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Intracellular Mechanisms of Adult Neural Progenitor Proliferation
and Self-Renewal

by

Joseph Charles Peltier

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemical Engineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor David V. Schaffer, Chair

Professor Douglas Clark

Professor John Ngai

Spring 2010

Abstract

Intracellular Mechanisms of Adult Neural Progenitor Proliferation and Self-Renewal

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Professor David V. Schaffer, Chair

Adult neurogenesis is the process by which the brain continuously generates and adds new neurons into specific regions, and the discovery and isolation of the progenitor cells responsible for this process has created exciting new possibilities for the treatment of multiple neurological diseases and injuries. Additionally, adult neurogenesis may play roles in learning, memory, stress, depression, and aging. Adult neural progenitor cells (NPCs) from the subgranular zone (SGZ) of the mammalian hippocampus have the ability to differentiate into the three major cell types of the brain: neurons, astrocytes, and oligodendrocytes. They also have a fourth option, which is to simply divide and remain as stem cells, a process known as self-renewal. It is this mechanism by which NPCs replenish themselves, and it appears to be a significant regulatory point of adult neurogenesis. Therefore, the goal of this work is to investigate the intracellular molecular regulatory mechanisms of NPC proliferation and self-renewal.

There are multiple signaling mechanisms/pathways that could potentially promote NPC proliferation and self-renewal. To determine which of these pathways may be most critical, we used a series of established pharmacological inhibitors. The inhibitors of the phosphoinositide 3-OH kinase (PI3K)/Akt pathway dramatically reduced NPC proliferation; and multiple NPC mitogens, including Sonic hedgehog (Shh) and basic fibroblast growth factor (FGF-2, the strongest NPC mitogen), activated Akt signaling. Additionally, retroviral vector-mediated overexpression of wild type Akt increased proliferation, while a dominant negative mutant decreased proliferation. Furthermore, wild type Akt over-expression reduced glial (GFAP) and neuronal (β -tubulin III) marker expression during differentiation, indicating that it inhibits cell differentiation.

Downstream of the Akt signal, we show that activation of the cAMP response element binding protein (CREB) occurs in cells stimulated by FGF-2, and it is limited when Akt signaling is inhibited, demonstrating a link between Akt and CREB. Overexpression of wild type CREB increases progenitor proliferation, whereas dominant negative CREB only slightly decreases proliferation. Most importantly, Akt overexpression promotes expression of the transcription factor SRY-related HMG-box 2 (Sox2), which is important for the self-renewal of multiple stem cell types, including NPCs. It is also widely used as a multipotent NPC marker. Akt drives Sox2 expression by increasing its mRNA concentration. Additionally, Akt inhibition

decreases Sox2 expression. Interestingly, however, Sox2 overexpression did not promote NPC proliferation. Taken together, these results indicate that Akt is a master promoter of proliferation and self-renewal, and that self-renewal is mediated by increased Sox2 expression.

Despite its demonstrated importance, the Akt signaling cascade is unlikely to be the only cascade responsible for NPC maintenance. Growth factor stimulation similar to that of FGF-2 is known to promote the activity of other key pathways, including Ras/mitogen activated protein kinase (MAPK) and calcium ion (Ca^{2+})-mediated pathways, in addition to Akt. There is also a great deal of cross-talk observed in multiple cell types between all three signaling modules. The precise importance of Akt signaling was investigated using tamoxifen-inducible, conditionally active Akt and PI3K mutants (Akt-ER and PI3K-ER). While these results show that Akt is sufficient for NPC proliferation, PI3K is not. Since Akt activation is mediated by two phosphorylation events, but only one of these events is driven by PI3K, these data indicate that Akt is acting as an AND gate and that the second phosphorylation of Akt is PI3K independent. Using pharmacological and genetic manipulation of Ras, calmodulin (CaM), CaM kinase II (CaMKII), CaM kinase kinase (CaMKK), CaM kinase IV (CaMKIV), and calcineurin, I investigated the importance of these pathways for NPC proliferation and self-renewal. The results of these studies provide no clear indication that any of these molecules are important for NPC proliferation and self-renewal. However, this is a critical step toward ruling out several major pathways.

Intracellular signaling and gene expression pathways can be very complex. In addition to the cross-talk mentioned above, they may involve intricate feed-forward loops, feedback loops, and parallel processing. Systems biology applies computational and experimental approaches to investigate the emergent behavior of collections of molecules and strives to explain how these numerous components interact to regulate molecular, cellular, and organismal behavior. Here, I also review systems biology, and in particular computational, efforts to understand the intracellular mechanisms of stem cell fate choice. These tools could potentially improve our understanding of NPC proliferation and self-renewal. I discuss deterministic and stochastic models that synthesize molecular knowledge into mathematical formalism, enable simulation of important system behaviors, and stimulate further experimentation. In addition, statistical analyses such as Bayesian networks and principal components analysis (PCA)/partial least squares (PLS) regression can distill large datasets into more readily-managed networks and principal components that provide insights into the critical aspects and components of regulatory networks. These computational tools, coupled with traditional experimentation may further our understanding of the critical networks governing NPC proliferation and self-renewal.

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ACKNOWLEDGMENTS

There are many people I'd like to thank for their contributions to this work. First, my advisor Dave Schaffer has been a great mentor and source of support throughout my dissertation research. He is always ready to help with advice and guidance, and without him this work would not be possible. I would also like to thank the other members of my dissertation committee, John Ngai and Doug Clark, for their helpful discussions.

For funding, I am thankful to the National Science Foundation, the National Institutes of Health, and the California Institute for Regenerative Medicine.

I owe many thanks to all members of the Schaffer lab, past and present. I am especially grateful to Ana O'Neill for the superb introduction to this project and Jolene Ignowski for taking time out of her busy sixth year to train a clueless first year. There are many others to thank for reagents, experimental advice, countless discussions, and occasional rounds of shots. The discussions may have been scientific, political, irritated, humorous, or just downright strange, but they were always interesting: Matt Robertson, Smita Agrawal, JT Koerber, John Burnett, Kris Saha, Phung Gip, Lauren Little, Priya Shah, Albert Keung, Ashley Fritz (aka Pooky), Stephanie Willerth, Dan Stone, Shawdee Eshghi, Randy Ashton, John Weinstein, Noem Ramey, Nicol Wilson, Josh Leonard, Julie Yu, Kwang-Il Lim, Jae Jang, Kathryn Miller-Jensen, Jonathan Foley, Jamie Bergen, and Paul Davis. Noem Ramey and Nicol Wilson did an amazing job of keeping the lab running smoothly and therefore keeping me sane, especially during my last two years. Lauren Little, Phung Gip, and Shawdee Eshghi have been great dinner company, providing good food and conversation. And Ashley Fritz was Pooky, who was always ready for an emergency trip to Arby's whenever necessary.

Outside of lab my friends and family have always been supportive. My parents Bill and Trudy are a constant source of encouragement in addition to my siblings: Chad, Adam, Dan, and Carrie. Alisyn Nedoma has been a great friend throughout my graduate career. She is always there with a sympathetic ear, a helping hand, a four course meal, a plate of cookies, or an ill-advised Halloween costume. Maria Goodrich, John Daniels, Megan Fox, Brian Mayer, Cari Dutcher, Dustin Nest, and Rebecca Dylla-Spears have been great friends, symphony dates, and/or prelim study buddies.

Lastly, I'd like to thank Nick Perry who I met while here at Berkeley. His love and support have enriched my life in many ways, and my time in graduate school wouldn't have been nearly as fulfilling without him.

CHAPTER 1

INTRODUCTION

Stem cells – first discovered in mouse bone marrow by Becker, Till, and colleagues [1, 2] – are defined by their two hallmark properties: (1) self-renewal, or extended maintenance and potentially proliferation in an undifferentiated state, and (2) differentiation into one or more specialized cell types. Pluripotent embryonic stem (ES) cells can give rise to any cell type in an adult organism, whereas multipotent adult stem cells are capable of generating a more limited set of cell types, typically ones in the tissue in which the stem cells reside. Their ability to self-renew or differentiate into multiple cell types makes stem cells promising therapeutic candidates for multiple injuries and diseases, including diabetes [3], spinal cord injury [4], and Alzheimer’s disease [5], among others. However, before we can harness stem cells’ therapeutic potential and guide their production of a desired cell type, we must first identify the factors and understand the mechanisms that govern their behavior and fate choices.

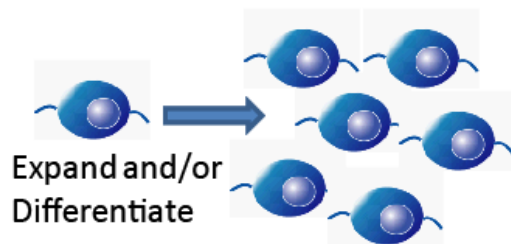
Potential Stem Cell Based Therapies

There are two potential therapeutic approaches utilizing stem/progenitor cells: *ex vivo* and *in vivo* (Figure 1). In *ex vivo* therapy, stem cells are isolated from a patient or donor and grown in culture outside the body. They are expanded and/or differentiated until the proper cell type and quantity is achieved for the desired therapy. These cells are then transplanted into a diseased or injured individual (as reviewed [6]). In some cases, these transplanted cells may replace the damaged tissue, for instance to replace β -cells for the treatment of diabetes [7], to replace dopaminergic neurons for the treatment of Parkinson’s disease [8, 9], or to remyelinate axons in a mouse model of multiple sclerosis [10]. In other instances the cells may influence the behavior of endogenous cells by secreting specific factors or otherwise directing their fate. Human ES cell transplantation has been used to treat radiation-induced cognitive impairment in rats [11]; and in a rat model of multiple sclerosis, transplanted neural progenitor cells are able to induce host oligodendrocyte progenitor cells to remyelinate damaged axons [12].

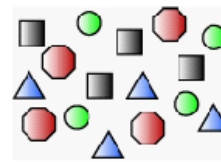
In *in vivo* therapy, small molecules, proteins, and/or gene therapy are used to modulate the behavior of endogenous adult stem/progenitor cells, eliminating the need to culture cells. By targeting endogenous stem cells, they can be directed to proliferate, differentiate, or express a specific factor that will promote the repair of diseased or damaged tissue (as reviewed [13]). For example, the mitogen Sonic hedgehog (Shh) is known to promote proliferation of adult neural stem/progenitor cells (NPCs) [14], and manipulation of Shh signaling with small molecule agonists promoted proliferation of telencephalic neural progenitors [15]. Additionally, adenovirus-mediated gene delivery of Shh promoted the proliferation of NPCs [14]. Direct protein infusion can also influence endogenous NPC behavior, as demonstrated by the chronic peripheral administration of insulin-like growth factor 1 (IGF-1), which enhanced the number of newborn neurons in the rat hippocampus [16].

Whether the therapies are *in vivo* or *ex vivo*, a deeper understanding of the intracellular molecular mechanisms that govern stem cell fate will yield insights into the normal roles stem cells play in adult tissues as well as enhance the development of regenerative medicine approaches that achieve desired therapeutic outcomes.

Ex Vivo Cell Replacement Therapy



In Vivo Therapy



Small Molecules,
Proteins, Genes

Implant

Targeted Delivery to
Endogenous Stem Cells



Figure 1: Potential stem-cell based therapies. *Ex vivo* therapy: cells are grown and/or differentiated in culture and then transplanted for treatment. *In vivo* therapy: small molecules, proteins, and/or gene therapy are targeted to endogenous stem cells to promote tissue repair.

Adult Neural Progenitor Cells

The discovery of the continuous generation of new neurons in several regions of the adult brain (adult neurogenesis) [17], and the isolation of the progenitor cells that generate these new neurons [18, 19], have created exciting new possibilities for the treatment of many neurological diseases and injuries. This excitement is further fueled by the fact that NPCs can generate nearly all major cell types within the mammalian brain, making them promising candidates for the treatment of conditions such as stroke, Alzheimer's, or Parkinson's disease. Additionally, neurogenesis may play roles in learning and memory [20-22], the effects of exercise on learning [22], stress and depression [23, 24], response to injury [25, 26], and aging [27]. However, before we can fully understand their natural roles in the brain, as well as pursue therapeutic applications, we must first understand the mechanisms governing NPC behavior.

There are two major neurogenic niches within the adult central nervous system: the sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) of the hippocampus, which is a brain region important for learning and memory. The biology of both cell types is comprehensively covered elsewhere [5, 28]. NPCs from the SGZ can differentiate into the three major cell types in the brain: neurons, astrocytes (support cells with multiple roles), and oligodendrocytes (support cells that improve neural transmission by providing electrical insulation) [29]. Recently, they have also been shown to generate endothelial cells [30]. They also have a fifth option, which is to simply divide and remain as stem cells, a process known as self-renewal. It is this mechanism by which NPCs replenish themselves, and it appears to be a significant regulatory point of adult neurogenesis [31, 32]. For this reason, the focus of this

work is to understand the intracellular mechanisms by which NPCs proliferate and self-renew rather than differentiate.

Cell Fate Determination: A Complex Intracellular Signal Processing System

A NPC constantly receives environmental cues in many forms: soluble cues from proteins such as mitogens and cytokines [14, 33], small molecules [34, 35], and nutrients [36, 37]; as well as “solid phase” cues such as cell-cell contacts and the biochemical and mechanical properties of the extracellular matrix, including signals immobilized to it [38-42]. These signals guide the cell towards specific behaviors, such as survival, apoptosis, self-renewal, or differentiation into one of multiple lineages (Figure 2). An instructive view of fate choice states that environmental cues initiate the intracellular signals that direct the cells to their fate, while a selective mechanism indicates that environmental factors merely support the survival of certain fates. It appears likely that both of these modes operate in different tissues and circumstances [43].

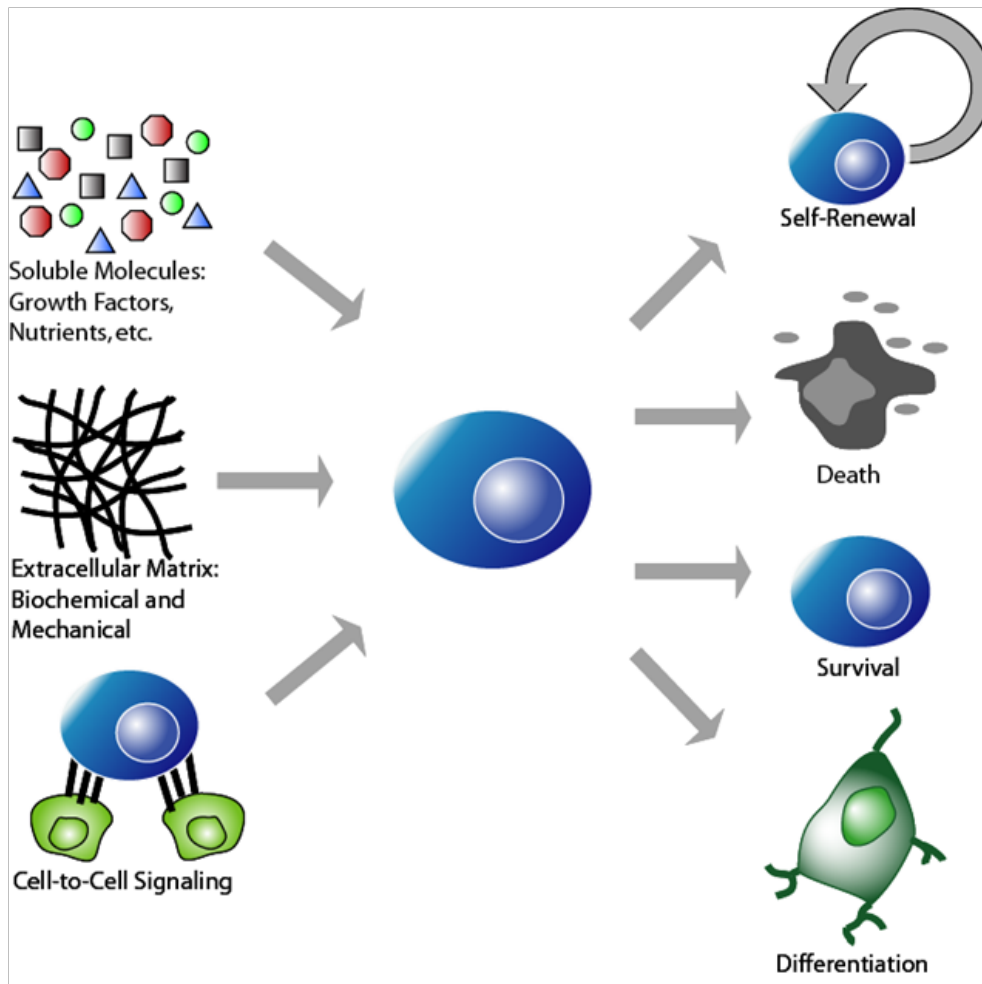


Figure 2: The NPC as a signal processor. NPCs receive multiple signals from their environment: soluble factors such as mitogens, cytokines, and nutrients; the biochemical and mechanical properties of the extracellular matrix; and contacts from neighboring cells. The cell processes these signals to determine its fate: self-renewal, survival, apoptosis, or differentiation. © Institution of Engineering and Technology. Reproduced with permission from [44].

Regardless of which mechanism is operating, however, NPC behavior is guided by molecular interactions and reactions involving receptors, signaling networks, and transcription factors. The signals for proliferation and self-renewal begin at the cell surface with the binding of mitogens critical for NPC maintenance. These growth factors include basic fibroblast growth factor (FGF-2) [33, 45], Shh [14], IGF-1 [46], vascular endothelial growth factor (VEGF) [47], and epidermal growth factor (EGF) [48]. The mitogenic (input) signal “enters” the cell when these molecules bind a receptor on the cell’s surface, inducing a conformational change and receptor activation. The signal is then transmitted to the cell interior and processed through a complex, non-linear network of chemical and physical interactions until it arrives at the cell nucleus and induces expression of genes that promote self-renewal and/or proliferation (outputs). Signal processing is mediated by two major types of molecular mechanisms: the covalent modification of proteins and the generation of small molecules known as second messengers. Second messengers can act by binding to and activating/inactivating multiple downstream effectors, or they can recruit proteins to a particular sub-cellular compartment where they are most/least bioactive. Covalent protein modification, however, is typically achieved through phosphorylation and de-phosphorylation events, which then modulate protein activity. Generally, a signal is transduced by a cascade of second messengers and/or phosphorylation events where each phosphorylation event activates/inactivates another protein. Information processing then continues within the nucleus where transcription factor networks control the expression of target genes required for execution of the self-renewal/proliferation program, which include enzymes, structural proteins, and other transcription factors.

The purpose of this work is to determine the important intracellular molecular mediators of NPC proliferation and self-renewal. The phosphoinositide 3-OH kinase (PI3K)/Akt pathway has emerged as a critical promoter of this process. Subsequent chapters demonstrate the importance of Akt signaling for NPC proliferation and differentiation (Chapter 2, [49]) and self-renewal (Chapter 3). One of the key ways Akt promotes self-renewal is by increasing expression of the transcription factor Sox2 (Chapter 3), which is important for the self-renewal of embryonic stem (ES) cells [50] and NPCs [51]. I also investigated the potential role of other signaling pathways, including the Ras/mitogen activated protein kinase (MAPK) cascade and calcium ion (Ca^{2+}) signaling (Chapter 4).

As noted above, signal processing networks can be very complex, including feedback loops, feed-forward loops, signal amplification, and crosstalk between multiple signaling cascades [52]. This can result in a number of rich behaviors, including switches and oscillations [53-55]. These behaviors are critical regulators of stem cell self-renewal and differentiation, and in many ways they are difficult to investigate and interpret intuitively without the aid of systems-level analysis and the accompanying mathematical tools. Therefore, the final chapter of my dissertation (Chapter 5) reviews the systems biology tools and techniques used to study the intracellular mechanisms of stem cell fate choice, highlighting how they may be helpful for future studies.

PI3K/Akt Signaling

The PI3K/Akt pathway (summarized in Figure 3) is known to play important roles in cellular proliferation, growth, survival, metabolism, and migration [56]. Receptor activation on the cell surface recruits PI3K to the cell membrane, where it catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits Akt and phosphoinositide-

dependent kinase 1 (PDK1) to the membrane. PDK1 phosphorylates Akt at the activation segment (T308), and a second phosphorylation event on the hydrophobic motif (S473) by PDK2 leads to complete activation of the molecule [57]. PDK2 is widely believed to be the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) [58]; however, other molecules have been implicated, including PDK1 [59], integrin-linked kinase (ILK) [60], Akt itself [61], and DNA-PK [62, 63].

The importance of Akt has been demonstrated in many stem cell types, including mouse ES cells [64, 65], primate ES cells [66], rabbit ES cells [67], mesenchymal stem cells [68], and hematopoietic stem cells [69, 70]. Here, I show the importance of Akt for NPC proliferation and inhibition of differentiation (Chapter 2, [49]) and self-renewal (Chapter 3). Others have shown the importance of signaling events downstream of Akt to NPC maintenance, including mTOR [71] FoxO [72] and GSK-3 [73]. However, very little work has investigated how and whether this signaling pathway interacts with the transcriptional machinery responsible for maintaining the multipotency of these cells. One result of my work is the demonstration that Akt activity drives the expression of the transcription factor Sox2, an important nuclear regulator of NPC self-renewal (Chapter 3).

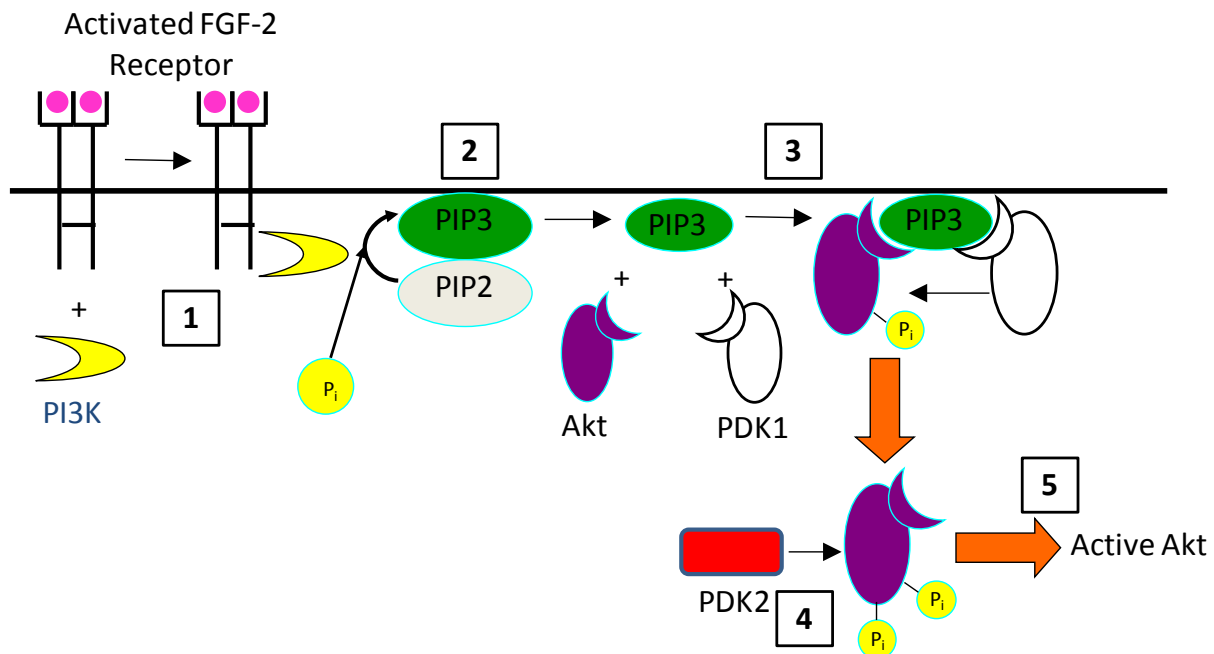


Figure 3: PI3K/Akt activation. 1) FGF-2 binds to and activates its receptor. The activated FGF-2 receptor recruits PI3K to the cell membrane where it is activated. 2) PI3K catalyzes the addition of a phosphate group to PIP2, generating PIP3. 3) PIP3 recruits Akt and PDK1 to the cell membrane where it phosphorylates Akt at T308. 4) PDK2 (widely believed to be mTORC2) also phosphorylates Akt at S473. 5) Fully activated Akt diffuses away from the cell membrane where it can catalyze downstream phosphorylation events.

Transcription Factors Important for NPC Self-Renewal

Within the nucleus, there are several transcription factors that are important for NPC self-renewal. The FoxO family of transcription factors, widely known to operate downstream of PI3K/Akt, controls adult neural stem cell homeostasis by promoting quiescence within the stem

cell pool [72, 74], and the proto-oncoprotein Myc is a transcription factor that promotes both proliferation and self-renewal of NPCs [75, 76]. Another modulator of NPC self-renewal is the Id family of transcription factors, which promote self-renewal in SVZ-derived adult neural stem cells [77, 78].

The two most widely studied transcription factors important for hippocampal NPC maintenance are TLX and Sox2. NPCs from TLX-null mice fail to proliferate or self-renew [79], and the mice have decreased performance in spatial learning tasks [80]. TLX is an orphan nuclear receptor, and the mechanisms controlling its expression are poorly understood; however, recent studies have revealed that microRNAs strongly regulate TLX [81, 82].

Another critical regulator of NPC maintenance is Sox2 [51], which is also generally used as an NPC marker [83]. In addition to NPCs, it is also important for the self-renewal of ES cells [50] and is one of the factors necessary for reprogramming and generation of induced pluripotent stem (iPS) cells [84, 85]. Lineage tracing studies have shown that Sox2-positive cells in the hippocampus can self-renew and generate differentiated progeny [86]. In chick embryos, Sox2 overexpression prevents differentiation [87]; however, hypomorphic murine Sox2 mutant cells grown in culture have impaired neuronal generation, indicating that Sox2 may also be important to prime cells for neuronal differentiation [88]. A nervous-system specific Sox2 knockout mouse revealed that one way Sox2 maintains the adult NPC population is through the upregulation of Shh [89], a morphogen known to control NPC maintenance [14]. Sox2 is also known to repress transcription of glial fibrillary acidic protein (GFAP), an important astrocytic marker [88].

