Title
The Role of AKT on Regulating Stem Cells in the Planarian Schmidtea Mediterranea

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The Role of AKT on Regulating Stem Cells in the Planarian Schmidtea Mediterranea

In

Quantitative Systems and Biology

By

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2014

DEDICATION

I would like to dedicate my thesis to my family and loved ones. Their support and pride in my education has provided in me the greatest motivation to better my academic career and achieve all the goals I have and will set out to achieve.

The thesis of Daniel Ramirez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2014

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ABSTRACT OF THE THESIS

The Role of AKT on Regulating Stem Cells in the Planarian Schmidtea Mediterranea

By

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In long-lived organisms like humans, stem cells (SCs) constantly divide to replace aged and damaged cells. Deregulation of this process leads towards aging and pathologies such as cancer and degenerative diseases. The mechanisms regulating this cellular turnover are poorly understood, mainly due to the difficulty in analyzing SCs in their natural environment. Here, we use a new approach and model organism, the planarian flatworm to study the process of SC regulation in the complexity of the whole organism. In particular, we analyze the role of signaling pathways that regulate SC response to tissue turnover and regeneration in the adult body. One such signaling pathway of interest involves the protein kinase AKT. This master regulator of cellular functions (cell survival, cell growth, protein synthesis, etc.) has been implicated in the systemic regulation SCs and disruption of this pathway has shown to be highly involved in human cancer. We have designed an effective strategy to downregulate AKT function with RNA-interference and have followed SC response during cellular turnover and tissue regeneration. This approach revealed that loss of AKT disrupts the systemic balance between SC proliferation and cell death during tissue renewal. Altogether, our results suggest a central role for AKT in regulating cell behavior during tissue renewal and repair. Our long-term goals seek to specifically use signaling pathways to control cellular behavior in vivo to customize tissue repair for therapeutic intervention in cancer and aging.

INTRODUCTION

In all living organisms, cellular functions are coordinated through signaling pathways. Cells contain sensory networks for these local and/or system signals and deregulation of these pathways have been implicated in degenerative diseases such as cancer. How signaling pathways coordinate systemic cell behavior is poorly understood. One of the main limitations is the lack of model systems that help us to explore cellular signaling pathways in vivo. Another limiting factor is the possibility to access specific cells such as stem cells that are modulated by signaling pathways. Stem cells (SCs) are a group of cells found within multi-cellular organisms and help to replace damaged/lost cells during normal physiological activities (1) (2). However, the molecular basis of stem cells and how these cells are regulated in vivo needs to be elucidated. Dissecting the molecular basis of SC regulation in vivo will provide new avenues for developing novel treatments and therapeutic approaches for degenerative diseases such as cancer.

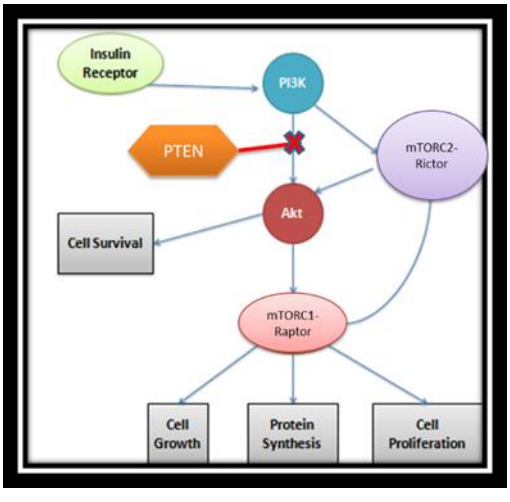


Figure 1: Akt-dependent cellular signaling involves regulation on protein synthesis, cellular proliferation, cell growth and cell survival.

Here, we use a new approach and model organism, the planarian flatworm to study the process of stem cells (SC) regulation in the complexity of the whole organism. These organisms contain pools of adult stem cells known as neoblasts that renders extraordinary regenerative capability and exceptionally high cell turnover rate (3) (4) (5). In particular, we analyze the role of signaling pathways that regulate SC response to tissue turnover and regeneration in the adult body. The protein kinase AKT regulates growth and metabolism and has the capability to activate several key molecules in

living cells (**Fig. 1**). It is unknown how AKT systemically regulates stem cells, however the dysregulation of this signaling pathway has been implicated in many cancers and degenerative diseases (6) (7). As part of my Master's thesis in Dr. Oviedo's lab, I am interested in studying how the AKT pathway regulates stem cells in vivo through utilizing the planaria flatworm model organism, *Schmidtea mediterranea.* An in depth look into various mechanisms through which AKT is associated will provide an overview of AKT functions and provide a premise to what may be expected during the absence of this master regulator.

