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Tissue and spine regeneration in the temperate sea urchin *Psammechinus miliaris*

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Abstract

The tissue regenerative capabilities of echinoderms are well known at the morphogenic level, yet significant knowledge gaps remain concerning the molecular control of these processes. This pilot study assayed two pharmacological agents (vincristine sulphate and DAPT) injected directly into the body cavity of specimens of the temperate echinoid *Psammechinus miliaris* which had had their spines and tube feet deliberately amputated. Vincristine sulphate, which is a generalised mitotic inhibitor, was used as a positive control, whereas DAPT is a γ -secretase inhibitor known to block sea urchin embryo development and suppress echinoid regeneration by interfering with Notch signalling pathways. Significant differences in regeneration rate became apparent in both treatments 29 days post amputation with both inhibitors slowing regeneration of tube feet (0.6 $\mu\text{g/g}$ vincristine sulphate by 44.4% relative to controls; 4 $\mu\text{g/g}$ DAPT by 55.6% relative to controls) and spines (0.6 $\mu\text{g/g}$ vincristine sulphate by 53.3% relative to controls; 4 $\mu\text{g/g}$ DAPT by 66.7% relative to controls). Vincristine sulphate was more clearly dose-dependent than DAPT. This initial inhibition-based approach allows inferences to be made concerning possible molecular pathways controlling regeneration within *P. miliaris* and adds further support to the hypothesis that Notch signalling plays a major role in regulating regeneration in echinoids.

Introduction

Regeneration, defined as the biological process of restoring removed or damaged cells, tissues or entire organs, is commonplace in echinoderms (Alvarado & Tsonis 2006; Hall & Olson 2006; Reinardy et al. 2015). It can range from the replacement of lost arms in ophiuroids and asteroids (Candia Carnevali 2006) to the regrowth of the entire nervous system in certain holothuroids (Mashanov et al. 2014). Echinoids are capable of both tissue regeneration through the regrowth of damaged or amputated tube feet, and biomineralisation following spine loss (Candia Carnevali 2006; Dubois & Ameye 2001).

The morphogenesis of echinoid skeletal spine regeneration is well studied (Dubois & Ameye 2001); however, less is known regarding the molecular basis of spine and tube feet regeneration (Candia Carnevali 2006; Reinardy et al. 2015). Spine regeneration begins with the deposition of amorphous calcium carbonate, a process directed by sclerocytes (Politi et al. 2004; Gorzelak et al. 2011; Yang et al. 2011; Cartwright et al. 2012; Sancho-Tomás et al.

2014), followed by a wound healing phase (Dubois & Ameye 2001; Politi et al. 2004). This is followed by the raising of small conical micro-spines from the wound site through magnesium calcite deposition (Dubois & Ameye 2001). Micro-spines extend vertically and release lateral, transversal bridges that connect, forming a tri-dimensional structure known as the stereom (Dubois & Ameye 2001; Politi et al. 2004; Vinnikova & Drozdov 2011a; Vinnikova & Drozdov 2011b; Tsafnat et al. 2012). Mineral deposition thickens the stereom and it extends outwards forming a new spine (Dubois & Ameye 2001). Tube feet are flexible appendages that secrete a temporary adhesive for use in movement and prey capture; they consist of a multi-layered glycocalyx encasing the outer epidermis, inner myomesothelium, connective tissue layer, and the nerve plexus (Santos and Flammang 2006; Lebesgue et al. 2016). Less is known regarding the morphogenesis of tube feet regeneration (Dubois & Ameye 2001).

Echinoids undergo a combination of regenerative processes; epimorphosis (proliferation of dedifferentiated cells or stem cells and typically involves the formation of a blastema), and morphallaxis (the rearrangement or remodelling of existing tissue with little cell proliferation) (Candia Carnevali 2006). As the molecular basis for echinoid regeneration is less understood, focus is now turning towards the signalling pathways that induce appendage regeneration, for example the Notch signalling pathway. The Notch signalling pathway is an evolutionary conserved pathway (Guruharsha et al. 2012; An et al. 2014) consisting of five known ligands (Jagged1 and -2 and Delta1, -3 and -4) and four receptors (NOTCH1, -2, -3 and -4) (An et al. 2014). The Notch pathway has been observed in vertebrates (Beck et al. 2003) and invertebrates, including numerous representatives of the Echinodermata (Burns et al. 2012; Mashanov et al. 2015; Reinardy et al. 2015).

