Inhibition of Runx by Ro5-3335 Affects *Nematostella* Regeneration

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ABSTRACT

The sea anemone, *Nematostella vectensis*, has the ability to fully regenerate amputated body parts. We hypothesize that Runx, a transcription factor, controls the cellular processes for regeneration, specifically the transition between cellular proliferation and cellular differentiation. A known inhibitor to the Runx pathway is Ro5-3335, a benzodiazepine. Inhibiting the Runx pathway by Ro5-3335 will help determine if Runx is necessary for proper regeneration in *Nematostella.* We introduced bisected *Nematostella* polyps to Ro5-3335 for a 24-48 period and observed regeneration of oral ends. Tentacle regeneration appeared delayed in treated polyps compared to the controls. It was not until three weeks post-treatment that the treated animals recovered normal regeneration. We conclude that Ro5-3335 appears to repress regeneration in the polyps which suggests that the Runx pathway is important for proper regeneration in *Nematostella*.

INTRODUCTION

The sea anemone, *Nematostella vectensis,* is a member of the cnidarian clade Anthozoa. They are burrowing anemones found in estuarine habitats with brackish waters (Reitzel et al., 2007). *Nematostella* are diploblastic with an outer and inner epithelial layer. Within their transparent body column are mesenteries that facilitate digestion. At one end of the body column is the physa, a rounded aboral structure used to burrow into the sand. The oral end, which includes the pharynx and tentacles, remains exposed to the water for feeding (Amiel et al., 2015). *Nematostella* is gaining increasing recognition as a model organism for developmental and genomic research due to the completion of its sequenced genome and the ability to spawn the animals in the laboratory (Reitzel et al., 2007).

Nematostella are capable of regeneration. Regeneration is thought to occur via epimorphosis, a mechanism in which cells proliferate to regenerate the lost body part. During this process a cluster of undifferentiated cells form the blastema at the site of injury before differentiation and patterning of cells. Almost immediately after bisection, wound healing starts "with the edges of the wound site coming together." For *Nematostella,* differentiation of cells typically occurs between 36 and 48 hours after bisection (Passamaneck and Martindale, 2012).

Runx is a family of transcription factors known to be involved in the regulatory mechanism of cell proliferation and differentiation, especially during development in metazoans (Coffman, 2003). When Runx heterodimerizes with CBFβ, "a non-DNA binding beta subunit," the DNA binding affinity of Runx is enhanced (Coffman, 2009). In adult *Nematostella,* Runx and CBFβ shows high expression in overlapping regions of the head and tentacles (Sullivan et al., 2008).

Mammals have three *Runx* genes that are also involved in developmental processes (Sullivan et al., 2008). However, mutations in mammalian Runx can be oncogenic or tumor suppressive (Coffman, 2009). The *Nematostella Runx* gene (*Nv-runx*) is 93% similar to RUNX1 in humans. *Nv-runx* also has a characteristic sequence that is conserved in protostomes and deuterostomes.

Ro5-3335, a benzodiazepine, is known to interact with the Runx/CBFβ complex. The drug was developed to "block HIV gene expression by inhibiting Tat-mediated transactivation" in HIV patients (Cunningham et al., 2012). However, the drug has also been identified as an inhibitor to CBF leukemia in zebrafish models. Ro5-3335 does not completely disrupt the heterodimerization between RUNX1-CBFβ, but "changes the conformation of their complex or increases the distance between RUNX1 and CBFβ in the complex" to inhibit their functions (Cunningham et al., 2012). The MCSFR (macrophage colony-stimulating factor receptor) promoter is activated by Runx/CBFβ. However, the activation of the promoter is significantly reduced in the presence of Ro5-3335 (Cunningham et al., 2012).

This study investigates the inhibition of Runx by Ro5-3335 and its effect on regeneration of oral ends of *Nematostella*. We hypothesize that *Nematostella* treated with Ro5-3335 will only be able to wound heal insofar as forming a blastema and differentiation into regenerated body parts will not occur while the animals are undergoing treatment with the drug.