Control of Sox2 activity and its expression in NPCs is poorly understood. Most Sox2-control mechanisms have been studied in other cell types. Post-translational modification of Sox2 by sumoylation negatively regulates its transcriptional activity by inhibiting DNA binding in mouse ES cells [90], and in human ES cells this sumoylation event is regulated by phosphorylation [91]. Additionally, poly(ADP-ribosylation) by PARP1 has been shown to regulate FGF-4 expression in human ES cells [92]. Transcriptional control of Sox2 is promoted by the Sox2 regulatory region 2 (SRR2), a Sox2 enhancer known to regulate its expression in the telencephalon [93]. Forming a positive feedback loop in ES cells, Sox2 forms a heterodimer with Oct4 that positively regulates Sox2 expression [94]. Despite these advances in our understanding of Sox2 regulation, little is known about the primary signaling pathways that connect signals from the cell surface to the nucleus and therefore drive Sox2 expression, particularly in NPCs. In this work (Chapter 3), I demonstrate that the Akt pathway is one of these critical regulators.

Future Directions: Computational Modeling

In addition to traditional experimental approaches, intracellular signaling pathways are also investigated with systems biology. Systems biology is a field that studies the collective behavior of multiple complex biological components as one whole system. This offers multiple advantages that complement and enhance traditional reductionist experimental techniques that focus on specific components more so than the whole system. Because biological systems such as cells involve many components that interact with one another in complex and often non-linear ways, systems biology often relies on computational models. Such models serve many uses. 1) They summarize our knowledge and assumptions about a system into a formal, mathematical statement. 2) They highlight gaps in our knowledge of a system. 3) They generate hypotheses about the behavior of the system that motivate experimentation and further modeling. 4) They

aid in the analysis of large datasets - such as those generated by genomic, transcriptomic, proteomic, and kinomic experimentation - and thus summarize the data and highlight important, potentially unintuitive, genes and/or signaling molecules for future experimentation. 5) And finally, they highlight critical loci within a system that can be manipulated to generate a desired outcome. For instance, a model can be used to pinpoint a druggable therapeutic target that directs endogenous stem cell pools to a desired fate; alternatively, a model could be used to optimize *ex vivo* expansion for cellular therapy [95].

In Chapter 5 [44], I discuss systems biology modeling approaches and techniques that are increasingly utilized to understand the intracellular mechanisms of stem cell fate choice in order to highlight techniques that could potentially be used to further study the NPC intracellular signaling networks responsible for proliferation and self-renewal. Deterministic and stochastic computational models have formally synthesized our molecular knowledge into mathematical statements, furthered our understanding of important network behaviors, and motivated future experimentation [53, 96, 97]. Statistical analyses such as Bayesian networks and principal components analysis have distilled large datasets into tractable networks and principal components that are then used to derive insight into the critical pieces of the fate choice network [98, 99]. Collectively, these efforts have furthered the stem cell field and brought us closer to the eventual goal of harnessing the therapeutic promise of stem cells [100]. Similarly, these techniques can also prove useful for understanding NPC fate choice and developing novel therapies derived from them.

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CHAPTER 2

PI3K/AKT AND CREB REGULATE ADULT NEURAL HIPPOCAMPAL PROGENITOR PROLIFERATION AND DIFFERENTIATION

This chapter is a postprint of a paper submitted to and accepted for publication as

Peltier J, O'Neill A, Schaffer DV. (2007). PI3K/Akt and CREB Regulate Adult Neural Hippocampal Progenitor Proliferation and Differentiation. *Dev Neurobiol* 67:1348-1361.

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The copy of record is available at

<http://www3.interscience.wiley.com/journal/117929398/grouphome/home.html>.

Abstract

The phosphoinositide 3-OH kinase (PI3K)/Akt pathway has been implicated in regulating several important cellular processes, including apoptosis, survival, proliferation, and metabolism. Using both pharmacological and genetic means, we demonstrate here that PI3K/Akt plays a crucial role in the proliferation of adult hippocampal neural progenitor cells. PI3K/Akt transduces intracellular signals from multiple mitogens, including FGF-2, Shh, and IGF-1. In addition, retroviral vector-mediated overexpression of wild type Akt increased cell proliferation, while a dominant negative Akt inhibited proliferation. Furthermore, wild type Akt overexpression reduced glial (GFAP) and neuronal (β -tubulin III) marker expression during differentiation, indicating that it inhibits cell differentiation. We also show that activation of the cAMP response element binding protein (CREB), which occurs in cells stimulated by FGF-2, is limited when Akt signaling is inhibited, demonstrating a link between Akt and CREB. Overexpression of wild type CREB increases progenitor proliferation, whereas dominant negative CREB only slightly decreases proliferation. These results indicate that PI3K/Akt signaling integrates extracellular signaling information to promote cellular proliferation and inhibit differentiation in adult neural progenitors.

Introduction

Since the first evidence for adult mammalian neurogenesis [1, 2] and the initial isolation of adult hippocampal neural progenitor cells (NPCs) from the dentate gyrus of the hippocampal formation [3, 4], considerable work has advanced our understanding of their function from a systems to a molecular level. Neurogenesis may play roles in learning and memory [5-7], the effects of exercise on learning [5], stress and depression [8, 9], response to injury [10, 11], and aging [12]. At a cellular level, neurons generated from NPCs differentiate to form functional connections with existing neurons in the adult brain [3], and the resulting mature neurons can persist for years after differentiation [13]. Furthermore, a number of extracellular molecules that regulate several stages in the process of adult neurogenesis have been identified, and the

proliferation or amplification of progenitors prior to differentiation appears to be a significant regulatory point [10, 14, 15].

In particular, basic fibroblast growth factor (FGF-2) [16, 17], vascular endothelial growth factor (VEGF) [18], insulin-like growth factor 1 (IGF-1) [19], epidermal growth factor (EGF) [20], Sonic hedgehog (Shh) [21], and glucose-dependent insulinotropic polypeptide (GIP) [22] have been discovered to regulate NPC proliferation. In contrast to this increasing knowledge of extracellular mitogens, however, comparatively little is known about the intracellular signaling cascades that transduce these signals to modulate cell proliferation. Nakagawa et al. demonstrated that cAMP plays a role in NPC proliferation *in vivo* [23], and Mantamadiotis et al. found that conditional knock-out of the cAMP response element binding protein (CREB) impairs *in vivo* proliferation [24]. It has also been demonstrated that opioids and IGF-1 potentially act through activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-OH kinase (PI3K) pathways [19, 25]. In addition, NPCs in a *Sox2(-/-)* mutant mouse exhibited reduced proliferative capability [26]. However, it is unclear whether numerous intracellular signaling pathways transduce and process the known extracellular mitogenic signals in parallel, or whether a common pathway integrates numerous upstream mitogenic signals. We have therefore investigated the intracellular pathways that mediate FGF-2 and Shh signals *in vitro*.

The FGF-2 signaling cascade begins when FGF-2 binds to the cell surface receptor FGFR-1 [27], a member of the receptor tyrosine kinase superfamily specific for FGF-2. Upon ligation, FGFR-1 is capable of initiating intracellular signal cascades through several downstream mechanisms, including the Ras/MAPK, p38 MAP, protein kinase C (PKC), phospholipase C (PLC), and PI3K/Akt pathways (as reviewed [28]). These cascades can be simultaneously stimulated, and crosstalk may occur between them, such as Akt mediated phosphorylation of Raf [29] and MAPK-activated protein kinase-2 (MAPKAPK2) mediated phosphorylation of Akt [30]. By contrast, the mitogen Shh, binds to the receptor Patched (Ptch), which relieves its inhibition of the membrane protein Smoothed (Smo). Smo then activates the Gli family of transcription factors. Recent work has shown that Shh is also capable of activating PI3K/Akt in endothelial cells, and that PI3K/Akt act synergistically with Shh to activate Gli [31].

Here, we investigate this complex signaling landscape using pharmacological and genetic techniques and demonstrate that the PI3K/Akt pathway, often associated with anti-apoptotic and survival functions [32], is a key transducer of mitogenic signals that drive proliferation and inhibit differentiation of adult hippocampal neural progenitors. Additionally, we show that Shh stimulation activates Akt but in a novel, translationally mediated manner.

Upon activation of PI3K by a given cell surface receptor, the kinase increases the membrane concentration of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits Akt, as well as kinases that act on Akt, to the cell membrane [33]. Once activated via phosphorylation, the three Akt isoforms (Akt1, Akt2, Akt3) are collectively capable of phosphorylating numerous downstream signaling proteins, including members of the Bad apoptosis machinery [34], the forkhead/FOXO [35], NF κ B [36, 37], p53 [38], and glycogen synthase kinase 3 β (GSK3 β) [39] pathways, as well as the mammalian target of rapamycin (mTOR), which modulates protein translation machinery [40, 41]. In addition, *in vitro* kinase assays have demonstrated that Akt is capable of phosphorylating and activating CREB [42], though no direct evidence of a direct intracellular link has yet been reported.

PI3K activity has been implicated in the function of stem and neural progenitor cells during development. The phosphatase and tensin homologue deleted on chromosome 10

(PTEN), a phosphatase that antagonizes PI3K's activity, has been shown to limit the proliferative capabilities of embryonic neural stem cells [43]. Akt has also been shown to play a role in IGF-1 signaling in fetal cerebellar precursors [44], in the self-renewal of embryonic stem cells [45, 46] and fetal neural progenitors [47], and in the survival of terminally differentiated neurons [48]. Also, *Akt3*(-/-) deficient mice have reduced brain size and weight, further indicating its crucial role in the development of the central nervous system [49]. However, there is limited evidence for an Akt role in neural stem or progenitor cells of adult organisms.

CREB is a transcription factor activated in part by the phosphorylation of serine 133, after which it is capable of inducing the transcription of many classes of genes under the control of the cAMP response element (CRE). Several kinases have been shown to phosphorylate CREB at this site, including protein kinase A (PKA) [50], PKC [51], MAPKAPK [52], potentially Akt [42], and other kinases under various conditions [53, 54].

Here, we demonstrate that Akt is activated in adult hippocampal neural progenitor cells downstream of FGF-2 and Shh and is involved in transducing the mitogenic activity of these factors, adding evidence to a developing hypothesis that Akt may play a general role in stem cell and progenitor proliferation or self-renewal [45-47]. Additionally, Akt overexpression inhibits progenitor differentiation into glial and neuronal lineages. Furthermore, Akt signaling is mediated in part through CREB. These results demonstrate an important mechanistic link between mitogens, PI3K/Akt, CREB, and ultimately, adult neural progenitor proliferation and differentiation.

Materials and Methods

Cell Culture

Adult neural progenitor cells were isolated from the hippocampi of 6-week-old female Fischer 344 rats as described [16]. Cells were cultured on tissue culture polystyrene coated with poly-ornithine and 5 µg/ml of laminin (Invitrogen) and grown in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) high-glucose medium (Invitrogen) containing N-2 supplement (Invitrogen) and 20 ng/ml recombinant human FGF-2 (Peprotech).

For inhibitor studies, adult neural progenitor cells were plated at low density (1000 cells/well) on 96-well poly-ornithine/laminin-coated tissue culture plates with or without 20 ng/ml FGF-2, 100 nM recombinant rat Shh produced as described [21], or 1 ng/ml FGF-2 plus 100 ng/ml IGF-1 (Sigma). In some cases, the following compounds were included (all from Calbiochem, unless otherwise noted): LY-294002 (10 µM), PD-98059 (10 µM), rapamycin (0.5 µM), SB-203580 (10 µM), API-2 (1 µM), 2-naphthol-AS-E-phosphate (25 µM, Fluka), GSK3β Inhibitor II (5 µM), z-VAD-FMK Caspase Inhibitor V (20 µM), and cycloheximide (0.1 mg/ml, Sigma). Fifty percent media changes were conducted daily. After 5 days in culture, cell number was quantified using the WST-1 assay following the manufacturer's instructions (Roche) and utilizing a standard curve generated with known cell numbers.

Akt and CREB Cell Lines

Progenitor cells constitutively expressing mutant and wild type proteins of Akt and CREB were generated by retroviral infection. cDNA encoding the following proteins were kind gifts: wild type murine CREB (K. Saeki, International Medical Center of Japan [55]), dominant negative murine CREB_{S133A} (M. Montminy, Salk Institute [56]), wild type murine Akt1 (S.

Ferguson, Roberts Research Institute, London, Ontario Canada), and dominant negative (Akt-AAA) bovine Akt1 (from J. Woodgett, Ontario Cancer Institute [57]). PCR products were subcloned into the MMLV retroviral vector CLPIT [58] containing the tetracycline-repressor element [59] and a puromycin selection gene. Correct products were confirmed by sequence analysis. Retroviral vectors were packaged using CMV gag-pol and CMV VSV-G envelope helper plasmids by calcium phosphate transfection as described [58]. Vectors were harvested, concentrated by ultracentrifugation, and titered on HEK 293Ts. Progenitor cells were infected at a multiplicity of infection of 1 IU/cell and were selected with 1 μ g/ml puromycin (Sigma). To assay proliferation, cells were grown in 0 or 1 ng/ml FGF in DMEM/F-12 + N-2 medium, in doses of tetracycline (tet) of 0, or 0.1 μ g/ml. Fifty percent media changes were conducted daily, and proliferation was quantified using the WST-1 assay after 5 days in culture.

Immunoblotting

After 24 hours of FGF-2 starvation, 20 ng/ml FGF-2 was added to progenitor cells, which were then lysed at various time intervals (0 to 24 hours) after addition. In some cases, samples were pre-incubated with small molecule inhibitors (listed in text and described above) for 2 hours prior to FGF-2 addition. Lysis solution contained IGEPAL (1%, Sigma), sodium dodecyl sulfate (SDS, 0.1%), phenylmethanesulfonylfluoride (PMSF, 0.1 mg/ml, Sigma), aprotinin (0.03 mg/ml, Sigma), and sodium orthovanadate (1 mM, Sigma) in PBS. Lysate protein concentrations were quantified by BCA Protein Assay Kit (Pierce). Fifteen μ g of protein from each lysate were electrophoretically separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). In cases where blots were stripped and re-probed, phosphorylated epitopes were probed first, as previously described [60]. Primary antibodies included: rabbit anti-CREB (1:1000), mouse anti-phospho-CREB (Ser133) (1:2000), rabbit anti-Akt (1:1000), and rabbit anti-phospho-Akt (Ser473) (1:500), all from Cell Signaling Technology. Secondary antibodies included donkey anti-rabbit IgG-HRP (1:5000) and goat anti-mouse IgG-HRP (1:5000) from Santa Cruz Biotechnology, Inc.

Akt Immunostaining

NPCs were seeded at 10,000 cells/well in Falcon 8-well chamber slides and starved of FGF-2 for 24 hours. Medium containing 20 ng/ml FGF-2 was added to each well, and cells were fixed in 4% paraformaldehyde. Samples were blocked as previously described [21] and incubated overnight with rabbit anti-phospho-Akt antibody (Cell Signaling, 1:100). Slides were then washed and incubated with Alexa 488 donkey anti-rabbit antibody and TO-PRO nuclear stain (Molecular Probes) for 2 hours at room temperature. Slides were mounted with Pro-long Anti-Fade reagent and imaged on a Leica TCS confocal microscope.

Quantitative RT-PCR

NPC's were seeded at 500,000 cells per plate on 6 cm culture dishes (Falcon) in DMEM/F-12 + N-2 medium containing either 20 ng/ml FGF-2 or 1% fetal bovine serum (Invitrogen) plus 1 μ M retinoic acid (Biomol) to induce differentiation. Medium was replenished on day 2, and on day 4 RNA was isolated by TRIzol (Invitrogen) according to manufacturer's instructions. cDNA's were then generated using Invitrogen's ThermoScript RT-PCR kit according to manufacturer's instructions. Using a BioRad iCycler, Taqman probe QPCR was performed for the astrocytic marker GFAP and the neuronal marker β -tubulin III with the 18S ribosomal subunit as an internal control. GFAP and β -tubulin III probes from

Biosearch Technologies contained FAM490 fluorophore with Black Hole Quencher (BHQ), while the 18S rRNA probe contained CAL610 fluorophore with BHQ. Primer and probe sequences were as follows: GFAP: 5'-GACCTGCGACCTTGAGTCCT-3', 5'-TCTCCTCCTTGAGGCTTTGG-3', probe 5'-FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ-3'; β -tubulin III: 5'-GCATGGATGAGATGGAGTTCACC-3', 5'-CGACTCCTCGTCGTCATCTTCATAC-3', probe 5'-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3'; 18S rRNA: 5'-GTAACCCGTTG AACCCCATTC-3', 5'-CCATCCAATCGGTAGTAGCGA-3', probe 5'-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3'.

Results

PI3K/Akt Mediate the Proliferative Signal from Multiple Mitogens

FGF-2 [16], Shh [21], and IGF-1 [19] are adult hippocampal neural progenitor mitogens. To determine which of the many possible downstream effectors of these factors are important for NPC proliferation, we utilized a series of established small molecule inhibitors. Cells were grown with 20 ng/ml FGF-2 or 100 nM Shh and inhibitors of PI3K (LY-294002), Akt (API-2), mTOR (rapamycin), MEK (PD-98059), p38 MAP (SB-203580), and GSK3 β (GSK3 β Inhibitor II). Results are shown in Figure 1A (FGF-2) and Figure 1B (Shh), normalized to the vehicle control.

Although the inhibitors of MEK, p38 MAP, and GSK3 β did significantly alter proliferation compared to the DMSO + FGF-2 control (Figure 1A), the inhibitors of known transducers in the PI3K pathway (LY-294002, API-2, and rapamycin) dramatically reduced cell proliferation (Figure 1A). In fact, addition of these drugs to cultures containing 20 ng/ml FGF-2 reduced proliferation levels to those seen in carrier control cultures without FGF-2.

As PI3K and Akt are known to function as anti-apoptotic signals [32, 33], the decreased cell numbers observed in the presence of PI3K pathway inhibitors could be due to either decreased cell proliferation or increased cell death. FGF-2 proliferation experiments were therefore also conducted in the presence of the caspase inhibitor z-VAD-FMK. The caspase inhibitor did not result in increased cell numbers, indicating that apoptosis did not play a role in the apparent anti-proliferative effects of these inhibitors (Figure 1A). Similarly, it should be noted that for all the PI3K pathway inhibitors with FGF-2, more cells were present after 5 days than were initially plated. Collectively, these data indicate that PI3K/Akt activity is important for robust cell proliferation in response to FGF-2. In addition, these results motivated a thorough analysis of the role of the PI3K/Akt pathway in NPC proliferation and FGF-2 signal transduction.

Accordingly, we investigated whether PI3K/Akt were equally important for other known NPC mitogens. Inhibitors of the PI3K/Akt pathway also caused a reduction in proliferation in Shh-stimulated cells (Figure 1B), indicating that this pathway may transduce proliferative signals from mitogens not normally thought to act through PI3K/Akt. Interestingly, in contrast to FGF-2 result, the MEK inhibitor PD-98059 inhibited Shh-stimulated NPC proliferation to a similar extent as the PI3K/Akt inhibitors, representing a possible novel mechanism of Shh action.

To further confirm the importance of PI3K/Akt in NPC proliferation, we tested its role in the transduction of other mitogenic signals. Cells were grown in 1 ng/ml FGF-2 plus 100 ng/ml IGF-1, or 1 ng/ml FGF-2 plus 100 ng/ml VEGF. IGF-1 induced a significant increase in NPC

proliferation over 1 ng/ml FGF-2 alone (Figure 1C), whereas IGF-1 alone was unable to appreciably enhance cell proliferation (data not shown). Additionally, in this assay VEGF did not induce a significant increase in proliferation (data not shown). Importantly, proliferation in FGF-2/IGF-1 was significantly attenuated by the presence of LY-294002 (Figure 1C). However, PI3K inhibition also attenuated cell proliferation in the 1 ng/ml FGF-2 control, such that the 1 ng/ml FGF-2-only and 1 ng/ml FGF-2 plus 100 ng/ml IGF-1 samples were statistically similar when grown in the presence of inhibitor. This result therefore demonstrates a PI3K pathway dependence for the proliferative effect of IGF-1 over FGF-2 alone.

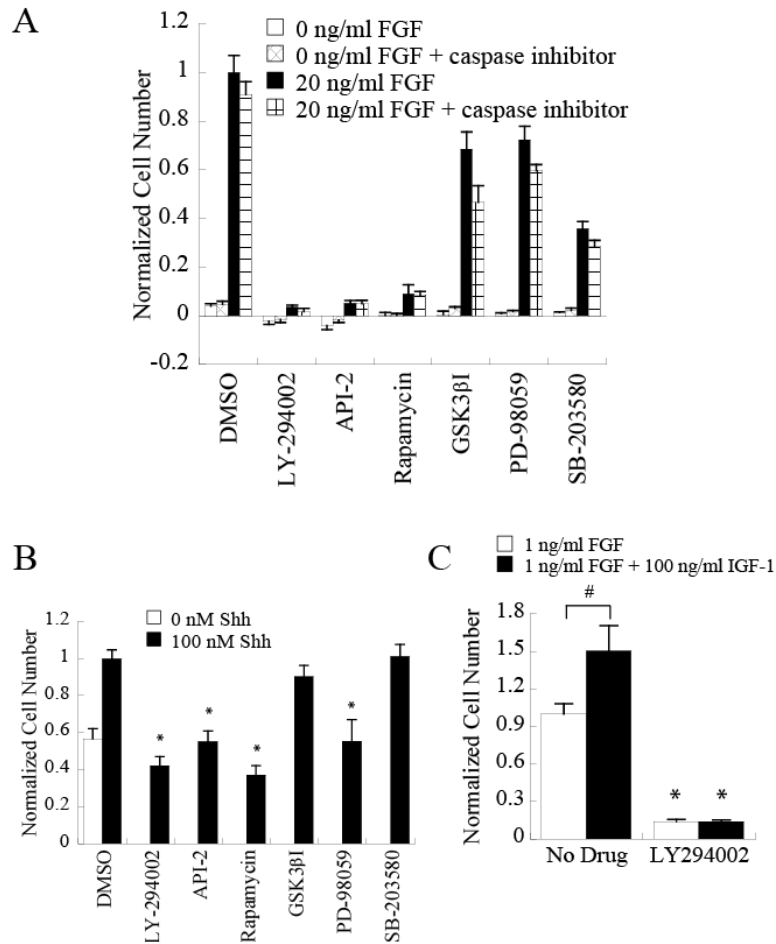


Figure 1: PI3K/Akt mediate the proliferative signal from multiple mitogens. (A) Cells were seeded in quintuplicate in 96-well laminin-coated plates at a density of 1000 cells/well in indicated media. Cells were counted by WST-1 assay after 5 days in culture. Drugs added include: LY-294002 (PI3K antagonist, 10 μ M), API-2 (Akt antagonist, 1 μ M), rapamycin (mTOR antagonist, 500 nM), GSK3 β Inhibitor II (GSK3 β I, 5 μ M), PD-98059 (MEK inhibitor, 10 μ M), SB-203580 (p38 MAP inhibitor, 10 μ M), and z-VAD-FMK (caspase inhibitor, 20 μ M), all dissolved in DMSO. Data are represented as cell number normalized to a DMSO carrier control sample with FGF-2 (FGF). Media conditions include: no mitogen, 20 ng/ml FGF-2, and 20 ng/ml FGF-2 with caspase inhibitor. (B) Cells were cultured as described in (A); however, media conditions instead include: no mitogen and 100 nM Shh. Data are represented as normalized to a DMSO carrier control with 100 nM Shh. (C) Cells were cultured as in (A) and (B) in medium containing 1 ng/ml FGF-2 or 1 ng/ml FGF-2 plus 100 ng/ml IGF-1. Data are represented as normalized to the 1 ng/ml FGF-2 only sample. For all panels, error is the 95% confidence interval, and asterisks indicate a statistically significant difference from appropriate control ($p < 0.05$), and (#) denotes a statistically significant difference ($p < 0.05$) between the indicated conditions.

Akt is Activated upon NPC Stimulation by FGF-2 and Shh

In the initial pharmacological screen, PI3K/Akt pathway inhibition reduced progenitor proliferation in response to several mitogens. Based on these results, we sought to confirm the importance of Akt in FGF-2 signaling and NPC proliferation. Immunoblotting was performed to determine Akt activation. Cells were deprived of FGF-2 for 24 hours, and at various time points after 20 ng/ml FGF-2 addition, cell lysate was collected and analyzed with antibodies against the phosphorylated, active form of Akt and total Akt (Figure 2A). Akt activation above baseline levels is observed within 10-15 minutes after FGF-2 addition, consistent with prior observations in murine fibroblasts [61]. This activation increased over the first hour of FGF-2 administration and peaked at 1-2 hours. Subsequently, at 24 hours post-stimulation the phospho-Akt signal decreased to a level greater than the baseline activation in the absence of FGF-2 (data not shown). Cells were also pre-incubated with LY-294002 and rapamycin prior to FGF-2 addition. As anticipated, PI3K inhibition decreased Akt activation at all time points relative to the corresponding inhibitor-free condition (Figure 2A). Conversely, Akt signaling was unaffected by the presence of rapamycin (data not shown) [41].

Interestingly, we also found that Akt is activated in response to Shh (Figure 2B). However, the time course of activation is delayed compared to FGF-2. As the slower response suggested that protein synthesis may be involved in this Akt activation, the experiment was also performed in the presence of cycloheximide. The protein synthesis inhibitor intriguingly substantially reduced Shh-induced activation of Akt (Figure 2B).

To confirm immunoblotting results, we next immunostained for phospho-Akt in culture. Cells were again deprived of FGF-2 for 24 hours. Twenty ng/ml of the growth factor was then added, and the cells were stained for phospho-Akt. Representative cells are shown in Figure 2C without FGF-2 and after 10 minutes of FGF-2 exposure, where the increased fluorescence and characteristic membrane localization of phosphorylated Akt are clearly visible at the later time point.