Notch signalling can interact with numerous other signalling pathways to regulate tissue homeostasis as well as regeneration in adult organisms (An et al. 2014). Studying Notch signalling within echinoids has become particularly relevant following the find that approximately 70% of *Strongylocentrotus purpuratus* genes have human homologues (Sodergren et al. 2006). The chemical agent N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) functions by inhibiting the γ -secretase complex that mediates proteolytic cleavage in the Notch signalling pathway and has been shown to suppress regeneration in the sub-tropical/tropical echinoid *Lytechinus variegatus* (Reinardy et al. 2015). The current study has attempted to determine whether vincristine sulphate and DAPT

treatments are similarly effective at inhibiting regeneration in the smaller temperate echinoid, *Psammechinus miliaris*.

Materials and methods

Psammechinus miliaris, collected from White Bay, Great Cumbrae, Scotland, UK (55°79'N and 4°91'W), were maintained at Newcastle University in an indoor recirculating seawater aquarium at 17 °C. Urchins were fed weekly with *Laminaria digitata* collected from Cullercoats Bay, Tyne and Wear, UK (55°03'N and 1°43'W). No permits or ethical approvals were required for the collection or manipulation of any organisms used in this study.

The regeneration assay was adapted from Reinardy et al. (Reinardy et al. 2015). Spines and tube feet from one ambulacral section were removed from the oral to the aboral surface using dissecting scissors. Animals were placed in a tank of shallow seawater one at a time while initial amputation took place and were then left to recover and extend their tube feet. Any tube feet missed in the first amputation were then removed.

22-oxo-vincaloeukoblastine sulphate (vincristine sulphate, Cayman Chemical, 11764) was diluted into calcium- and magnesium-free artificial seawater (460 mM NaCl, 10 mM KCl, 7 mM Na₂SO₄, 2.4 mM NaHCO₃) for preparation for injections at concentrations of 0, 0.2, 0.4 and 0.6 µg of vincristine sulphate per gram body weight in 200 µl injections. Six urchins were injected for each of the vincristine sulphate concentrations. N-[2S-(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethylethyl ester-glycine (DAPT, Cayman Chemical, 13197) was diluted in dimethyl sulfoxide for injection at concentrations of 0, 1, 2 and 4 µg per gram body weight, in injections of 100 µl with five urchins being injected for each DAPT concentration. For both treatments the urchins were injected twice weekly over a four week period for a total of nine injections. Injections were delivered through the peristomial membrane and into the body cavity.

The regeneration of spines and tube feet were measured weekly (one, eight, 15, 22 and 29 days post-amputation) using underwater photography. Individuals were placed in a shallow seawater tank with a ruler adjacent to the body, and photographed using an Olympus stylus TG-3 underwater camera with the body and ruler included within the frame. The best quality images were selected (one per animal) and uploaded onto Fiji ImageJ [(Fiji Is Just) ImageJ,

ImageJ1.149b,] (Schindelin et al. 2012). The free-hand drawing tool was used to draw a line over un-amputated and amputated tube feet that were measured by the software to the scale set by the ruler image. Ten uncut and cut tube feet were measured within each photo to calculate a mean length and mean percentage of regeneration. The straight line tool was used to measure spine length, six spine measurements were taken from each image. Full length spine measurements for each urchin were taken at one and 29 days post amputation; the two measurements were averaged and used to generate percentage spine regeneration values. All statistics were conducted using IBM® SPSS® Statistics (Version 22). A Kolmogorov-Smirnov test was conducted to ensure data conformed to normal distribution before further analysis. Data were tested for equal variance using Levene's test. A general linear model (GLM) analysis was conducted to establish whether time and concentration had an overall effect on regeneration. One-way ANOVAs with post hoc Tukey tests were used to test for significant differences between concentrations at every time point. The rates of spine and tube feet regrowth over the course of the experiment were tested by linear regression. Data are presented as means $\pm$ standard error.

