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Pten Function in Postnatal Brain Development and Tumorigenesis

Abstract

PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is a tumor suppressor gene that is commonly mutated in multiple types of human cancers including endometrial, prostate, breast cancers and glioblastoma multiforme (GBM). *PTEN* functions as a phospholipid phosphatase antagonizing phosphorylation by phosphatidylinositol-3-OH kinases (PI3Ks) and thus serves as a major negative regulator of the PI3K pathway. Homozygous *Pten* knock-out mice die during embryogenesis. Conditional *Pten* knock-out in the nervous system showed that *Pten* negatively regulates self-renewal and proliferation of neural stem cells, negatively controls neuronal size, is required for proper neuronal positioning and is involved in the development and maintenance of normal function for oligodendrocytes and astrocytes.

Neuronal precursors (neuroblasts) are generated every day throughout life in the subventricular zone (SVZ) and migrate through the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into mature interneurons. The signaling pathways instructing when and where these tangentially migrating neuroblasts stop are largely unknown. In this study, we found that the PI3K-Akt-mTorc1 pathway is selectively inactivated in the migrating neuroblasts in the wild-type subventricular zone and rostral migratory stream and activated when they reach the olfactory bulb. Postnatal deletion of *Pten* in the SVZ resulted in aberrant activation of the PI3K-Akt-mTorc1 pathway and enlarged SVZ and RMS. This was caused by premature termination of migration and differentiation of neuroblasts, and could be rescued by inhibition of mTorc1. Although this is consistent with previous studies in lower organisms showing that *Pten* was required for directional migration, live imaging of acute brain slices showed that the migrating *Pten*-deficient neuroblasts were not defective in speed and showed appropriate directional movement. Therefore, the apparent "migration defect" in *Pten*-deficient neurons was secondary to ectopic differentiation rather than an intrinsic defect in directional migration.

Inactivation of *Pten* in the neural stem/progenitor cells at birth also revealed a novel perivascular proliferative niche in the cerebellum without full-blown tumor formation. Co-deletion of *Pten* and *Trp53*, two commonly mutated tumor suppressor genes in human brain tumors, could synergize in generating high-penetrant medulloblastoma with a growth pattern centering around blood vessels and extensive neuronal differentiation. The *Pten* and *Trp53* double knock-out medulloblastomas spontaneously lost chromosome 7, 13 and 16 and had mutations in the gene *Ptch1*. Therefore the PI3K and Shh pathways cooperate in generating medulloblastomas.

Our results show *Pten* is required for appropriate positioning of neuronal differentiation and preventing ectopic neural stem/progenitor proliferation and medulloblastoma genesis.

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**PTEN FUNCTION IN POSTNATAL BRAIN DEVELOPMENT AND
TUMORIGENESIS**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Guo Zhu
May 2011

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DEDICATION

To my wife Lingli Zhang
To my father Ya-Zong Zhu
To my mother Guo-E Li
To my brother Ye Zhu

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ABSTRACT

PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is a tumor suppressor gene that is commonly mutated in multiple types of human cancers including endometrial, prostate, breast cancers and glioblastoma multiforme (GBM). *PTEN* functions as a phospholipid phosphatase antagonizing phosphorylation by phosphatidylinositol-3-OH kinases (PI3Ks) and thus serves as a major negative regulator of the PI3K pathway. Homozygous *Pten* knock-out mice die during embryogenesis. Conditional *Pten* knock-out in the nervous system showed that *Pten* negatively regulates self-renewal and proliferation of neural stem cells, negatively controls neuronal size, is required for proper neuronal positioning and is involved in the development and maintenance of normal function for oligodendrocytes and astrocytes.

Neuronal precursors (neuroblasts) are generated every day throughout life in the subventricular zone (SVZ) and migrate through the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into mature interneurons. The signaling pathways instructing when and where these tangentially migrating neuroblasts stop are largely unknown. In this study, we found that the PI3K-Akt-mTorc1 pathway is selectively inactivated in the migrating neuroblasts in the wild-type subventricular zone and rostral migratory stream and activated when they reach the olfactory bulb. Postnatal deletion of *Pten* in the SVZ resulted in aberrant activation of the PI3K-Akt-mTorc1 pathway and enlarged SVZ and RMS. This was caused by premature termination of migration and differentiation of neuroblasts, and could be rescued by inhibition of mTorc1. Although this is consistent with previous studies in lower organisms showing that *Pten* was required for directional migration, live imaging of acute brain slices showed that the migrating *Pten*-deficient neuroblasts were not defective in speed and showed appropriate directional movement. Therefore, the apparent “migration defect” in *Pten*-deficient neurons was secondary to ectopic differentiation rather than an intrinsic defect in directional migration.

Inactivation of *Pten* in the neural stem/progenitor cells at birth also revealed a novel perivascular proliferative niche in the cerebellum without full-blown tumor formation. Co-deletion of *Pten* and *Trp53*, two commonly mutated tumor suppressor genes in human brain tumors, could synergize in generating high-penetrant medulloblastoma with a growth pattern centering around blood vessels and extensive neuronal differentiation. The *Pten* and *Trp53* double knock-out medulloblastomas spontaneously lost chromosome 7, 13 and 16 and had mutations in the gene *Ptch1*. Therefore the PI3K and Shh pathways cooperate in generating medulloblastomas.

Our results show *Pten* is required for appropriate positioning of neuronal differentiation and preventing ectopic neural stem/progenitor proliferation and medulloblastoma genesis.

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LIST OF ABBREVIATIONS

aCGH	array comparative genomic hybridization
aCSF	artificial cerebrospinal fluid
bHLH	basic helix loop helix
BAC	bacterial artificial chromosome
BrdU	5-Bromo-2'-deoxyuridine
BRRS	Bannayan-Riley-Ruvalcaba syndrome
CB	cerebellum
CNS	central nervous system
CS	Cowden syndrome
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DPI	days post induction
EGL	external granule layer
FISH	fluorescent in situ hybridization
FRB	FKBP-rapamycin-binding
GABA	γ -Aminobutyric acid
GAP	GTPase-activating protein
GBM	glioblastoma multiforme
GCL	granule cell layer
GTP	guanosine triphosphate
hPLAP	human placental alkaline phosphatase
HBSS	Hank's balanced salt solution
IGL	internal granule layer
IRES	internal ribosomal entry site
LDD	Lhermitte-Duclos disease
LV	lateral ventricle
mTORC	mammalian target of rapamycin complex
ML	molecular layer
MMAC	mutated in multiple advanced cancers
OB	olfactory bulb
PBD	phosphatidylinositol-4,5-bisphosphate binding domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	phosphoinositide-dependent kinase 1
PDZ	postsynaptic density protein–Drosophila disc large tumor suppressor–zonula occludens 1 protein
PEST	proline, glutamic acid, serine, threonine
PFA	paraformaldehyde
PH	pleckstrin homology
PHTS	PTEN hamartoma tumor syndrome
PI3Ks	phosphatidylinositol-3-OH kinases
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate

PNET	primitive neuroectodermal tumours
PNS	peripheral nervous system
PSL	Proteus-like syndrome
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RMS	rostral migratory stream
RTKs	receptor tyrosine kinases
RT-PCR	reverse transcriptase polymerase chain reaction
SGZ	subgranular zone
SHH	sonic hedgehog
SVZ	subventricular zone
TEP1	transforming growth factor β -regulated and epithelial cell enriched phosphatase 1
TM	tamoxifen
TUNEL	terminal deoxynucleotidyl transferase-mediated nick end labeling
WHO	World Health Organization

CHAPTER 1. GENERAL INTRODUCTION

PTEN and its Signaling Pathway

Identification of *PTEN*

PTEN (Phosphatase and **T**ensin homolog deleted on chromosome **10**) also known as *MMAC* (mutated in multiple advanced cancers) or *TEP1* (transforming growth factor β -regulated and epithelial cell enriched phosphatase 1) was originally identified in 1997 as a putative tumor suppressor gene on chromosome 10q23.3, a genomic region commonly deleted in several types of human cancers including endometrial, prostate, breast cancers and glioblastoma multiforme (GBM) (Li and Sun 1997; Li et al. 1997; Steck et al. 1997).

