The role of Forkhead transcription factors in the proliferation, differentiation, and apoptosis of neural stem cells

Neurogenesis during embryonic and postnatal development is a complex process regulated at many key stages to enable proper nervous system formation. The current dogma subdivides this regulatory process at three levels: (1) a cellular clock that limits the progenitor cell’s intrinsic ability to undergo multiple cell divisions, (2) extracellular signaling factors acting as mitogens to enhance proliferation by promoting DNA replication, and (3) intra or extracellular factors that inhibit mitosis by inducing cell cycle arrest thereby providing a pool of quiescent progenitor cells [1]. Ultimately the proper formation of the mature nervous system is an interactive function of cell proliferation, differentiation and apoptosis [2]. While much progress has been made in understanding the individual components regulating neurogenesis a gap still exists in deciphering a common mechanism that integrates proliferative, differentiative and apoptotic signals to properly drive neural formation. The winged helix or Forkhead box (FOX) family of transcription factors has been implicated in an array of cellular functions, including cell cycle arrest, differentiation, and apoptosis [3]. The FOXO family constitutes a subclass of the Forkhead family that functions as transcriptional activators. In mammals this family consists of four members: FOXO1, FOXO3a, FOXO4, and FOXO6 [4,5]. FOXO proteins are known to transcribe the p27KIP1, p21CIP1 and BIM target genes involved in cell cycle arrest, differentiation, and apoptosis respectively [6-8]. FOXO proteins are negatively regulated by the phosphoinositide 3 kinase (PI3K)/Akt pathway [9]. The PI3K/Akt pathway is activated by growth and survival factors including insulin-like growth factor-1 (IGF-1). Akt phosphorylates FOXO proteins, subsequently forcing them out of the nucleus and preventing the transcription of cytostatic and differentiative target genes [10]. Consistent with this observation, the p13K/Akt pathway has been shown to control cellular survival in a wide range of neuronal cell types [11]. Recently, FOXO proteins have also been found to control differentiation in pre-adipocytes, myoblasts, and pancreatic β cells, through the upregulation of various target genes including BTG1 [12-14]. However, despite the high expression of FOXO3a in invertebrate and vertebrate neurons, the role of FOXO in cell fate determination has not been investigated in the nervous system [15]. Emerging evidence indicate that FOXO transcription factors are also regulated by intrinsic factors that play a role in cell fate determination. Another member of the Forkhead family, FOXG1 (formerly named Brain Factor-1) is a transcriptional repressor that is highly expressed in the neuroepithelial progenitor population in the telencephalon of the developing mouse [16]. Lack of FOXG1 in mice causes severe hypoplasia and loss of differentiation [17]. In contrast, over-expression of FOXG1 promotes overgrowth of the neural tube [18]. These studies indicate that FOXG1 serves as a transcriptional repressor to protect neuroepithelial progenitor cells from cytostatic signals [19]. Recent studies have shown that FOXG1 negatively regulates the transcription of the cytostatic target gene p21CIP1, by interacting with FOXO transcription factors in the nucleus even after activation by extracellular transforming growth factor β (TGF-β) signaling [20,21]. Therefore, FOXO and FOXG1 establish a link between extrinsic and intrinsic signaling that regulates proliferation, differentiation and apoptosis at the cellular level and therefore may serve as active components of the synergistic mechanism underlying cell fate determination in the nervous system.

Proposal:

The purpose of this proposal is to determine whether the regulative process underlying neurogenesis is controlled through Forkhead signaling pathway. Specifically my hypothesis is that neural stem/progenitor cell fate (proliferation, differentiation, and apoptosis) is regulated by FOXO3 and FOXG1 during development.

Aim 1: To investigate FOXO3 and FOXG1 expression in embryonic neural stem cells in vivo and in culture

Preliminary data from Anne Brunet’s lab at Stanford has shown that FOXO3a is highly expressed in cultured embryonic cortical progenitor cells at E13 in rats [22]. To thoroughly investigate the role FOXO3a may play during neurogenesis, I will first use immunocytochemistry to determine the expression and subcellular localization of FOXO3a in coronal mouse forebrain sections at E10.5-E18.5. The lab has generated an antibody to FOXO3 that recognizes endogenous FOXO3 in tissue sections. My previous experience with immunostaining at equivalent embryonic ages in the mouse spinal cord [23] will facilitate the proposed set of experiments. The FOXO3a immunostaining will allow us to determine the expression patterns of FOXO3 throughout development. These experiments will also reveal changes in FOXO3 subcellular localization between the cytoplasm and nucleus where transcription of cytostatic target genes may occur. In a second set of experiments, using double staining with the antibodies to FOXO3 in combination with commercially available goat polyclonal antibodies to FOX1, I will investigate the co-expression of FOXO3a and FOXG1 to determine areas of possible interaction between these transcription factors. Finally, I will analyze the expression of FOXO3a and FOXG1 in vitro in cultured embryonic neuronal progenitor cells in the presence or absence of growth factors for these stem cells such as basic fibroblast growth factor (bFGF) and insulin like growth factor 1 (IGF-1). My prediction is that FOXO3 should be localized in the nucleus when these cells are deprived of growth factors. Taken together, these experiments will allow me to determine the conditions when FOXO3 and FOXG1 are both present in the nucleus, and thereby can affect programs of gene expression and cellular function.