Results

No mortalities occurred over the course of the vincristine sulphate treatment. Combining time and concentration had no overall statistically significant effect on spine or tube feet regeneration (GLM, $F = 0.74$, $df = 9$, $P > 0.05$); however, when considered independently, there was a statistically significant effect of time (GLM, $F = 24.12$, $df = 3$, $P < 0.05$) and concentration (GLM, $F = 4.16$, $df = 3$, $P < 0.05$). For tube feet, there were no statistically significant differences between vincristine sulphate concentrations at eight, 15 or 22 days post amputation; however, by day 29 the control animals had a statistically higher regeneration rate than urchins in the 0.6 μg treatment (mean = $37.44 \pm 2.59\%$ versus $24.35 \pm 1.56\%$ respectively) (One-way ANOVA, $F = 6.372$, $df = 3, 18$, $P < 0.05$) (Figure 1a). The mean regeneration rate of *P. miliaris* spines showed no significant differences between concentrations at each time point, again until day 29 whereupon the control treatment had a higher regeneration rate (mean = $35.23 \pm 3.29\%$) than both the 0.4 $\mu\text{g/g}$ (mean = $26.72 \pm 2.29\%$) and 0.6 $\mu\text{g/g}$ (mean = $23.98 \pm 1.26\%$) treatments (One-way ANOVA, $F = 11.392$, $df = 3, 18$, $P < 0.001$) (Figure 1b). Further, the 0.2 $\mu\text{g/g}$ treatment (mean = $33.26 \pm 1.29\%$) was also significantly greater than the 0.6 $\mu\text{g/g}$ treatment.

Linear regression analysis revealed that, for spines, the regrowth rate declined from the control rate of 0.075 ± 0.27 mm/d ($0.84 \pm 1.8\%$, $t = 5.763$, $R^2 = 68.3$, $P < 0.05$) to 0.04 ± 0.36 mm/d ($0.29 \pm 6.99\%$, $t = 3.726$, $R^2 = 46.3$, $P < 0.05$) in animals injected with $0.6 \mu\text{g/g}$ of vincristine sulphate. Likewise, the rate of tube feet regrowth was highest in the control group at 0.09 ± 0.35 mm/d ($1.01 \pm 7.8\%$, $t = 4.928$, $R^2 = 86.3$, $P < 0.05$) and declined to a rate of 0.04 ± 0.26 mm/d ($0.44 \pm 1.64\%$, $t = 7.332$, $R^2 = 64.7$, $P < 0.05$) in the $0.6 \mu\text{g/g}$ treatment.

In the DAPT experiment, two mortalities occurred prior to measurement in the control treatment at day 29. As with the vincristine sulphate treatments, combining time and concentration had no overall statistically significant effect on spine or tube feet regeneration (GLM, $F = 0.64$, $\text{df} = 9$, $P > 0.05$); however, when considered independently, there was a statistically significant effect of time (GLM, $F = 27.37$, $\text{df} = 3$, $P < 0.001$) and DAPT concentration (GLM, $F = 3.81$, $\text{df} = 3$, $P < 0.05$) on regeneration rate.

For tube feet, there were no significant differences until day 29 (One-way ANOVA, $F = 4.485$, $\text{df} = 3$, 14 , $P < 0.05$) (Figure 2a) when the control treatment (mean = $35.83 \pm 1.5\%$) had a greater regeneration rate than the $4 \mu\text{g/g}$ treatment (mean = $26.93 \pm 1.9\%$). Likewise, spine regeneration showed no statistically significant differences until day 29 (One-way ANOVA, $F = 13.373$, $\text{df} = 3$, 14 , $P < 0.001$) (Figure 2b) when the control treatment (mean = $36.66 \pm 1.34\%$) was significantly greater than each of the DAPT treatments (Figure 2b).