PTEN functions as a phosphatase

The structure of PTEN protein organizes into four functional domains: N-terminal phosphatidylinositol-4,5-bisphosphate (PIP₂)-binding domain (PBD), a phosphatase domain, a C2 domain for membrane recruitment and a C-terminus containing two PEST sequences for proteolytic degradation and a PDZ-binding motif for protein-protein interaction (Figure 1-1A), (Georgescu et al. 1999; Lee et al. 1999; Georgescu et al. 2000; Vazquez et al. 2001; Das et al. 2003). As indicated in its structure, PTEN shares sequence homology with the family of protein-tyrosine phosphatases and can dephosphorylate serine, threonine, and tyrosine residues on peptide substrates in vitro (Myers et al. 1997). However, no protein substrate has been convincingly identified in vivo so far and the best characterized function of PTEN is associated with its lipid phosphatase activity with which PTEN catalyzes dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) at position 3 on the inositol ring to generate PIP₂ (Maehama and Dixon 1998). This lipid phosphatase activity is direct antagonism to that of phosphatidylinositol-3-OH kinases (PI3Ks) which phosphorylates PIP₂ to PIP₃ and activate downstream pathway (Figure 1-1B).

PTEN negatively regulates PI3K/AKT/mTOR pathway

PI3Ks are an evolutionarily conserved family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides, the crucial regulators of a variety of cellular processes (Engelman et al. 2006). PIP₂, one kind of substrates for PI3Ks, is phosphorylated in response to activation of receptor tyrosine kinases (RTKs) or G-protein coupled receptors on the plasma membrane creating PIP₃ (Engelman et al. 2006). PIP₃ acts as a second messenger recruiting the serine/threonine kinase AKT to the membrane via its pleckstrin homology (PH) domain. AKT is phosphorylated at threonine 308 by another serine/threonine phosphoinositide-dependent

Figure 1-1. Schematic PTEN secondary protein structure and biochemical function.

(A) The structure of PTEN protein organizes into four functional domains: N-terminal PIP2-binding domain (PBD), a phosphatase domain, a C2 domain for membrane recruitment, and a C-terminus containing two PEST sequences for proteolytic degradation and a PDZ-binding motif for protein-protein interaction. (B) PTEN functions as a phosphatase which dephosphorylates membrane-bound phospholipid PIP3 at position 3 on the inositol ring to generate PIP2. This dephosphorylation is a reverse reaction to the phosphorylation by PI3K downstream RTK activation. Modified with permission. Chalhoub, N. and Baker, S.J. 2009. PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* 4: 127-150. Courtesy of Dr. Suzanne Baker.

kinase 1 (PDK1) recruited by binding to PIP3 as well (Alessi et al. 1997). To be fully activated, AKT needs also to be phosphorylated at serine 473 by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbasov et al. 2005). By converting PIP3 back to PIP2, PTEN antagonizes PI3K activity, limits and ultimately terminates PI3K-AKT signaling in cells and this lipid phosphatase ability has been shown to be essential for PTEN tumor suppressor function (Sansal and Sellers 2004) (Figure 1-2).

Activated AKT is able to phosphorylate a diverse set of substrates such as GSK3 β , p27KIP1, MDM2, BAD, FOXOs, IKK, TSC2 and PRAS40 to regulate cell cycle progression, cell survival, cell growth and cell metabolism (Engelman et al. 2006; Shaw and Cantley 2006). Among the variety of pathways downstream of AKT, mTORC1 (one of two complexes nucleating mTOR, the other is mTORC2) is essential in regulating cell growth through coordinating anabolism including macromolecule synthesis and nutrient storage and catabolism like autophagy and utilization of energy stores in response to growth factors, nutrients, energy and oxygen level etc. environmental cues (Sengupta et al. 2010). mTORC1 is regulated by direct modification of its components or modulation of Rheb, a small GTPase that binds and activates mTORC1 when bound to GTP. AKT phosphorylates PRAS40 and prevents its binding and inhibition of mTORC1 (Sancak et al. 2007; Vander Haar et al. 2007). AKT could also activate mTORC1 by phosphorylating and suppressing TSC2 which, together with its partner TSC1, is a GTPase-activating protein (GAP) for Rheb (Sancak et al. 2007) (Figure 1-2).

mTORC1 promotes protein synthesis through two of its substrates, the S6 kinases (S6Ks) and 4E-BPs which enhance ribosome assembly and mRNA translation. mTORC1 inhibits autophagy by modulating Atg1/ULK activity in the presence of nutrients and growth factors. mTORC1 also controls numerous metabolic pathways including glycolysis, sterol and lipid biosynthesis as well as nucleotide metabolism (Sengupta et al. 2010). Upon activation by growth factors, mTORC1 limits the extent of upstream growth factor signaling by a “negative feedback loop”. The best characterized mechanism of the “negative feedback loop” is through S6K1 phosphorylation of IRS1 which interferes with IRS1 binding to insulin receptor and promotes its degradation, thus preventing over-activation of the pathway (Harrington et al. 2004; Shah and Hunter 2006). Many mTORC1 functions were first discovered by use of the small-molecule rapamycin, an allosteric inhibitor of mTOR. Rapamycin forms a complex with protein FKBP12 which binds to FRB domain of mTOR and somehow only inhibits mTORC1 activity in acute treatment (Huang et al. 2003). For long-term or high dose treatment, rapamycin could also inhibit mTORC2 activity by interfering with its assembly (Sarbasov et al. 2006). Inhibition of mTORC1 by rapamycin releases the “negative feedback loop” and results in enhanced PI3K signaling to AKT (O'Reilly et al. 2006).

Deletion of PTEN will lead to unopposed and constitutive activation of the PI3K-AKT-mTORC1 pathway. Increases in phosphorylation of AKT serine 473 and threonine 308, phospho-S6 serine 235/236 (substrate of S6K) and phospho-4EBP1 threonine 37/46 are often used as the readouts of activated PI3K-AKT-mTORC1 pathway.

Figure 1-2. Schematic PI3K signaling pathway.

The RTK is activated when binding to its ligand and recruits PI3K which phosphorylates membrane-bound PIP2 to PIP3. PIP3 recruits AKT and PDK1 to the membrane through their PH domain. AKT is fully activated by phosphorylations by PDK1 and mTORC2. Activated AKT phosphorylates a diverse set of substrates such as TSC1/2, MDM2, FOXO, BAD, NF- κ B and GSK3 to regulate processes including cell growth, cell-cycle apoptosis, cell metabolism and autophagy. By converting PIP3 back to PIP2 antagonizing PI3K activity, PTEN limits and terminates PI3K-AKT signaling. Reprinted with permission. Chalhoub, N. and Baker, S.J. 2009. PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* 4: 127-150. Courtesy of Dr. Suzanne Baker.

PTEN and Human Diseases

Germline *PTEN* mutations have been found to occur in several inherited cancer and developmental syndromes including 80% of Cowden syndrome (CS), 60% of Bannayan-Riley-Ruvalcaba syndrome (BRRS), up to 20% of Proteus syndrome (PS), 50% of Proteus-like syndrome (PSL) (Eng 2003) and 83% of Lhermitte-Duclos disease (LDD) (Zhou et al. 2003). These seemingly unrelated hereditary syndromes are now categorized under a unifying molecular-based diagnosis PTEN Hamartoma Tumor Syndrome (PHTS) since they share the clinical manifestation of multiple hamartomas and have overlapping mutational spectra of *PTEN* (Eng 2003). Several important clinical manifestations of PHTS patients include macrocephaly, multiple hamartomas and cancer predisposition in Cowden disease to breast, thyroid, endometrial, colorectal, and renal cancers (Riegert-Johnson et al. 2010).

Besides germline mutation, *PTEN* is also found to be frequently mutated in a wide variety of sporadic human cancers. The list of human cancers that harbor PTEN mutation has expanded from endometrial, prostate, breast cancers and GBM initially reported in 1997 to more than ten types (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>), placing it as the second most frequently mutated gene in human cancer. In no doubt this list will keep expanding with the advancement of whole genome sequencing technology.