AKT Structure and Signal Transduction

The AKT serine/threonine kinase is also known as PKB. All conserved structures of this master regulator protein kinase include three functional domains: an N-terminal Pleckstrin Homology (PH) domain, a central kinase domain and a C-terminal regulatory domain containing the hydrophobic phosphorylation (HM) site (8) (9). This architecture is conserved among species spanning from yeast and drosophila to mouse and human.

Largely, AKT activity is induced following activation of the phosphoinositide-3 kinase (PI3K) through receptor tyrosine kinase mediated signaling cascades. Induced by ligands such as insulin, amino acids, and other growth factors, this signaling hub has shown to have an extremely complex transduction network leading many of which utilize AKT (10) (11). Downstream of the activated (and auto-phosphorylated) PI3K, AKT may translocate from the cytoplasm to the plasma membrane initiating the binding of the PH domain to PI3K. AKT is then activated by a multistep process that requires phosphorylation of both Thr308 in the activation loop of the kinase domain by PDK1 (12) and Ser437 within the HM of the regulatory domain by the TORC2 protein (among others) (13) (14) . Once activated at the plasma membrane, phosphorylated AKT can translocate to the cytosol or nucleus where it

may carry out its regulatory roles in metabolism, cell survival, motility, protein synthesis, and cell cycle progression.

Several phosphatases negatively regulate AKT activity such as the tumor suppressor phosphatase and tensin homology deleted on chromosome ten (PTEN) and SH2-domian containing inositol polyphosphate 5-phosphatase (SHIP). Both act indirectly at the plasma membrane deactivating PI3K signaling (15) (16). Interestingly, AKT induced pathways are subject to modulation through other pathways, further highlighting the complexity of AKT signaling regulation.

AKT role in metabolism

Several mechanisms mediating the regulation of insulin-dependent glucose homeostasis are AKT-dependent. These mechanisms include suppression of glycogenolysis and the insulin-mediated increase in glycogen synthesis post AKT phosphorylation and inactivation of Glycogen synthase kinase- 3 (GSK3) (17). AKT activation further effects cellular metabolism by phosphorylating and activating 6-phsophofrcto-2-kinase, a key regulatory kinase involved in the progression of glycolysis (18). Other metabolic functions regulated by AKT include increasing lipogenesis through ATP-citrate lyase phosphorylation (19) and increasing the transcription of the glucose transporters GLUT1 and GLUT4 thus increasing glucose uptake into cells (20) (21).

Cell-cycle regulation through AKT

AKT involvement in regulating cell-cycle progression has been well established. AKT activation has been shown to affect two key cell-cycle regulator proteins; $p21^{Cip1/WAF1}$ and $p27^{Kip1}$. Phosphorylation of these two proteins leads to activation of proliferating cell nuclear antigen (PCNA), promotion of the assembly of the cyclin D1: cyclin-dependent

kinase 4 (CDK4), and resistance to cytokine-mediated G1 arrest (22) (23). The p53 tumor suppressor is also a downstream target of AKT signaling. AKT phosphorylates the ubiquitin ligase mouse double minute homolog 2 (Mdm2) allowing this protein to enter the nucleus to inhibit p53, thus maintaining cell-cycle progression (24).

Cell Survival & Apoptosis

The AKT association with cellular metabolism and survival go hand in hand with its direct involvement in apoptosis suppression. AKT has been shown to directly or indirectly inhibit pro-apoptotic proteins. One of the earliest known AKT pro-apoptotic targets identified was the BCL2-antagonist of death (BAD) protein. While BAD is active, it inhibits anti-apoptotic Bcl-2 family members. However, after phosphorylation by AKT, active sites of BAD form high affinity binding sites for cytoplasmic 14-3-3 molecules thus retaining the BAD protein in the cytosol where it cannot complete its functions (25) (26).