Linear regression analysis revealed that, for spines, the regrowth rate declined from the control rate of 0.06 ± 0.46 mm/d ($0.79 \pm 2.7\%$, $t = 6.689$, $R^2 = 76.2$, $P < 0.05$) to 0.04 ± 0.4 mm/d ($0.29 \pm 6.99\%$, $t = -2.526$, $R^2 = 28.5$, $P < 0.05$) in urchins injected with $4 \mu\text{g/g}$ DAPT. Similarly, the rate of tube feet regrowth was highest in the control group at 0.09 ± 0.59 mm/d ($1.01 \pm 12\%$, $t = 7.398$, $R^2 = 79.6$, $P < 0.05$) and declined to 0.05 ± 0.38 mm/d ($0.53 \pm 2.93\%$, $t = 5.022$, $R^2 = 58.3$, $P < 0.05$) for the highest DAPT treatment group.

Discussion

Regeneration of the spines and tube feet was successfully inhibited by applying the mitotic inhibitor vincristine sulphate and the γ -secretase inhibitor DAPT over 29 days of treatment. This confirms that regeneration in *P. miliaris* can be manipulated by direct injection of

inhibitors into the body cavity and that neither inhibitor was directly toxic to the urchins at the dosages administered.

DAPT inhibits an integral component of the Notch signalling pathway that mediates proteolytic cleavage prior to ligand and receptor binding and the release of the Notch intracellular domain (Guruharsha et al. 2012; An et al. 2014). Currently, detailed understanding of Notch signalling in echinoids is limited to embryonic non-skeletal mesoderm (Croce & McClay 2010), with only Reinardy et al. (Reinardy et al. 2015) providing some preliminary information for adult tissue. Although Notch signalling is not the only target of γ -secretase (Purow 2012), in the echinoid *Lytechinus variegatus* three Notch target genes, *hey*, *hes* and *gataC*, are downregulated during tube feet regeneration following DAPT treatment (Reinardy et al. 2015). Reinardy et al. (Reinardy et al. 2015) showed a dose dependant decrease in tube feet and spine regeneration over the course of 29 days, a pattern not observed in this experiment. Further, in Reinardy et al. (Reinardy et al. 2015) the tube feet and spines in the control group had regenerated to approximately 80% of their full length by day 29, whereas in the control *P. miliaris* group the tube feet and spine length had regrown to less than 40% of the full length after 29 days. This disparity could be directly linked to the effect of temperature on echinoid regeneration. The lower rate of regeneration of *P. miliaris* resulted in less pronounced differences in appendage length at each time point. Future measurement of regeneration rate in temperate echinoid species should therefore be taken over a longer period of time. Using *Strongylocentrotus purpuratus* collected from northern and southern U.S. populations, Pespeni et al. (Pespeni et al. 2013) showed that latitude has a significant effect on echinoid regeneration rates with a faster spine biomineralisation rate in southern populations, related to the warmer sea surface temperature. This difference in regeneration rate is thought to be controlled by differences in gene regulation and expression that are genetically controlled for adaptive benefit that alters between the environments. In the southern population, the expression of 66 genes associated with biomineralisation, ribosomal function, and regeneration rate were higher than those of the northern population.

Neurogenesis, the growth and development of nerve tissue, is fundamental to echinoderm regeneration (Burns et al. 2013; Mashanov et al. 2015; Pirotte et al. 2016); for example, removing the radial nerve in *Asterina gibbosa* makes arm regeneration impossible (Candia Carnevali 2006). It has been hypothesised that Notch signalling regulates the undifferentiation of neural cells by reducing the activity of pro-neural proteins (Holmberg et

