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Aurora kinase inhibitors delay regeneration in *Stentor coeruleus* at an intermediate step

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Abstract

The giant unicellular ciliate Stentor coeruleus can be cut into pieces and each piece will regenerate into a healthy, full-sized individual. The molecular mechanism for how Stentor regenerates is a complete mystery, however, the process of regeneration shows striking similarities to the process of cell division. On a morphological level, the process of creating a second mouth in division or a new oral apparatus in regeneration have the same steps and occur in the same order. On the transcriptional level, genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during regeneration. This suggests that there may be some common regulatory mechanisms involved in both regeneration and cell division. If the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in Stentor. Here we show that two well-characterized Aurora kinase A+B inhibitors that affect the timing of regeneration. ZM447439 slows down regeneration by at least one hour. PF03814735 completely suppresses regeneration until the drug is removed. Here we provide the first direct experimental evidence that Stentor may harness the cell division machinery to regulate the sequential process of regeneration.

Introduction

The ability to heal wounds and regenerate damaged structures is essential for an organism's survival. Multicellular organisms mostly rely on cell division to patch wounds and regenerate lost structures with newly proliferated cells, but when a single cell is damaged, it must be able to recognize and repair that damage without being able to rely on other cells. Nowhere is this challenge more dramatic than in the giant unicellular ciliate Stentor coeruleus, for when cut into pieces, each piece will fully regenerate into a healthy, full-sized individual. Stentor cells are a millimeter long with a wine glass shape, and have a

complex and intricate ultrastructure. Stentor are binucleate ciliates with two morphologically distinct nuclei. The micronuclei is used for germline reproduction and the macronucleus is transcriptionally active throughout the cell cycle. Stentor has an oral pouch, a cilia-lined pore to intake food at its wide anterior and a holdfast, the structure by which the cell attaches to a surface, at its posterior. Connecting these two are a series of microtubule rows called cortical rows that resemble pinstripes. The oral pouch and the holdfast can each fully regenerate if removed, and a bisected cell can regenerate two normal looking cells [1]. The molecular mechanism for how Stentor regenerates missing parts is a complete mystery. This study focuses on regeneration of the oral apparatus, which consists of a circular band of cilia-based structures known as the membranellar band, connected to an oral pouch located at a defined position. During feeding, the membranellar band creates a fluid flow to bring food to the anterior end of the cell, where it is engulfed through the oral pouch.

Regeneration in Stentor coeruleus can be induced by sucrose shock [1]. This leads to shedding of the oral apparatus, which is comprised of the oral pouch and membranellar band (Figure 1A). After sucrose shocking, Stentor look tear-drop shaped and stay stationary for approximately three hours. After three or four hours of regeneration, Stentor begin to form a membranellar band in the middle of the cell body, initially oriented parallel to the body axis. The membranellar band grows simultaneously towards the top and bottom of the cell. At the top of the cell, the membranellar band will continue growing across the top. After six or seven hours of regeneration, the posterior end of the membranellar band will begin to curl to form the oral pouch and a physical indentation of the cell surface can be seen. Within the last two hours of regeneration, the oral pouch will be moved to the top of the cell along with the membranellar band. Stentor usually completes regeneration within 8 hours.

The process of regeneration shows striking similarities to the process of cell division. When a Stentor cell divides asymmetrically along its vertical axis, the anterior daughter cell retains the oral apparatus from the mother cell and the posterior daughter inherits a de novo oral apparatus that forms just prior to cytokinesis. This de novo creation of an oral apparatus during regeneration proceeds through a series of morphological steps virtually identical to those seen during the creation of a new oral pouch during division [1], namely, the formation of a membranellar band parallel to the body axis, curling of the band, and formation of the oral pouch. During division, the macronucleus undergoes a dramatic shape change from a moniliform string of small spherical nodes into a short tube, when then re-elongates just prior to mitosis. This same nuclear shape change also takes place during regeneration, further suggesting a similarity of the two processes [2].