AKT anti-apoptotic regulations extend also to maintaining mitochondrial integrity to keep *Cytochrome c* and other apoptogenic factors inside the mitochondrial membrane (27). Additionally, stress-activated kinase cascades have been linked to induce apoptosis. AKT activity has been shown to suppress these cascades in the JNK and p38 pathway (28) by regulating apoptosis signal-regulating kinase-1 (ASK1) (29).

AKT involvement in cancer

With AKT having a role in a wide array of cellular functions, aberrant signaling involving AKT has been implicated in several cancer causing pathways. There are numerous studies on oncogenic amplifications of different AKT genes in primary human tumors and cancer cell lines (30) (31). Constitutive high levels of AKT activity occur through a variety of mechanisms including amplification of AKT genes and mutations in components of the

PI3K subunits. PTEN is often mutated in human cancer and silencing of PTEN is frequently observed in primary tumors and cancer cell lines (32) (33).

Even aberrant growth factor receptors and Ras can induce AKT hyperactivity independent of PI3K activation in tumor cells (34). Because AKT hyper-activation is found more so in poorly differentiated tumors, these cancers are inclined to be more invasive, grow faster, and respond less to treatment thus leading to a negative prognosis marker for disease outcome (35). However, AKT alone is believed to be insufficient for tumorigenesis but is more likely to contribute to cancer progression by promoting the proliferation, cell survival and metabolic capacity of cells and increased cell size (36) (37).

Planarian Model:

There are limitations to studying essential signal transduction pathways in model organisms such as mice. For instance, knocking out AKT in mice has shown to cause death at birth (38). Additionally, analyzing the effects AKT has on various cell signaling pathways involving stem cell and tissue regeneration in mice may not be the most efficient and/or cost effect approach.

One way to circumvent these limitations would be to utilize the model organisms, freshwater planarian flatworms. *Schmidtea mediterranea* flatworms are an attractive model for research on conserved signaling pathways as these animals may also be used to study the underlying mechanisms controlling tissue regeneration and physiological cellular turnover. Cellular turnover is the process by which older differentiated cells undergo apoptosis and are replaced by the dividing progeny of adult stem cells (5) (3). These planarian adult stem cells are known as neoblasts and it is due to the vast pool of neoblasts that the planarian flatworm has the remarkable ability of tissue regeneration. Only a small fragment of amputated

planarian tissue (anywhere below the photoreceptors) is all that is needed to regenerate an entire animal (39) (40).

Studies on planarian regeneration have been carried out for over two centuries (41) (42) and many studies on the planarian neoblast have demonstrated stem cell regulation and function as well as cell lineage commitment. (43). For a brief overview on planarian tissue regeneration, upon amputation, an accumulation of undifferentiated cells aggregates at the site of the wound forming a mass of tissue named the blastema. This blastema formation is considered an imperative center of cellular signaling leading to the hyper-proliferation and migration of neoblasts to the wound to commence the regeneration process of the missing tissue (44). Methods to analyze the progression of the neoblast response have been developed to track the patterning and quantification of stem cell expansion (45) (46).

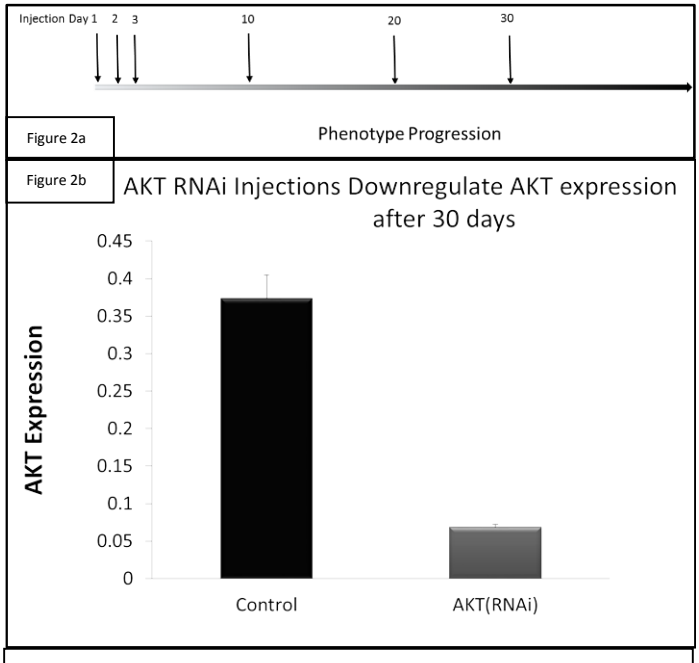


Figure 2: (a) AKT-dsRNA injection scheduled was optimized for maximal aberration of AKT signaling on the stem cell population. RNAi protocol was optimized until it achieved reproducible maximum abrogation (5-6 microinjections of double-stranded RNA (dsRNA)). (b) qPCR analysis of AKT expression indicates a marked downregulation of AKT post 5-6 dsRNA microinjections.