PTEN Knock-out Mouse Models outside of the Central Nervous System

Pten function in development and tumor suppression has been well characterized using traditional germline and conditional gene knock-out strategies. Homozygous deletion of *Pten* resulted in embryonic lethality by day 6.5-9.5 because of disrupted development of germ layers, while heterozygous mutant mice are predisposed to a variety of tumors including lymphomas, intestinal polyps, endometrial hyperplasia, prostate intraepithelial neoplasia and thyroid neoplasms (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999). This spectrum of neoplasias is similar to those in PHTS patients.

Cre-LoxP system has allowed people to bypass the embryonic lethality of *Pten*^{-/-} mice and to study Pten function in a tissue-specific manner. Deletion of *Pten* in T cells resulted in hyperproliferation, auto-immunity and T-cell lymphoma (Suzuki et al. 2008). *Pten* deletion in skin lead to epidermal hyperplasia, hyperkeratosis, squamous papillomas, squamous cell carcinomas, sebaceous carcinomas and sweat gland adenocarcinomas (Suzuki et al. 2008). Hepatocyte-specific *Pten* knock-out caused massive hepatomegaly, steatohepatitis, liver adenomas and hepatocellular carcinomas (Suzuki et al. 2008). Deletion of *Pten* in urothelial cells resulted in urothelial hyperplasia and hypertrophy and transitional cell carcinomas (Suzuki et al. 2008). Most of mice with bronchioalveolar epithelium-specific inactivation of Pten died of hypoxia at birth and the rest developed spontaneous lung adenocarcinomas (Suzuki et al. 2008). Pten is also required for normal blood vessel remodeling and suppressing tumor angiogenesis (Suzuki et al. 2008). Pten has an essential role in restricting the activation of hematopoietic stem

cells and preventing leukaemogenesis. Inactivation of Pten in the bone marrow resulted in a short-term expansion but long-term decline of hematopoietic stem cells and eventual leukemia formation (Yilmaz et al. 2006; Zhang et al. 2006). Oocyte-specific deletion of *Pten* causes premature activation of the primordial follicle pool and premature ovarian failure (Reddy et al. 2008). Conditional *Pten* knock-out mice have also been generated that develop teratomas, pancreatic cancers, cholangiocellular carcinomas and leiomyosarcomas (Suzuki et al. 2008).

PTEN in Neuropathology and Neural Development

Pten function in neurons

PTEN inactivation in the cerebellum causes LDD or dysplastic gangliocytoma of the cerebellum, a hamartomatous lesion in the cerebellum. LDD patients manifest with progressive macrocephaly, seizure, ataxia and mental retardation. The typical pathological feature of LDD is the diffusely hypertrophic and disorganized cerebellar folia with replacement of the Purkinje and granule cells by hypertrophic ganglionic neurons (Abel et al. 2005). The features of LDD were recapitulated by mouse models in which Pten was conditionally deleted in cerebellar granule cells during development. Pten deletion resulted in progressive increase in brain size, seizures, neuronal hypertrophy and migration defect demonstrating that Pten regulates neuronal size and migration (Backman et al. 2001; Kwon et al. 2001). PHTS patients also exhibit autistic behavior and mental retardation with decreased or delayed learning (Goffin et al. 2001; Eng 2003). Interestingly, a mouse model deleting *Pten* in differentiated neurons in the cerebral cortex and hippocampus showed similar autistic features to human patients including abnormal social interaction, anxiety-like behavior and decreased learning (Kwon et al. 2006). These neurological and behavioral manifestations could be presumably explained by the abnormalities in neuronal structure and function caused by Pten deficiency such as hypertrophic and ectopic dendrites, increased dendritic spine density, increased axonal synapses, severe myelination defects, weakened synaptic transmission and synaptic plasticity (Kwon et al. 2006; Fraser et al. 2008).

Pten function in glia

The axons of neurons are usually ensheathed by myelin which is formed by oligodendrocytes in the central nervous system (CNS) or Schwann cells in the peripheral nervous system (PNS). Abnormalities in myelination underlie a variety of human diseases such as multiple sclerosis, Guillain-Barré Syndrome, central pontine myelinosis, inherited demyelinating diseases like Leukodystrophy and Charcot Marie Tooth (Suter and Scherer 2003). One histopathological feature in LDD patients is the abnormal myelination in the cerebellar molecular layer (Abel et al. 2005) suggesting Pten might be involved in the regulation of myelination. Recent studies in mouse models just started to reveal Pten function in the oligodendrocytes and Schwann cells. Pten deletion in either

developing or differentiated oligodendrocytes and Schwann cells resulted in dramatic hypermyelination (Fraser et al. 2008; Goebbels et al. 2010; Harrington et al. 2010). The hypermyelination was caused by oligodendrocyte hypertrophy and increased thickness of myelin sheath but not change in the number of oligodendrocyte in the CNS. In the PNS, however, Pten loss caused an increased number of Schwann cells and de novo myelination of normally non-myelinated nerve fibers as well as increased thickness of myelin sheath. Rapamycin treatment strongly impeded the volume increase of the white matter indicating that the AKT/mTOR pathway is one critical downstream effector in the differentiation of oligodendrocytes (Goebbels et al. 2010). There was also massive axon degeneration associated with progressive myelin sheath abnormalities. However, there was no detectable improvement in remyelination following white matter injury. These studies indicate that Pten is required to regulate myelin thickness and preserve axon integrity but dispensable during myelin repair (Harrington et al. 2010). Pten is also required for the Dlg1-mediated myelination brake whose failure causes Schwann cell hypermyelination with focally folded myelin, a hallmark of one type of Charcot-Marie-Tooth disease (Cotter et al. 2010). In addition, Pten also inhibits oligodendrocyte progenitor cell proliferation and promotes their differentiation (Paintlia et al. 2010).

Astrocytes are another large population of glial cells that provide structural support, regulate water balance and ion distribution and maintain the blood-brain barrier etc (Rowitch and Kriegstein 2010). Only very few studies have been conducted on Pten function in the development of astrocytes in their physiological environment. A mouse model inactivating Pten in both astrocytes and neurons during development showed increased cell size and proliferation in the astrocytes in contrast to the increase in cell size only in the neurons (Fraser et al. 2004). However, whether or not the hypertrophy and hyperproliferation in the astrocytes are cell-autonomous still needs further investigation because astrogliosis (an abnormal increase in the number of astrocytes) is a known phenomenon of astrocytes in response to environmental stresses such as neuron damage. Deletion of Pten in astrocytes in the adult brain resulted only in a slightly increased Gfap expression (Chow et al. 2011).

Pten function in neural stem cells

Inactivation of Pten by a *Nestin* promoter driven *Cre* in the neural stem/progenitor cells at midgestation demonstrated that mutant mice exhibit enlarged, histoarchitecturally disrupted brains, which resulted from increased cell proliferation, decreased cell death and enlarged cell size. Neurosphere cultures further showed increased proliferation and self-renewal capacity for Pten deficient neural stem/progenitor cells with no deficit in differentiation (Groszer et al. 2001; Groszer et al. 2006). These studies indicate Pten negatively controls proliferation and self-renewal of neural stem/progenitor cells during early brain development. However, the *Nestin-Cre;Pten^{loxP/loxP}* mice die soon after birth precluding investigation of Pten function in neural stem/progenitor cells in early postnatal days and adulthood.

PTEN and Brain Tumorigenesis

Postnatal brain development and neural stem cells in adulthood

The bulk of brain structure changes relatively little after birth except the cerebellum, hippocampus and subventricular zone (SVZ) where neurogenesis and neuronal migration continue in postnatal days and adults. Although the establishment of the cerebellar territory and generation of Purkinje neurons and deep cerebellar nuclei happen during embryogenesis, most granule neurons are generated postnatally. Granule neuron progenitors migrate superficially from rhombic lip to form the external granule layer (EGL) on the surface of the cerebellum at embryogenesis. These cells continue proliferating and generate granule cells which descend into the cerebellum and form the internal granule layer (IGL) until postnatal day 21 in mouse and up to year 2 in human (Figure 1-3A). Active postnatal development makes cerebellum very susceptible to mutagenesis. It is the most common site of origin for pediatric solid tumors, accounting for 15.6% of all primary brain and CNS tumors in childhood (CBTRUS 2010).

