denervated at 27 DPA and collected at 36 DPA. All experiments were conducted with the approval of and in accordance with the Northeastern University Institutional Animal Care and Use Committee.

Drug treatment

Mubritinib (TSZ Scientific) stock solution (10 mM in DMSO) was diluted in salamander housing solution to 500 nM for juveniles [3.5-6 cm snout-to-vent length (SVL)] and 10 µM for adults (20-25 cm SVL), which are more capable of tolerating the drug. Juvenile animals were bathed in mubritinib starting at either 0 or 16 DPA, and adult animals were treated from 6-23 DPA before tissues were collected and prepared for immunohistochemistry. AG1478 (Tocris) stock solution (10 mM in DMSO) was diluted to 10 µM and animals were bathed in either 500 nM mubritinib, 10 µM AG1478 or 10 µM DMSO for 6 days prior to tissue collection. BrdU (20 mg/ml, Sigma) was injected intraperitoneally at 1 mg BrdU/1 g animal. Limbs were collected at 24 h post-injection.

Immunohistochemistry and histology

Tissues were fixed in 10% neutral buffered formalin at 4°C overnight, washed 2× in PBS, incubated in 10% EDTA at 4°C for 48 h, processed for paraffin embedding, and sectioned at 10 µm. Sections were de-paraffinized and hydrated, pressure-cooked in 10% citrate buffer for 20 min (Cuisinart electric pressure cooker CPC-600), washed for 5 min in PBS, blocked for 30 min in 1.5% normal goat serum, incubated at 4°C overnight in primary antibody, washed, and incubated for 30 min at room temperature in Alexa Fluor 488 and 594 secondary antibodies (1:400; Life Technologies, A11037, A11034, A21044, A11032, A11006), then mounted and coverslipped with Slowfade Diamond Antifade Mountant with DAPI (Life Technologies). Slides stained for ErbB2 were soaked for 30 min in 0.05% saponin (Sigma) then washed 3×10 min in PBS prior to the blocking step. Primary antibodies are listed in Table S1. Picrosirius (Polysciences) and Masson's trichrome (Thermo Scientific) stains were performed according to the manufacturers' protocol. Alcian Blue staining was performed according to Lee and Gardiner (2012).

RT-PCR analysis and *in situ* hybridization

Total RNA was extracted from uninjured and 21 DPA limbs (Qiagen RNeasy Kit), converted to cDNA template (Life Technologies Maxima H Minus First Strand cDNA Synthesis Kit), and PCR amplified (2× PCR Master Mix; Thermo Scientific) with 10 ng cDNA template and 0.5 µM of isoform-specific primers. PCR products were cloned into pGEM-T (Promega), sequence verified (Genewiz), and vectors used to generate digoxigenin-labeled probes. Limbs were collected at 16 DPA and ISH performed on 35 µm thick cryosections according to Monaghan et al. (2012). See Table S2 for primer sequences.

Western blot

NRG1 primary antibody was diluted to 1:10,000; secondary was horseradish peroxidase-conjugated goat anti-rabbit antibody at 1:5000

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(115-035-003, Jackson ImmunoResearch). Mouse anti-alpha tubulin (1:5000; Sigma) was used as the loading control and was detected with goat-anti-mouse HRP (1:5000; Jackson ImmunoResearch). All antibodies were incubated in 5% bovine serum albumin and 0.1% Tween 20 in TBS.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
J.E.F. and J.R.M. designed the research. J.E.F., P.D.F., D.M.B. and J.L.W. performed experiments and provided materials. J.E.F. and J.R.M. analyzed the data. J.E.F. wrote the paper with contributions from J.R.M., D.M.B. and J.L.W.

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Supplementary information
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