MATERIALS AND METHODS

ANIMAL CARE

The *Nematostella* were kept in filtered seawater (12ppt) at room temperature. For the first 24-48 hours, the polyps were kept in wells of microtiter plates with varying concentrations of Ro5-3335, DMSO, and seawater (see below). After treatment the polyps were transferred to glass dishes with seawater that were covered to prevent evaporation. Menthol crystals were used to relax the *Nematostella* prior to bisection and before capturing images. *Nematostella* were fed using hatched brine shrimp. After treatment, relaxation, or feeding, the polyps had a change in seawater.

PREPARATION AND TREATMENT

The experiment was conducted with adult polyps, and they were bisected transversely across the oral/aboral axis using a scalpel. All cuts were made below the pharynx, at approximately the same location on each polyp. Each aboral end retained some presence of mesenteries. Once all the cuts were made, the aboral fragments were placed into the wells of a microtiter plate with seawater, varying concentrations of Ro5-3335 (0.5μM, 5μM, 25μM, and 50μM in DMSO). All animals were exposed to the drug for 24 hours (except for the 50μM concentration, where some were exposed for 48 hours). The control consisted of eighteen polyps which were only treated with seawater and 25μL of DMSO (the same amount of DMSO found in the 50uM treatment). There were eight polyps for all concentrations of inhibitor, except the solution with 50μM of Ro5-3335 which consisted of a sample of fifteen polyps (5 treated for 24 hours, 10 treated for 48 hours).

DEVELOPMENTAL AND FUNCTIONAL OBSERVATIONS

The polyps were observed at least once daily for a period of seven days following exposure to the drug. For 10 of the polyps treated for 48 hours in 50μM of Ro5-3335 (see above), we also exposed the regenerating animals to brine shrimp and observed for the ability to feed. Images were captured using a camera attached to a dissecting microscope.

RESULTS

MORPHOLOGICAL OBSERVATIONS

To observe the effect of Ro5-3335 on *Nematostella* regeneration, we looked for regeneration of the oral end, including development of the mouth and pharynx and tentacle growth. Although tentacle growth was not quantified, qualitative observations were made. After one day, the polyps appeared to have wound healing. After three days, all of the control polyps show signs of tentacle regeneration. By contrast, only some of the treated polyps displayed tentacle buds (Fig. 1). After four days, polyps from each concentration all had tentacle growth (Fig. 2), but the tentacles were shorter compared to controls. After seven days, polyps from each concentration of Ro5-3335 treatment showed regeneration of tentacles (Fig. 3). However, many of the polyps displayed tentacles that were stubbier compared to the tentacles of the control.

FUNCTIONAL OBSERVATIONS

A feeding assay was conducted to see if the tentacles were fully operative following regeneration. For this assay, there were ten polyps for the control, and ten polyps that underwent treatment in 50μM Ro5-3335. The feeding sessions lasted between thirty minutes to one hour to allow for all polyps to have a chance to exhibit ingestion. Two days after treatment, three

controls ate while no treatments ate. Four days after treatment, all ten controls exhibited tentacles, and seven were able to ingest the brine shrimp. For the ten 50µM Ro5-3335 treated polyps, nine showed tentacles, and only two ate (Table 1).

FIGURES AND TABLES

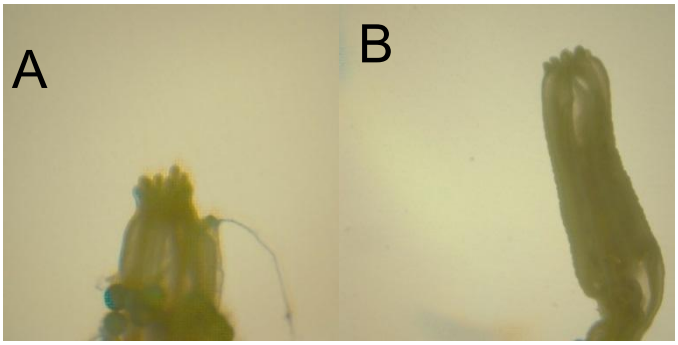

Fig. 1. Tentacle development three days after treatment. **A.** A control polyp. **B**. A polyp from 0.5µM treatment.