Through techniques utilizing Fluorescence Activated Cell Sorting (FACS), based on DNA content and cell size, three cell populations have been identified within the planaria, two of which are X-ray sensitive (47) (48). One of the X-ray sensitive groups, the X_1 population, is composed of S/M-phase cells considered pure neoblasts. The X_2 population is composed of G1-phase neoblast early division progeny cells and is also sensitive to

irradiation. Lastly, the $X_{insenstive}$ fraction is composed of differentiated cells which can all tolerate irradiation (49) (48). Many cellular markers have been identified to specifically label these populations. Smedwi-1 expression is characteristic of neoblasts (X_1 population), *Smed-NB.21.11e* expression is characteristic of early-division progeny (X_2 population), and Smed-Agat-1 expression is characteristic of late-division progeny (X_{ins} population) (43).

Given these preliminary data, we have chosen to investigate the roles of the AKT signal transduction pathway within the planarian model. Previously cloned by Oviedo et al 2008, AKT was shown to be ubiquitously expressed throughout the planarian body, and, through FACS-isolated cells, expressed in approximately 50% of X_1 and $X_{insenstive}$ cells and approximately in 20% of the X_2 population (33). This type of AKT expression suggests its conserved widespread roles in regulating planarian homeostasis through its involvement in the aforementioned regulatory behaviors. We have optimized an RNA-interference protocol to efficiently downregulate the expression of the AKT kinase and examine the effects of AKT loss in the processes of tissue regeneration and maintenance of homeostasis during physiological cellular turnover. (**Fig. 2 a-b**)

EXPERIMENTAL PROCEDURES

Planarian Culture

The clonal lines of the diploid asexual strain of Schmidtea mediterranea was used in all experiments. Animals were kept and maintained at room temperature (21-22º C) in dechlorinated tap water supplemented with 2 mM NaCl, 0.1 mM KCl, 0.1 mM $MgSO_4$, 0.12

mM $NaHCO_3$ and fed beed liver paste once each week. Animals were starved for 1 week prior to all labeling experiments.

dsRNA and microinjections

dsRNA synthesis was carried out as previously described in (33). Microinjections were carried out as described in figure 2a.

TUNEL assay

The apoptotic staining TUNEL assay was performed as previously described in (50). Briefly, this technique utilizes reagents that recognize double stranded DNA breaks, enzymatically reconfigure the broken ends, and label those double stranded breaks with fluorescent tags.

In Situ hybridization

Riboprobes for in situ hybridization were synthesized using T3 or T7 polymerase and digoxigenen-labeled ribonucleotide mix with specific PCR templates as previously described (33). Animals were killed in 5% NAC solution. Whole-mount and Fluorescent in situ hybridizations were performed as previously described (51).

H3P staining and immunofluorescence

Animals were Carnoys fixed and immunostaining was performed as previously described (33). Mitotic H3P positive cells were counted and normalized to the area (mm^2) using NIS element software (Nikon). Statistical analysis were performed using Microsoft Excel.

FACS

Planarians were dissociated as previously described (49). DAPI was used to stain cell nuclei and the data were collected using an LSRII flow cytometer (BD Biosciences) with DIVA software. Flow cytometry analyses were performed with FlowJo software, Version 8 (www.flowjo.com)

qRT-PCR

RNA extraction and quantitative real-time PCR (qPCR) reactions were performed as previously described (33) (49). Trizol was used to isolate RNA from control and experimental animals. qPCR reactions were performed using the SYBER Green Master Mix in a 7500 Fast Real Time PCR cycler (Applied Biosystems). Gene expressions are relative to the ubiquitously expressed clone *H.55.12e* (49).

Measurements of planaria and image processing

Nikon AZ-100 multizoom microscope and NIS Elements AR 3.2 software was used to record animal behavior collect digital pictures. Brightness and contrast were adjusted with Adobe Photoshop.