Similarity between regeneration and division has also been reported at the transcriptional level, based on studies of the RNA transcriptome during regeneration. Genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during the later stages of regeneration compared to the earlier stages of regeneration [3]. Such similarities suggest that there may be some common regulatory mechanisms involved in both regeneration and cell division. Since aurora kinase signaling indicates that a spindle is properly assembled [4], a similar mechanism could be at work in Stentor to signal the correct assembly of one or more structures during regeneration. But it is also possible that the similarity has nothing to do with regeneration

and instead plays some other role. For example, the micronuclei undergo mitosis during both cell division and regeneration [5], so perhaps the transcriptional changes in cell cycle-related genes have only to do with the micronuclear mitosis and not regeneration itself. However, if the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in Stentor. Here we show that two well-characterized Aurora kinase A+B inhibitors slow or stop regeneration in Stentor, providing the first direct experimental evidence that Stentor may harness the cell division machinery to regulate the sequential process of regeneration.

Objective

The general objective is to learn whether regeneration and division may harness conserved molecular mechanisms. The specific objective is to test whether inhibition of the Aurora kinases, well known regulators of cell division, alters the process of regeneration in Stentor.

Results & Discussion

Compared to the timing of events in untreated cells (Figure 1B), addition of Aurora kinase A+B inhibitor ZM447439 [4] caused regeneration to be delayed by at least one hour. 10% of treated cells did not form a membranellar band until 4 hours into regeneration (Figure 1C). Treated cells spent more time forming a membranellar band and the first oral pouch did not appear until seven or eight hours after starting regeneration, compared to untreated cells where oral pouches appear in the 6.5 hour time point. The first fully regenerated Stentor did not form until eight and a half hours later (Figure 1C). We have observed the same pattern of delay three times in separate experiments (data not shown).

Although ZM447439 is known to be a highly specific inhibitor of Aurora kinases in mammalian cells, any chemical inhibitor can show off-target effects, especially when applied in a different cell type. One type of off-target effect would be a fortuitous interaction of a drug to another target via a binding interaction that had no relation to the binding modality used with the intended target. This type of off-target interaction is less likely to be seen if one tests a second drug with a distinct chemical structure. To rule out this type of off-target effect and confirm our result that Aurora inhibition delays regeneration, we tested a second highly specific and reversible Aurora kinase A+B inhibitor, PF03814735 [6], which has a different chemical structure from ZM447439, and which also has the advantage of being reversible in many systems. We found that with this inhibitor, regeneration was suspended at the membranellar band stage (Figure 1D). 38% of Stentor still had no oral pouch by six hours, and none of the Stentor had regenerated by ten and a half hours. Regeneration was paused at the membranellar band stage for the duration of the experiment. This result suggests that the delay of regeneration with ZM447439 may not have been an off-target effect, but our results cannot rule out an off-target effect that involves a binding site resembling the site present on Aurora kinase itself.

PF03814735 is reversible in other systems, therefore we questioned whether the block on regeneration could be reversed after the inhibitor is removed. After a two hour incubation and three subsequent washes, Stentor were able to regenerate in a timely fashion, forming

membranellar bands after five hours, oral pouches after seven hours and fully regenerating in ten hours (Figure 1E).

Our results indicate that Aurora kinase function may normally be required to drive a specific step of regeneration that takes place after the membranellar band has formed but before it moves to the anterior of the cell and forms an oral pouch. Such a temporal requirement is reminiscent of the requirement of Aurora kinases for specific stages of mitotic progression. As with the cell cycle, the ability to reversibly arrest regeneration and then analyze timing of events after the arrest is alleviated may, in the future, provide a way to determine whether regeneration is timed by a series of domino-like events, each triggering the next, or a master clock like that used in the cell cycle. Actinomycin D [7], puromycin [7], concanavalin A [8] and DNA synthesis inhibitors [9] have previously been shown to affect cell growth or regeneration in Stentor, but the Aurora inhibitors reported here have the advantage that they target a very specific signaling pathway.