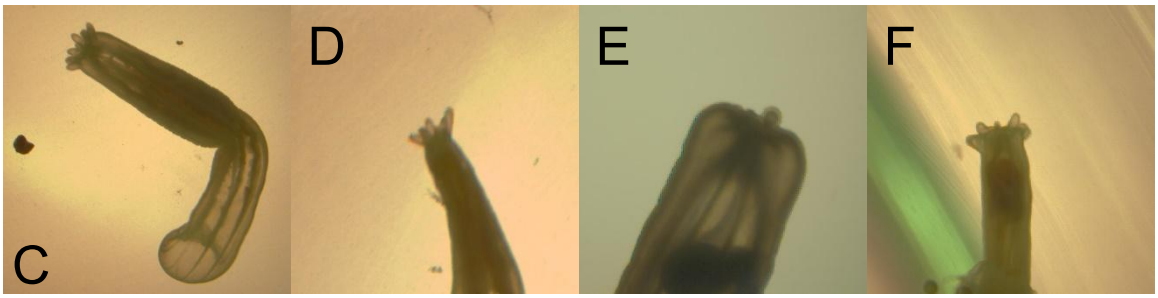

Fig. 2. (C-F) Tentacle development four days after treatment. From left to right: control, 0.5µM, 5.0µM, and 25µM.

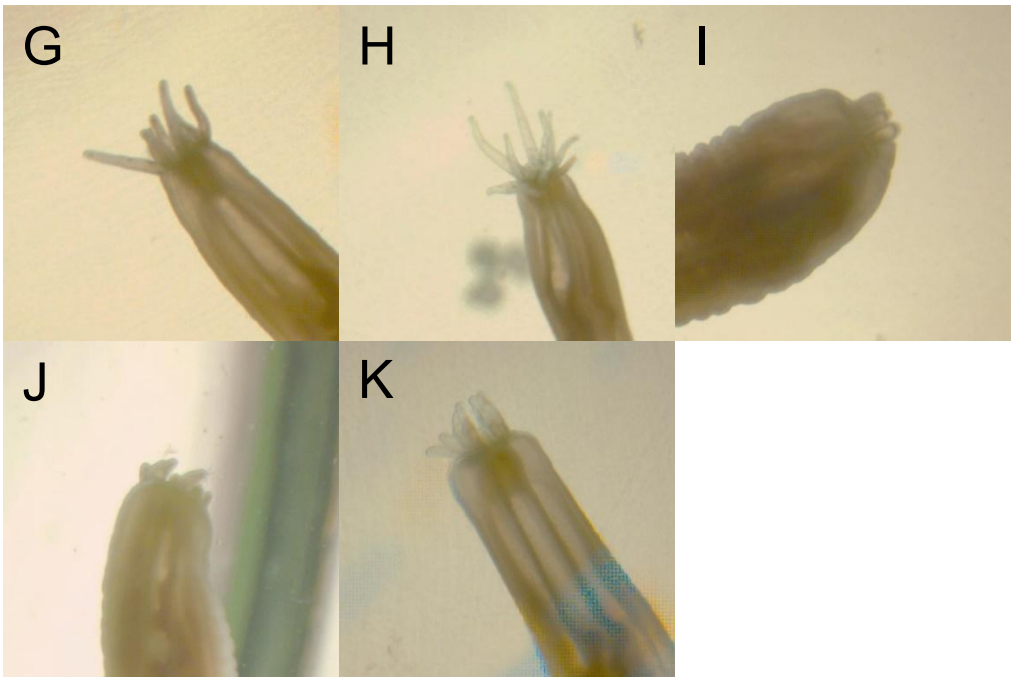

Fig. 3. (G-K) Tentacle growth on polyps seven days after treatment. Although regenerated, the tentacles are shorter than in the controls. Top: control, 0.5µM, 5.0µM. Bottom: 25µM and 50µM.