Neural stem cells or neural precursor cells in adulthood are mainly restricted to the hippocampus and SVZ in the brain where neurogenesis occurs throughout life. In mouse, it has been shown that thousands of new neuronal precursors are born every day in the SVZ and migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into interneurons and are integrated into local circuits. Migrating neuronal precursor cells or neuroblasts (Dcx+) are differentiated from Mash1+ transit amplifying cells which in turn are generated by Gfap+ neural stem cells in the SVZ (Alvarez-Buylla and Garcia-Verdugo 2002) (Figure 1-3B, C). SVZ has also been found to be conserved in human brain (Quinones-Hinojosa et al. 2006; Curtis et al. 2007). However whether or not the RMS is present in human brain is still controversial (Sanai et al. 2004; Curtis et al. 2007; Guerrero-Cazares et al. 2011). In hippocampus, new neurons are continuously generated in the subgranular zone (SGZ) of dentate gyrus and migrate outward for a short distance to be incorporated into dentate gyrus. Neural stem/progenitor cells in the SVZ-RMS-OB and the SGZ are thought to play an important role in damage repair and the process of memory formation (Gage 2002; Ming and Song 2005; Ghashghaei et al. 2007). They might also serve as cells of origin for brain tumors because of their great proliferative capacity.

Unlike the neurogenesis which occurs before gliogenesis during brain development and peaks at embryonic day 14 (E14), astrocytes and oligodendrocytes, two major glial cells in the brain are generated mainly postnatally peaking at P2 and P14, respectively (Sauvageot and Stiles 2002). Mature astrocytes and oligodendrocytes are able to proliferate in physiological condition or pathologically in response to injury. They are thought to be the other candidate cells of origin for primary brain tumors besides neural stem/progenitor cells. It is still largely unclear whether gliomas arise from neural stem/progenitor cells or from fully differentiated astrocytes or oligodendrocytes, the two most possible cell sources maintaining proliferation capacity in the brain for malignant

Figure 1-3. Postnatal development of mouse brain.

(A) Nissl staining of sagittal mouse brain at age P4. Neurogenesis is still actively ongoing in postnatal brain in two places. First, new neuronal progenitors (or neuroblasts) arise from neural stem cells in the subventricular zone (SVZ) in the lateral walls of lateral ventricles (LV), migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they terminally differentiate and are integrated into local neural circuits. Second, granule cell progenitors actively proliferate in the external granule layer (EGL) of the cerebellum (CB), then migrate inward and differentiate forming the internal granule layer (IGL) during early postnatal days. (B) Nissl staining of coronal mouse brain of age P28 shows the SVZ. (C) Cell lineage in the SVZ. Neural stem cells (B cells) give rise to transit amplifying cells (C cells) which in turn generate neuroblasts (A cells). E cells are ependymal cells lining the LV. Panel C is Modified with permission. Alvarez-Buylla, A. and Garcia-Verdugo, J.M. 2002. Neurogenesis in adult subventricular zone. *J Neurosci* **22**(3): 629-634.

transformation. The identification of cell origins of gliomas will not only improve our knowledge but also help develop therapeutic strategies of this disease.

Primary brain tumors

In the United States in the year 2010, there was an estimated 62,930 new cases of primary non-malignant and malignant brain and CNS tumors (CBTRUS 2010). Primary malignant brain and CNS tumors account for 1.4 percent of all cancers, 2.3 percent of all cancer deaths and 21.3 percent of pediatric cancers (ACS 2010). Meningioma, gliomas and pituitary tumor are the most common histology types of primary brain tumors in adults. Gliomas including astrocytoma, glioblastoma, oligodendroglioma, ependymoma and mixed gliomas etc. represent 32% of all primary brain tumors and 80% of malignant primary brain tumors. GBM (WHO grade IV) is the most malignant form and accounts for 53.8% of all gliomas (CBTRUS 2010) (Figure 1-4A). The median survival of GBM patients ranges from 9 to 12 month despite combined treatment of surgery, radiotherapy and chemotherapy. The survival rate for GBM has not been improved and no effective agents have emerged for over two decades (Furnari et al. 2007).

In children, brain tumors are the most common type of solid tumor and the incidence is about 4.7 per 100,000 person-years in the US. Pilocytic astrocytoma, malignant glioma and medulloblastoma are the most common individual histologies. Gliomas are the most frequent primary brain tumors in childhood accounting for about 50% of all tumors and 70% of malignant tumors. Embryonal tumors including medulloblastoma (WHO grade IV) are the second most common malignant form and account for 13.7% of all pediatric brain tumors among 0-14 years olds (CBTRUS 2010) (Figure 1-4B). While the 5-year survival rate of medulloblastoma patients has achieved 60% after treated with radio- and chemotherapy, the patients often suffer from side effects such as cognitive impairment and psychiatric disorders.

Overall, malignant glioma (WHO grade III and IV) and medulloblastoma, the most common primary brain tumors in adults and children, pose a big challenge to healthcare and await novel therapies.

***PTEN* mutations in brain tumors**

One genetic abnormality that is frequently seen in adult and pediatric brain tumors is the loss of chromosome 10q where *PTEN* is located. Losses of chromosome 10q have been reported to occur in 60-80% of GBM (Ohgaki and Kleihues 2007) and 25-30% of medulloblastoma (McCabe et al. 2006; Rossi et al. 2006; Lo et al. 2007).

PTEN is reported to be mutated in 15-40% of GBM but is rare in low-grade gliomas (Ohgaki and Kleihues 2009) suggesting *PTEN* mutation is a late event in glioblastomagenesis. This is in contrast to some other mutations such as *TP53* which occurs at the same frequencies in both low and high grade gliomas. Besides *PTEN*, 88%

Figure 1-4. Distributions of primary brain and CNS tumor by histology. (A) Distribution of all primary brain and CNS tumors by histology. (B) Distribution of childhood primary brain and CNS tumors by histology (Ages 0-14). Source: CBTRUS. 2010. *CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2004-2006*. Central Brain Tumor Registry of the United States, Hinsdale.

of GBM samples harbored at least one genetic abnormality in the RTK/PI3K pathway including EGFR mutation and amplification (45%), *ERBB2* mutation (8%), *PDGFRA* amplification (13%), *PIK3CA* (15%), *NF1* mutation (18%) and other less common genetic changes (TCGA 2008). RTK/PI3K pathway together with p53 (87%) and RB (78%) tumor suppressor pathways comprised the three core pathways that were altered in GBM (Parsons et al. 2008; TCGA 2008).

Numerous human genomic analyses and mouse modeling studies have underscored the pivotal role of the sonic hedgehog (SHH) pathway in medulloblastoma pathogenesis. The binding of SHH to its receptor PTCH1 relieves its inhibition on SMO and allows the release of the transcription factor Gli from the inhibitory complex containing SUFU trans-activating downstream effector genes in the nucleus. SHH signaling plays an important role in driving proliferation of granule cell progenitors in the EGL of cerebellum. Inactivating mutations of *PTCH1* and *SUFU* and activating mutations of *SMO* have been found in 15-25% of sporadic medulloblastoma (Raffel et al. 1997; Reifenberger et al. 1998; Taylor et al. 2002; Parsons et al. 2011). The second signaling pathway that is frequently altered in the development of medulloblastoma is the Wnt pathway in which mutations in *APC*, *AXIN* or *CTNNB1* occur in 20% of the samples (Zurawel et al. 1998; Huang et al. 2000; Baeza et al. 2003; Parsons et al. 2011). Recent whole genome sequencing analysis also revealed new genetic alterations in this disease such as mutations in the histone-lysine N-methyltransferase *MLL2* and *MLL3* (20%) and *PTEN* (3/88) as well as other genetic changes like *TP53* (6/88), *MYC* (3/88) and *OTX2* (2/88) which have been implicated in medulloblastoma previously. Interestingly, three of the medulloblastomas with *PTEN* mutation also had either *TP53* or *PTCH1* mutation suggesting potential co-operational effect among them (Parsons et al. 2011).