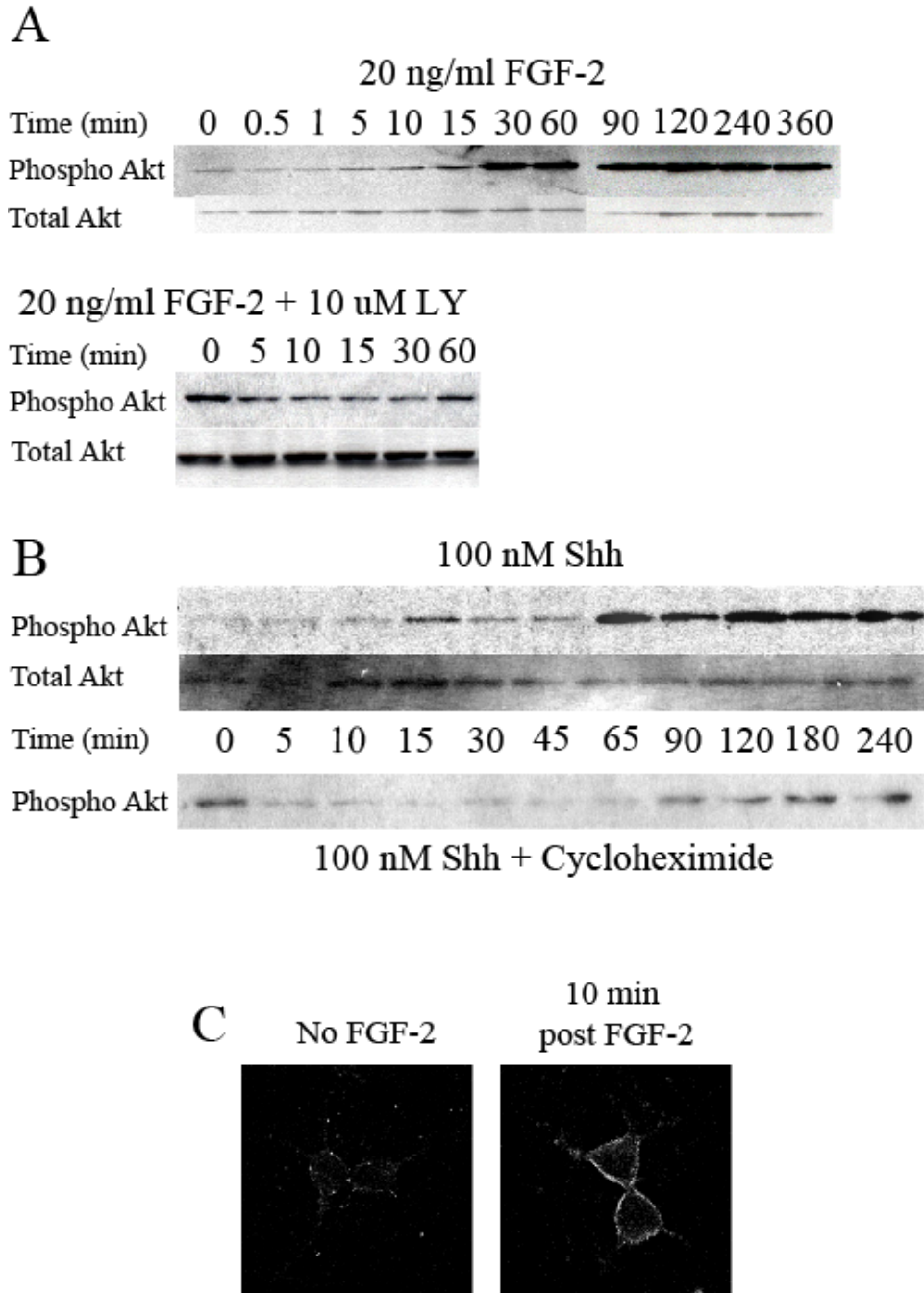


Figure 2: Akt is activated upon NPC stimulation by FGF-2 and Shh. (A) Cell lysates were analyzed for activated Akt levels in cultures pre-incubated with or without LY-294002 (10 μ M, PI3K inhibitor). Lysates were collected at indicated times (in minutes) after addition of FGF-2, and immunoblots were performed for Ser473 phospho-Akt and total Akt. All samples were exposed to 20 ng/ml FGF-2. (B) Shh immunoblots were performed as in (A). Conditions include 100 nM Shh with or without 0.1 mg/ml cycloheximide. (C) NPCs were fixed after 24 hours of FGF-deprivation (No FGF-2) or after 10 minutes of exposure to 20 ng/ml FGF-2. Samples were stained with an antibody to Ser473 phosphorylated Akt and imaged by confocal microscopy.

Akt Promotes NPC Proliferation and Inhibits Differentiation

To provide genetic evidence to complement the pharmacological and immunoblotting data, we overexpressed wild type (wt) Akt. The cDNA was inserted into a retroviral vector under the control of a tetracycline-regulated promoter (where transgene expression decreases with increasing tetracycline concentration [58]). Akt overexpression was confirmed by Western blot analysis (Figure 3A), and proliferation of the mutant cell line, along with a cell line created with an empty control vector, was tested in a 5-day growth assay. Cells were cultured with or without 0.1 $\mu\text{g/ml}$ tetracycline (tet) (Figure 3B). Even in the complete absence of FGF-2 these Akt overexpressers exhibited a dramatic increase in proliferation compared to cells infected with an empty vector control. Furthermore, the addition of tetracycline mitigated the proliferative effect.

We also overexpressed a dominant negative (dn) version of Akt containing alanine substitutions in the kinase domain and at sites of enzymatic activation by phosphorylation [57]. dnAkt overexpression was confirmed by Western blot analysis of total Akt expression, while probing the same blot for Akt phosphorylated at Ser473 demonstrated reduced active Akt (Figure 3C). Cells were cultured either with or without 0.1 $\mu\text{g/ml}$ tetracycline (tet) and with or without 1 ng/ml FGF-2 (Figure 3D). Proliferation of control cells cultured with FGF-2 was approximately 4-fold higher than the FGF-2-free condition. Decreased proliferation was observed in the cells expressing dominant negative Akt, and tetracycline addition restored proliferation to levels comparable to control. It is interesting to note that dominant negative Akt expression did not completely halt proliferation, indicating a possible role of other signaling pathways.

We next analyzed Akt's effect on neural progenitor differentiation. Cells overexpressing wild type or dominant negative Akt were grown under proliferative conditions (20 ng/ml FGF-2) or stimulated to differentiate into astrocytic and neuronal lineages with 1% fetal bovine serum + 1 μM retinoic acid, as previously described [16]. Quantitative RT-PCR of lineage markers was used to analyze cell differentiation, as we have previously reported [62] and analogous to the use of promoter-luciferase constructs to quantify lineage marker expression [63]. Under proliferative conditions, GFAP, a marker highly expressed in astrocytes, was undetectable in all cell types (Figure 3E). Although the neuronal marker β -tubulin III was detected, there was no significant difference between all cell types under proliferative conditions (Figure 3F). As expected, fetal bovine serum and retinoic acid addition strongly upregulated lineage marker expression in naïve cells. However, cells overexpressing wild type Akt exhibited a drastic (~50X) decrease in GFAP expression and a more moderate (~2.5X) decrease in β -tubulin III expression compared to empty vector control, indicating that Akt overexpression inhibits the ability of NPCs to differentiate into glial and neuronal lineages. Interestingly, cells overexpressing the dominant negative Akt exhibited lineage marker levels significantly greater than cells overexpressing wild type Akt, but still below empty vector control cells.

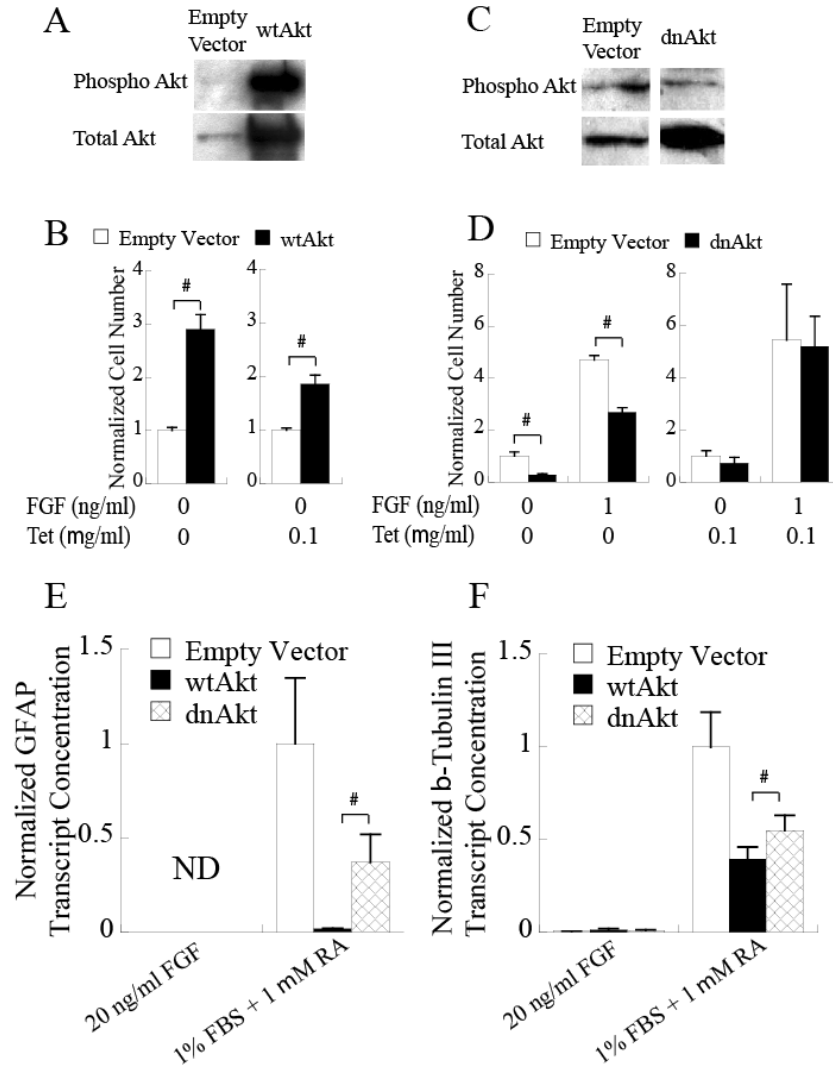


Figure 3: Akt promotes NPC proliferation and inhibits differentiation. (A) Cells were stably infected with an empty retroviral vector or one containing wild type Akt (wtAkt), and Western blot analysis confirmed wtAkt overexpression. The blot was probed with Ser473 phosphorylated Akt antibody, stripped and reprobed for total Akt. (B) Increased proliferation was observed in cells expressing wtAkt. Cells were seeded in quintuplicate 96-well laminin-coated plates at a density of 1000 cells/well in the indicated media and counted after 5 days in culture. Data in each panel are represented as cell number normalized to the empty vector control sample. (C) Western blot analysis confirmed overexpression of dominant negative Akt (dnAkt). The blot was probed with Ser473 phosphorylated Akt antibody, stripped, and reprobed for total Akt. All bands are from the same gel, but intervening lanes have been removed for clarity. (D) Decreased proliferation is observed in cells expressing dnAkt. Cells were stably infected with an empty retroviral vector or one containing dnAkt. Cells were seeded in quintuplicate in 96-well laminin-coated plates at a density of 1000 cells/well in indicated media and counted after 5 days in culture. Data in each panel are represented as cell number normalized to the empty vector control without FGF-2. (E, F) Cells were cultured in triplicate under proliferating (20 ng/ml FGF-2) or differentiating conditions (1% fetal bovine serum + 1 μ M retinoic acid) for 4 days. As measured by quantitative RT-PCR, GFAP (E) and β -tubulin III (F) expression are lower in cells overexpressing wild type Akt, while modestly lower GFAP and β -tubulin III expression is seen in cells expressing dominant negative Akt. Data are represented as transcript concentration normalized to the empty vector control sample. For all panels, error is the 95% confidence interval, and (#) denotes a statistically significant difference ($p < 0.05$) between the indicated conditions. ND: not detected.

CREB Promotes NPC Proliferation and Acts Downstream of Akt

Akt has a number of potential downstream effectors [28, 34-41]. However, because CREB has been implicated as a regulator of progenitor proliferation [64], we probed potential connections between Akt and CREB. First, we found that both FGF-2- and Shh-induced proliferation were completely halted in the presence of the CREB inhibitor 2-naphthol-AS-E-phosphate (Figures 4A, 4B). We next analyzed the time course of CREB activation upon the administration of FGF-2 and Shh to NPCs (Figure 4C) and found that it increased over 4 hours, similar to Akt activation but with a later onset. We next measured CREB activation in FGF-2-stimulated cells in the presence of signal transduction inhibitors (Figure 4D). When cells were pre-treated with LY-294002 or the Akt inhibitor API-2, CREB activation was substantially decreased compared to drug-free control. There was no discernable long-term effect of rapamycin on FGF-2-induced CREB activation (data not shown).

To further analyze whether CREB is necessary or sufficient for cell proliferation, we also analyzed the effects of wild type and dominant negative CREB overexpression, which were confirmed by Western blot analysis (Figure 4E). Cell proliferation was assayed as in Figure 3, and the proliferation of control cells cultured with FGF-2 was approximately 3-fold higher than the FGF-2-free condition, slightly different but consistent with the prior result (Figure 3D). In contrast to Akt, wild type CREB overexpression did not significantly alter proliferation in the absence of FGF-2. However, with just 1 ng/ml FGF-2, proliferation increased more than 3-fold relative to empty vector control (Figure 4F), whereas tetracycline reversed proliferation to levels comparable with control. By contrast, the mutant CREB inhibited NPC proliferation by approximately half in the absence of FGF-2 (Figure 4G). However, that difference was less pronounced in the presence of 1 ng/ml FGF-2, indicating that the proliferative signal is not solely mediated by CREB. Finally, full recovery to levels indistinguishable from control cells was observed with tetracycline addition regardless of FGF-2 supplementation.

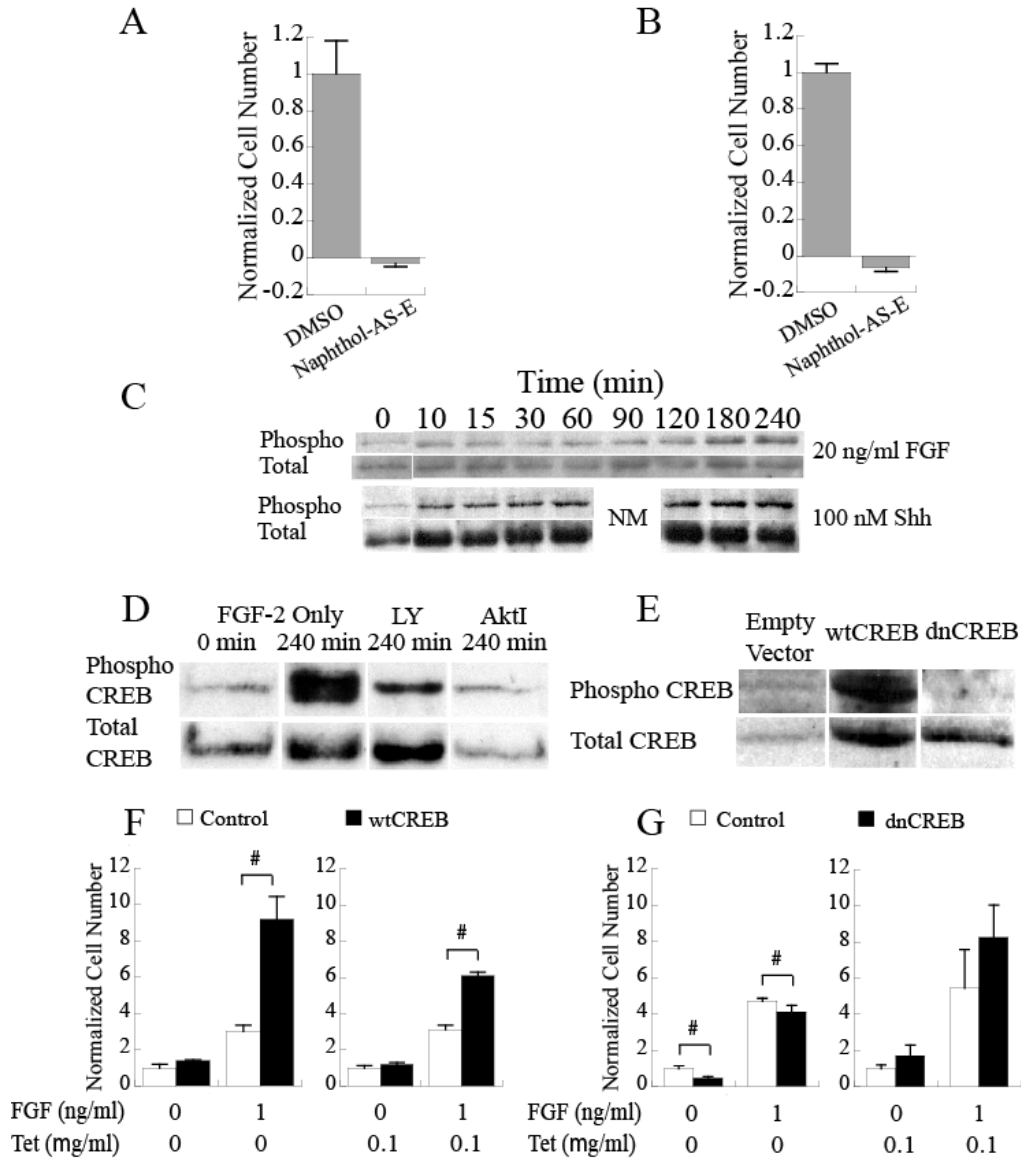


Figure 4: CREB promotes NPC proliferation and acts downstream of Akt. (A, B) Cells were seeded in quadruplicate in media containing either 20 ng/ml FGF-2 (A) or 100 nM Shh (B) and counted after 5 days in culture with 2-naphthol-AS-E-phosphate (CREB antagonist, 25 μ M). Data are represented as cell number normalized to a DMSO carrier control sample. (C) Cell lysates were analyzed for levels of activated CREB. Lysates were collected at indicated times after addition of 20 ng/ml FGF-2 or 100 nM Shh, and immunoblots were performed for phospho-CREB and total CREB. NM: not measured. (D) CREB immunoblots were performed as above with cells that had been pre-incubated for 2 hours with either LY-294002 (LY, 10 μ M, PI3K inhibitor) or API-2 (AktI, 1 μ M, Akt inhibitor). All bands are from the same gel, but intervening lanes have been removed for clarity. (E) Cells were stably infected with an empty retroviral vector, the vector containing wild type CREB (wtCREB), or the vector carrying a dominant negative CREB (dnCREB). Western blot analysis confirmed overexpression of wtCREB and dnCREB. The blot was probed with a Ser133-phosphorylated CREB antibody, stripped, and reprobed for total CREB. All bands are from the same gel, but intervening lanes have been removed for clarity. (F, G) Increased proliferation is observed (F) in cells overexpressing wtCREB, while decreased proliferation is observed in (G) cells expressing dnCREB. Cells were seeded in quintuplicate in the indicated media and counted after 5 days in culture. Data in each panel are represented as cell number normalized to the empty vector control without FGF-2. For all panels, error is the 95% confidence interval, and (#) denotes a statistically significant difference ($p < 0.05$) between the indicated conditions.

Discussion

Although an increasing number of extracellular factors have been found to regulate the proliferation of adult hippocampal neural progenitors [16-22], the intracellular transducers that control NPC proliferation have not been extensively studied, with several exceptions [23-25, 65]. Elucidating the signaling mechanisms that regulate NPC proliferation and differentiation will enhance our understanding of how the adult brain regulates neurogenesis, as well as lead to potential longer term exploration of modulating neurogenesis for therapeutic application (as reviewed [66]). Our results are the first to demonstrate the importance of PI3K/Akt in adult hippocampal progenitor proliferation driven by multiple mitogens (FGF-2, Shh, and IGF-1).

IGF-1 has been shown to both stimulate NPC proliferation and activate PI3K/Akt [19], and our results build upon this finding with chemical and genetic evidence that Akt is a central regulator of NPC proliferation and an inhibitor of cell differentiation. It has recently been shown in non-neural cells that Shh can activate a PI3K signal within 5-15 minutes [31, 67]; however, Shh stimulation of NPCs induced a delayed, protein synthesis dependent Akt activation (Figure 2B), indicating a novel mechanism. For example, Shh could potentially upregulate growth factor signaling components, such as the platelet-derived growth factor receptor PDGFR α that is up-regulated by Shh stimulation of C3H10T1/2 cells [68]. In addition, the Shh receptor Patched can regulate insulin-like growth factor-2 (IGF-2) expression [69], which could potentially activate PI3K in an autocrine fashion. Since PI3K/Akt has recently been shown to act synergistically with Shh to stimulate Gli2 [67] and N-myc [70], this novel result of Shh transcriptional activation of Akt may represent a positive feedback loop that can further reinforce Shh effects on cells. Future work may elucidate the mechanism of Shh activation of Akt, investigate this potential positive feedback loop, and determine the specific Akt isoform(s) activated in NPCs.

We also found that MEK does not strongly mediate FGF-2-induced NPC proliferation (Figure 1A), similar to results reported in embryonic carcinoma cells [65], but contrasting with other findings in NPCs [19] and with known roles for MEK and p38 MAP pathways in the proliferation of other cell types [71, 72]. Similar to our findings, primate and murine embryonic stem cell proliferation and self-renewal rely on Akt [45, 46], and MEK pathway inhibition actually promotes murine embryonic stem cell self-renewal [73]. MEK pathway inhibition, however, did attenuate Shh activity (Figure 1B), a novel result that should be explored in future work.

Akt also plays a role in NPC differentiation. When cultured under media conditions that strongly drive cell differentiation [21, 74], NPCs overexpressing wild type Akt exhibited substantially lower (~50X) expression of the astrocytic marker GFAP (Figure 3E). GFAP expression was also modestly lower in cells expressing dominant negative Akt but was still below the control (~2.5X), perhaps consistent with the finding that Akt activation is involved in astrocytic differentiation induced by ciliary neurotrophic factor [75]. The expression of the neuronal marker β -tubulin III was also lower (~2.5X) after exposing cells overexpressing Akt to differentiation conditions (Figure 3F). Again, this marker was modestly lower in cells expressing dominant negative Akt, consistent with Akt's role in neuronal differentiation and survival [48, 76]. Together, these data support a model that high levels of Akt activation inhibit cell differentiation, whereas low Akt activation levels may be permissive or necessary for cell differentiation.

PI3K and Akt have a number of downstream effectors. mTOR functions downstream of Akt in embryonic cortical neural progenitor cells [47], consistent with our observed rapamycin inhibition of adult NPCs (Figure 1A). However, we also investigated CREB's role in FGF-2

induced proliferation in detail. A small molecule CREB inhibitor inhibited NPC proliferation, while a dominant negative form of the protein moderately decreased the cells' proliferative capacity (Figure 4), indicating that CREB is not the sole mediator of proliferation. Moreover, wild type CREB overexpression sensitized cells to FGF-2, a result that also indicates that CREB still relies upon upstream signals to promote proliferation. Further studies will be necessary to quantitatively understand CREB's role in FGF-2 intracellular signaling and its relative importance compared to other transcription factors.

There are conflicting reports on PI3K/Akt pathway activation of CREB. An *in vitro* kinase assay suggests a direct phosphorylation of CREB by Akt [42], and cellular data in PC12 cells [77], neonatal cardiomyocytes [78], rat pituitary tumor cells [79], and striatal neurons [80] that imply that CREB is positively regulated by Akt. However, other studies suggest that Akt may repress CREB, since Akt phosphorylates and inactivates GSK3 β [39] which may otherwise phosphorylate (at serine 129) and activate CREB [81]. However, since other reports indicate that GSK3 β inhibits CREB [82], it is unclear whether Akt inhibition should positively or negatively influence CREB via GSK3 β . Future work will therefore be necessary to analyze the mechanism of Akt activation of CREB.

There are numerous downstream transcriptional targets of Akt, CREB, and the Shh-responsive transcription factor Gli that could influence or mediate proliferative effects. These include cell cycle components such as cyclin A [53] and cyclin D2, which has been shown to be important for adult neurogenesis [83]. CREB can also activate targets such as *c-fos*, CREB itself, and more than 100 other genes (as reviewed in [53]). In addition, since the overexpression of dominant negative forms of CREB and Akt do not completely halt NPC proliferation, other pathways are likely involved in proliferation.

Further work should also focus on downstream targets of Akt that inhibit NPC differentiation, particularly the dramatic inhibition of astrocyte differentiation (Figure 3E). This pathway may influence Sox family transcription factors such as Sox2, which is required for embryonic development and is necessary for NPC maintenance and proliferation [26].

Further work is required to analyze the role of Akt in adult neurogenesis *in vivo*, but these results do have several potentially intriguing implications. For instance, Robles et al. have found that Akt expression in the dentate gyrus is upregulated in rats performing spatial discrimination learning exercises [84], behavioral conditions that in another study have been shown to upregulate adult neurogenesis [85]. In addition, the PI3K/Akt pathway has a well documented role in cancer progression that makes it an attractive therapeutic target [86, 87]; however, our results indicate that Akt inhibition *in vivo* may have the unintended side effect of inhibiting the development of new neurons in the adult brain. Future work to determine the relative importance of different Akt isoforms to NPC proliferation may provide opportunities to target tumors without affecting neural progenitor function.

In summary, this work demonstrates that Akt is an important regulator of adult hippocampal neural progenitor cell proliferation and differentiation, as well as implicates CREB as a downstream effector. Recent work demonstrating a role for Akt in the proliferation of embryonic stem cells [45, 46] and stem cells in the developing nervous system [44, 47], in concert with our work with FGF-2, Shh and IGF-1, makes Akt an attractive potential target for understanding regulatory mechanisms of proliferation and self-renewal in various stem and progenitor cell types.