Aim 2: To determine the importance of FOXO3 for neural proliferation, differentiation and apoptosis

After determining the spatiotemporal pattern of FOXO3a and FOXG1 during development, I will functionally knock down FOXO3a expression in cultured embryonic neural stem cells, in the presence and absence of growth factors, using an RNA interference (RNAi) approach. The Brunet lab has generated a lentivirus expressing a small hairpin RNA (shRNA) that allows the specific degradation of FOXO3 mRNA [24]. I will assess the effect of knock-down FOXO3 expression on cell proliferation, neural differentiation, and apoptosis. To study cell proliferation I will use bromodeoxyuridine (BrdU), as BrdU is known to be incorporated in DNA during the S phase of cell division. I will add BrdU into the neural stem cell culture system and determine changes in proliferation 48-72 hours after lentiviral infection with FOXO3 shRNA compared to control cultures.
In a second set of experiments, I will investigate neuronal differentiation by examining the expression of early (Map2) and late (NeuN) neuronal markers by immunostaining in the neural stem cells that have been infected by FoxO3 shRNA for 48-72 hours. Finally, I will analyze cell death by examining the presence of cleaved caspase 3, an apoptotic marker, in the 48-72hr incubated cells by immunostaining with antibodies that specifically recognize the cleaved form of caspase 3. These experiments will indicate if FoxO3 plays a critical role in neural stem cell fate in vitro. Based on the conserved cytostatic role of FoxO3a in other cell types and the preliminary data indicating that this protein is present in neural progenitor cells, I expect FoxO3a inhibition by shRNA to promote cell proliferation and prevent apoptosis and differentiation, thereby expanding a pool of stem cells. Additionally, if I find that the presence of IGF-1 trigger similar neural cell fate as exposing the neuronal stem cells to FoxO3a shRNA, these results will indicate that FoxO3a regulation may occur extrinsically via the PI3K/Akt pathway in neural stem cells. By analyzing changes in proliferation, differentiation or apoptosis we can better understand whether these processes are regulated concomitantly or individually by this mechanism. It is possible however, that the inhibition of FoxO3a expression may not clearly alter neural stem cells function, maybe due to the functional redundancy with other FoxO family members. In this case, I will use shRNA to down-regulate the other FoxO family members and determine the effects of abrogating the expression of the FoxO family as a whole on neural stem cell fates. To investigate further the role of the FoxO transcription factors, I will infect cultured neural stem cells with a lentivirus encoding a constitutively active form of FoxO3, made by replacing the three AGT phosphorylation sites of FoxO3 (Threonine 32, Serine 253, and Serine 315) with non-phosphorylatable alanine residues. These experiments will indicate whether an active form of FoxO is sufficient to repress proliferation, and promote differentiation in neural stem cells in vitro. I expect FoxO activation to repress cell proliferation, but promote differentiation and if very active to also promote apoptosis. Differentially altering the neural stem cell activity through the down-regulation and up-regulation of FoxO proteins will reveal if these proteins are both necessary and sufficient to control neural stem cell proliferation, differentiation and/or apoptosis.

**Aim 3: To explore the functional interactions between FoxO3 and FoxG1**

To investigate the functional interaction between FoxO3a and FoxG1 in cultured neural stem cells, I will perform coimmunoprecipitation experiments using an antibody against FoxO3a and determine whether FoxG1 is co-precipitated in complex with FoxO3a using western blot analysis with antibodies to FoxG1. To further understand the relationship between FoxO3a and FoxG1, I will investigate the activity of FoxO3a in the presence or absence of FoxG1. To this end, I plan to collaborate with Dr. Susan McConnell’s lab at Stanford that has generated a conditional knockout mouse model of FoxG1 [25]. I will inhibit FoxO3a expression using shRNA in neural stem cell cultures from wild-type and knock out FoxG1 mice to determine the effects of FoxG1 on FoxO3a mediated proliferation, differentiation and apoptosis. I expect the control stem cell cultures from FoxG1 knockout mice to sustain little cell proliferation but increased differentiation and apoptosis, while cultures containing the FoxO3 shRNA will display the reversed effect. If the alterations in cell proliferation, differentiation and/or apoptosis differ in the presence and absence of FoxG1, then it can be concluded that FoxG1 is an intrinsic factor that negatively regulates the FoxO3a dependent activation of cytostatic target genes at the nuclear level. If on the other hand, neural stem cell fates are not altered by changes in FoxG1, it may mean that FoxO3 transcriptional activity during development is primarily regulated extrinsically through the PI3K/Akt pathway.

**Conclusion:**

The present proposal aims to decipher a mechanism that can regulate proliferation, differentiation and apoptosis in neuronal stem/progenitor cells. By linking these different processes to a common FoxO mediated pathway, this project will help to investigate the conditions under which cells preferentially choose individual fates at different stages in development. Furthermore, in my long-term experiments, I will assess the importance of the FoxO mediated pathway in neuronal stem cell populations in vivo through the use of currently available genetic knock-out mouse models. Consequently, this developmental model may ultimately be applied to other systems involved in neurogenesis. In particular, the FoxO mechanism may help reveal why neural stem cell activity is limited in the adult nervous system. Thus, this project offers wide applications beyond the development of the nervous system. The proposed set of experiments will be conducted in the lab of Dr. Anne Brunet at Stanford University. Dr. Brunet is a pioneer in elucidating the biochemical pathway under which Forkhead proteins regulate cell cycle arrest, proliferation, differentiation and apoptosis. Additionally she has investigated the link between these cellular processes and aging. Therefore her lab will facilitate my future applications of this developmental model in adult neurogenesis systems.

**References:**

[22] Anne Brunet, personal communication.  