Specific PTEN functions in brain tumors

Numerous studies in the past using glioma cell lines and xenograft models have revealed various aspects of PTEN tumor suppressor functions. For instance, re-expression of PTEN in the PTEN-deficient glioblastoma cell line could suppress proliferation in vitro by arresting cells in the G1 phase of the cell cycle through a p27Kip1-dependent manner (Furnari et al. 1998; Gottschalk et al. 2001). Reintroduction of PTEN into human glioma cell lines could inhibit the in vitro invasiveness which is associated with reduced activity of matrix metalloproteinases including MMP2 and MMP9 (Koul et al. 2001; Kubiakowski et al. 2001). PTEN reconstitution also decreased the growth of orthotopic transplants of human glioma cell lines in nude mouse brain by reducing angiogenesis. This was due to PTEN function in induction of thrombospondin1 expression which inhibited angiogenesis and inhibition of HIF-1 α that induces angiogenesis (Wen et al. 2001; Pore et al. 2006). However most human glioma cell lines have been maintained and passaged in tissue culture for decades. They have adapted to animal serum that is different from the brain environment they originally arise from. These cell lines often do not show the classical histologic appearance of human gliomas any more (Lee et al. 2006a) and are not predictive for response for clinical trials (Voskoglou-Nomikos et al.

2003). In addition, cell culture system is unable to model cancer invasion, angiogenesis, metastasis and the role of microenvironment in drug response.

Because of the limitations of cell line studies, genetically engineered mouse models with a close genetic resemblance to human disease have been utilized to better model human brain tumors. Several genetic mouse models of human glioma and medulloblastoma by knocking out *Pten* in combination with engineering of other disease-relevant genes have been published in recent years that provide new insights into these diseases as well as *Pten* tumor suppressor function in the brain tumors. Specific deletion of *Trp53* and *Pten* with Cre/loxP system in the mouse CNS generated spontaneous high-grade gliomas resembling human GBM. Inactivation of p53 and *Pten* promotes maintenance of mouse neural and glioma stem/progenitors in an undifferentiated state with high renewal potential by increasing *Myc* expression (Zheng et al. 2008). Haploinsufficiency of *Pten* could accelerate development of grade III astrocytomas generated by concomitant deletion of *Trp53* and *Nf1* and loss of *Pten* heterozygosity promoted progression into GBM (Kwon et al. 2008). Further study demonstrated specific inactivation of *Trp53*, *Nf1* and *Pten* in neural stem/progenitor cells in the SVZ was both necessary and sufficient to induce astrocytoma formation suggesting neural stem/progenitor as cells of origin for human GBM (Alcantara Llaguno et al. 2009). Another study provides similar evidence by showing that brain tumors arose from deleting *Rb;Trp53*, *Rb;Trp53;Pten* or *Pten;Trp53* in adult SVZ neural stem cells but not in mature astrocytes. This study also demonstrates that genetic mutations might specify brain tumor phenotypes since inactivation of *Pten;Trp53* gave rise to gliomas whereas deletion of *Rb;Trp53* or *Rb;Trp53;Pten* generated primitive neuroectodermal tumours (PNET) (Jacques et al. 2010). However, in another study all these combinations of tumor suppressor inactivation give rise to high-grade gliomas but not PNET (Chow et al. 2011). This discrepancy might be explained by the different strategies of gene inactivation between these two studies. The high-grade gliomas generated by Chow et al. (2011) mimic the expression profiling of human disease and arise from within and outside of the proliferative niches which demonstrates the relevance of the genetically engineered mouse models to human gliomas and also suggest that cells other than neural stem cells could serve as the origin of human gliomas.

In a mouse medulloblastoma model driven by *Shh* overexpression, *Pten* loss is shown to confer radiation resistance to cancer stem cells in the perivascular niche that is responsible for the tumor recurrence after irradiation (Hambardzumyan et al. 2008). *Pten* loss could also promote tumorigenesis in a mouse model of medulloblastoma driven by constitutive activation of *SmoA1* and is associated with poor prognosis with clinical patients (Castellino et al. 2010). Interestingly, *Pten* is spontaneously lost in combination with biallelic inactivation of *Ptch1* in the mouse medulloblastoma initiated with disrupted homologous recombination indicating a cooperation effect of PI3K and *Shh* pathway in medulloblastoma pathogenesis (Frappart et al. 2009).

Although past work has revealed various *Pten* functions in brain development and tumorigenesis, many questions still remain. How does loss of *Pten* cause the ectopic positioning of neurons? Is *Pten* involved in regulating directional migration as it is in

Dictyostelium? How does the migration of Pten-null neuronal progenitors change compared to their wild-type counterparts? How will inactivation of Pten affect the neural stem/progenitor cells postnatally? Is loss of Pten in the neural stem/progenitors sufficient for brain tumorigenesis? What are other cells of origins for brain tumors besides neural stem cells in the SVZ? In the present study, we deleted *Pten* as well as other tumor suppressor genes in neural stem/progenitors using *Nestin-CreER^{T2}* transgenic mice to address these questions.

CHAPTER 2. PTEN DELETION IN THE SUBVENTRICULAR ZONE CAUSES MTORC1-DEPENDENT ECTOPIC DIFFERENTIATION OF NEUROBLASTS WITHOUT DISRUPTING DIRECTIONAL MIGRATION

Introduction

In rodent brain, thousands of new neuronal precursors arise every day in the SVZ, the postnatal neural stem cell niche in the lateral walls of the lateral ventricles. Neuroblasts migrate from the SVZ through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into interneurons and are integrated into local circuits. Multiple cell types reside in this niche including Gfap-expressing neural stem cells which generate Mash1-expressing transit amplifying cells. Transit amplifying cells further differentiate into migrating neuronal precursor cells or neuroblasts that express the microtubule associated protein, Dcx (Alvarez-Buylla and Garcia-Verdugo 2002). In human brain, the SVZ niche harboring neural stem cells is conserved (Quinones-Hinojosa et al. 2006; Curtis et al. 2007), however the presence and cellular composition of the RMS remains somewhat controversial (Sanai et al. 2004; Curtis et al. 2007; Guerrero-Cazares et al. 2011). Although work conducted during recent years has unraveled many signals regulating the tangential migration from the SVZ to the OB, the extracellular cues and signaling pathways instructing when and where these tangentially migrating interneurons stop are largely unknown.

PI3Ks are an evolutionarily conserved family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides, essential regulators of diverse cellular processes including proliferation, survival, apoptosis, autophagy, growth, metabolism and cell migration (Engelman et al. 2006). Downstream of PI3K, Akt-mTorc1 signaling is a crucial branch of the pathway controlling protein synthesis, cell growth, autophagy and metabolism. Deregulation of the PI3K-Akt-mTorc1 pathway is associated with a variety of human genetic diseases, for instances, inherited mutations in *TSC1* or *TSC2* cause tuberous sclerosis; *NF1* mutation results in neurofibromatosis type1; and *PTEN* mutations underlie CS, BRRS, LDD and Proteus syndrome (Inoki et al. 2005; Rosner et al. 2008). Patients with these inheritable diseases frequently manifest neurological abnormalities indicating that proper PI3K-Akt-mTorc1 pathway activity is important in normal neural development and/or function. Conditional mouse models knocking out the individual component genes in the brain recapitulate human diseases and further underscore the importance of PI3K-Akt-mTorc1 pathway regulation of neural development. Mice with neuron-specific ablation of *Nf1* have relatively normal neuronal development but display a reduced cerebral cortex and extensive reactive astrogliosis (Zhu et al. 2001). Deletion of *Nf1* in astrocytes resulted in glial hyperplasia and optic nerve glioma (Bajenaru et al. 2003; Zhu et al. 2005) which is a hallmark of neurofibromatosis type1 patients. Mice deleted of *Tsc1* or *Tsc2* in neural progenitor or neurons demonstrated disrupted cortical and hippocampal lamination, ectopic dysplastic neurons as well as abnormal myelination and astrogliosis which recapitulate the neuropathology in tuberous sclerosis patients (Meikle et al. 2007; Way et al. 2009).

Pten, an important tumor suppressor gene, is a major negative regulator of the PI3K pathway. It functions as a lipid phosphatase antagonizing the activity of PI3Ks by catalyzing dephosphorylation of PIP3 to PIP2. Pten plays a number of roles in brain development including negatively regulating self-renewal and proliferation of neural stem cells (Groszer et al. 2001) and controlling neuronal size (Backman et al. 2001; Kwon et al. 2001). A pronounced phenotype in the brain of Pten knock-out mice is the disrupted lamination and ectopic differentiation of neurons, which had been attributed to failed neuronal migration (Backman et al. 2001; Groszer et al. 2001; Kwon et al. 2001; Marino et al. 2002; Yue et al. 2005). However, the underlying mechanism of how Pten is involved in neuronal migration is currently unknown.