Acknowledgments

We thank K. Lai, S. Agrawal, and J. Haugh for helpful discussions, and J. Haugh, J. Woodgett, K. Walsh, K. Saeki, R. Goodman, S. Ferguson, and M. Montminy for the kind gift of reagents.

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CHAPTER 3

AKT INCREASES SOX2 EXPRESSION IN ADULT HIPPOCAMPAL NEURAL PROGENITOR CELLS BUT INCREASED SOX2 DOES NOT PROMOTE PROLIFERATION

Abstract

We have previously demonstrated that Akt is important for the proliferation of adult hippocampal neural progenitor cells (NPCs). Here we extend that work to demonstrate that in addition to proliferation, Akt promotes expression of the transcription factor Sox2, a critical regulator of self-renewal in multiple stem/progenitor cell types, including NPCs. Akt promotes Sox2 expression by increasing mRNA concentration. Notably, increased Sox2 expression does not promote NPC proliferation, indicating that Akt-mediated NPC proliferation is Sox2 independent and that Akt acts as a master regulator of NPC proliferation and self-renewal.

Introduction

Neural progenitor cells (NPCs) from the adult hippocampus have the potential to maintain their population, a process called self-renewal, as well as to undergo lineage commitment and differentiation into the three major cell types of the mammalian brain: neurons, astrocytes, and oligodendrocytes [1]. The regulation of these processes is central to adult neurogenesis [2, 3], which in turn contributes to cognition and mood regulation [4, 5] and may be important for learning and memory [6-10]. A number of extracellular factors have been shown to modulate NPC proliferation and self-renewal, including basic fibroblast growth factor (FGF-2) [11], epidermal growth factor (EGF) [12], and sonic hedgehog (Shh) [13]. However, the intracellular signaling cascades mediating these extracellular signals have only recently been explored. We have demonstrated that the PI3K/Akt signaling pathway is an important mediator of the effects of extracellular factors on NPC proliferation and differentiation [14]. Others have demonstrated the importance of mTOR [15], GSK3 [16], and Wnt/ β -catenin [17, 18] signaling for neural stem/progenitor cell proliferation; however, some of these studies did not necessarily investigate hippocampal progenitors. Nevertheless, very little is known about which pathways modulate downstream transcriptional targets important for cell functions such as cell maintenance in a multipotent state.

The PI3K/Akt pathway is known to play important roles in cellular proliferation, growth, survival, metabolism, and migration [19]. The activation of cell surface receptors, such as tyrosine kinase receptors and adhesion receptors, recruits PI3K to the cell membrane where it catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits Akt and PDK1 to the membrane. PDK1 phosphorylates Akt at the activation segment (T308), and a second phosphorylation event on the hydrophobic motif (S473) leads to complete activation of the enzyme [20]. This second phosphorylation event is widely believed to be mediated by the mTOR complex 2 (mTORC2) [21]. The importance of Akt has been demonstrated in many stem cell types, including mouse embryonic stem (ES) cells [22, 23],

primate ES cells [24], rabbit ES cells [25], mesenchymal stem cells [26], and hematopoietic stem cells [27, 28]. Additionally, we have previously reported the importance of Akt for NPC proliferation and inhibition of differentiation [14]. Others have shown the importance of signaling events downstream of Akt to NPC maintenance, including mTOR [15] FoxO [29] and GSK-3 [16]. However, very little work has investigated how and whether this signaling pathway interacts with the transcriptional machinery responsible for maintaining the multipotency of these cells.

The SRY-related HMG-box 2 (Sox2) transcription factor is important for self-renewal of ES cells [30] and is one of the factors necessary for reprogramming and generation of induced pluripotent stem (iPS) cells [31]. It is also a critical factor for NPC maintenance [32] and has increasingly been utilized as an NPC marker [33]. Furthermore, lineage tracing studies have shown that Sox2-positive cells in the hippocampus can self-renew and generate differentiated progeny [34]. In chick embryos, Sox2 overexpression prevents differentiation [35]; however, hypomorphic Sox2 mutant cells from the mouse sub-ventricular zone grown in culture have impaired neuronal generation, indicating that Sox2 may also be important to prime cells for neuronal differentiation [36]. A nervous-system specific Sox2 knockout mouse revealed that one way in which it maintains the adult NPC population is through the upregulation of Shh [37], a morphogen known to control adult NPC proliferation [13]. Sox2 is also known to repress the transcription of glial fibrillary acidic protein (GFAP), an important astrocytic marker [36].

Control of Sox2 activity and its expression in NPCs is poorly understood, though some Sox2 control mechanisms have been studied in other cell types. Post-translational modification of Sox2 by sumoylation negatively regulates its transcriptional activity by inhibiting DNA binding in mouse ES cells [38], and in human ES cells this sumoylation event is regulated by phosphorylation [39]. Additionally, poly(ADP-ribosyl)ation by PARP1 has been shown to regulate FGF-4 expression in human ES cells [40]. Transcriptional control of Sox2 is promoted by the Sox2 regulatory region 2 (SRR2), a Sox2 enhancer known to regulate its expression in the telencephalon [41]. Furthermore, Sox2 forms a heterodimer with Oct4 that positively regulates Sox2 expression in human ES cells [42], but Oct4 is not expressed in most cell types. Despite these advances in our understanding of Sox2 regulation, little is known about the signaling pathways that drive Sox2 expression, particularly in NPCs. Given the importance of Akt and Sox2 to pluripotency [22-25] and multipotency [26-28], it is important to better investigate whether Sox2 expression is linked to Akt activity.

Here we extend our previous work with Akt [14] to demonstrate that it both enhances cell proliferation via a Sox2-independent mechanism, as well as promotes the expression of the transcription factor Sox2 to support self-renewal. Through retroviral transduction we demonstrate that increased Sox2 expression is promoted by Akt activity via an increase in Sox2 transcripts. Interestingly, we also show that increased Sox2 protein levels, while inhibiting differentiation, do not increase proliferation. This indicates that Akt serves as an important master regulator in NPC maintenance by independently promoting downstream cell proliferation and Sox2-dependent self-renewal.

Materials and Methods

Cell Culture

Adult neural progenitor cells isolated from the hippocampi of 6-week-old female Fischer 344 rats as described [43] were cultured on tissue culture polystyrene coated with poly-ornithine and 5 $\mu\text{g/ml}$ of laminin (Invitrogen). Cells were grown in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) high-glucose medium (Invitrogen) containing N-2 supplement (Invitrogen) and 20 ng/ml recombinant human FGF-2 (Peprotech).

Mutant Cell Lines

Progenitor cells constitutively expressing wild-type Akt or Sox2 were generated by retroviral infection. Wild type murine Akt1 cDNA (Akt) was a kind gift from S. Ferguson (Robarts Research Institute, London, ON, Canada), and wild type murine Sox2 cDNA was purchased from Stemgent (Cambridge, MA). Both cDNAs were subcloned into the MMLV retroviral vector CLGPIT, which is a variant of CLPIT [44]; however, the puromycin resistance gene puromycin N-acetyl transferase (PAT) is replaced with a gene encoding a GFP-PAT fusion [45] (a kind gift from M. McVoy, Governor's School for Government and International Studies, Richmond, VA). An empty control vector was also produced. Correct products were confirmed by sequence analysis. Retroviral vectors were packaged using CMV gag-pol and CMV VSV-G envelope helper plasmids by calcium phosphate transfection as described [44]. Vectors were harvested, concentrated by ultracentrifugation, and titered on HEK 293Ts. Progenitor cells were infected at a multiplicity of infection of 1 IU/cell and subsequently selected with 0.6 $\mu\text{g/ml}$ puromycin (Sigma) for four days.

Proliferation Assay

NPCs overexpressing wild type Sox2 or an empty vector control were plated at 1000 cells/well on 96-well poly-ornithine/laminin-coated tissue culture plates with either 0 or 1 ng/ml FGF-2 in DMEM/F-12 + N-2 medium. Each condition was cultured in biological quintuplicate, and fifty percent media changes were conducted daily. After 5 days in culture, cell number was quantified using the WST-1 assay following the manufacturer's instructions (Roche) and utilizing a standard curve generated with known cell numbers.

Quantitative RT-PCR

NPCs were seeded at 200,000 cells per well in 6-well poly-ornithine/laminin coated culture plates in DMEM/F-12 + N-2 medium containing either 1 ng/ml FGF-2 to sustain multipotency or 1% fetal bovine serum (FBS) plus 1 μM retinoic acid (RA) (Biomol) to induce differentiation. Medium was replenished daily, and on day 5 RNA was isolated by TRIzol (Invitrogen) according to manufacturer's instructions. cDNA's were then generated using Invitrogen's ThermoScript RT-PCR kit according to manufacturer's instructions. Using a BioRad iCycler, Taqman probe QPCR was performed for the astrocytic marker GFAP, the neuronal marker β -Tubulin III, or Sox2 with the 18S ribosomal subunit as an internal control. GFAP, β -Tubulin III, and Sox2 probes from Biosearch Technologies contained the FAM490 fluorophore with Black Hole Quencher (BHQ), while the 18S rRNA probe contained the CAL610 fluorophore with BHQ. Table 1 lists primer and probe sequences.

	Sequence
β-Tubulin III	
Forward Primer	5'-GCATGGATGAGATGGAGTTCACC-3'
Reverse Primer	5'-CGACTCCTCGTCGTCATCTTCATAC-3'
Probe	FAM490-TGAACGACCTGGTGTCTGAG-BHQ
GFAP	
Forward Primer	5'-GACCTGCGACCTTGAGTCCT-3'
Reverse Primer	5'-TCTCCTCCTTGAGGCTTTGG-3'
Probe	FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ
Sox2	
Forward Primer	5'-CGAGTGGAAACTTTTGTCCGAGAC-3'
Reverse Primer	5'-CGGGAAGCGTGTACTTATCCTTCTT-3'
Probe	FAM490-CTCTGCACATGAAGGAGCACC-BHQ
18S	
Forward Primer	5'-GTAACCCGTTGAACCCCATTC-3'
Reverse Primer	5'-CCATCCAATCGGTAGTAGCGA-3'
Probe	CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ

Table 1: Primer and probe sequences for quantitative RT-PCR.

Western Blotting

NPCs were lysed by adding lysis solution directly to the culture plate. Lysis solution contained IGEPAL (1%, Sigma), sodium dodecyl sulfate (SDS, 0.1%), phenylmethanesulfonylfluoride (PMSF, 0.1 mg/ml, Sigma), aprotinin (0.03 mg/ml, Sigma), and sodium orthovanadate (1 mM, Sigma) in PBS. Lysate protein concentrations were quantified by BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Equal amounts of protein from each lysate were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). In cases where blots were stripped and re-probed, phosphorylated epitopes were probed first, as previously described [46]. Primary antibodies included rabbit anti-Sox2 (Abcam, ab15830, 1:2000), rabbit anti-GAPDH (Abcam, ab9485, 1:2000), rabbit anti-histone H1 (Santa Cruz, sc-10806, 1:200), rabbit anti-phosphoT308 Akt (Cell Signaling, 4056, 1:1000), rabbit anti-phosphoS473 Akt (Cell Signaling, 9271, 1:1000), and rabbit anti-total Akt (Cell Signaling, 9272, 1:2000). HRP-conjugated, goat anti-rabbit secondary antibody (Pierce, 31460, 1:10,000) was used to develop the blots.

Immunofluorescence

NPCs were seeded onto Falcon 8-well chamber slides coated with poly-ornithine and 10 μ g/ml laminin. Cultures grown in 1 ng/ml FGF-2 were seeded with 20,000 cells/well, while cultures grown with 1% FBS + 1 μ M RA or with 1 μ M API-2 (Akt inhibitor, dissolved in DMSO, EMD Biosciences) were seeded with 40,000 cells/well. Cells were grown for 5 days with 50% media replenishment daily, and then fixed for 10 min with 4% paraformaldehyde. After four PBS washes, cells were blocked/permeabilized for 2 hr with blocking buffer containing 0.3% Triton X-100 + 5% donkey serum in PBS. Cells were stained with goat anti-Sox2 (Santa Cruz, sc-17320, 1:200) in blocking buffer for approximately 72 hr at 4°C. After four PBS washes, slides were stained with Cy3-conjugated, donkey anti-goat secondary antibody

(Jackson ImmunoResearch, West Grove, PA, 705-165-147, 1:250) for 2 hrs in blocking buffer. Slides were washed four more times, and the final wash contained 5 $\mu\text{g/ml}$ of the nuclear stain DAPI (Invitrogen, D21490). Slides were mounted with Cytoseal 60 (Fisher Scientific). Four random images from each well were used for quantification. Using the freely available image analysis software CellProfiler [47], primary objects/nuclei were identified using the DAPI stain, and the pixel intensity of Sox2 staining within each nucleus was then measured. In all cases, Sox2 staining remained localized to the nucleus.

Results

Sox2 Expression and Akt Activation Increase under Proliferating Conditions

Given the importance of Sox2, we sought to determine how its expression is regulated in NPCs. We first confirmed that Sox2 protein levels increase under culture conditions known to support NPC self-renewal and proliferation. We cultured NPCs with varying FGF-2 concentrations for 5 days, lysed, and probed for Sox2 via immunoblotting (Figure 1A). Sox2 expression increases with increasing FGF-2 concentration. We also performed the opposite experiment whereby we differentiated the cells for 6 days with 1 μM RA + 1% FBS and measured Sox2 protein expression by immunoblotting (Figure 1B). Consistent with its role as a NPC marker, Sox2 expression decreases upon differentiation, which also halts proliferation, and this result was confirmed quantitatively by immunostaining (Figure 1C).

Our previous results showed that Akt stimulates NPC proliferation and inhibits differentiation [14]; therefore, we sought to determine whether Akt activation had a similar pattern to Sox2 expression. NPCs starved of FGF-2 overnight were stimulated with various FGF-2 concentrations for 10 min, lysed, and probed for phosphorylated, active Akt (Figure 1D). Increasing FGF-2 concentrations resulted in higher Akt phosphorylation at both the S473 and T308 sites, which are critical for full activation of the molecule. These data indicate a correlation between Akt activation and Sox2 expression within NPCs.

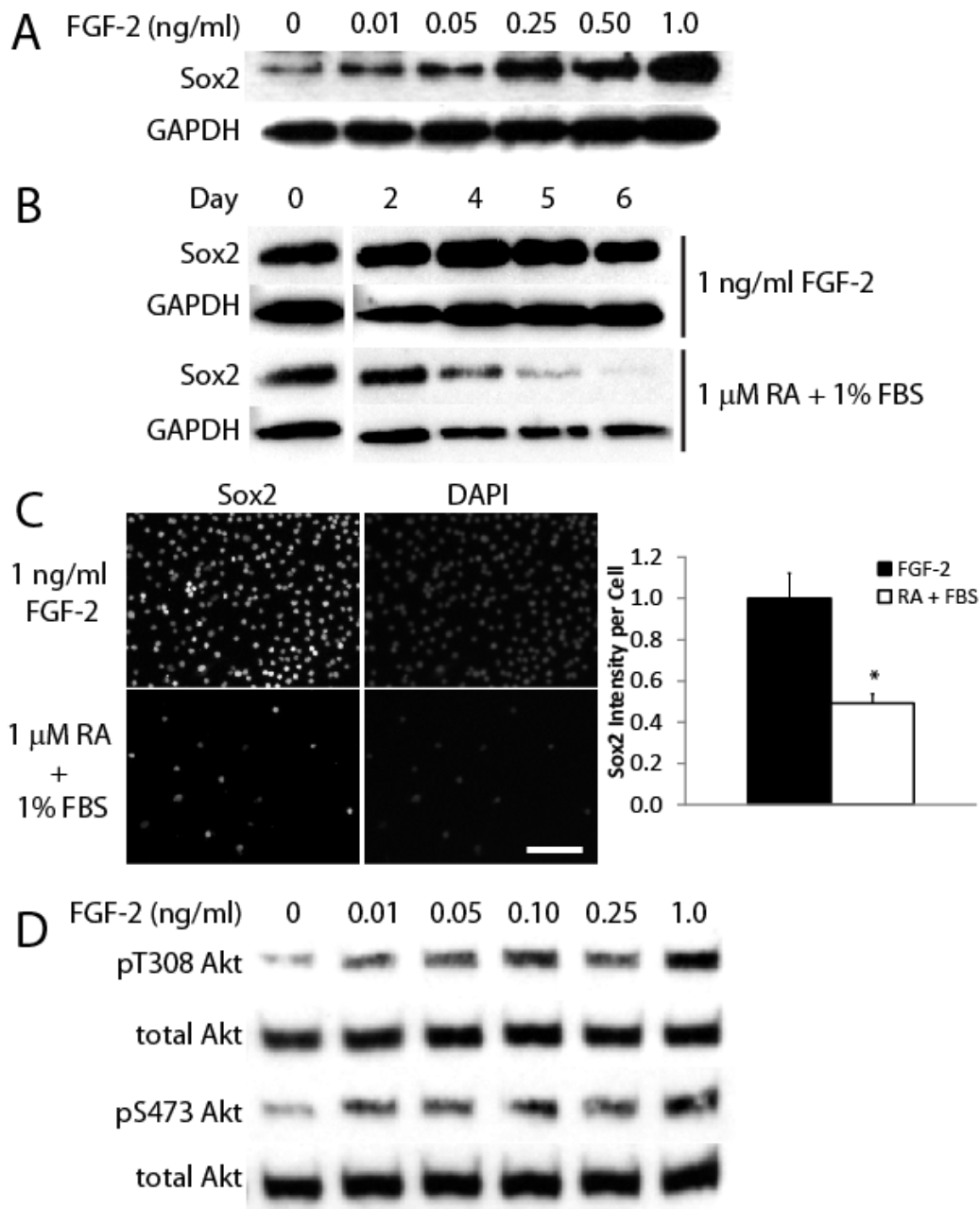


Figure 1: Sox2 expression and Akt activation both increase under proliferating conditions. (A) Cells were cultured with the indicated concentrations of FGF-2 for 5 days. Lysates were analyzed by immunoblotting for Sox2, using GAPDH as a loading control. (B) Cells were cultured for the indicated number of days in media containing either 1 ng/ml FGF-2 or 1 μM retinoic acid + 1% FBS. Lysates were analyzed for Sox2, using GAPDH as loading control. All bands are from the same blot; however, intervening lanes have been removed for clarity. (C) Left panel: Cells were cultured for 5 days in the indicated media and stained for Sox2 and DAPI. Scale bar: 200 μm. Right panel: Quantification of Sox2 staining intensity under proliferative (1 ng/ml FGF-2) and differentiating (1 μM retinoic acid + 1% FBS) conditions, using the image analysis software CellProfiler. Error bars are 95% confidence intervals, and * indicates $p < 0.05$. (D) Cells were FGF-2-starved overnight, then stimulated with indicated concentrations of FGF-2 for 10 min. Lysates were analyzed by immunoblotting for Akt phosphorylated at S473 and T308. Total Akt was used as loading control.

Akt Promotes Sox2 Expression

To test whether the relationship between Akt activity and Sox2 expression is correlative or causal, we overexpressed wild-type Akt in NPCs via retroviral transduction. Cells were cultured under either proliferative (1 ng/ml FGF-2) or differentiation (1 μ M RA + 1% FBS) conditions. After five days in culture, the intensity of Sox2 expression was measured via immunofluorescence and compared to empty vector infected control cells and control cells cultured with the Akt inhibitor API-2/triciribine (1 μ M) (Figure 2A-B). Cells overexpressing Akt had increased Sox2 expression under both proliferation and differentiation conditions. However, the effect was more pronounced under differentiation conditions. Surprisingly, cells overexpressing Akt cultured under differentiation conditions had Sox2 expression levels comparable to control cells cultured in proliferation conditions—a complete rescue of Sox2 expression.

Additionally, empty vector control cells cultured with API-2 had decreased Sox2 expression under both proliferation and differentiation conditions. Again, the effect was more pronounced under differentiation conditions, where Sox2 expression was almost completely suppressed. This partial but not complete elimination of Sox2 expression in the presence of API-2 indicates that Akt is important, but it is likely not the sole mediator of FGF-2 upregulation of Sox2 expression.

To further confirm the immunofluorescence results, we repeated the experiment and probed cell lysates for Sox2 levels via Western blotting (Figure 2C). Again, Akt overexpression increased Sox2 protein expression, whereas Akt inhibition decreased expression. This was observed under both proliferation and differentiation conditions. However, because the basal Sox2 protein level was much lower under differentiation conditions (lower panel), extended exposure was required, resulting in high background and lower resolution. Nevertheless, the result that Akt promotes Sox2 expression was confirmed.

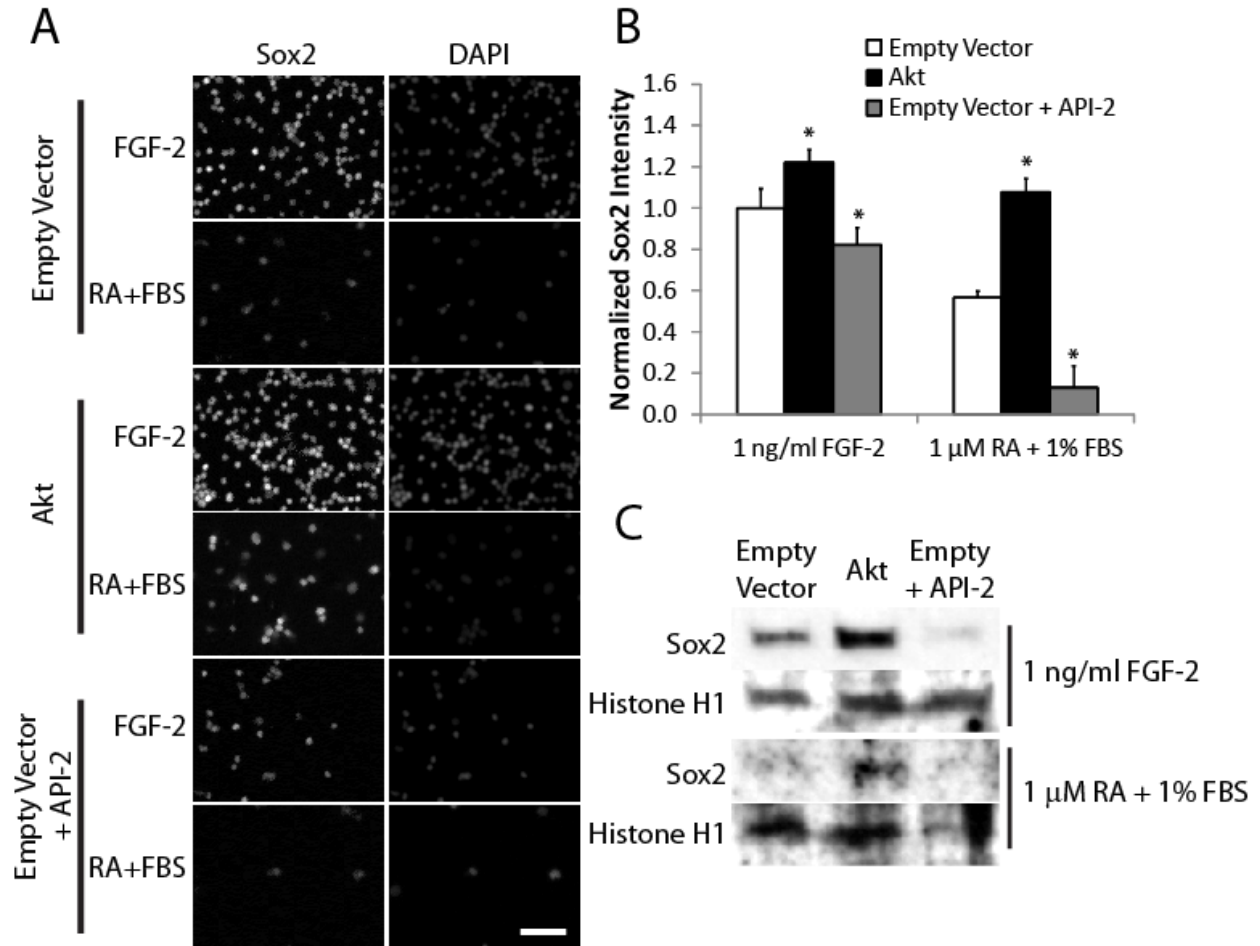


Figure 2: Akt promotes Sox2 expression. (A) Cells stably infected with retroviral vector containing wild type murine Akt1, empty vector control, or empty vector control cultured with Akt inhibitor (1 μ M API-2/triciribine) were seeded in media containing either 1 ng/ml FGF-2 (proliferative) or 1 μ M retinoic acid + 1% FBS (differentiating) and cultured for 5 days. Cells were fixed and stained for Sox2 and DAPI. Scale bar: 200 μ m. (B) The resulting images were quantified for Sox2 staining intensity using the image analysis software CellProfiler. Error bars are 95% confidence intervals, and * indicates $p < 0.05$. (C) The same cells as above were cultured for 5 days in the indicated media. Lysates were analyzed by immunoblotting for Sox2, using histone H1 as loading control.

Akt Increases Sox2 mRNA Concentration

To test whether Akt promotes Sox2 expression by increasing mRNA concentration, we again cultured Akt-overexpressing NPCs and empty vector control cells for five days under proliferation and differentiation conditions. RNA was isolated, and quantitative RT-PCR for Sox2 was performed on the resulting cDNAs (Figure 3A). As expected, Sox2 transcript concentration decreased upon differentiation of the control cells. However, Sox2 expression did not decrease in Akt-overexpressing cells under the same conditions, demonstrating rescue of Sox2 by the Akt activity, similar to the rescue of Sox2 protein expression (Figure 2). Unlike Sox2 protein expression, however, Akt did not statistically increase Sox2 mRNA concentration under proliferation conditions compared to empty vector control.