Conclusions

Our results indicate that regeneration in Stentor takes place in two separately regulated steps, with Aurora kinase possibly regulating the second step. These results support the idea that regeneration in Stentor is regulated by components of the cell division machinery, suggesting that the similarity between the two processes is more than just a superficial coincidence. These small molecule inhibitors provide new tools to perturb the process and study its effects.

Limitations

Bioinformatic analysis of the aurora kinase family in Stentor indicates that there are 44 different aurora kinases [10], and that these cannot be clearly mapped onto the Aurora classes A, B, and C in mammals. Consequently, it is not currently clear which of the Aurora kinases in Stentor is actually being affected by the inhibitors during regeneration. We also note that because both aurora kinase inhibitors were developed to target mammalian kinases, the possibility that the drugs have off target effects in Stentor cannot be excluded.

Conjectures

Both mitosis and regeneration proceed through a series of distinct steps that must take place in the correct order, and each step must not start until the preceding steps are completed. We conjecture that the cell cycle machinery, which has evolved to regulate the sequential steps of division, may provide the necessary timing and ordering of events that allows proper regeneration. For example, regeneration might require a series of checkpoints, one of which is mediated by Aurora signaling. Early observations of washing out the competitive inhibitor suggested that subsequent events took place more synchronously. However, measurements of regeneration timing will be needed to confirm this impression.

Methods

Sucrose shock

Sucrose shock was performed according to Tartar 1961 [1]. Cells were gathered by pipette individually and washed with pasteurized spring water (PSW; Carolina Biological Supply). An equal volume of 25% (w/v) sucrose was added to cells in PSW to give a final concentration of 12.5% sucrose. Cells were incubated for approximately 3 minutes or until the membranellar band was shed. Sucrose was then diluted 50x by addition of PSW. After 20–30 minutes, cells that have rounded up (indicating imminent death) or that still had membranellar bands present were discarded.

Identification of stages

Cells were examined at 30 minute or one hour intervals, using a Zeiss Stemi 2000 at 5x to identify cells that retained a non-spherical shape. Three hours after sucrose shock, the presence of a membranellar band was assessed by looking for a faint band of randomly beating cilia in the middle of the cell. Since these may be facing away from the camera lens, it was important to look at the other side of Stentor. If the cells had been starved, the membranellar band was more likely to be visible through the cell. To locate the oral pouch, the most posterior part of the membranellar band was examined for the presence of an indentation that represents the oral pouch. It was observed that immediately before the oral pouch first appeared, the membranellar band began curling. Cells were considered to have completed regeneration if the oral pouch was present and the membranellar band had migrated to the anterior end of the cell. Bar graphs in Figure 1-B–E depict the distribution of regeneration stages as a function of time. The number of cells analyzed in the four panels were 42 (control), 32 (ZM447439), 35 (PF03814735), and 21 (ZM447439 with washout). Percentages were obtained by dividing the number of cells at each stage of regeneration by the total number of cells in each experiment. Experiments were done at 21C in room light.

Inhibitor treatment

The inhibitors ZM447439 and PF03814735, purchased from Selleck Chemicals, were dissolved in DMSO at concentrations of 5.0mM and 2.1mM respectively. These stock solutions were then diluted to final concentrations of 0.1nM and 42pM in wells containing Stentor cells in PSW.