In the present study, we demonstrate that Pten inhibits downstream activation of mTorc1 in normal migrating neuroblasts in the RMS. Deletion of Pten in the SVZ and RMS caused ectopic differentiation of neuroblasts without disrupting the directional migration in the RMS.

Experimental Procedures

Mice

Nestin-CreER^{T2} mice (Cicero et al. 2009) were generated using a transgene containing the *Nestin* promoter and second intron (Zimmerman et al. 1994) to direct expression of CreER^{T2} (Metzger et al. 1995; Feil et al. 1996; Feil et al. 1997), an internal ribosomal entry site (IRES), and human placental alkaline phosphatase (hPLAP) specifically in progenitor cells in the central nervous system. To map tamoxifen induction of Cre activity, *Nestin-CreER^{T2}* transgenic mice were bred with *R26R-LacZ* (Soriano 1999) or *R26R-EYFP* (Srinivas et al. 2001) mice to generate *Nestin-CreER^{T2};R26R-LacZ* or *Nestin-CreER^{T2};R26R-EYFP* mice. Cre reporter activity was detected by X-gal staining or EYFP IF staining on frozen tissue sections. *Pten* conditional knock-out mice (*Nestin-CreER^{T2}; Pten^{loxP/loxP}*, or *R26R-EYFP;Pten^{loxP/loxP}* for Cre electroporation experiments) were generated by intercrossing *Nestin-CreER^{T2}* or *R26R-EYFP* transgenic mice to *Pten^{loxP}* mice (Suzuki et al. 2001), a gift from Tak Mak (University of Toronto, Toronto). For all analyses including *Nestin-CreER^{T2}*, the transgene was hemizygous to avoid possible variation from transgene dosage. Littermate *Pten^{loxP/loxP}* mice without the *Nestin-CreER^{T2}* transgene were used as control and processed the same way as *Pten^{CKO}* mice. All procedures were reviewed and approved by the Animal Care and Use Committee at St. Jude Children's Research Hospital.

Genotyping polymerase chain reaction assays

Polymerase chain reaction (PCR) was used to genotype mice. Primers used are: *Cre*, forward primer: 5'AGCGATCGCTGCCAGGAT; reverse primer: 5'ACCAGCGTTTTCGTTCTGCC; expected product: 157 base pairs. *Pten*, forward

primer: 5'TTATCTGGATCAACTTTGGGCC; reverse primer: 5'TCCCACCAATGAACAAACAGT; expected products: 146 (wild-type) and 244 (loxP) base pairs.

Induction of Cre activity

Tamoxifen (TM) (Sigma) was dissolved at 20mg/ml or 5 mg/ml at 37°C in corn oil (Sigma) for Cre induction in adult mice or pups respectively. Dissolved TM was sterilized by filtering and stored at 4°C in the dark for up to 10 days. Pups were injected with 3 mg/40g TM intraperitoneally (i.p.) daily from P0-1 or P11-12 as indicated. Adult mice were injected with 9 mg/40g TM i.p. daily from P28-30. An equivalent volume of sterile filtered corn oil alone was used for vehicle injections. Multiple injections in the same mouse were separated by 24 hours.

5-Bromo-2'-deoxyuridine injection and rapamycin treatment

5-Bromo-2'-deoxyuridine (BrdU) (Sigma) was dissolved at 5 mg/ml in sterile phosphate buffered saline (PBS) and stored at -20°C. BrdU was injected i.p. at a dose of 50 µg/g body weight every 2 hours for 5 injections for short-term BrdU labeling or every 12 hours for 6 times from P4-P6 for BrdU birthdating experiment.

Rapamycin (LC Laboratories) was dissolved at 20 mg/ml in sterile dimethyl sulfoxide (DMSO), stored at -20°C and diluted in 5.2% Tween80 as working solution before use. Rapamycin was injected i.p. at 1.5 µg/g body weight daily from P8-P31 of age. An equivalent volume of sterile DMSO+Tween80 alone was used for vehicle injections. When TM was given on the same day, the two drugs were separated by 6 hours.

Histochemistry

Mice were anesthetized and perfused transcardially with PBS followed by 2% paraformaldehyde (PFA) in PBS. Following dissection, tissues were post-fixed overnight in 2% PFA in PBS at 4°C, and then equilibrated in 25% sucrose in PBS for an additional 24 hours at 4°C. Tissues were embedded in TBS embedding media (Triangle Biomedical Sciences) on dry ice and cryosectioned at thickness of 12 µm. Tissue slides were equilibrated at room temperature for 20 minutes then washed three times in PBS prior to staining.

For detection of β -galactosidase activity, slides were washed in Rinse A (100 mM NaPO₄, pH 7.3; 2 mM MgCl₂; 5 mM EGTA) for 30 min followed by Rinse B (100 mM NaPO₄ pH 7.3; 2 mM MgCl₂; 0.01% sodium deoxycholate; 0.02% NP-40) for 5 min. Slides were then stained at 37°C for overnight in Developing Buffer (1 mg/ml X-gal; 100 mM NaPO₄; pH 7.3; 2 mM MgCl₂; 0.01% sodium deoxycholate; 0.02% NP-40; 5 mM

K₃Fe(CN)₆; 5 mM K₄Fe(CN)₆). After washing in PBS, slides were counterstained with Nuclear Fast Red (Vector Labs).

Immunohistochemistry and immunofluorescence

Cryosections were prepared as described above. For paraffin sections, mice were processed the same way as above except the PFA concentration was 4%PFA. Following perfusion, tissues were post-fixed for an additional 24 hours in 4% PFA, embedded in paraffin, and cut into 5 µm sections. Primary antibodies used for immunostaining were against GFP (1:1000, Invitrogen A6455; 1:2000, Abcam #13970), Pten (1:100 for immunohistochemistry (IHC), 1:500 for immunofluorescence (IF) with Tyramide amplification, Cell Signaling #9559), p-Akt S473 (1:50, Cell Signaling #9271), p-S6 S235/236 (1:500, Cell Signaling #2211), Dcx (1:4000 for IHC, 1:500 for IF, Chemicon AB5910), Mash1 (1:100, BD #556604), NeuN (1:500, Chemicon), Map2 (1:5000, Sternberger SMI52), Calretinin (1:2000, Chemicon AB149), Gfap (1:200, Sigma G3893), Ki67 (1:5000, Novocastra NCL-Ki67p), BrdU (1:1000, AbDSerotec OBT0030CX) and active Caspase3 (1:1000, BD #559565). For IHC, microwave antigen retrieval was performed for all antibodies and biotinylated secondary antibodies were used in conjunction with horseradish peroxidase-conjugated streptavidin (Elite ABC, Vector Laboratories). Tyramide Signal Amplification (Perkin Elmer) was used to improve Pten IF and p-Akt S473 signal. Color development was conducted with substrates NovaRed, DAB or VIP (Vector Laboratories) and counterstaining with hematoxylin or methyl green (Vector Laboratories), respectively. For IF, Alexa Fluor 488, 647 (Invitrogen) and cyanine 3, 5 (Jackson ImmunoResearch) conjugated secondary antibodies were employed along with Vectashield mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was conducted with the ApopTag peroxidase in situ apoptosis detection kit (Chemicon S7100) according to the manufacturer's protocol.

Ki67 and BrdU positive cell quantification

Ki67 or BrdU immunostaining from anatomically matched sections were chosen for quantification. For proliferation analyses, the Bioquant system (R&M Biometrics) was used to count all the Ki67+ cells in the SVZ. For the BrdU birthdating experiment, BrdU+ cell number and granule cell density in a field of 20x objective in the same areas of the OB granule cell layer (GCL) or total number of BrdU+ cell in the SVZ were counted with ImageJ v1.44 cell counter. Four controls and four *Pten*^{CKO} brains were analyzed for each group and positive cells were counted from three sections from each brain for each staining. Student's t-test was used for statistical significance analysis.