RESULTS

AKT-RNAi leads to a marked decrease in proliferative and mitotic cells

As the AKT kinase is highly involved in cell proliferation and cell cycle progression, Aim 1 of this thesis is to identify any difference in the levels of mitotic and proliferating cells throughout the system post AKT-RNAi. After 30-40 days of 6 microinjections, we see an approximate 95% decrease in mitotic H3P+ cells (**Fig. 3a-b**). To identify whether these AKT-independent H3P+ cells are actually cycling through the s-phase, we further tested cell cycle progression through Bromo-deoxyuridine (BrdU) incorporation (**Fig. 3b**). Our results show an atypical staining pattern indicative to no true BrdU incorporation when compared to the controls. FACS analysis confirms that AKT-RNAi cells have a clear decrease in S-phase cells (**Fig. 3c**).

Fig. 3a

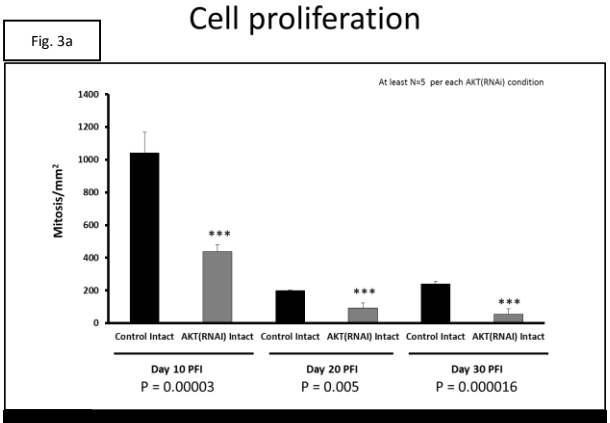


Fig. 3b

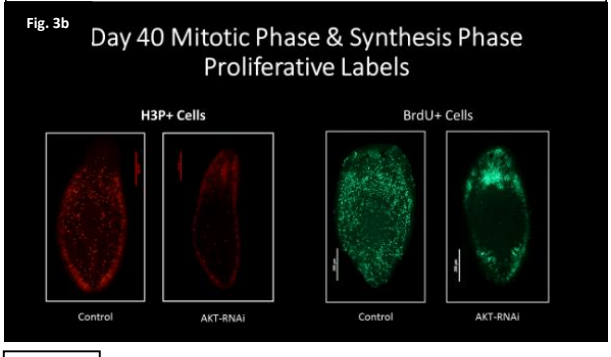


Fig. 3c

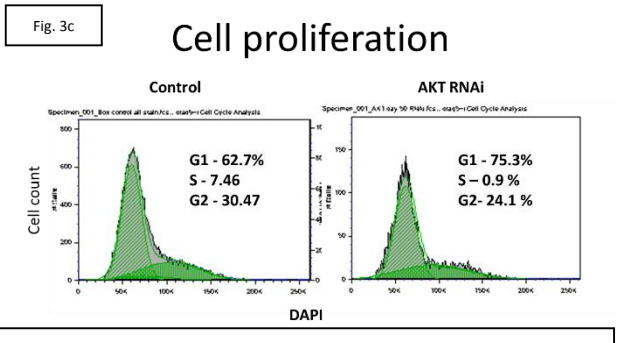
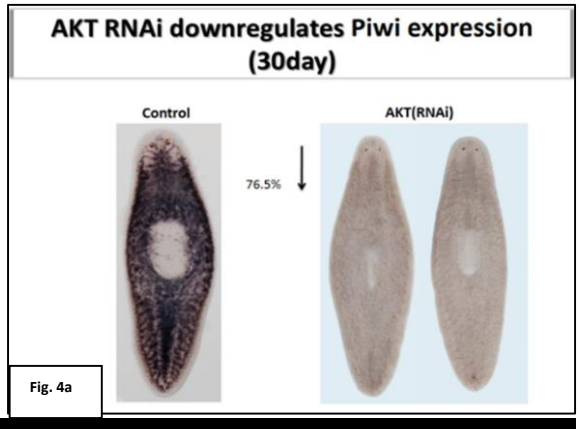


Figure 3: (a) Mitotic cell quantification over animal area performed over a 30 day time course. Three independent experiments, n ≥ 30 animals (b) Anti-H3P and Anti-BrdU fluorescent staining's performed post 40 days. (c) Cell-cycle analysis for DNA content performed on 40 day dissociated animals.