To further confirm that Akt does not promote Sox2 protein stability, we again used the Sox2-overexpressing NPCs, where Sox2 is constitutively transcribed. These cells were cultured

for five days with 1 ng/ml FGF-2 and 1 μ M API-2 and analyzed for Sox2 protein expression by Western blot (Figure 3B) and immunofluorescence (Figure 3C-D). Compared to carrier control (DMSO), API-2 did not decrease Sox2 protein expression. In fact, immunofluorescence quantification revealed a slight but statistically significant increase in Sox2 intensity per cell when cultured with the Akt inhibitor (Figure 3D). The fact that Akt inhibition did not decrease Sox2 protein in cells constitutively transcribing Sox2 indicates that Akt's effect on Sox2 protein expression is not post-translationally mediated. Taken together, these results show that Akt drives Sox2 expression by increasing the concentration of its mRNA, rather than preventing Sox2 protein degradation.

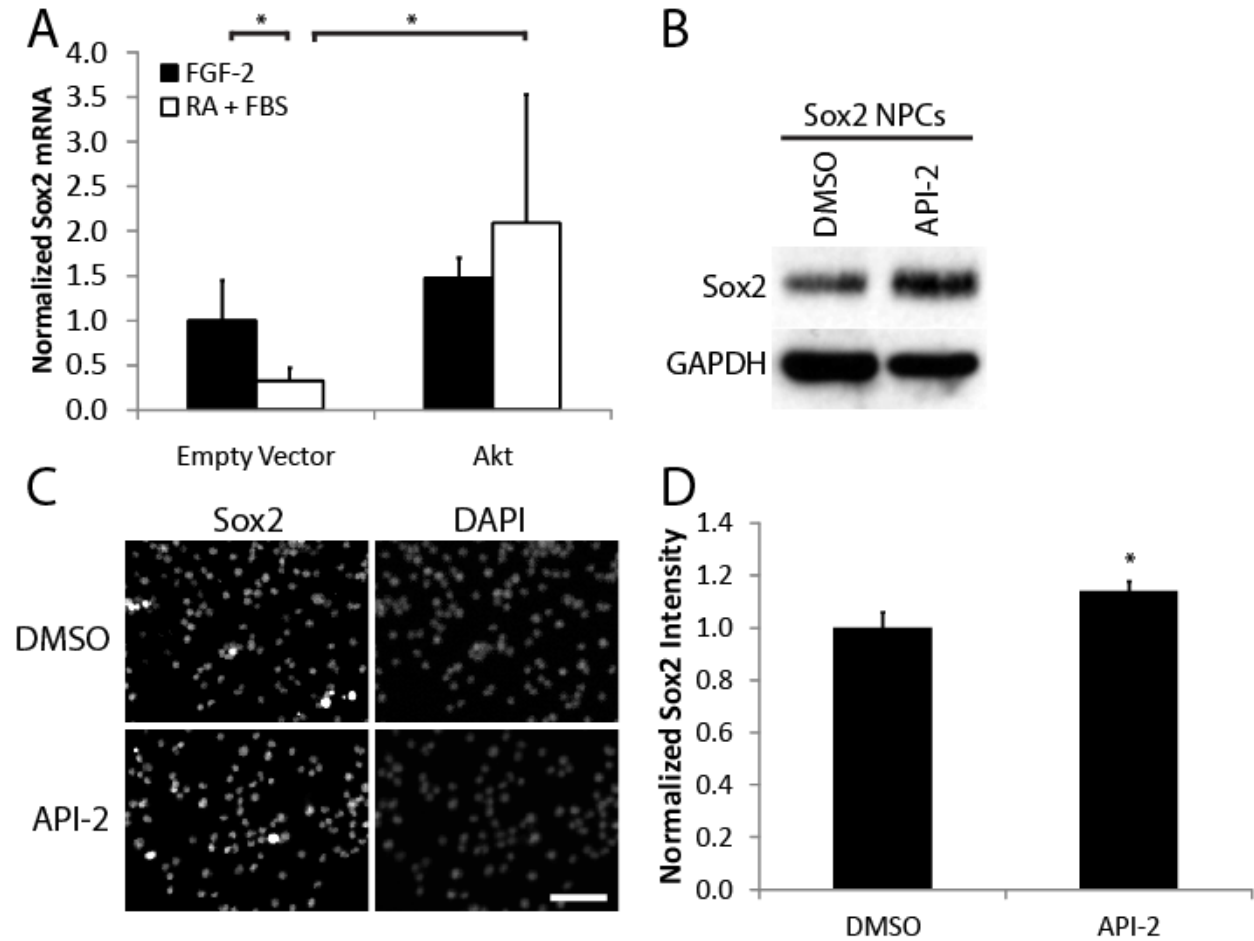


Figure 3: Akt increases Sox2 mRNA concentration. (A) Akt overexpressing NPCs and empty vector control were cultured with either 1 ng/ml FGF-2 or 1 μ M RA + 1% FBS for 5 days. RNA was extracted, reversed transcribed, and quantitative PCR was performed on the resulting cDNAs for Sox2. Samples were normalized to the 18S ribosomal subunit as an internal control. (B) Sox2 overexpressing NPCs were cultured for 5 days in 1 ng/ml FGF-2 with 1 μ M API-2 (Akt inhibitor) or DMSO carrier control. Lysates were analyzed by immunoblotting for Sox2, using GAPDH as loading control. (C) Similar to (B) Sox2-overexpressing cells were cultured with API-2 or DMSO carrier control, fixed, and stained for Sox2 and DAPI. Scale bar: 200 μ m. (D) The resulting images were quantified for Sox2 intensity using the image analysis software CellProfiler. Error bars are 95% confidence intervals, and * indicates $p < 0.05$.

Sox2 Inhibits Differentiation but does not Promote Proliferation of NPCs

We have shown that Sox2 expression is Akt-mediated, but it is unclear whether Sox2 maintains NPC multipotency, promotes proliferation, or both. To demonstrate that Sox2 inhibits differentiation, we stably overexpressed wild type Sox2 in NPCs using a retroviral vector, as confirmed by Western blot analysis (Figure 4A). Both the Sox2 cell line and an empty vector control cell line were infected in parallel and differentiated with 1 μ M RA + 1% FBS, a condition that promotes the generation of both neurons and astrocytes. After 5 days, quantitative RT-PCR of lineage markers was used to analyze cell differentiation as previously reported [14, 48] (Figure 4B). Compared to control, mutant cells overexpressing Sox2 had significantly decreased expression of both the neuronal marker β -tubulin III and the astrocytic marker GFAP; however, GFAP expression was more strongly inhibited than β -tubulin III, similar to previous results in cells overexpressing wild type Akt [14]. We have also previously shown that Akt strongly upregulates NPC proliferation [14]. To determine whether Sox2 has a similar effect, we cultured the cells in a 5 day proliferation assay with and without FGF-2 (Figure 4C). Surprisingly, Sox2 overexpression had no effect on proliferation in the absence of FGF-2. Furthermore, it moderately decreased proliferation in the presence of the growth factor. Taken together, these results indicate that Sox2 inhibits NPC differentiation, which is consistent with its known role in self-renewal [32, 34], but it does not promote proliferation.

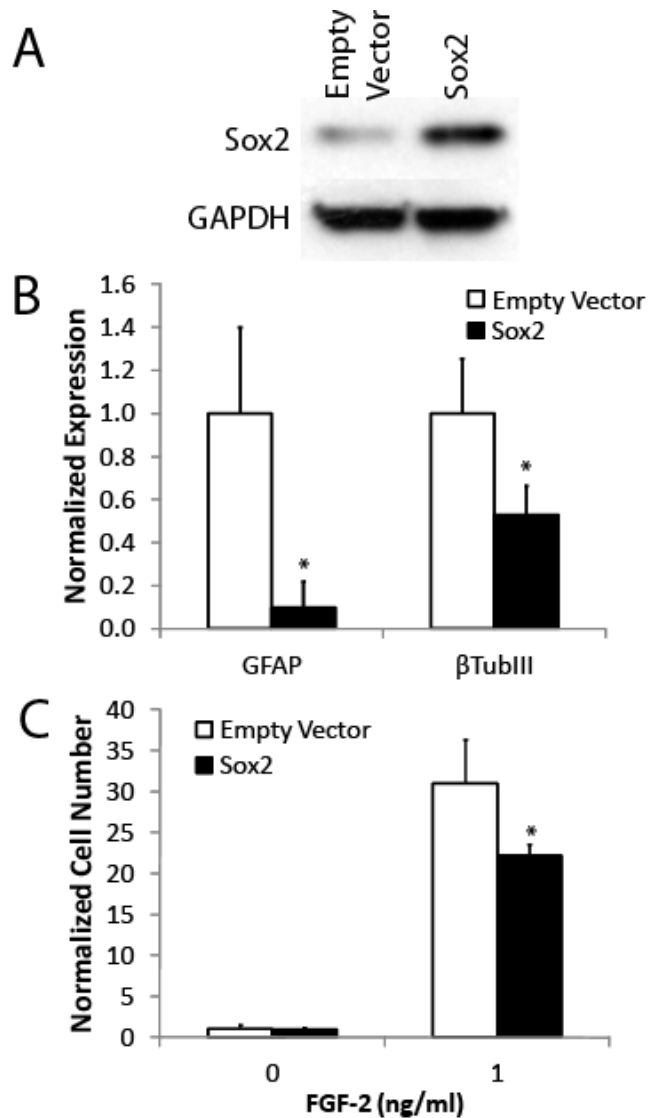


Figure 4: Sox2 inhibits differentiation without affecting proliferation in NPCs. (A) Cells stably infected with retroviral vector containing wild type murine Sox2 or empty vector control were lysed, electrophoretically separated, and probed to confirm Sox2 overexpression. GAPDH was used as loading control. (B) Cells overexpressing Sox2 or empty vector control were cultured under differentiating conditions (1% fetal bovine serum + 1 μ M retinoic acid) for 5 days. RNA was extracted, reverse transcribed, and quantitative PCR was performed on the resulting cDNAs for GFAP, and β TubIII. Samples were normalized to the 18S ribosomal subunit as an internal control. (C) Sox2 overexpressing cells and control cells were seeded in media containing either 0 or 1 ng/ml FGF-2 and counted after 5 days in culture by WST-1. Data are represented as cell number normalized to the empty vector control sample. Error bars are 95% confidence intervals, and * indicates $p < 0.05$.

Discussion

Sox2 is an important regulator of ES cell and NPC self-renewal [32-35], and it is regulated as a part of the core transcriptional circuitry controlling ES cell self-renewal [42, 49, 50]. However, very little is known about how it is regulated by upstream signaling pathways. One upstream pathway important for the maintenance of numerous stem cell populations is the PI3K/Akt pathway [22-28]; however, it is unknown how or even whether these two critical

elements of stem cell regulatory machinery interact. Here we demonstrate that Akt promotes Sox2 expression by increasing its mRNA concentration, thereby promoting Sox2 protein expression and NPC self-renewal. However, increased Sox2 expression did not increase NPC proliferation. Given our previous work showing that Akt drives NPC proliferation [14], this indicates that Akt is an important regulator of both proliferation and self-renewal in NPCs.

Akt overexpression completely rescued differentiation-induced loss of Sox2 expression (Figure 2). Additionally, Akt increased Sox2 expression in cells cultured under proliferative conditions, although this was not a large effect presumably due to the presence of a strong FGF-2-mediated mitogenic signal. Interestingly, Akt inhibition did not strongly inhibit Sox2 expression in cells cultured with FGF-2; however, this result was statistically significant. This indicates the unlikelihood that Akt is the only mediator of FGF-2-induced Sox2 expression. There is some evidence that Wnt/ β -catenin signaling is important for NPC self-renewal [17, 18]; however, a link between the Wnt signal and Sox2 expression was not established in either of these studies. Additional studies may reveal interesting links between Sox2 and other signaling pathways.

It is well known that Akt promotes translation by activating its downstream effector mTORC1 [19]. However, one interesting result of our work is that Akt promotes expression of Sox2 by increasing the concentration of the Sox2 message. This was demonstrated by Akt-mediated rescue of Sox2 transcript expression in differentiating cultures (Figure 3A). Additionally, pharmacological Akt inhibition did not affect Sox2 protein levels in a constitutively expressing Sox2 mutant (Figure 3B-D), indicating that Akt inhibition did not modify protein levels by decreasing Sox2 translation or increasing protein degradation. One potential mechanism for Akt-induced Sox2 transcription is through stabilization of c-Myc [51, 52], a transcription factor shown to modulate Sox2 expression in mouse ES cells [50].

In addition to NPCs, Akt is also known to promote the proliferation and self-renewal of ES cells [22-25]; therefore, investigating the effects of Akt on Sox2 expression in ES cells could have important implications for the development of more efficient ES cell culture systems and perhaps eventually ES cell based therapies. Furthermore, pharmacological enhancement of Akt signaling may improve the efficiency of reprogramming and the generation of iPS cells, potentially eliminating the need for one or more of the canonical Yamanaka factors (for review see [53]). Recent work has demonstrated that pharmacological inhibition of ERK and GSK3 β can generate iPS cells from neural stem cells using only two of the four Yamanaka factors, eliminating the need for exogenous Sox2 expression [54]. Other studies have also used GSK3 β inhibition along with ERK and ALK5 inhibition to promote reprogramming [55]. Because GSK3 β activity is directly inhibited by Akt, these findings taken together with our results may indicate that modulation of the Akt pathway could improve reprogramming efficiencies by upregulating expression of endogenous Sox2.

NPCs overexpressing Sox2 had a decreased ability to differentiate as measured by quantitative RT-PCR (Figure 4B). In particular, upon exposing cells to conditions strongly favoring astrocytic and neuronal differentiation, Sox2-overexpressing cells had ~10X lower expression of the astrocytic marker GFAP and ~2X lower expression of the neuronal marker β -tubulin III. This result, similar to that seen in NPCs overexpressing Akt [14], further supports observations in chick embryos constitutively expressing Sox2, which also experienced impaired neuronal differentiation [32]. Notably, however, Sox2 is still required for proper neuronal differentiation and development. Cells derived from Sox2 hypomorphic mice and cultured *in vitro* generated abundant β -tubulin III positive cells, but those cells failed to mature [36].

Therefore, a minimal threshold amount of Sox2 appears to be required for proper neuronal development.

Although Sox2 overexpression inhibited differentiation, it did not increase NPC proliferation (Figure 4C) and in fact slightly reduced proliferation in the presence of the growth factor FGF-2. This is consistent with the observation that Sox2-positive cells within the subgranular zone of the hippocampus are able to remain quiescent for extended periods [34]. Additionally, it indicates that although Sox2 is important for NPC self-renewal, it is not sufficient for their proliferation. This is not the case in other cell types. Sox2 promotes proliferation of tracheal and airway stem cells [56], and as an oncogene it is necessary for the proliferation and transformation of lung or esophageal squamous cell carcinomas [57].

In summary, this work demonstrates that in addition to being important for NPC proliferation [14], Akt is also an important promoter of Sox2 expression, thereby driving NPC self-renewal. Importantly, however, Sox2 itself does not promote NPC proliferation, but it does inhibit neuronal and glial differentiation. Given that it is a key regulator of NPC proliferation and self-renewal, Akt is an attractive target for understanding the maintenance of other stem cell types, particularly ES cells and the generation of iPS cells.

Acknowledgments

We thank S. Ferguson and M. McVoy for the kind gift of reagents and Ashley Fritz for molecular biology assistance. This work was funded by a training grant fellowship from the California Institute for Regenerative Medicine (T1-00007) (to JP).

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CHAPTER 4

EFFECTS OF RAS AND CALCIUM SIGNALING ON ADULT NEURAL PROGENITOR PROLIFERATION AND SELF-RENEWAL

Abstract

Akt is an important mediator of basic fibroblast growth factor (FGF-2)-mediated proliferation and self-renewal in adult hippocampal neural progenitor cells (NPC). It is activated by two phosphorylation events; one is mediated by phosphoinositide 3-OH kinase (PI3K), and the other is likely mediated by mTOR complex 2 (mTORC2), representing a potential AND gate for NPC proliferation. Despite its demonstrated importance, the Akt signaling cascade is unlikely to be the only cascade responsible for NPC maintenance. Growth factor stimulation similar to that of FGF-2 is known to promote the activity of other key pathways, including Ras/mitogen activated protein kinase (MAPK) and calcium ion (Ca^{2+})-mediated pathways, in addition to Akt. There is also a great deal of cross-talk observed in multiple cell types between all three signaling modules. Here, I investigate the precise importance of Akt signaling using tamoxifen-inducible, conditionally active Akt and PI3K mutants (Akt-ER and PI3K-ER). While these results show that Akt is sufficient for NPC proliferation, PI3K is not, indicating that Akt is acting as an AND gate and that the second phosphorylation of Akt is PI3K independent. Using pharmacological and genetic manipulation of Ras, calmodulin (CaM), CaM kinase II (CaMKII), CaM kinase kinase (CaMKK), CaM kinase IV (CaMKIV), and calcineurin, I investigated the importance of these pathways for NPC proliferation and self-renewal. Constitutively active Ras (RasG12V) overexpression actually halted NPC proliferation and promoted differentiation. Although multiple pharmacological inhibitors of various Ca^{2+} signaling components inhibited NPC proliferation (including CaM, CaMKII, CaMKK, calcineurin, and Ca^{2+} channels), genetic overexpression of CaM, CaMKIV, and constitutively active CaMKK (CaMKKca) did not improve NPC proliferation or affect NPC differentiation or self-renewal. Similarly, CaM, CaMKII, and CaMKKca overexpression did not increase Akt phosphorylation. Notably, CaMKII overexpression did promote proliferation, but so did overexpression of its endogenous inhibitor CaMKIIN. Finally, calcineurin overexpression promoted astrocytic differentiation with no effect on neuronal differentiation. Although these results provide no clear indication that any of the investigated signaling cascades are important for NPC proliferation and self-renewal, it is still a critical step toward ruling out several major pathways, and this brings us closer to understanding the mechanisms behind this important feature of the central nervous system.

Introduction

The serine/threonine kinase Akt is critical for adult neural hippocampal progenitor cell (NPC) proliferation and self-renewal (Chapters 2 & 3 [1]) Its activity is necessary and sufficient for NPC proliferation. Additionally, Akt promotes expression of the transcription factor Sox2, which is a critical factor for NPC self-renewal [2], and it also functions as an NPC marker [3]. In NPCs, Akt is activated by the extracellular mitogen basic fibroblast growth factor (FGF-2) (Chapter 2 [1]). The FGF-2 receptor is a receptor tyrosine kinase (RTK); and like most signaling

events, binding of FGF-2 to its receptor on the cell surface can potentially trigger multiple downstream signals. For the FGF receptor, these include the Ras/mitogen activated protein kinase (MAPK) cascade and calcium ion-induced signaling events, in addition to the Akt signal [4, 5].

RTK signaling begins when FGF-2 binds to its receptor on the cell surface, causing the receptors to dimerize and autophosphorylate tyrosine residues in their intracellular domains. The phosphorylated tyrosine residues serve as binding sites for proteins with Src-homology 2 (SH2) domains, which fall into one of three categories: enzymes, adaptors, and docking proteins. It is this repertoire of potential binding partners that allows RTKs to activate multiple intracellular signaling pathways [6].

To activate the Akt pathway, RTK activation recruits phosphoinositide 3-OH kinase (PI3K) to the cell membrane where it catalyzes the production of the membrane-bound second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits Akt and PDK1 to the membrane. PDK1 phosphorylates Akt at the activation segment (T308), and a second phosphorylation event on the hydrophobic motif (S473) leads to complete activation of the molecule [7]. This second phosphorylation event is widely believed to be mediated by the mTOR complex 2 (mTORC2) in many cell types [8]. The Akt pathway is known to play important roles in cellular proliferation, growth, survival, metabolism, and migration [9]. The importance of Akt has been demonstrated in many stem cell types, including mouse embryonic stem (ES) cells [10, 11], primate ES cells [12], rabbit ES cells [13], mesenchymal stem cells [14], and hematopoietic stem cells [15, 16]. Additionally, we have previously reported the importance of Akt for NPC proliferation and self-renewal (Chapters 2 & 3, [1]). Others have shown the importance of signaling events downstream of Akt to NPC maintenance, including mTOR [17], FoxO [18], and GSK-3 [19].

The Ras/MAPK signal cascade begins when RTK activation at the cell membrane allows the adaptor Grb2 to bind. This recruits the guanine nucleotide exchange factor (GEF) Sos, which exchanges GDP for GTP on Ras. This GTP-bound, active form of Ras is then free to interact with and activate the kinase Raf through a complex process involving membrane interactions, phosphorylation, and de-phosphorylation events (reviewed in [6]). Once activated, Raf initiates a phosphorylation cascade by activating MEK, which in turn phosphorylates and activates ERK [6]. Additionally, there is the potential for cross-talk, because Ras can activate PI3K [20].

The Ras/MAPK cascade is well-known to regulate many cellular functions including proliferation, differentiation, and apoptosis [21]. It has been studied in many stem cell types and has different, cell type-specific effects. ERK inhibition promoted differentiation of rabbit embryonic stem (ES) cells [13]; however, ERK inhibition in mouse ES cells promoted self-renewal [22, 23]. In neural systems, there is conflicting evidence describing the effect of the Ras/MAPK cascade. Overexpression of constitutively active Ras in PC12 cells, a pheochromocytoma cell line often used as a model for neuronal differentiation, halts proliferation and forces them to differentiate into neurons [24]. Similarly, in a converse experiment, we observed that MEK inhibition only caused mild reduction in NPC proliferation (Chapter 2, [1]) and no obvious differentiation phenotype (data not shown). However, MEK inhibition in neural progenitor cells isolated from the sub-ventricular zone of the lateral ventricles did inhibit proliferation and increase apoptosis [25]. Furthermore, other groups working with hippocampal NPCs (my cells of interest) found that ERK mediates opioid-induced proliferation [26] and insulin-like growth factor (IGF-1)-induced proliferation [27]. Most of the above data was based on pharmacological inhibition of the Ras/MAPK pathway, which can

cause unintended off-target effects. Therefore, in this chapter I sought to rectify these conflicting results by overexpressing constitutively active Ras in NPCs and determining its effect on their proliferation and differentiation.

Calcium ion (Ca^{2+}) signaling impacts almost every cellular function. In the nervous system this includes neuronal development, synaptic transmission, and synaptic plasticity [28]. The three major signaling cascades potentially activated by Ca^{2+} influx are the protein kinase C (PKC), calmodulin (CaM)/CaM kinase II (CaMKII), and the CaM/CaM kinase kinase (CaMKK) pathways. To activate PKC signaling, phospholipase C- γ (PLC γ) associates with active RTKs via its SH2 domain. Then, it cleaves membrane bound phosphatidylinositol-4,5-bisphosphate into two second messengers: 1,2 diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG remains membrane associated, while IP3 is free to diffuse into the cytosol where it causes the release of Ca^{2+} stored in the endoplasmic reticulum. The cytoplasmic Ca^{2+} , together with DAG, activates PKC, allowing it to phosphorylate its substrates [5, 29].

In addition to PKC signaling, Ca^{2+} potentiates other signaling events through the small adaptor protein CaM. Ca^{2+} binding to CaM causes a conformational change that allows it to interact with other molecules [28]. One of these binding partners is CaMKII. Ca^{2+} /CaM binding relieves CaMKII autoinhibition and allows for autophosphorylation at T286, locking the molecule in an active conformation. This is considered one of the biochemical mechanisms of memory formation [30]. Opposing CaMKII activity is an endogenous inhibitor of CaMKII, called CaMKIIN [31] that is known to function in the central nervous system and elsewhere throughout the body [32].

The CaMKK cascade consists of CaMKK, CaM kinase I (CaMKI), and CaM kinase IV (CaMKIV). It is similar to the CaMKII cascade in that all of its players are activated by Ca^{2+} /CaM. When CaMKI or CaMKIV are bound to Ca^{2+} /CaM an activation loop phosphorylation site is exposed, which is phosphorylated by CaMKK for full activation [32].

In addition to promoting kinase cascades, Ca^{2+} /CaM can also activate the phosphatase calcineurin. Intracellular Ca^{2+} along with Ca^{2+} /CaM binds to calcineurin and relieves autoinhibition [33]. Calcineurin is important for immune response, cardiovascular function, and apoptosis, among others [34]. Intriguingly, a pharmacological inhibitor of calcineurin, cyclosporin A, has recently been shown to increase the survival of NPCs both *in vitro* and *in vivo* without affecting their proliferation or differentiation [35].

FGF-2-mediated Ca^{2+} is known to be important for proliferation and self-renewal of rat embryonic neural stem cells [36, 37]. Additionally, opioid-induced proliferation of adult hippocampal NPCs is Ca^{2+} -mediated [26]. However, Ca^{2+} influx in hippocampal NPCs due to GABAergic excitation promoted differentiation [38]. Further downstream, retinoic acid (a strong inducer of differentiation) down-regulates expression of CaMKK and CaMKIV in neuroblastoma cells, accelerating their differentiation [39]. Furthermore, CaMKIV can phosphorylate and activate the transcription factor CREB at S133 [40, 41], which we have shown is important for NPC proliferation (Chapter 2, [1]); however, other MAPK-mediated mechanisms can also phosphorylate CREB at the same site [42].

While there is evidence that each of these pathways alone could be important for NPC proliferation and self-renewal, a great deal of cross-talk is known to occur. This indicates many signaling pathways may be working in concert. Because genetic and/or pharmacological inhibition of Akt did not completely halt NPC proliferation or Sox2 expression (Chapters 2 & 3, [1]), our results show that other pathways are likely important for these critical NPC functions. CaM is known to mediate survival signaling in embryonic cortical neurons by activating Akt

[43]. Other studies have shown that $\text{Ca}^{2+}/\text{CaM}$ can bind directly to Akt [44] and that CaMKK can phosphorylate and activate Akt at T308 [45], representing a potential mechanism for CaM-mediated survival. Additionally, CaMKIIN, the endogenous CaMKII inhibitor, suppresses tumor growth by promoting cell cycle arrest [46] and inhibiting Akt signaling [47]. $\text{Ca}^{2+}/\text{CaM}$ can also activate the MAPK cascade through CaMKK/CaMKIV [48]. Furthermore, mTORC2, the kinase complex responsible for phosphorylating Akt at S473, can also phosphorylate PKC and promote its activity [49, 50].