In vivo electroporation

In vivo electroporation was performed using a similar approach to previous reports (Boutin et al. 2008; Chesler et al. 2008). 2 μ l of endotoxin-free *pCAG-Cre* expression plasmid (0.65 μ g / μ l, in PBS containing 1% fast green) was injected into the lateral ventricle of P2 pups with a 30G needle Hamilton syringe using the midpoint between Bregma and Lambda and 1mm lateral to the midline suture as landmarks for injection. Fast green dye allowed visual confirmation of correct injection into lateral ventricles. Only successfully injected animals were subjected to five square-wave electrical pulses (50 ms separated by 950 ms intervals, 150v). Electroporated pups recovered on a 37°C heating pad before returning to the mother.

Brain slice preparation, time-lapse live imaging and migration analysis

Mouse brains were quickly removed and placed in sterile filtered cold (4°C) dissecting Complete Hank's Balanced Salt Solution (Complete HBSS) containing 1x HBSS (GIBCO), 2.5 mM Hepes (pH 7.4, GIBCO), 30 mM D-glucose, 1 mM CaCl₂, 1 mM MgSO₄, and 4 mM NaCO₃ (Polleux and Ghosh 2002). After dissection, brains were embedded in 3% low-melting point agarose (Promega) and cut into 300 μ m thick sections sagittally with a vibratome. Slices containing the RMS were identified by EYFP expression, equilibrated for 2 hours in artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 20 mM D-glucose (285-295 mOsm) with 95% O₂ / 5% CO₂ at room temperature and then transferred into the submerged recording chamber and superfused (1-2 ml/min) with warm (36-37°C) aCSF. Time-lapse live imaging was acquired by two-photon laser-scanning microscopy equipped with an Ultima imaging system (Prairie Technologies), a Ti:sapphire Chameleon Ultra femtosecond-pulsed laser (940 nm) (Coherent), and a 20x 0.95 numerical aperture water-immersion infrared objective (Olympus). Z stacks (512 x 512 pixels, 0.079 μ m/pixel, 60-100 sections per stack, 1 μ m/section) were collected at the elbow of the RMS every 5 minutes for 3-6 hours.

Average projections of 20 consecutive sections in the middle of z-stacks of time-lapse live imaging were analyzed using SlideBook v5.0 (Intelligent Imaging Innovations). Cells that exhibited bipolar morphology and moved at least two cell body lengths were counted as "bipolar migrating cells". All other cells were considered "stationary or non-polar cells". For each movie, all the EYFP+ cells were manually tracked but only those that could be observed more than 5 consecutive frames were included in the analysis. The average speeds (total displacement / time) or endpoint speeds (the distance between the first and last timepoints of the path / time) were binned in 0.005 μ m/second interval to obtain a distribution. χ^2 analysis or Student's t-test was used to assess if the distributions or means, respectively, of average speeds (or endpoint speeds) were significantly different.

Results

PI3K pathway activity in the wild-type SVZ-RMS-OB

To understand how PI3K signaling is regulated in the SVZ-RMS-OB, we examined expression of several key components of the pathway. Levels of *Pten* expression were heterogeneous in the SVZ and all of the cells expressing *Pten* were Dcx+ neuroblasts (Figure 2-1A). In the SVZ and proximal RMS, the majority of cells expressing p-S6, a downstream indicator of PI3K/mTorc1 pathway activity, were Mash1+ transit amplifying cells, and not Dcx+ migrating neuroblasts (Figure 2-1B and data not shown). In contrast, in the terminal RMS at the OB core, a substantial population of Dcx+ type A cells expressed p-S6 (Figure 2-1C). This indicates that the PI3K pathway was not strongly active in migrating neuroblasts until these cells reached their destination in the OB.

Postnatal deletion of *Pten* caused ectopic differentiated neurons in an expanded SVZ and proximal RMS

To evaluate the function of the PI3K pathway regulator *Pten* in postnatal neural stem/progenitors, we generated *Nestin-CreER^{T2}* mice with inducible Cre activity in the SVZ (Figure 2-2), and bred them to *Pten^{loxP/loxP}* (Suzuki et al. 2001) to generate inducible *Pten* conditional knockout mice (hereafter *Pten^{CKO}*). Induction of *Pten* deletion in adult SVZ by TM injection in *Pten^{CKO}* mice at P30-P32 did not cause obvious neurological abnormalities throughout a normal lifespan except for occasional seizures in some aged animals. Analysis of all *Pten^{CKO}* brains (n=24) showed dramatic expansion of the SVZ (Figure 2-3A) and proximal RMS (Figure 2-4). The expansion was most pronounced at the dorsal junction of the SVZ abutting the corpus callosum and the ventral tip where it formed a bulb-like structure. The expansion started to appear as early as 20 days post induction (DPI) and was similar at 60, 150, 300, and 540 DPI without progressive enlargement at later time points. IHC analysis showed the expanded SVZ was uniformly negative for *Pten* staining with marked elevation of p-Akt indicating overall activation of the PI3K pathway in the entire region (Figure 2-3B, C).

When conditional *Pten* deletion was induced in newborn *Pten^{CKO}* mice by TM injection at P0 and P1, similar SVZ and RMS expansion were consistently detected by P10-30 in the absence of other obvious abnormalities (data not shown), although the ventral expansion of the SVZ was not detected at this age.

Close examination of the cell morphology in the SVZ revealed that in contrast to wild-type control brains in which the SVZ was mainly composed of well-organized spindle-like progenitor cells with scant cytoplasm, the expanded SVZ in *Pten^{CKO}* mice contained a central region of cells with large round nuclei interspersed with isolated progenitor cells, and with clusters of progenitor cells at the edge (Figure 2-3A). This expanded region of the SVZ and RMS was composed of Dcx+ and NeuN+ cells which

Figure 2-1. The PI3K-mTorc1 pathway was inactive in migrating SVZ neuroblasts, but activated in OB neuroblasts.

IF staining in the SVZ (A and B) and OB (C) of coronal brain sections from 6-month old wild-type mice. (A) Pten (green) and Dcx (red) double IF labeling of the SVZ. Nuclei in the SVZ are shown with DAPI (white). Pten was expressed in Dcx+ cells in the SVZ (overlay of all channels in the far right panel). (B and C) Mash1 (white), Dcx (red) and p-S6 (green) triple IF labeling of the SVZ (B) and OB (C), overlay shown in far right panels. p-S6 was expressed in Mash1+ transit amplifying cells but not Dcx+ migrating neuroblasts in the SVZ (B) while in the OB, a substantial population of Dcx+ type A cells expressed p-S6 (C). The white dashed lines in the overlay mark the boundary of the SVZ in panels A and B. Scale bar: 20 μ m.

Figure 2-2. Nestin-CreER targeted neural stem/progenitor cells in the SVZ.

(A) Schematic diagram of the *Nestin-CreER* transgene used to target neural stem/progenitor cells. (B and C) X-gal staining of sagittal (C and B left and center panels) or coronal (B, right panel) sections from *Nestin-CreER;Rosa26R-LacZ* reporter mice. (D) EYFP IF of SVZ from *Nestin-CreER;Rosa26R-EYFP* reporter mice. The mice were injected with vehicle or tamoxifen (TM) daily between P30-32 (B) or between P0-1 (C and D) and brains were collected 5 days after the first injection. The most uniform Cre activity was induced in the SVZ, RMS and the inner layer of the DG where neural stem/progenitors reside. The vast majority of cells in the SVZ were positive for the Cre reporters X-gal or EYFP. Cre reporter activity was also detected at low levels in scattered cells throughout other brain regions outside of neurogenic niches. This activity outside of the neural stem cell niche was more robust when Cre was induced in neonates (C). (E) Gfap (red) + EYFP (green), Mash1 (red) + EYFP (green) and Dcx (red) + EYFP (green) double IF staining in the SVZ showed overlap of EYFP+ cells with the neural stem cells (Gfap+), transit amplifying cells (Mash1+) or neuroblasts (Dcx+) in the far right panels. Scale bar: D=100 μ m, E=10 μ m. Nuclei were visualized with DAPI (blue).

Figure 2-3. Postnatal *Pten* deletion caused ectopic differentiated neurons in an expanded SVZ.