Loss of AKT removes stem cell, early division and late division cell markers

With the tremendous loss of cycling cells throughout the system post AKT-downregulation, Aim #2 was to identify whether or not stem cells are depleted from the system or if they are present but stalled somewhere in the cell cycle. Strikingly, loss of AKT produced major down-regulations of the characteristic stem cell marker *Smedwi-1* (Piwi), early-division cell marker *NB.21.11e*, and even late-division (differentiated) cell marker *Agat-1* (**Fig. 4a-b**).



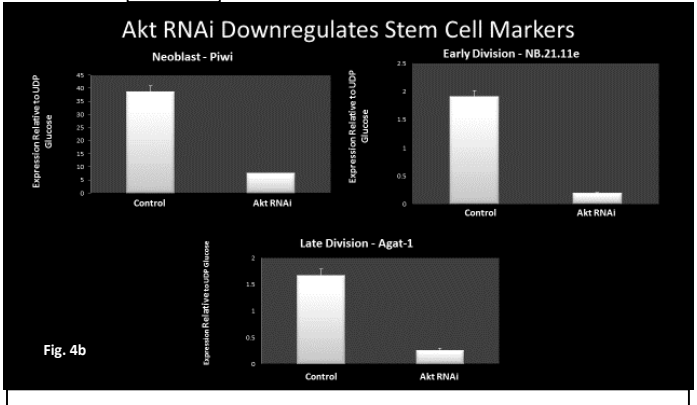
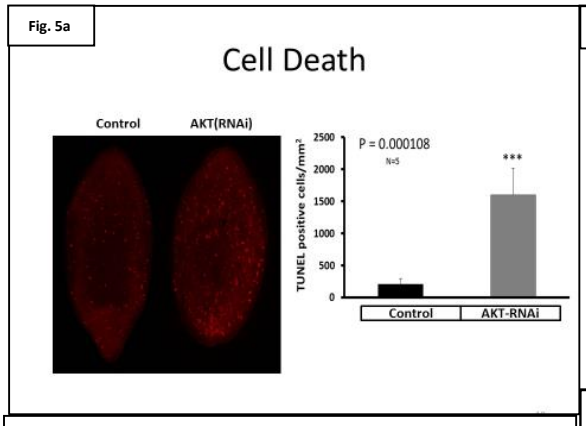
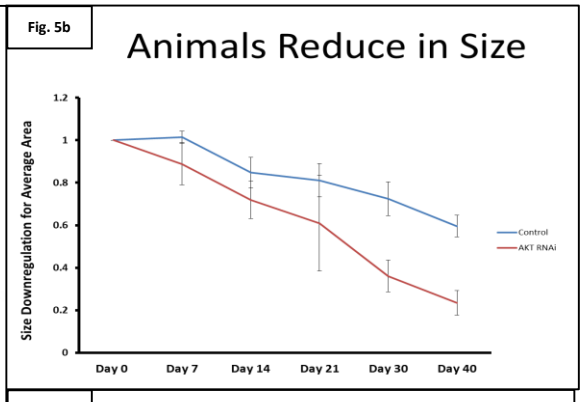


Figure 4: (a) Piwi-Wish performed on AKT-RNAi 30 days. (b) Cell population markers show major downregulation. Q-PCR performed at 30 days.

AKT plays a major role in cell survival and apoptosis

Through the apparent overall loss of dividing, early-division and late-division cells, we suspect that without AKT regulating the cell-survival signal, the loss of these cellular markers suggests that many, if not all, cell types within the animal are dying off due to either programmed cell death or necrosis. Aim #3 was to identify the abundance of cell death along with any pattern of animal size over the progression of the AKT-RNAi phenotype. Through the use of the TUNEL assay, we recognized an 8X increase in cellular death throughout the animal following the loss of AKT (**Fig. 5a**). Moreover, the