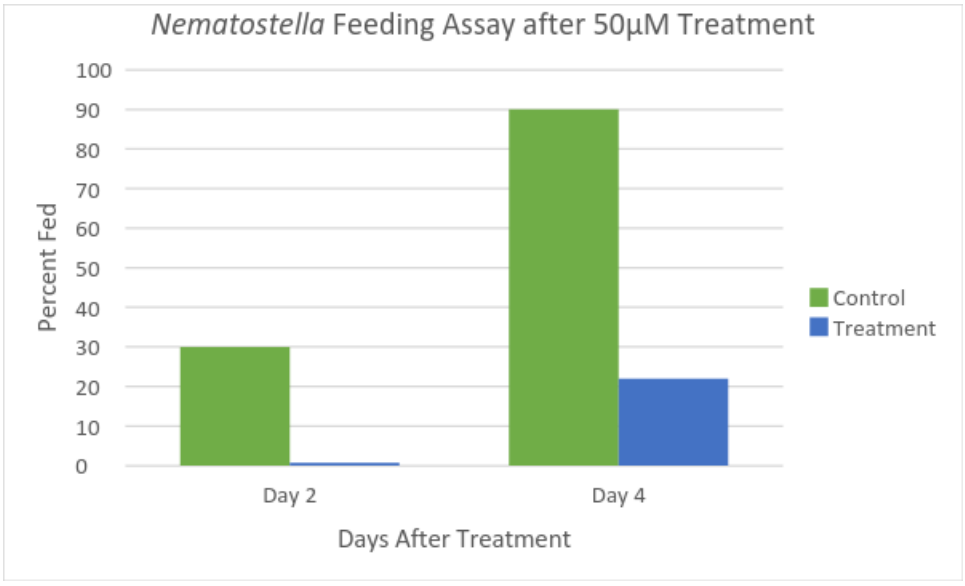

Table 1. Percentage of polyps that ate after showing tentacle regeneration.

DISCUSSION

Runx is thought to be involved in the transition between cell proliferation and differentiation during development and regeneration (Coffman, 2003). Ro5-3335 is known to be an inhibitor to Runx, so we wanted to see if Ro5-3335 has any effect on regeneration in *Nematostella*. Our study shows that Ro5-3335 treatment does not completely inhibit regeneration, but that it does slow down the process. Differentiation and patterning normally occurs after 36-48 hours (Passamaneck and Martindale, 2012), but our observations indicate that treated animals took approximately 72 hours to regenerate. Even though the tentacles on the control polyps exhibited more regeneration after seven days compared to polyps with Ro5-3335 treatment, they appeared to be thinner and shorter when compared to normal regenerating *Nematostella.* After twenty-three days the treated polyps looked fully regenerated, likely due to the decreased effects of the drug.

Our data shows that 0.5μM of Ro5-3335 is sufficient to temporarily repress the Runx pathway and delay regeneration. Because the low concentrations of inhibitor show no significant effect, we conducted a second trial in order to increase the sample size and produce a kill curve. When 50μM of Ro5-3335 is used to treat the *Nematostella,* it does not demonstrate lethal effects but has an effect on tentacle function.

The feeding assay was conducted to determine if treated *Nematostella* have fully functioning tentacles. After two days of 50μM treatment, zero treated polyps ate compared to thirty percent of the controls. Four days following treatment displayed more significant results. Ninety percent of controls ate while only twenty-two percent of the treatments ate. During the feeding assay, we

noticed that the treated polyps were able to sense the brine shrimp but were unable to retract them with their tentacles for ingestion. This suggests that high concentrations of inhibitor have a significant effect on tentacle function and may underlie the production of nematocysts.

Since we did not determine *Nv-runx* expression, it cannot be certain that Ro5-3335 inhibited *Nv-runx* in the polyps. Our data indicate that Ro5-3335 causes a delay in complete and normal regeneration in *Nematostella* which can be due to inhibiting the transition between cell proliferation and differentiation that the Runx pathway is known to regulate. We also show that inhibiting Runx with Ro5-335 likely affects tentacle regeneration. A greater sample size is needed to validate the significance of our data, so the results of this study should be viewed as preliminary.

The conservation of Runx among a range of species suggest that it plays vital roles in the transition between cell proliferation and cellular differentiation (Coffman, 2009). The function of Runx and its role in developmental signaling is not fully understood. Future research might focus on only regeneration of tentacles since that is a region with high expression of Runx. A comparison can also be done with *Hydra*. It is a species within the cnidarian phylum capable of regeneration. However, regeneration is mediated by morphallaxis for *Hydra*. Further research of Runx will help understand why it has been conserved after independent diversification and its role in developmental signaling.

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