al. 2008). Pro-neural proteins induce the production of new neuron cells by promoting original neuron cells to differentiate. Two pro-neural proteins that Notch signalling can repress are the basic helix-loop-helix (bHLH) proteins and E-proteins (Holmberg et al. 2008). Notch signalling can achieve this through a processes dependent on the transcriptional factor Hes, which was downregulated following DAPT treatment during *L. variegatus* regeneration (Reinardy et al. 2015), and controls the balance of undifferentiated versus differentiated cells (Burns et al. 2012). However, Notch signalling also requires the activity of SoxB1 proteins. SoxB1 transcription factors also inhibit pro-neurone proteins independent of Notch (Burns et al. 2012) thereby preventing stem cells from terminally differentiating into neurones (Bylund et al. 2003; Holmberg et al. 2008). Therefore, suppression of Notch signalling alone does not necessarily remove the block to differentiation if SoxB1 proteins are still being transcribed. In holothuroids, Sox transcription factors target the radial glial glands that produce new and functional neurones following traumatic lesions (Mashanov et al. 2013). During the distal regeneration of the ophiuroid *Amphiura filiformis*, three Notch1 proteins and a Sox1 transcriptome factor involved in neurogenesis are upregulated (Burns et al. 2012). Likewise, in the asteroid *Echinaster sepositus* neurogenesis occurs alongside skeletogenesis between one and six weeks following arm amputation (Ben Khadra et al. 2015). Neurogenesis involving Notch signalling and *SoxB1* during sea urchin embryonic development is well documented and is being used to develop an overall model for metazoan neurogenesis (Burke et al. 2014).

There is increasing evidence that echinoid tube feet have a photosensory neuron system (Burke et al. 2006; Agca et al. 2011) with numerous neuromuscular genes associated with the tube feet. Therefore, although there is no direct evidence that Notch signalling regulates neurogenesis in *P. miliaris*, as tube feet and spines are neuromuscular appendages connected independently to sensory-interneuron-motor reflex arcs (Burke et al. 2006; Agca et al. 2011), it may be reasonable to hypothesise that tube feet regeneration is driven by Notch signalling.

One of the most researched interactions with Notch signalling is the negative crosstalk that occurs between Notch and the Wnt signalling pathway (An et al. 2014). The Wnt signalling pathway is more complex than Notch (Croce et al. 2006; An et al. 2014), and a thorough analysis of the *S. purpuratus* genome found eleven of thirteen known Wnt subfamilies and three Wnt signalling pathways (Croce et al. 2006). The Wnt signalling pathway is also associated with regenerative processes in other echinoderms (Sun et al. 2013; An et al. 2014)

including intestinal regeneration of the holothuroid *Apostichopus japonicas* and during *Paracentrotus lividus* embryogenesis (Robert et al. 2014).

This study utilised a novel sea urchin regeneration assay developed by Reinardy et al. (Reinardy et al. 2015), and found that treatment with the dipeptide γ -secretase inhibitor DAPT and the mitotic inhibitor vincristine sulphate caused a significant reduction in regeneration following 29 days of treatment. The results support the view that Notch signalling is likely to be a crucial molecular pathway involved in echinoid regeneration; however, it is likely that Notch signalling works in concert with other processes deemed vital to the regenerative cellular plasticity (Beck et al. 2003; Odelberg 2004; Katoh 2007). Further study identifying gene expression during *P. miliaris* regeneration is needed. As DAPT is a Notch signalling inhibitor, it is concluded that Notch signalling is likely to be involved in the molecular processes that drive echinoid regeneration. Taken together with previous work on *L. variegatus*, the results of the current study support the conclusions of Holmberg et al. (Holmberg et al. 2008) who suggest that Notch signalling may interact with pro-neural proteins to regulate neurogenesis.

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Figure legends

Figure 1. Mean ($\pm$ standard error of the mean) regeneration rate (%) for a) tube feet and b) spines of *Psammechinus miliaris* injected with an increasing dose of vincristine sulphate (n = six per concentration). Treatments that do not share a superscript letter on day 29 are statistically different (one-way ANOVA).

Figure 2. Mean ($\pm$ standard error of the mean) regeneration rate (%) for a) tube feet and b) spines of *Psammechinus miliaris* injected with an increasing dose of DAPT (n = five per concentration). * = statistically significant reduction relative to the control treatment on day 29 (one-way ANOVA).

Fig. 1

Fig. 2