Given this complex landscape of potential signaling mechanisms, I wanted to determine the precise importance of Akt signaling and whether other signaling cascades such as Ras/MAPK at Ca^{2+} were important to NPC proliferation and self-renewal. Through the use of conditionally active PI3K and Akt mutants, I found that while Akt activity is sufficient to promote NPC proliferation, PI3K activity is not. PI3K mediates only one (T308) of the two phosphorylation events required for Akt activation; however, FGF-2 stimulates both phosphorylation events. This indicated that some other kinase was responsible for S473 phosphorylation independent of PI3K. Since RTKs can also activate Ras/MAPK and Ca^{2+} signals in addition to Akt, and there is a great deal of cross-talk between these pathways, I then investigated whether Ras and Ca^{2+} signaling components could promote proliferation, self-renewal, and/or Akt activation. Overall, these experiments did not yield consistent clues that point to a critical mechanism for NPC proliferation or self-renewal; however, they do rule out the potential importance of several major signaling cascades and bring us closer to understanding the rules governing NPC fate choice.

Materials and Methods

Cell Culture

Adult neural progenitor cells isolated from the hippocampi of 6-week-old female Fischer 344 rats as described [51] were cultured on tissue culture polystyrene coated with poly-ornithine and 5 $\mu\text{g}/\text{ml}$ of laminin (Invitrogen) and grown in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) high-glucose medium (Invitrogen) containing N-2 supplement (Invitrogen) and 20 ng/ml recombinant human FGF-2 (Peprotech).

Mutant Cell Lines

Progenitor cells constitutively expressing mutant and wild-type versions of various signaling molecules were generated by retroviral infection. cDNAs encoding the following proteins were either purchased or given as kind gifts: constitutively active PI3K-estrogen receptor fusion mp110*-ER (PI3K-ER) (A. Klippel [52]), constitutively active Akt-estrogen receptor fusion mAkt-ER (Akt-ER) (B. Nelson [53]), constitutively active human HRas (RasG12V) (P. Khavari [54]), wild type rat calmodulin (CaM) (Harvard Institute of Proteomics—PlasmID), wild type rat CaMKII (T. Soderling [31]), wild type rat CaMKIIN (T. Soderling [31]), constitutively active rat CaMKK lacking the C-terminal regulatory domain (CaMKKca) (T. Soderling [55]), wild type rat CaMKIV (T. Soderling [32]), and constitutively active human calcineurin A containing only the catalytic subunit. Calcineurin residues 1-398 were amplified from the pET15a-CnACnB plasmid from Addgene [56] to generate the constitutively active calcineurin previously reported [57]. Additionally, a constitutively active PI3K (mp110*) PCR product with stop codon was amplified from the mp110*-ER fusion. All

cDNAs were subcloned into the MMLV retroviral vector CLGPIT, which is derived from CLPIT [58]; however, the puromycin selection gene is replaced with a gene encoding a GFP-puromycin fusion protein (M. McVoy [59]). An empty control vector was also produced. Correct products were confirmed by sequence analysis. Retroviral vectors were packaged using CMV gag-pol and CMV VSV-G envelope helper plasmids by calcium phosphate transfection as described [58]. Vectors were harvested, concentrated by ultracentrifugation, and titered on HEK 293Ts. Progenitor cells were infected at a multiplicity of infection of 1 IU/cell and were selected with 0.6 µg/ml puromycin (Sigma) for four days.

Proliferation Assays

For inhibitor studies, NPCs were plated in quintuplicate at 1000 cells/well on 96-well poly-ornithine/laminin-coated tissue culture plates with 20 ng/ml FGF-2 in DMEM/F-12 + N-2 medium. Where indicated, the following compounds were included (all from EMD Biosciences): KN-93 (CaMKII inhibitor, 10 µM), 2-APB (internal Ca²⁺ channel inhibitor, 75 µM), SKF-96365 (external Ca²⁺ channel inhibitor, 10 µM), bisindolylmaleimide (PKC inhibitor, 1 µM), U-73122 (PLC inhibitor, 0.5 µM), and W-7 (calmodulin inhibitor, 200 µM). Fifty percent media changes were conducted daily. After 5 days in culture, cell number was quantified using the WST-1 assay following the manufacturer's instructions (Roche) and utilizing a standard curve generated with known cell numbers.

Proliferation of mutant cell lines was also assessed by WST-1 in 96-well plate format, similar to above. Depending on the experiment, cells were grown in 0, 1, or 5 ng/ml FGF-2 with or without 200 nM 4-hydroxytamoxifen (4-OHT) (Sigma) in DMEM/F-12 + N-2 medium. A cell line infected with an empty control vector was used as a control. Fifty percent media changes were conducted daily, and proliferation was quantified using the WST-1 assay after 5 days in culture, as described above.

Quantitative RT-PCR

NPCs were seeded at 200,000 cells per well in 6-well poly-ornithine/laminin coated culture plates in DMEM/F-12 + N-2 medium containing 1% fetal bovine serum plus 1 µM retinoic acid (Biomol) to induce differentiation. Medium was replenished daily, and on day 5 RNA was isolated by TRIzol (Invitrogen) according to manufacturer's instructions. cDNA's were then generated using Invitrogen's ThermoScript RT-PCR kit according to manufacturer's instructions. Using a BioRad iCycler, Taqman probe QPCR was performed for the astrocytic marker GFAP, the neuronal marker β-Tubulin III, or the multipotent marker Sox2 with the 18S ribosomal subunit as an internal control. GFAP, β-Tubulin III, and Sox2 probes from Biosearch Technologies contained FAM490 fluorophore with Black Hole Quencher (BHQ), while the 18S rRNA probe contained CAL610 fluorophore with BHQ. Primer and probe sequences were as follows:

GFAP:

Sense: 5'-GACCTGCGACCTTGAGTCCT-3'
Antisense: 5'-TCTCCTCCTTGAGGCTTTGG-3'
Probe: 5'-FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ-3'

β -Tubulin III:

Sense: 5'-GCATGGATGAGATGGAGTTCACC-3'
Antisense: 5'-CGACTCCTCGTCGTCATCTTCATAC-3'
Probe: 5'-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3'

Sox2:

Sense: 5'-CGAGTGGAAACTTTTGTCCGAGAC-3'
Antisense: 5'-CGGGAAGCGTGTACTTATCCTTCTT-3'
Probe: 5'-FAM490-CTCTGCACATGAAGGAGCACC-BHQ-3'

18S rRNA:

Sense: 5'-GTAACCCGTTGAACCCCATTC-3'
Antisense: 5'-CCATCCAATCGGTAGTAGCGA-3'
Probe: 5'-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3'

Immunoblotting

NPCs were lysed by adding lysis solution directly to the culture plate. Lysis solution contained IGEPAL (1%, Sigma), sodium dodecyl sulfate (SDS, 0.1%), phenylmethanesulfonylfluoride (PMSF, 0.1 mg/ml, Sigma), aprotinin (0.03 mg/ml, Sigma), and sodium orthovanadate (1 mM, Sigma) in PBS. Lysates were clarified by centrifugation and protein concentrations were quantified by BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Equal amounts of protein from each lysate were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Blocking buffer was either 5% nonfat milk or 5% BSA in TBS + 0.1% Tween-20, according to the primary antibody manufacturer's instructions. After blocking, antibody was diluted in blocking buffer and incubated on the membrane overnight at 4°C. The following day, blots were rinsed and probed with secondary antibody in blocking buffer for 1 hr at room temperature. After another set of rinses, blots were incubated with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and imaged on Hyperfilm ECL (Amersham). In cases where blots were stripped and re-probed, phosphorylated epitopes were probed first, as previously described [60]. Blots were stripped with 0.5 M acetic acid + 0.5 M NaCl for 10 min, neutralized with 0.5 M NaOH, and rinsed twice with TBS before adding the second primary antibody in blocking buffer. Primary antibodies included rabbit anti-estrogen receptor α (Santa Cruz, sc-542, 1:200), rabbit anti-GAPDH (Abcam, ab9485, 1:2000), rabbit anti-phosphoT308 Akt (Cell Signaling, 4056, 1:1000), rabbit anti-phosphoS473 Akt (Cell Signaling, 9271, 1:1000), and rabbit anti-total Akt (Cell Signaling, 9272, 1:2000). Goat anti-rabbit HRP (Pierce, 31460, 1:10000) was used as a secondary antibody.

Results

PI3K Activity is not Sufficient for Full Akt Activation and NPC Proliferation

Because the PI3K/Akt pathway is important for NPC proliferation and self-renewal (see Chapters 2 & 3, [1]), I sought to determine the precise importance of the two major players:

PI3K and Akt. Constitutively active versions of either PI3K or Akt fused to a mutant murine estrogen receptor (ER) were overexpressed in NPCs. The constitutively active PI3K (mp110*) is a myristoylated chimera of the p110 catalytic subunit fused at the N-terminus to the inter SH2 (iSH2) domain of the p85 regulatory subunit via a flexible glycine linker [61]. Akt was made constitutively active by adding a myristoylation signal [62]. The ER is responsive only to 4-hydroxytamoxifen (4-OHT); therefore, adding 4-OHT to cultures expressing either of these fusion proteins (PI3K-ER [52] or Akt-ER [53]) directly stimulates kinase activity.

After retroviral transduction of NPCs with the fusion proteins, proper expression was confirmed by Western blotting and probing for the estrogen receptor (Figure 1A). To test the importance of PI3K and Akt in NPC proliferation, I cultured cells expressing these fusions in a 5 day proliferation assay in the presence of 200 nM 4-OHT, which directly stimulates either PI3K or Akt kinase activity. Ethanol was used as a carrier control, and all cultures were without FGF-2 (Figure 1B). When compared to cells expressing an empty control construct, there is no significant increase in proliferation of cells expressing PI3K-ER when cultured with 4-OHT. This is despite an increase in Akt phosphorylation at T308, the phosphorylation site directly influenced by PI3K activity (Figure 1C). However, cells expressing Akt-ER proliferated nearly 3-fold higher than control when cultured with 4-OHT, confirming previous results with wild type Akt (Chapter 2, [1]). This leads to the intriguing hypothesis that T308 phosphorylation is not sufficient for NPC proliferation and leaves the mechanism driving S473 phosphorylation unknown. Given that FGF-2 stimulation causes phosphorylation at both T308 and S473 [1], it appears likely that FGF-2 is independently activating the two separate kinases responsible for T308 and S473 phosphorylation.

Because the cells expressing PI3K-ER had a significant increase in proliferation when cultured with only carrier control, I sought to determine whether this was simply an experimental artifact. I also sought to confirm the 4-OHT result where proliferation was unaffected. Therefore, mp110* was PCR-amplified out of the PI3K-ER fusion construct and expressed in NPCs by retroviral expression. Proliferation of the resulting cells was measured along with empty vector control cells (Figure 1D). Only mp110* cells cultured with 1 ng/ml FGF-2 had a slight increase in proliferation compared to control. mp110* cells cultured with 0 or 5 ng/ml FGF-2 were no different from control. This confirms the above 4-OHT result that PI3K is not sufficient for NPC proliferation and that some other kinase downstream of FGF-2 must be responsible for phosphorylating Akt at S473 independently of PI3K.

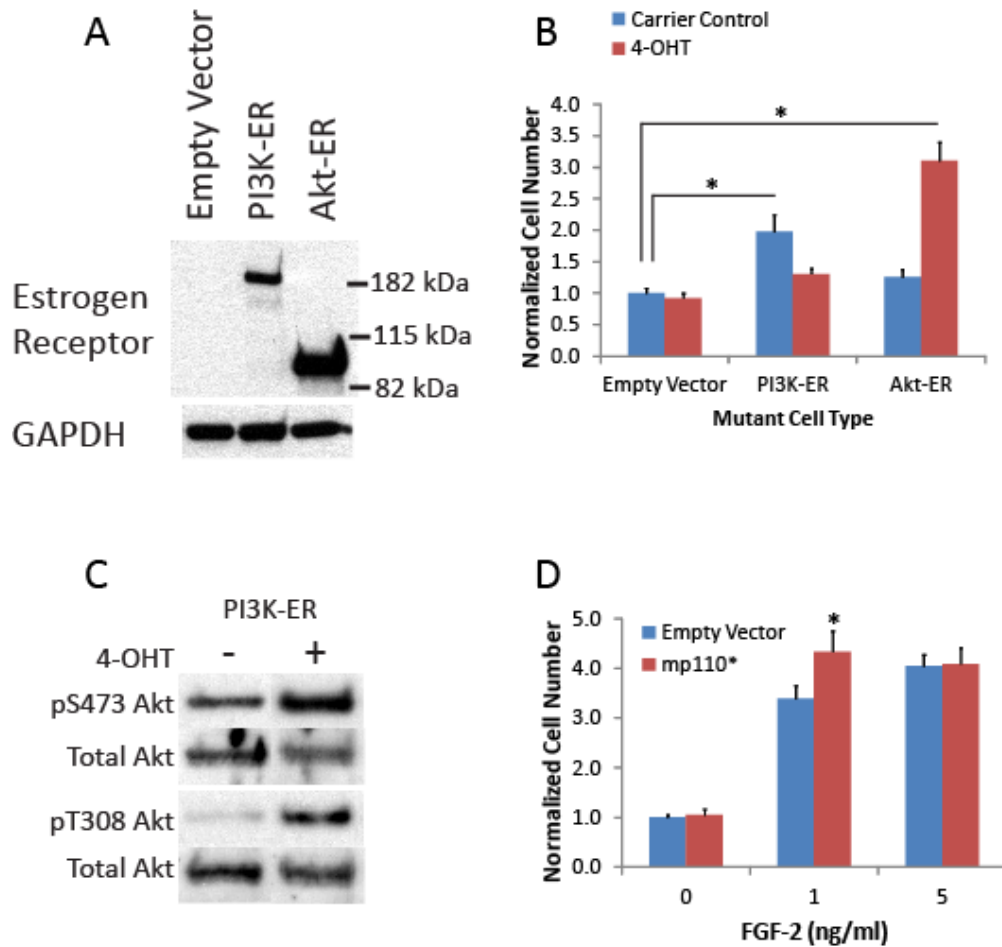


Figure 1: PI3K activity is not the only input necessary for full Akt activation and subsequent NPC proliferation. (A) Cells stably infected with retroviral vectors containing PI3K-ER, Akt-ER, or empty vector control were lysed and analyzed by immunoblotting for estrogen receptor to confirm expression. Blots were stripped and reprobbed for the loading control GAPDH. (B) Empty vector, PI3K-ER, and Akt-ER infected cells were seeded in media containing either 200 nM 4-OHT or carrier control (ethanol) without FGF-2. After 5 days, cultures were counted by WST-1. (C) PI3K-ER expressing cells were FGF-2-starved overnight, stimulated with 200 nM 4-OHT for 90 min, and the resulting lysate was probed for phosphorylated Akt at T308 and S473. Blots were stripped and reprobbed for total Akt. (D) Cells stably infected with constitutively active PI3K (mp110*) or empty vector were cultured in media containing 0, 1, or 5 ng/ml FGF-2. After 5 days, cells were counted by WST-1. Error bars are 95% confidence intervals, and * indicates $p < 0.05$.

Ras Overexpression does not Promote Proliferation or Self-renewal

Given that PI3K is not sufficient to promote NPC proliferation and that some other kinase downstream of FGF-2 is required for full Akt activation, I began investigating whether other pathways downstream of the FGF receptor could promote proliferation and/or Akt activation. In addition to the PI3K pathway, the Ras/MAPK pathway and Ca^{2+} signaling pathways can be activated by RTKs similar to the FGF receptor [4]. Pharmacological MEK inhibition does not hamper NPC proliferation as strongly as PI3K or Akt inhibition (Chapter 2, [1]), but Ras operates upstream of the MEK signal. To determine whether Ras signaling affects NPC proliferation, constitutively active Ras (RasG12V) was overexpressed. Despite culturing the cells with 20 ng/ml FGF-2, a strongly proliferative condition, cell growth completely halted 5

days after infection. Additionally, the cells displayed an elongated phenotype resembling differentiating cells (Figure 2A).

To test whether RasG12V expression was actually promoting differentiation, similar to that observed in PC12 cells [24], RasG12V cells cultured for 5 days with 20 ng/ml FGF-2 were lysed and analyzed for β -tubulin III (neuronal marker), GFAP (astrocytic marker), or Sox2 (multipotent marker) expression by quantitative RT-PCR (Figure 2B). Expression of both differentiation markers significantly increased in RasG12V expressing cells, while Sox2 expression markedly declined. This confirms the hypothesis that RasG12V is actually promoting differentiation instead of differentiation and self-renewal. Because my goal is to investigate the mechanisms of proliferation and self-renewal, rather than differentiation, I will not pursue this interesting result further.

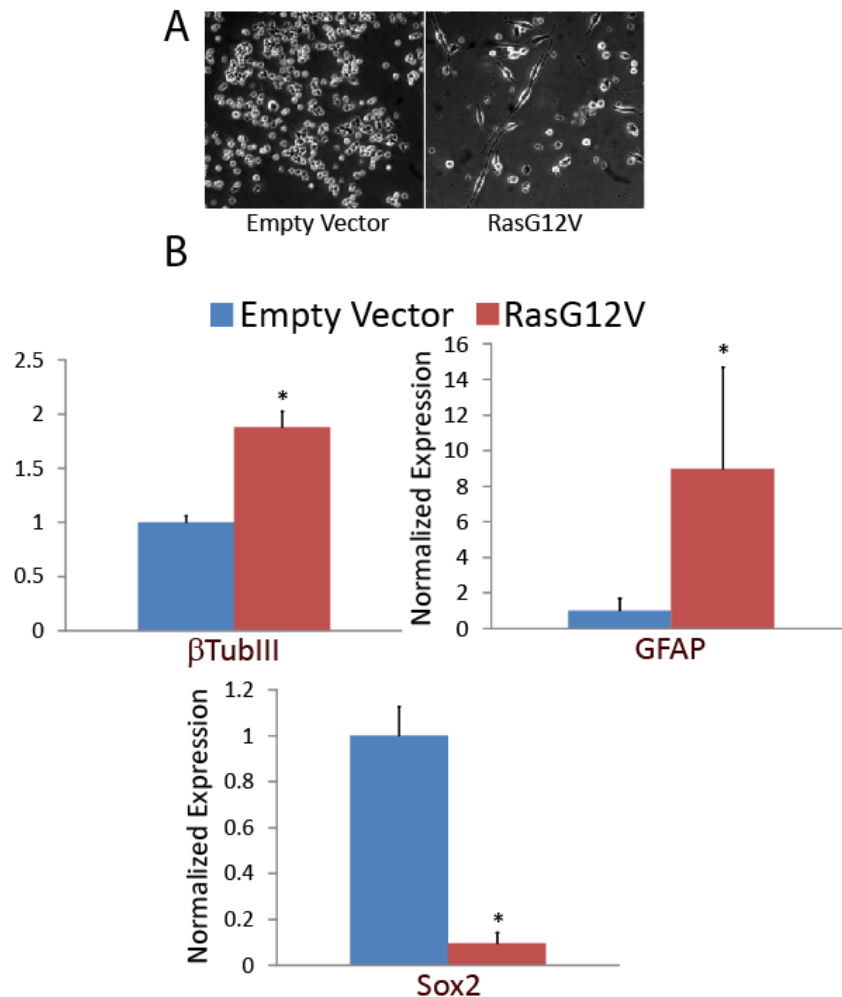


Figure 2: Ras overexpression does not promote proliferation or self-renewal. NPCs growing in 20 ng/ml FGF-2 were infected at MOI 1 IU/cell with retroviral vectors containing constitutively active Ras (RasG12V) or empty vector control and selected with puromycin. (A) After 5 days with 20 ng/ml FGF-2, RasG12V proliferation had ceased and cell morphology had significantly changed. (B) Cells were lysed, RNA was extracted, reverse transcribed, and the resulting cDNAs were analyzed for β -Tubulin III, GFAP, and Sox2 expression by quantitative RT-PCR. All samples were normalized to the 18S ribosomal subunit as an internal control. Error bars are 95% confidence intervals, and * indicates $p < 0.05$.

Various Ca²⁺ Signaling Inhibitors Prevent NPC Proliferation

As an initial screen to determine which, if any, Ca²⁺ signaling components may be involved in FGF-2-mediated NPC proliferation, I used a series of established inhibitors. Cells were cultured with 20 ng/ml FGF-2 and one of the following drugs along with the appropriate carrier controls: KN-93 (CaMKII inhibitor, 10 μM), 2-APB (internal Ca²⁺ channel inhibitor, 75 μM), SKF-96365 (external Ca²⁺ channel inhibitor, 10 μM), bisindolylmaleimide (PKC inhibitor, 1 μM), U-73122 (PLC inhibitor, 0.5 μM), W-7 (CaM inhibitor, 200 μM), and STO-609 (CaMKK inhibitor, 1 μg/ml) (Figure 3A-D).

Except for the PKC inhibitor bisindolylmaleimide, all drugs significantly reduced proliferation. Inhibitors of CaMKII (KN-93), internal Ca²⁺ channels (2-APB), external Ca²⁺ channels (SKF-96365) (Figure 3A), calmodulin (W-7) (Figure 3C), and CaMKK (STO-609) (Figure 3D) caused the most dramatic decreases in cell proliferation. Unsurprisingly, the strongest response came from the calcium channel inhibitors and the CaM inhibitor. These molecules are among the initial effectors of multiple Ca²⁺ signaling events including the PLC/PKC, CaMKII, and CaMKK pathways. Therefore, it is unsurprising that their inhibition should have such a profound effect. Collectively, these results indicate that Ca²⁺ signaling may be important for NPC proliferation. In particular, the CaMKII and CaMKK pathways appear to be most important, because their inhibition caused strong proliferation reductions. Therefore further analysis of proliferation and differentiation was focused on these two Ca²⁺ signaling pathways. However, because PKC inhibition did not affect proliferation, it was not included in subsequent analyses.

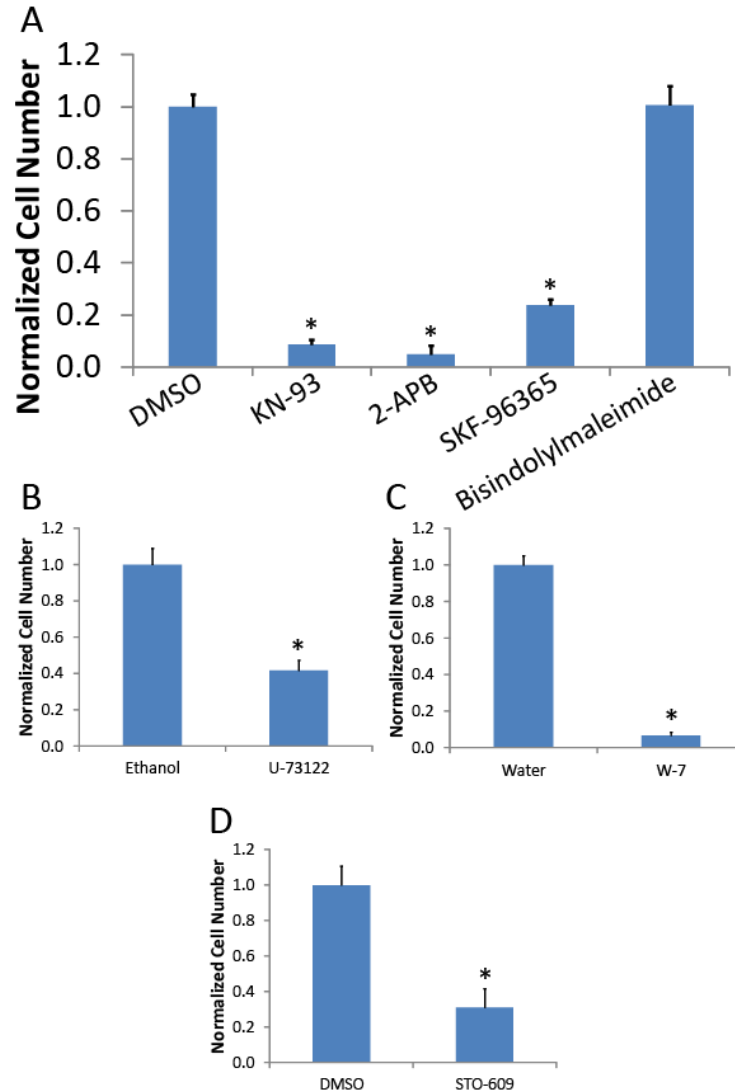


Figure 3: Various Ca^{2+} signaling inhibitors prevent NPC proliferation. NPCs were cultured with 20 ng/ml FGF-2 and one of the following drugs: (A) KN-93 (CaMKII inhibitor, 10 μ M), 2-APB (internal Ca^{2+} channel inhibitor, 75 μ M), SKF-96365 (external Ca^{2+} channel inhibitor, 10 μ M), bisindolylmaleimide (PKC inhibitor, 1 μ M), (B) U-73122 (PLC inhibitor, 0.5 μ M), (C) W-7 (calmodulin inhibitor, 200 μ M), and (D) STO-609 (CaMKK inhibitor, 1 μ g/ml) (B) U-73122 was dissolved in ethanol, (C) W-7 was dissolved in water, and (A,D) all others were dissolved in DMSO. After 5 days, cells were counted by WST-1. Error bars are 95% confidence intervals, and * indicates $p < 0.05$.

Genetic Overexpression of Ca^{2+} Signaling Proteins do not Consistently Improve NPC Proliferation

Because pharmacological inhibitors of signaling components are known to have unintended and unforeseen off-target effects, the CaMKII and CaMKK inhibitor data must be confirmed by genetic manipulation of these molecules' activities. Therefore, I retrovirally expressed multiple wild type and mutant versions of these proteins and measured their proliferation compared to control at various FGF-2 concentrations. The proteins studied included wild type CaM, wild type CaMKII, wild type CaMKIIN (an endogenous inhibitor of CaMKII activity), constitutively active CaMKK (CaMKKca), and wild type CaMKIV.