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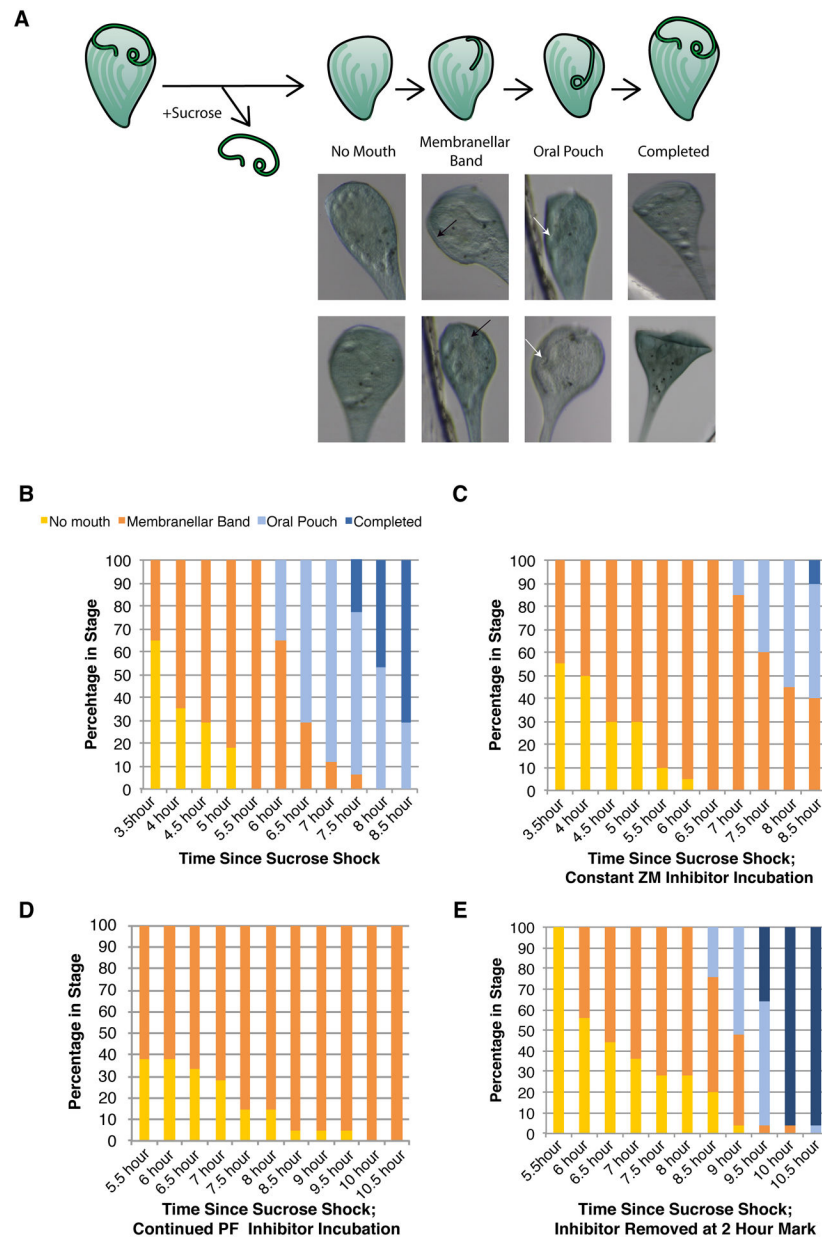
Figure 1. Aurora kinase A + B inhibitors slow or stop regeneration.

(A) Stentor exhibits three, distinct, chronological, morphologies during regeneration. After sucrose shocking, they first adopt a tear-drop shape, then form a membranellar band (dotted line) parallel to their body axis. Next, they form an oral pouch (arrow) at the posterior end of the membranellar band. Finally, they move the oral pouch to the top of the cell. At each stage, photos of two different individuals are shown in each column.

(B) Under normal conditions, Stentor need approximately eight hours to regenerate. After about three hours a membranellar band starts to appear, and after another three hours the oral pouch becomes visible, after which two more hours are spent moving the membranellar band and the pouch to the correct position to complete regeneration. Data shown are from 42 control cells.

(C) Aurora kinase A+B inhibitor, ZM447439, has little effect on the first phase of regeneration, formation of the membranelle band, but dramatically slows down the second phase of regeneration, formation of the oral pouch. Data shown are from 32 cells treated with ZM447439.

(D) Aurora kinase A+B inhibitor, PF03814735, permits formation of the membranellar band but completely blocks regeneration at the stage of oral pouch formation. Data shown are for 35 cells treated with PF03814735.

(E) Aurora kinase A+B inhibitor, PF03814735, can be removed and regeneration occurs subsequently within eight hours. Data shown are for 21 cells treated with PF03814735 followed by washout.