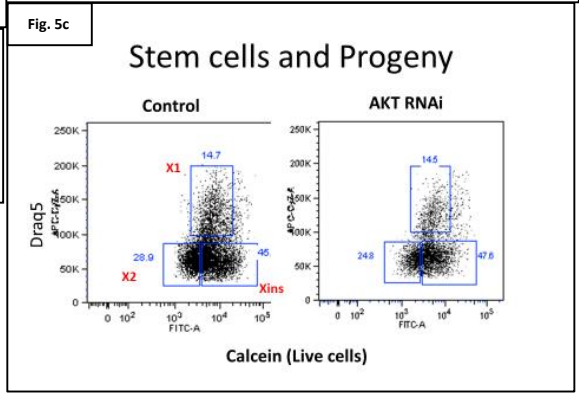
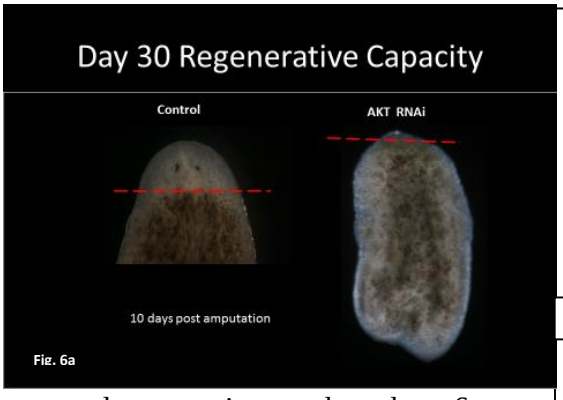


Figure 5: (a) TUNEL Assay performed at day 30. Quantification performed with cell count over area (mm^2). (b) Animal area taken over a 40 day time course. (c) Decrease in frequency of X1, X2, Xinc cells however overall balance of these cells in maintained

Control starved animals were approximately 2X the size of the AKT-RNAi animals after 40 days (**Fig. 5b**). FACS analysis confirmed the large decrease in overall cell count for each of the cell populations through Calcein and Draq5 labeling (**Fig. 5c**).

AKT-RNAi animals are unable to regenerate lost tissue

Proliferation at the site of injury within the planarian model has been widely studied and has shown to illicit a proliferative reaction post amputation or minor cuts. Aim #4 was to generate a proliferative response to amputation of the head (above the pharynx) and analyze the regenerative capabilities after loss of AKT. Our results indicate that without functional AKT, these animals are unable to form the typical blastema and regenerate missing tissue (**Fig. 6a**). After completing an H3P+ analysis of a regeneration time course, we identified that these animals were incapable of eliciting a proper mitotic response towards the site of the wound (**Fig. 6b**). Typically, control animals show a steady incline of mitotic cell count with two peaks at 30 hours and 7 days post amputation. The AKT-RNAi animals however,



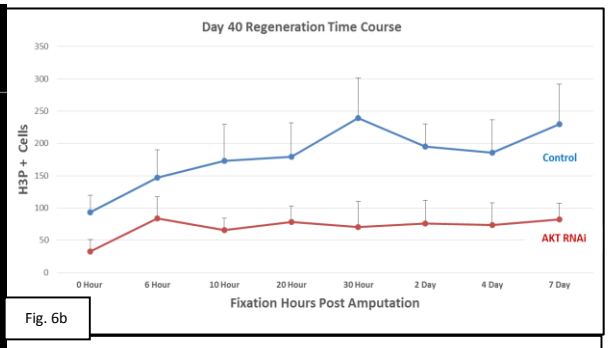
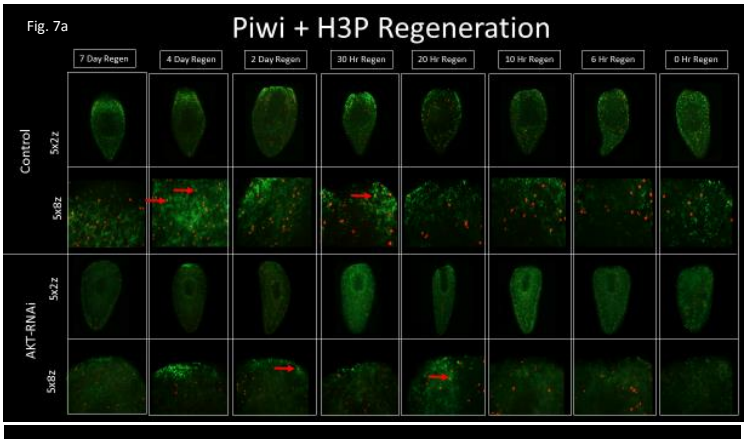


Figure 6: (a) Regeneration sites post head amputation. Red line indicates site of cut. (b) Mitotic (H3P) cell counts recorded through an amputation time course. Quantification performed with cell count over area (mm^2).

mounted a very minor peak at about 6 hours post amputation but never fully recovered when compared to the controls.