Overexpressing CaM, which is the upstream regulator of both CaMKII and CaMKK/CaMKIV, had no positive effect on NPC proliferation (Figure 4A). In fact, it slightly reduced proliferation when cultured with 5 ng/ml FGF-2. Because CaM has multiple downstream effects that could be both positive and negative, I investigated other downstream molecules to specifically identify a potentially critical player in NPC proliferation.

Intriguingly, CaMKII and its natural inhibitor CaMKIIN both significantly improved NPC proliferation but only in the presence of FGF-2 (Figure 4B). Although the CaMKII result was encouraging and agreed with the drug results, the CaMKIIN data was puzzling. It could potentially indicate that either high or low levels of CaMKII signaling can potentiate NPC proliferation, while the moderate levels encountered under normal conditions do not. It is also interesting to note that the positive effects were only observed with FGF-2, indicating that an extracellular proliferative signal is required for proper activation.

Another pathway highlighted by the drug screen was the CaMKK pathway. Neither CaMKKca overexpression (Figure 4C) nor overexpression of its downstream effector CaMKIV (Figure 4D) improved NPC proliferation. In fact, when cultured with FGF-2, CaMKIV reduced proliferation.

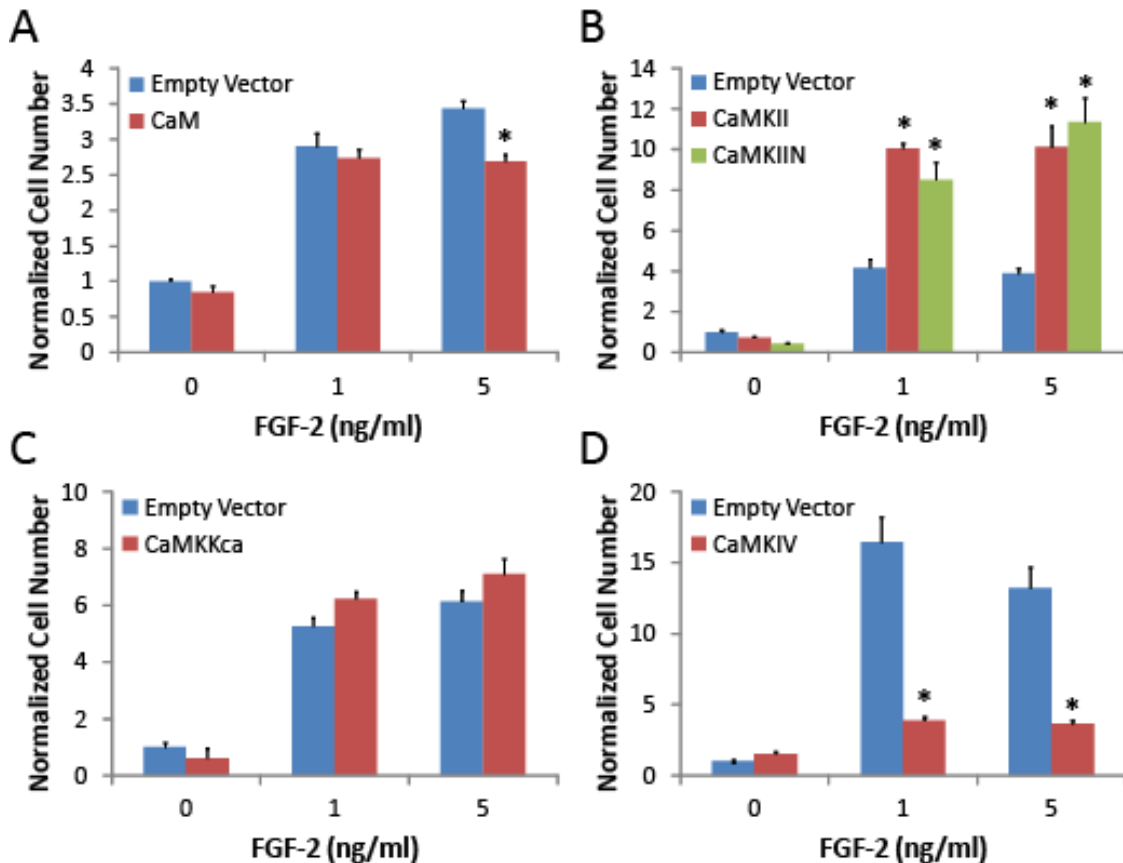


Figure 4: Genetic overexpression of Ca²⁺ signaling proteins do not improve NPC proliferation. Cells stably infected with retroviral vectors containing (A) calmodulin (CaM), (B) CaMKII, CaMKIIN, (C) constitutively active CaMKK (CaMKKca), (D) CaMKIV, or empty vector control were cultured with the indicated FGF-2 concentrations. After 5 days, cells were counted by WST-1. Error bars are 95% confidence intervals, and * indicates p < 0.05.

Key Ca²⁺ Signaling Molecules do not Promote Akt Activation, Self-Renewal, or Differentiation

Because none of the investigated proteins consistently improved NPC proliferation, I wanted to confirm that they had no effect on activation of Akt, a molecule that is important for NPC proliferation and self-renewal (Chapters 2 & 3, [1]). CaM, CaMKII, CaMKKca, and empty vector control cells were cultured overnight without FGF-2, lysed, and probed for phosphorylated Akt at T308 and S473 (Figure 5A-C). Akt phosphorylation did not increase for any of the above conditions. Cells overexpressing CaM actually had reduced phosphorylation at both T308 (site controlled by PI3K) and S473, while CaMKKca caused reduced phosphorylation at only S473. There was no change in cells overexpressing CaMKII. Given that Akt is critical for NPC proliferation, these results may help explain the lack of proliferation seen above.

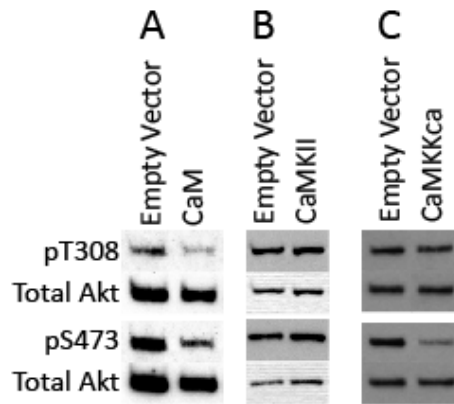


Figure 5: Key Ca²⁺ signaling molecules do not promote Akt activation. Cells stably infected with retroviral vectors containing (A) CaM, (B) CaMKII, (C) CaMKKca, or empty vector control were cultured overnight with 0 ng/ml FGF-2 and lysed. Lysates were analyzed by immunoblotting for phospho-T308 and phospho-S473 Akt. Blots were then stripped and reprobed for total Akt as loading control.

Given the lack of proliferation and Akt activity seen with the CaM, CaMKII, and CaMKKca overexpressing cells, perhaps these molecules have an effect on NPC differentiation. Cells were cultured under differentiation conditions strongly favoring the production of both neurons and astrocytes (1 μ M retinoic acid + 1% FBS). After 5 days, quantitative RT-PCR of lineage markers and the multipotency marker Sox2 was used to analyze differentiation as previously reported [63] (Figure 6A-C). Compared to empty vector control cells, mutant cells had unchanged expression of both the neuronal marker β -tubulin III and the astrocytic marker GFAP, compared to empty vector control cells. Similarly, Sox2 expression was unchanged compared to control. These results indicate that neither the CaMKII nor CaMKK signaling pathways affect NPC differentiation or self-renewal.

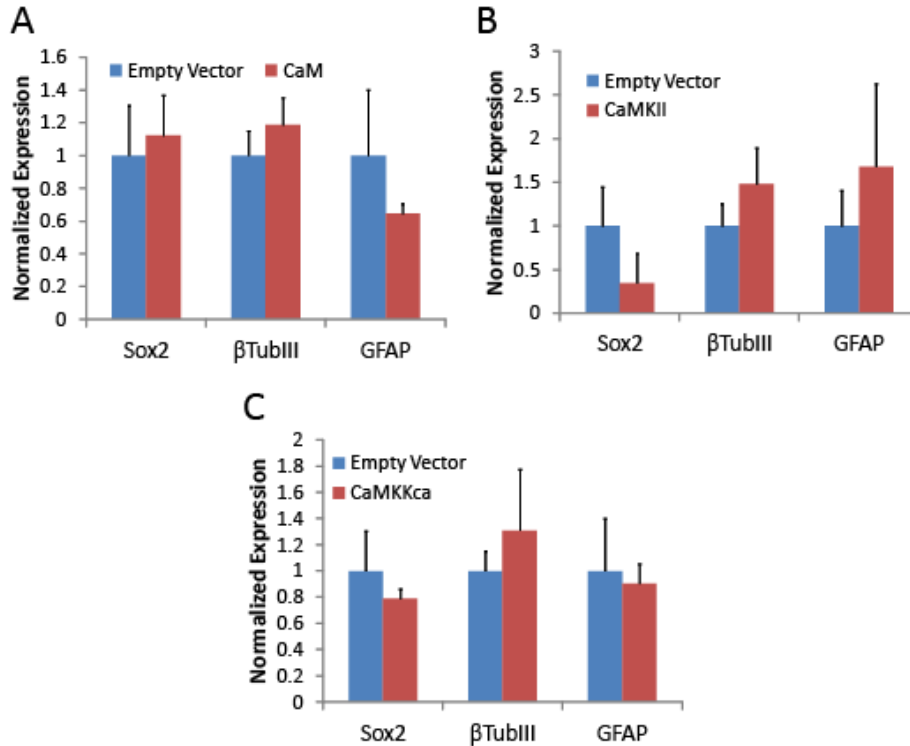


Figure 6: Key Ca²⁺ signaling molecules do not promote self-renewal or inhibit differentiation. Cells stably infected with retroviral vectors containing (A) CaM, (B) CaMKII, (C) CaMKKca, or empty vector control were cultured under differentiating conditions (1 μM retinoic acid + 1% FBS) for 5 days. RNA was extracted, reverse transcribed, and quantitative PCR was performed on the resulting cDNAs for Sox2, β-tubulin III, and GFAP. All samples were normalized to the 18S ribosomal subunit as an internal control. Error bars are 95% confidence intervals, and * indicates p<0.05.

Calcineurin Overexpression does not Promote Proliferation or Self-Renewal

The above experiments focused on the effects of Ca²⁺/CaM-regulated kinases, but Ca²⁺/CaM also regulates the physiologically important phosphatase, calcineurin [33]. Cyclosporin A, a known calcineurin inhibitor, inhibited NPC proliferation (Figure 7A) in contrast to a recent study showing that cyclosporin A promotes NPC survival [35]. Despite these cyclosporin effects, calcineurin overexpression had no effect on NPC proliferation (Figure 7B). Additionally, Sox2 expression in cells cultured under differentiation conditions was unaffected by calcineurin (Figure 7C), indicating no effect on self-renewal. Although the neuronal marker β-tubulin III was unaffected by calcineurin overexpression, the astrocytic marker GFAP was upregulated approximately 10-fold. This may indicate that calcineurin is an important mediator of astrocytic differentiation, and further investigation may be warranted.

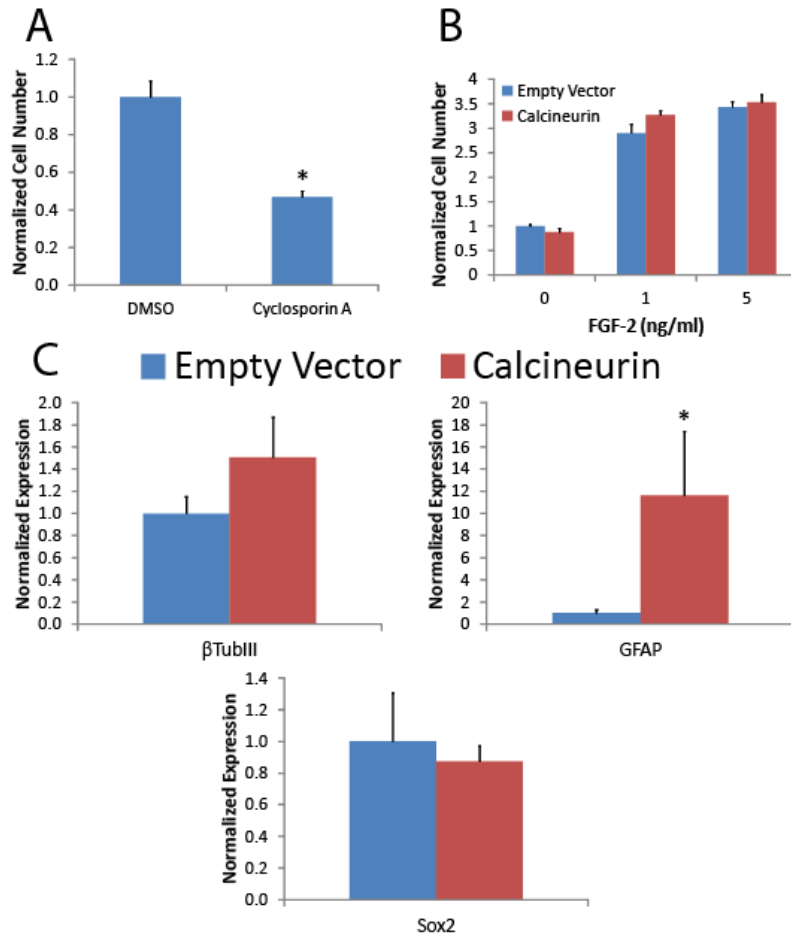


Figure 7: Calcineurin overexpression does not promote proliferation or self-renewal. (A) NPCs were cultured with 1 ng/ml FGF-2 and 0.5 μ M cyclosporin A (calcineurin inhibitor) or carrier control (DMSO). After 5 days, cells were counted by WST-1. (B) Cells stably infected with retroviral vectors containing calcineurin or empty vector control were cultured with the indicated FGF-2 concentrations. After 5 days, cells were counted by WST-1. (C) Cells expressing calcineurin or empty vector control were cultured 5 days under differentiating conditions (1 μ M retinoic acid + 1% FBS). RNA was extracted from lysates, reverse transcribed, and quantitative PCR was performed on the resulting cDNAs for β -tubulin III, GFAP, and Sox2. All samples were normalized to the 18S ribosomal subunit as an internal control. Error bars are 95% confidence intervals, and * indicates $p < 0.05$.

Discussion

Although direct stimulation of Akt via a conditionally active mutant confirmed that it is sufficient for NPC proliferation, PI3K activity is not. This indicates that Akt is acting as an AND gate with inputs from PI3K and some other kinase independent of PI3K. Because RTK signaling can potentially activate Ras/MAPK and Ca^{2+} signaling in addition to Akt, and there is a great deal of cross-talk, I investigated the effects of these pathways on NPC proliferation, differentiation, and self-renewal. Overall, no clear trends emerged. Pharmacological inhibitors of multiple Ca^{2+} signaling pathways inhibited proliferation, highlighting their potential importance, but genetic manipulation of those pathways did not support that assertion. Furthermore, neither CaM/CaMKK nor CaM/CaMKII promoted Akt activation or self-renewal. Ras overexpression actually halted NPC proliferation, despite strongly proliferative culture conditions, and it promoted astrocytic and neuronal differentiation. Overall, this leaves us with a

mystery as to what signaling events other than Akt could be promoting NPC proliferation and self-renewal.

Akt phosphorylation at T308 is mediated by PI3K/PDK, and the conditionally active PI3K mutant confirmed this in NPCs. However, complete Akt activation requires a second phosphorylation event at S473, which is mediated by mTORC2 [8, 64]. Although growth factor stimulation promotes S473 phosphorylation, the mechanisms controlling mTORC2 remain a mystery. My data with conditional PI3K and Akt mutants show that this mechanism must be PI3K independent. There is evidence that other kinases can phosphorylate S473, including PDK1 [65], integrin-linked kinase (ILK) [66], Akt itself [67], and DNA-PK [68]; therefore, the potential exists for redundancy with mTORC2. This is a major reason why I chose to investigate the effects of other common signaling pathways downstream of FGF-2. Based on pharmacological inhibitor data, Ca^{2+} /CaM-mediated signaling seemed most promising, but genetic overexpression could not promote S473 phosphorylation. Therefore, the mechanisms transmitting the signal from FGF-2 receptor to S473 phosphorylation remain a mystery.

I also investigated the effects of Ras overexpression, which promoted differentiation and halted proliferation, despite evidence that RasG12V is an oncogene [69]. Given my previous data showing the importance of PI3K/Akt signaling for NPC proliferation and self-renewal (Chapters 2 & 3, [1]), it appears unlikely that this phenotype is caused by Ras-mediated PI3K activation [20]. Nevertheless, the ability of this constitutively active Ras to promote NPC differentiation may warrant further investigation.

Pharmacological inhibition of many Ca^{2+} -mediated signaling events inhibited NPC proliferation; however, these drugs can have unintended off-target effects. For instance, the CaMKII inhibitor KN-93 is known to also inhibit members of the CaM/CaMKK cascade [32]; therefore, it is crucial to confirm these results via genetic manipulation. Overexpression of CaM, CaMKK, CaMKIV, or calcineurin did not improve NPC proliferation. In fact, CaMKIV overexpression actually reduced NPC proliferation in the presence of FGF-2, despite its known ability to activate CREB [40], which we have shown promotes proliferation (Chapter 2, [1]). Furthermore, expression of these Ca^{2+} -regulated genes had no effect on NPC differentiation or self-renewal as measured by quantitative RT-PCR. Notably, however, calcineurin overexpression did promote astrocytic differentiation, and that may be worthy of future investigation.

One notable exception is CaMKII. Its overexpression promoted NPC proliferation in the presence of FGF-2; however, overexpression of its endogenous inhibitor CaMKIIN had a similar effect. The likelihood of off-target CaMKIIN effects is low (as reviewed [32]); therefore, this puzzling result may indicate that either low or high CaMKII activity promotes NPC proliferation. Others have reported in ovarian cancer cells that CaMKIIN can downregulate Akt activity [47], but that mechanism appears unlikely to function in NPCs. Rectifying these seemingly contradictory results could potentially be an intriguing future study.

While this work has yielded some interesting results, there is still no clear indication of what other signaling mechanisms may be important for NPC proliferation and self-renewal. It seems that Ca^{2+} -mediated signaling plays only a minor role. However, CaMKII/CaMKIIN yielded results that may be worth investigating, and Ras signaling seems to promote differentiation rather than proliferation. Despite the lack of a clear mechanism, I have still taken the critical step of ruling out these well-known, important signaling cascades, and these results bring us closer to better understanding the mechanisms of NPC proliferation and self-renewal.

Acknowledgments

For the kind gift of reagents, I thank the following: A. Klippel for PI3K-ER; B. Nelson for Akt-ER; P. Khavari for RasG12V; T. Soderling for CaMKII, CaMKIIN, CaMKKca, and CaMKIV; and M. McVoy for the GFP-puromycin fusion.

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CHAPTER 5

SYSTEMS BIOLOGY APPROACHES TO UNDERSTANDING STEM CELL FATE CHOICE

This chapter is a postprint of a paper submitted to and accepted for publication as

Peltier J, Schaffer DV. (2010). Systems Biology Approaches to Understanding Stem Cell Fate Choice. IET Syst Biol 4:1-11.

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The copy of record is available at <http://www.ietdl.org/IP-SYB>.

Abstract

Stem cells have the capability to self-renew and maintain their undifferentiated state or to differentiate into one or more specialized cell types. Stem cell expansion and manipulation *ex vivo* is a promising approach for engineering cell replacement therapies, and endogenous stem cells represent potential drugable targets for tissue repair. Before we can harness stem cells' therapeutic potential, however, we must first understand the intracellular mechanisms controlling their fate choices. These mechanisms involve complex signal transduction and gene regulation networks that feature, for example, intricate feed-forward loops, feedback loops, and cross-talk between multiple signaling pathways. Systems biology applies computational and experimental approaches to investigate the emergent behavior of collections of molecules and strives to explain how these numerous components interact to regulate molecular, cellular, and organismal behavior. Here we review systems biology, and in particular computational, efforts to understand the intracellular mechanisms of stem cell fate choice. We first discuss deterministic and stochastic models that synthesize molecular knowledge into mathematical formalism, enable simulation of important system behaviors, and stimulate further experimentation. In addition, statistical analyses such as Bayesian networks and principal components analysis (PCA)/partial least squares (PLS) regression can distill large datasets into more readily-managed networks and principal components that provide insights into the critical aspects and components of regulatory networks. Collectively, integrating modeling with experimentation has strong potential for enabling a deeper understanding of stem cell fate choice and thereby aiding the development of therapies to harness stem cells' therapeutic potential.

Introduction

Stem cells – first discovered in mouse bone marrow by Becker, Till, and colleagues [1, 2] – are defined by their two hallmark properties: (1) self-renewal, or extended maintenance and potentially proliferation in an undifferentiated state, and (2) differentiation into one or more specialized cell types. Pluripotent embryonic stem (ES) cells can give rise to any cell type in an adult organism, whereas multipotent adult stem cells are capable of generating a more limited set

of cell types, typically ones in the tissue in which stem cells reside. Their ability to self-renew or differentiate into multiple cell types makes stem cells promising therapeutic candidates in cell replacement therapies for multiple injuries and diseases, including diabetes [3], spinal cord injury [4], and Alzheimer's disease [5], among others. However, before we can harness stem cells' therapeutic potential and guide their production of a desired cell type, we must first identify the factors and understand the mechanisms that govern their behavior and fate choices.

Whether in a multicellular organism or a culture dish, a stem cell constantly receives environmental cues in many forms: soluble cues from proteins such as mitogens and cytokines [6-9], small molecules [10, 11], and nutrients [12, 13]; as well as "solid phase" cues such as cell-cell contacts and the biochemical and mechanical properties of the extracellular matrix, including signals immobilized to it [14-17]. These signals guide the stem cell towards specific behaviors, such as survival, apoptosis, self-renewal, or differentiation into one of multiple lineages (Figure 1). An instructive view of stem cell fate choice states that environmental cues initiate the intracellular signals that direct the cells to their fate, while a selective mechanism indicates that environmental factors merely support the survival of certain fates. It appears likely that both of these modes operate in different tissues and circumstances [18]. Regardless of which mechanism is operating, however, stem cell behavior is guided by molecular interactions and reactions involving receptors, signaling networks, and transcription factors. In particular, signal processing networks that relay input signals from the cell surface to the nucleus feature complex, nonlinear components such as feed-forward and feedback loops, signal amplification cascades, and crosstalk between multiple signaling pathways. Information processing continues within the nucleus where transcription factor networks control the expression of themselves and each other, in addition to the target genes required for execution of fate choice. The result is a complex, multi-level, non-linear system that can exhibit a number of rich behaviors, including switches and oscillations [19-22]. These behaviors are critical regulators of stem cell self-renewal and differentiation, and in many ways they are difficult to investigate and interpret intuitively without the aid of systems-level analysis and the accompanying mathematical tools. A topic closely related to stem cell fate choice, organismal development, is also studied by systems biologists. However, we will focus on the molecular mechanisms of fate choice within single cells and refer readers to an excellent review of models of multi-cellular pattern formation [23].

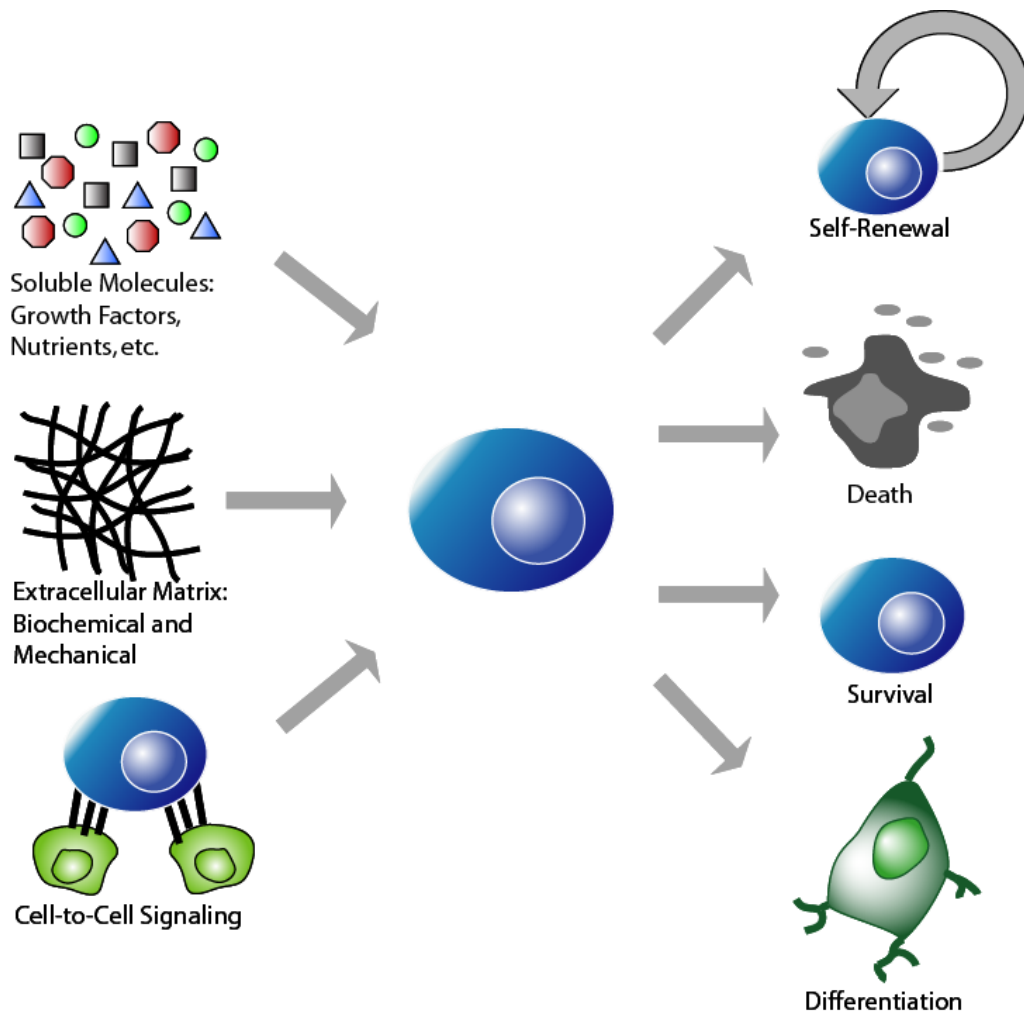


Figure 1: A stem cell as a signal processor. A stem cell receives multiple signals from its environment: soluble factors such as mitogens, cytokines, and nutrients; the biochemical and mechanical properties of the extracellular matrix; and contacts from neighboring cells. The cell processes these signals to determine its fate: self-renewal, survival, apoptosis, or differentiation.