Remaining mitotic cells show similar overlay with stem cell fluorescent insitu markers as controls

Despite the major loss of overall cellular proliferation, AKT-RNAi animals still maintain a small amount of H3P+ cells after 40 days and illicit a minute mitotic response post amputation. Our final Aim #5 was to identify which of the cell populations these mitotic cells belong to. Are these mitotic cells still expressing neoblast markers (*Piwi*) or are these cells possibly



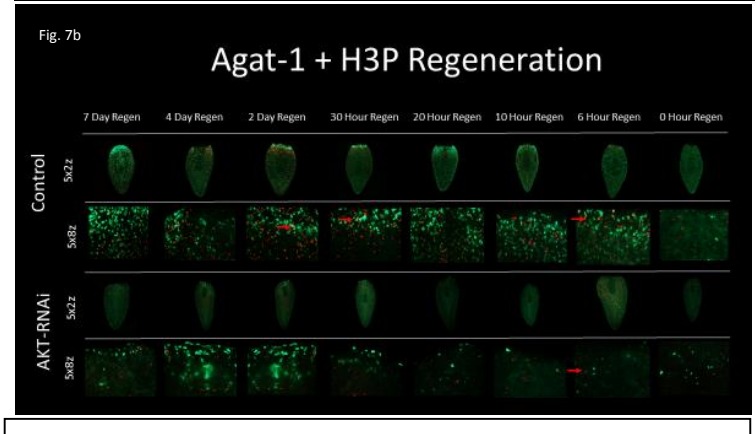


Figure 7. (a-b) Piwi FISH and Agat-1 FISH performed post amputation time course. H3P staining followed. Red arrows indicate overlay cells. All low magnification images taken at 5x2z and high magnification images at 5x8z. All overlays performed on Adobe Photoshop.

dedifferentiating late division progeny cell who have reverted back to neoblast characteristics in response to tissue insult? After completing amputations, fluorescent insitu hybridization (FISH) for the *Piwi* neoblast marker followed by the mitotic cell staining (H3P), the results indicate that the AKT-RNAi animals had very similar overlay patterning (**Fig. 7a**) and similar counts (data not shown) as the control animals. Consistently, overall cell number of AKT-RNAi animals was low in comparison to controls, however overlay between mitotic and neoblast marker cells showed no deviance. We repeated this technique with the FISH for *Agat-1* late division marker with similar patterning (**Fig. 7b**) and similar counts (data not shown).

DISCUSSION

The serine/threonine AKT kinase has been implicated in several cellular functions ranging from metabolism, cell-cycle regulation, and cell survival. These functions have shown to be conserved across several models and has now been shown to carry similar functions within the Planarian model. It is significant to continue study the AKT roles within adult models as pathways involving this master regulator have been highly characterized in aberrant cell signaling leading to degenerative diseases such as cancer. AKT is thus an attractive therapeutic target for cancer treatments, however fully understanding AKT function and loss within a model will provide insight onto what we may expect as side effects post downregulation.

Here, we have shown that loss of AKT from the system induces severe loss of adult stem cells and overall tissue throughout the body. The results also indicate the major increase in cellular death and overall loss of tissue over 40 days. Mitotic staining revealed that remaining neoblasts were unable to respond to amputation and differentiated tissue was incapable to sealing off the wound area through the formation of a proper blastema.

Low cell BrdU incorporation along with Cell-Cycle Analysis further supported the loss of cell-cycle progression post AKT-RNAi. Furthermore, characteristic cell populations (X_1, X_2, X_{ins}) post AKT-RNAi were highly downregulated with substantial loss of *Piwi, NB.21.11e,* and *Agat-1* cell markers indicated through *Insitu Hybridizations*. Additionally, remaining mitotic cells overlaid with *Piwi* expression or *Agat-1* expression further indicated the inability for proper cell-cycle progression, signaling and responses to amputation.

Conclusively, major defects in cell survival, cellular turnover and regenerative responses follow loss of AKT from the system. This data provides support for the well-established AKT functions within other models and may provide a better indication of what to expect with its loss during mutation or post therapeutic targeting during treatments of degenerative disease for humans.

ACKNOWLEDGEMENTS

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