Systems biology is a field that studies the collective behavior of groups of complex, interacting biological components. Its approaches offer advantages that complement and enhance traditional reductionist experimental avenues that tend to focus more on individual components than on interactions occurring within a larger scale system. Systems biology analyses of large biological systems such as cells often rely on computational models, which serve many uses: 1) they summarize our knowledge of and assumptions about a system into formal, mathematical statements; 2) they highlight gaps in our knowledge of a system; 3) they generate hypotheses about the behavior of the system that motivate experimentation and further modeling; 4) they aid in the analysis of large datasets - such as those generated by genomic, transcriptomic, proteomic, and kinomic experimentation - and thus summarize the data and highlight important, potentially unintuitive behavior for future experimentation [24-27], and 5) they highlight critical loci within a system that can be manipulated to generate a desired outcome. As examples of the last point, a model can be used to pinpoint drugable therapeutic

targets that direct endogenous stem cell pools to a desired fate or to enable the design of strategies to optimize *ex vivo* expansion for cellular therapy [28, 29].

In this review, we discuss systems biology modeling approaches and techniques that are increasingly utilized to understand the intracellular mechanisms of stem cell fate choice. Deterministic and stochastic computational models have formally synthesized our molecular knowledge into mathematical statements, furthered our understanding of important network behaviors, and motivated future experimentation. Statistical analyses such as Bayesian networks and principal components analysis have distilled large datasets into tractable candidate networks and principal components that are then used to derive insight into the critical pieces of the fate choice network. Collectively, these efforts have furthered the stem cell field and brought us closer to the eventual goal of harnessing the therapeutic promise of stem cells.

Deterministic Models

A deterministic model, one that always yields the same result given the same set of initial conditions, often consists of a set of either ordinary or partial differential equations that typically model the mass action of molecular species. Ordinary differential equations (ODEs) are used in situations where the system (i.e. the intracellular or extracellular region) is spatially homogeneous. This assumption can be relaxed somewhat by compartmentalizing the system into several homogeneous sub-systems with transport between them. Partial differential equations (PDEs) are used when spatial heterogeneity in species concentrations or other dependent variables becomes more complex and must be captured in order to accurately model the system. These ODE or PDE based models can be analyzed at steady state or dynamically, yielding either algebraic equations or spatially dependent PDEs/ODEs, respectively.

Deterministic models are often used to simulate the intracellular chemical reactions and interactions regulating stem cell fate choice, including ligand-receptor dynamics, signal transduction pathways, and transcription factor networks. To do so the modeler must have a precise understanding of these constituent molecular interactions within the network and knowledge of their rate and binding constants. The latter in particular is often limiting, as in many systems there is a dearth of experimentally measured constants, often requiring estimation of constants based on analogous systems.

Models are most often solved numerically, and the resulting output is kinetic information about the quantities of network species as a function of time and/or at steady state. This enables an investigation of how different parameters that describe molecular interactions (e.g. binding or catalysis) affect system behavior, driving the formation of new hypotheses. Additionally, models may be used to identify sensitive loci within the network, i.e. locations where small perturbations can exert a strong effect on cell behavior. These could represent “drugable targets” for therapeutic intervention or failure points where natural mutations may adversely affect system function and lead to disease.

In an early effort to quantitatively understand the molecular basis for stem cell fate choice, a ligand-receptor signaling threshold (LIST) model was proposed [30, 31]. This model posited that a threshold level of ligand-receptor signaling is an important determinant of stem cell fate and was useful in predicting cellular responses to various cytokine concentrations. It even demonstrated that a cytokine’s ability to maintain pluripotent ES cells was dependent on its receptor binding properties, such as heterodimerization vs. homodimerization. The intracellular

signal transduction and gene regulation processes, however, were not treated within the scope of this model.

Several papers have used deterministic models to mathematically investigate the role of intracellular signal transduction and transcription factor networks in stem cell fate choice. One behavior that emerges is network bistability, where as an input parameter is continuously varied, the system output transitions between one, then two, then one stable steady state solution (Figure 2). The two bifurcation points, where the number of stable solutions transitions between one and two, represent quantitative input threshold levels where the system qualitatively switches state. Bifurcation thus serves as an analog-to-digital converter to translate a graded input signal into an unambiguous, “all or nothing” behavioral response (e.g. self-renewal vs. differentiation). The behavior also serves a second important function. As discussed below (Stochastic Models), noise is a feature of many biological systems, and bistable systems exhibit hysteresis, where for example the input level at which the system switches from the first to the second state is higher than that at which it flips back from the second to the first (Figure 2). Hysteresis thus filters the noise within a system to prevent potentially deleterious rapid switching between states that would otherwise result from noise in the input parameter or signal. The initial example of such behavior in a stem cell regulatory network was the Sonic hedgehog (Shh) signaling system [19]. Shh, which patterns tissues during development and is an important mitogen for adult neural stem cells [6], drives expression of the transcription factor Gli1, which positively regulates its own transcription. Furthermore, Gli1 upregulates the expression of Patched, a repressor of Shh signaling. This nested positive and negative feedback renders an intuitive understanding of the pathway behavior difficult, and a Shh network model found that Gli1 expression exhibits bistability as a function of the input Shh concentration [19]. This outcome demonstrates one possible mechanism by which stem cells commit to a specific fate, i.e. once Shh concentration exceeds a threshold, and Gli1 expression switches to a “high” state, a moderate decrease in Shh will not return Gli1 expression to the “low” state.

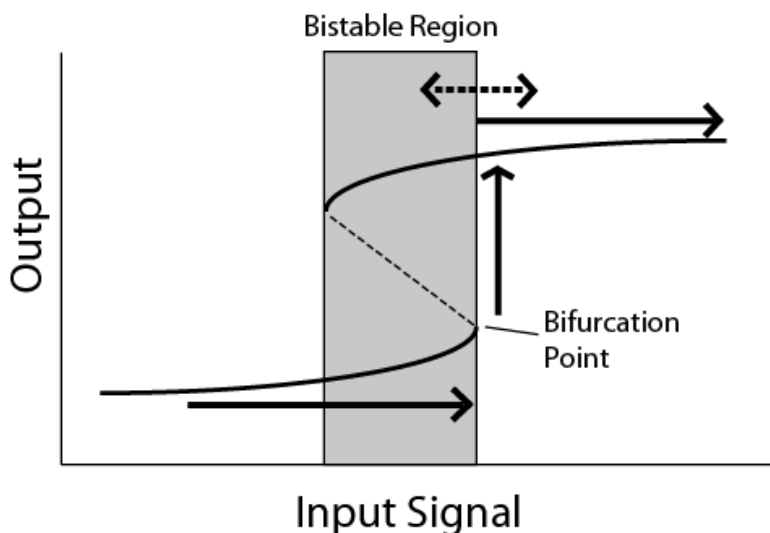


Figure 2: Bistability acts as a cell fate switch. In a bistable system, a range of input values generates two stable output solutions (the bistable region). When the input value increases to the bifurcation point the system switches state and “jumps” to the upper portion of the curve. That is, the system converts the analogue input signal to a digital output (i.e. fate choice). Once the system has switched states, hysteresis allows it to robustly resist the effects of input noise (represented by dashed double arrow). The system is unable to “jump” back to the lower curve unless the input signal decreases below the bistable region.

Several additional efforts have modeled transcription factor networks that exhibit bistability [20, 21]. Chickarmane *et al.* [20] modeled the interplay between three canonical pluripotency transcription factors crucial for ES cell self-renewal: Oct4, Sox2, and Nanog. Each of these genes positively regulates the expression of the others in addition to itself, as well as downstream target genes that either maintain pluripotency or induce differentiation [32-34]. This network gives rise to bistable expression of Oct4, Sox2, and Nanog, leading to a plausible mechanistic explanation for pluripotency maintenance. Since the publication of this work, however, other work has discovered additional transcription factors that play a role in maintaining pluripotency [35]. Future investigations of this updated network should prove interesting.

One genetic switch in hematopoietic stem cells involves the transcription factors GATA-1 and PU.1. Low GATA-1 and PU.1 expression maintain the cell in an undifferentiated state, while dominant expression of GATA-1 promotes the erythroid/megakaryocyte lineage, and PU.1 promotes the myeloid lineage. GATA-1 and PU.1 both stimulate their own transcription and inhibit that of the other, resulting in a network that generates a bistable, genetic toggle switch [21]. Huang *et al.* examined the same GATA-1/PU.1 system to determine how a cell transitions from a progenitor state to one of the two possible differentiated progeny [36]. They used a simple model of the GATA-1/PU.1 network, consisting of two ODEs, to generate what they termed an “attractor” landscape, which is loosely analogous to a potential landscape where the stable cell states (progenitors or their differentiated progeny) reside in alternate “wells.” Using this model, they could investigate how the shape of this landscape varies with levels of transcription factor auto-stimulation, cross-inhibition, and decay. They then analyzed possible mechanisms by which the landscape could be altered by the changes in transcription factor expression and activity that are triggered by differentiation signals. They found that the paradigm that resulted in the best match between the model results and experimental measurements of mRNA levels was one in which a differentiation signal altered the transcription factor landscape such that the “progenitor point” was no longer in a valley but rather on a peak. This rendered the progenitor state unstable and forced the cell into a stable differentiated state. The authors found that this landscape shift could be accomplished by a reduction in transcription factor auto-stimulation, an increase in transcription factor decay, or both.

In addition to bistability, recent work has shown that network oscillations may also play a role in stem cell fate choice, specifically in the maintenance of adult neural stem cells by Notch signaling [22]. Activation of the Notch pathway stimulates expression of the Hes family of transcription factors, which then inhibit their own transcription. This pathway can act as a switch that is important for developmental pattern formation [37, 38] or as an oscillator [39, 40] that is important for stem cell maintenance [22]. We have recently developed a single mathematical model that demonstrates that the Notch network can operate as a switch or an oscillator depending on the value of one key parameter. Specifically, tuning a single factor – the extent to which Hes1 binding reduces its expression – causes the network to transition between functioning as a bistable switch and an oscillator [41].

There have been additional efforts to mathematically model common signaling pathways downstream of key growth factors and cytokines [42, 43]. These include the effects of neurotrophin-3 (NT-3) on the MAPK pathway in embryonic stem cell-derived neural progenitors. The results provided insight into threshold levels of NT-3 stimulation and MAPK activity required for neuronal differentiation [42]. A similar study quantitatively studied the intracellular response to the cytokine leukemia inhibitory factor (LIF), which is crucial for

murine ES cell self-renewal. This work uncovered a likely positive feedback loop that stimulated production of components of the LIF signaling pathway during LIF signaling; however, removal of LIF causes lower expression of LIF signaling factors, thereby decreasing the cell's overall sensitivity to LIF. The authors demonstrated that this desensitization was a precursor to differentiation and loss of ES cell markers [43].

Furthermore, a systems-level analysis of stem cell fate choice can highlight potentially non-intuitive therapeutic targets for stem cell control and enable optimization of *ex vivo* stem cell production (as reviewed [28]). Zandstra and colleagues [29] developed a mass action model of the JAK/STAT3 pathway, which stimulates murine ES cell self-renewal. Upon binding of LIF to the LIF receptor (LIFR) and glycoprotein-130 (gp130), the resulting receptor complex triggers signaling and leads to phosphorylation and activation of the transcription factor STAT3. The authors experimentally verified the prediction that gp130 overexpression actually decreases STAT3 activation, because the excess gp130 binds and sequesters LIFR into non-signaling heterodimers. Additionally, a sensitivity analysis predicted and experimental results confirmed the additive importance of two individually “innocuous” parameters (STAT3 nuclear export rate and JAK-mediated receptor activation rate), demonstrating the utility of such a model in generating novel hypotheses and potential therapeutic interventions. Finally, as predicted by the model, continual LIF stimulation causes desensitization to ligand stimulation. The model enabled the design and experimental validation of a LIF addition protocol to maximize STAT3 phosphorylation, representing a useful application of a model for maximizing *ex vivo* production of pluripotent ES cells for potential therapeutic use.

Deterministic models of the intracellular mechanisms of stem cell fate choice aid in interpreting complex network interactions, highlight new research avenues and potential therapeutic interventions, and can potentially be applied to improve process development efforts for *ex vivo* cell production. However, detailed knowledge of the signal transduction and transcription factor networks of interest are required, including quantitative knowledge of the kinetic and equilibrium constants involved. A further limitation of these models is their failure to account for the “noisy” behavior that can arise in biological systems due to slow chemical reactions and/or small numbers of molecules, and the next section focuses on efforts to take these effects into account when modeling stem cell fate choice.

Stochastic Models

Ever since stem cells were first discovered in the hematopoietic system, researchers have been studying the role of stochastics in stem cell fate choice [44-46]. When investigating the intracellular mechanisms governing stem cell fate, stochastics become important when the regulatory networks involve slow biochemical reactions and/or a small number of constituent molecules (e.g. transcripts, proteins, second messengers, etc.) within a cell. Since landmark theoretical work by McAdams and Arkin sparked interest in stochastic effects in gene expression [47], a considerable amount of work has focused on the implications of noise in multiple biological processes (see review [48]), including phage infection [49], *B. subtilis* stress response mechanisms [50], circadian rhythm control [51], lymphocyte activation [52], and others [53]. Stochastic effects are sometimes able to explain phenomena not predicted by deterministic models. In fact, some work indicates that precise circadian rhythms are actually dependent on noise [51]. Furthermore, a stochastic model based on T cell antigen response has described

situations where a bimodal output is observed, whereas a deterministic model predicts only a single, intermediate solution [54].

To date, very little work has investigated stochasticity in intracellular stem cell signaling pathways. As mentioned above, our model of the Shh signaling system predicts that expression of the transcription factor *Gli1* exhibits deterministic bistability as a function of an input Shh signal. We also implemented stochastic simulations to show that stochastic effects near bifurcation points can lead to random switching between states, undermining deterministic switch-like behavior in the network (similar to subsequent observations in other systems [55]). However, in the Shh network, the effects of noise are moderated by the *Gli1*-driven expression of the Shh repressor *Patched*. Specifically, while positive feedback amplifies noise, this negative feedback loop functions to dampen noise, resulting a robust switch that reliably directs stem cell fate despite inherent stochastic effects [19].

There is also experimental evidence that stochasticity may be important in networks that control stem cell behavior. Recent studies show that populations of stem cells can exist in multiple metastable states, and that cells within the population are capable of switching between these states (reviewed in [56]). For instance, in one study approximately 80% of murine ES cultures expressed the transcription factor *Nanog*. This observation could be readily explained if the other 20% were differentiated; however, this same 80/20 distribution is re-established when high or low marker populations are separated and cultured in isolation. Furthermore, ES cells with high *Nanog* expression are less likely to differentiate than low *Nanog* expressers, indicating that the system is more complex than simple bimodal *Nanog* expression [57]. Similar switches are observed in hematopoietic stem cells where the surface marker *Sca-1* is expressed in a broad distribution. Analogous to *Nanog* expression in ES cells, after being separated by flow cytometry, high or low *Sca-1* expressing populations re-establish the original distribution within several population doublings. Additionally, low *Sca-1* expressing cells preferentially differentiate into the erythroid lineage, whereas high *Sca-1* expressers favor the myeloid lineage [58]. The mechanistic basis of these switches is not yet understood, but a mathematical model involving stochastic state transitions between multiple stable states indicates that stochastic gene expression likely plays a role [58]. Elucidating the true nature of these noisy transitions and their effects on fate choice will require further studies that utilize a systems-level approach to complement experimental investigations.

Bayesian Networks

The above approaches to understanding stem cell fate choice are applicable when the signaling network of interest is relatively well understood (such as Shh or JAK/STAT3 signaling). However, in many situations, the underlying molecular interactions are not yet well characterized or in large part unknown, but systems biology approaches can potentially help elucidate such unknown networks through the analysis of large datasets. One such technique is Bayesian network analysis (for mathematical details, see recent reviews [59, 60]), which can help reverse engineer signal transduction cascades from an “-omic” dataset (proteomic, transcriptomic, kinomic, etc.) and deduce candidate causal relationships between measured variables or quantities. The result is a graphical map of probable interactions that provides a physical/biochemical interpretation of the dataset and aids in the formulation of hypotheses for future experimentation. The network analysis is probabilistic in that it treats each measurement (i.e. mRNA concentration, protein phosphorylation level, etc.) as an uncertain estimate and

therefore incorporates measurement noise in a systematic way. It is important to note that connections between species represent a causal but not necessarily a direct biochemical relationship, i.e. two connected species may not have a direct physical interaction but may instead be separated by several intermediate steps. Whether the analysis detects these intermediate steps is dependent on the quality, size, and detail of the dataset being analyzed. Another limitation of a Bayesian network is that it does not provide information on the stepwise progression of the interactions. Rather, it predicts the likelihood of finding a species in a particular state given the states of the surrounding species. Finally, although a traditional requirement is that Bayesian network structures are acyclic and are therefore unable to capture feedback, recent work has developed a new technique to circumvent this requirement and recover feedback loops within a signal cascade [61, 62].

Bayesian networks have been used to predict microRNA targets [63], regulatory relationships between genes [64], and the effects of single nucleotide polymorphisms on the clinical outcome of sickle cell anemia [65]; however, comparatively little work has utilized Bayesian networks for the analysis of stem cell fate control. A Bayesian network model has been used to analyze a large proteomic dataset from mouse ES cells containing measurements of the phosphorylation states of several signaling molecules under multiple cytokine and extracellular matrix conditions [24]. The resulting model provided good agreement with several previously described stem cell signaling pathways despite the complete lack of *a priori* assumptions regarding these signaling systems, including the LIF/JAK/STAT3 pathway and the MAPK/ERK pathway. Additionally, the model predicted several novel links. For instance, the rate of conversion from undifferentiated to differentiated cells was found to be most dependent on the phosphorylation states of Adducin α and ERK2. Furthermore, the model predicted that Raf1 and PKC ϵ would exert an effect on differentiated cell growth. The authors went on to experimentally verify these results, demonstrating that Bayesian networks can provide useful insights into the complex biological processes underlying stem cell fate choice. However, the authors caution that care must be taken when implementing these models, because, like many other analyses, the output results depend strongly on the quality and breadth of input data [24]. For instance, the analysis predicted that the rate of undifferentiated cell proliferation depended on the cytokine LIF without depicting any intermediate steps, when in reality there are numerous molecular intermediates that were not measured in the original dataset.

As large datasets needed for meaningful model predictions – which are resource- and time-intensive to generate – become increasingly available, Bayesian network analysis may become more utilized in the stem cell field to make novel predictions and drive new hypotheses regarding the signaling events that control stem cell fate.

Principal Components Analysis and Partial Least Squares Regression

Other techniques to analyze large datasets include principal components analysis (PCA) and partial least squares (PLS) regression (reviewed in [66]). Each measured quantity within a dataset (e.g. phosphoprotein and/or transcription factor concentrations) can be depicted as an axis within “signaling space” – analogous to how time and concentration are typically the x - and y -axes when graphing data. The resulting data space can have dozens (perhaps hundreds) of axes or dimensions, one for each measured quantity. PCA reduces this large number of dimensions to a few new axes called principal components. Each principal component represents a combination of the original signaling axes that have high covariance with one other. This

reduces the data space to just a few tractable dimensions, allowing the researcher to more easily search for trends within the data. PLS is an extension of PCA that generates a predictive relationship between independent and dependent principal components.

PCA has found uses in many biological applications, including analyzing neuronal decision-making processes [67], microarray data [68], libraries of chemical inhibitors [69], and signal transduction pathways [70], in addition to stem cell fate choice [25, 26]. As one example, Sharov *et al.* [25] amassed a large collection (nearly 250,000) of expressed sequence tags (ESTs) from public and other sources to produce a database of the mouse transcriptome. They then analyzed this database for differences in EST frequency between cell types of varying potency. PCA identified a principal component that effectively represented a cell's developmental potential, from totipotent oocytes to fully differentiated newborn tissues. The expression levels of a set of 88 genes were closely associated with this principal component axis. All 88 genes followed a general trend of decreased expression with increased differentiation. Similar work analyzed global gene expression changes during neuronal differentiation [26]. This analysis – which included ES cells, adult neural stem cells, and neurons – identified a principal component axis composed of several genes that described the cell's level of neuronal commitment. It seems likely that similar axes will be found for other tissues, and the genes associated with these principal components are potential targets of research into the molecular mechanisms of differentiation.

PLS, a predictive extension of PCA, only recently found use in biological research when it was first used as a predictor of apoptosis resulting from various molecular perturbations [71, 72]. It has since been used to generate a protein signature consistent with metastatic breast cancer [73] and to investigate the migration and proliferation of mammary epithelial cells [74]. Recently, PLS regression has been used to demonstrate that multiple cell types process upstream kinase signals through a similar “effector-processing” system to generate cell-specific responses to the same extracellular stimulus [75]. PLS was also used to analyze murine ES cell fate choice [27]. Using results from the same dataset as Woolf *et al.* [24] (see Bayesian Networks above), which included phospho-protein measurements from multiple signaling pathways under multiple culture conditions, they correlated phosphorylation state to ES cell differentiation and self-renewal. The analysis revealed a set of seven signaling molecules closely associated with ES cell fate choice, including PKC ϵ . Additionally, the PLS-generated model predicted that PKC ϵ inhibition would slow the proliferation of differentiated cells. To test this prediction, the authors experimentally inhibited PKC ϵ activity and found that it did indeed inhibit proliferation in differentiated cells with little effect on undifferentiated cells. This effect of PKC ϵ inhibition was potentially moderate, however, because it involved blocking only one component of a much larger network. At any rate, this work successfully utilized systems biology to highlight key contributors to ES cell differentiation and self-renewal, and similar analyses may in the future determine whether the same signaling molecules play the same roles in other stem cell types and aid the development of *ex vivo* expansion technologies and therapeutic strategies.

Summary and Future Directions

The molecular mechanisms of stem cell fate choice involve multiple, interacting signaling pathways and transcription factor networks. The resulting signal processing circuitry is extremely complex and difficult to investigate solely through reductionist approaches; therefore, increasing numbers of efforts have pursued a systems approach to understanding stem cell fate

choice. The resulting models provide considerable insight into stem cell fate choice (summarized in Table 1). Several models have highlighted the importance of bistability and switches [19-21], as well as oscillations [22, 41]. Modeling also highlights gaps in our understanding, generates new hypotheses about network function and behavior, and highlights critical control points that can be manipulated for cellular expansion and control. Network manipulation for cellular control has already met with success in some stem cell types [28, 29], and we anticipate these successes will continue as regulatory mechanisms of other stem cell types is subjected to systems analysis. Another interesting avenue of future research is how stochasticity affect stem cell function, both for endogenous stem cell behavior during development and adulthood, as well as in culture when extraction of a stem cell from its “comfortable” niche may render it more susceptible to stochastic behavior. There is evidence that the expression levels of some key genes that control stem cell self-renewal fluctuate considerably, and that the underlying mechanisms governing these switches are susceptible to stochastic effects [56-58]. Complementary experimental and modeling studies may yield further insights into this apparent randomness in the regulation of stem cell behavior.

Advances in high-throughput experimental techniques have created large -omic datasets that are difficult to interpret without statistical analyses such as Bayesian networks and/or PCA/PLS. Some work has already utilized these techniques to highlight novel molecular interactions and key genes regulating stem cell fate [24-27], and future application should help further our understanding of these systems. The role of these candidates can then be tested experimentally to expand our knowledge of fate choice mechanisms. Furthermore, statistical analyses performed to date have primarily focused on murine ES cells, and future analyses in other stem cell types such as human pluripotent stem cells should prove equally fruitful.

In closing, the application of systems biology to the problem of stem cell fate choice is still young, and opportunities abound. Collectively, these efforts will bring us closer to a molecular understanding of stem cell fate choice and may aid the development of therapies for many debilitating injuries and diseases.

	Deterministic	Stochastic	Bayesian	PCA/PLS
Advantages	-Insight into complex networks -Aids therapeutic/process development	-Systems with low numbers of molecules and/or slow biochemical reactions -Behaviors not predicted by deterministic models	-Reverse engineers network structure with no <i>a priori</i> knowledge	-Condenses large dataset to several key parameters. -Predicts network outputs with no knowledge <i>a priori</i> of network structure.
Requirements	-Detailed knowledge of pathway, including kinetic data	-Detailed knowledge of pathway	-Large, high quality datasets	-Large, high quality datasets
Key Stem Cell Studies	-Bistable switches in Shh and transcription factor networks [19-21] -Switches and oscillations in Notch signaling [41] -Common signaling pathways [42, 43] -Optimization of <i>ex vivo</i> cell production [29]	-Stochastic effects near bifurcation points [19] -Stochastic switching between multiple metastable states [58]	-Analysis of proteomic data from mouse ES cells to highlight novel network links [24]	-Analysis of transcriptome data from cells of varying potency [25, 26] -Murine ES cell fate choice from analysis of phospho-protein data [27]

Table 1: Summary of the model types used for analyzing stem cell fate choice and the key studies that employed these models.

Acknowledgments

This work was supported by NIH R21EB007295, NIH R21DE018044, and a training grant fellowship (to JP) from the California Institute for Regenerative Medicine (Training Grant Number T1-00007).

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