Representative H&E (A), IHC (B-E) and IF staining (F) of the SVZ from matched coronal brain sections of mice injected with TM on P30-32 and analyzed more than 70 days later (n=24). A, D and E upper and lower panels show dorsal and ventral SVZ, respectively. (A) H&E staining showed that the *Pten*^{cKO} (cKO) SVZ was expanded compared to wild-type (WT). Black box on the cKO panel indicates the expanded SVZ region shown at higher magnification on the right panel. Scale bar: left panel=100 μ m, higher mag panel=10 μ m. (B-E) IHC staining of WT and cKO SVZ for Pten (B), p-Akt (C), NeuN (D) and Dcx (E). The expanded cKO SVZ was deficient for Pten, strongly positive for p-Akt and composed of NeuN⁺ and Dcx⁺ cells in contrast to the WT SVZ which had only thin layers of Dcx⁺ cells and lacked NeuN⁺ cells. In panel D, the NeuN⁺ cells adjacent to the wild-type SVZ were from surrounding striatum and the black line marks the edge of the expanded SVZ in cKO brain. Scale bar: B and C=300 μ m, D and E=100 μ m. (F) Mash1 (white), p-S6 (green) and Dcx (red) triple IF labeling of the expanded cKO SVZ showed a substantial overlap of p-S6⁺ and Dcx⁺ cells in the right bottom panel. Scale bar: 20 μ m.

Figure 2-4. Expansion and ectopic neuronal differentiation in the RMS.

IHC staining of the RMS from matched coronal brain sections of mice injected with TM on P30-32 and analyzed at 6 months of age (A and B) or mice injected with TM on P0-1 and analyzed at 18 days of age (C). (A) Pten IHC staining of both the WT and cKO RMS showed the cKO RMS was expanded and deficient for Pten. The arrow points to a thin layer of RMS in the WT whereas the solid black line outlines the cKO RMS. Scale bar: 200 μ m. (B) NeuN IHC staining of both the WT and cKO RMS showed the cKO RMS was expanded and composed of NeuN⁺ cells, whereas the WT RMS was a thin layer devoid of NeuN⁺ cells. Red dashed lines outline both the WT and cKO RMS. Scale bar: 100 μ m. (C) Dcx IHC staining of WT and cKO RMS showed expansion of the cKO RMS and formation of projections, whereas in the WT control, DCX expression was in a small rim tightly surrounding nuclei. Scale bar: 100 μ m.

mark migrating neuroblasts and differentiated neurons respectively (Figure 2-3D, E and Figure 2-4B, C). p-S6 staining in the Dcx+ neuroblasts of the *Pten*^{CKO} SVZ (Figure 2-3F) shows aberrant activation of the PI3K pathway in contrast to the absence of p-S6 expression in SVZ neuroblasts in wild-type mice (Figure 2-1B). The Dcx+ cells in the expanded SVZ and RMS showed evidence of neuronal differentiation including abnormal formation of projections (Figure 2-3E and Figure 2-4C), and Map2 expression, which marks dendrites in polarized neurons (Figure 2-5). The expression of Dcx decreased and NeuN expression increased in the ectopic cells as mice aged from 150 DPI to 300 DPI (data not shown). The expression of mature neuronal markers in Dcx+ cells in the SVZ indicates ectopic differentiation of neuroblasts that normally remain undifferentiated until they reach the OB. Furthermore, the ectopic cells also expressed calretinin (Figure 2-5), a marker expressed in mature GABAergic interneurons of the OB arising from neuroblasts that migrate from the SVZ through the RMS to populate the GCL and the periglomerular cell layer of the OB (Kohwi et al. 2005; Kohwi et al. 2007; Alvarez-Buylla et al. 2008).

Consistent with a failure of migrating neuroblasts to reach the OB, the diameter of the terminal RMS at the core of the OB and the cell density in the GCL were markedly reduced in *Pten*^{CKO} mice (Figure 2-6 and Figure 2-7A, C).

Pten loss did not induce substantial changes in proliferation or survival in the expanded *Pten*^{CKO} SVZ

To determine how loss of Pten affected the proliferation in the intact SVZ in vivo, we used both Ki67 IHC and short-term BrdU labeling, which gave consistent results (Figure 2-8A and data not shown). Because the cell numbers in the SVZ of *Pten*^{CKO} mice were increased due to the presence of ectopic post-mitotic differentiated cells, considering the percentage of proliferating cells would underestimate the proliferation capacity of progenitors in the *Pten*^{CKO} SVZ. Therefore, we counted the absolute numbers of Ki67+ cells from matched SVZs of both wild-type and *Pten*^{CKO} mice. There was no statistically significant difference in the proliferation rate in the *Pten*^{CKO} SVZ compared to control littermates (Figure 2-8A, C).

Activated PI3K signaling can also enhance cell survival, therefore we used active caspase3 and TUNEL staining to determine if Pten loss altered apoptosis in the SVZ (Figure 2-9A). The endogenous level of apoptosis was very low, and not significantly different in wild-type and *Pten*^{CKO} SVZ. Consistent with the resistance of differentiated neurons to radiation induced death (Frappart and McKinnon 2008), the expanded portion of the *Pten*^{CKO} SVZ containing differentiated neurons as well as the parenchyma surrounding the SVZ were resistant to irradiation-induced apoptosis (Figure 2-9B). However, the progenitor cells of both wild-type and *Pten*^{CKO} underwent apoptosis 5 hours after exposure to 4 Gy of irradiation. Therefore, Pten-deficient neuroblasts did not show enhanced survival.

Figure 2-5. Ectopic cells in the expanded cKO SVZ expressed markers of mature neurons.

Map2 (A) and Calretinin (B) IHC staining of the SVZ from matched coronal brain sections of WT (left) or cKO (right) mice injected with TM on P0-1 and analyzed at 18 days of age showed strong expression of these markers of neuronal differentiation in the expanded cKO SVZ. Scale bar: 100 μ m.

Figure 2-6. Reduced diameter of the terminal RMS and decreased granule cell density in the Pten^{cKO} OB.

Pten IHC staining from matched sagittal brain sections of the forebrain (A) or coronal brain section of the OB (B and C) from WT and cKO mice injected with TM on P0-1 and analyzed at P18 (A and B) or injected with TM on P30-32 and analyzed at 11 months of age (C). The cKO SVZ was expanded and deficient for Pten (indicated by arrows in panel A). The diameter of the terminal RMS in the cKO OB was diminished (indicated by arrowheads in panel A and dashed circles in panel B). The cell density of the GCL in the cKO OB was reduced (C). Scale bar: A=500 μ m, B=300 μ m, C=100 μ m.

Figure 2-7. *Pten*^{cKO} neuroblasts terminated tangential migration prematurely in the SVZ.

Immuno-staining (A-C) and quantification of BrdU+ cells (D) of matched sagittal brain sections from WT (left) and cKO (right) mice. Cre activity was induced by tamoxifen injection at P0 and 1, BrdU was injected on P4, 5, and 6, and tissue was analyzed at P18. (A and B) BrdU IHC staining. (A) In the OB, there were reduced numbers of BrdU+ cells in the granule cell layer of the cKO OB compared to WT. (B) In the SVZ, there was a significant accumulation of BrdU+ cells in the expanded cKO SVZ. (C) BrdU (red) and NeuN (green) double IF labeling of the SVZ from WT and cKO mice showed a substantial number of BrdU+ cells in the expanded cKO SVZ were NeuN+, while there were no NeuN+ cells in the WT SVZ. Black dashed lines in panel B and white dashed lines in panel C mark the boundary of the SVZ. Scale bar: 50 μ m. (D) Quantification of the granule cell density in OB (OB-Density, $p=0.001$), BrdU+ cell in the OB (OB-BrdU+, $p<0.0001$) and the percentage of BrdU+ cells in the OB (OB-%BrdU+, $p=0.008$) were all significantly reduced in the GCL of the cKO OB, whereas the total number of BrdU+ cells in the cKO SVZ (SVZ-BrdU, $p=0.003$) was markedly increased ($n=4$, for each genotype).

Figure 2-8. mTorc1 inhibition did not affect proliferation or apoptosis in the SVZ. Ki67 IHC staining in the SVZ of matched coronal brain sections from WT or cKO mice treated with (A) vehicle or (B) rapamycin. Mice were treated with rapamycin or vehicle from P8-P31, Cre activity was induced by TM injection at P11 and P12, and tissue was analyzed at P31. Scale bar: 100 μ m. (C) Quantification of Ki67+ cells in the SVZ showed no significant difference between vehicle-treated control and cKO or between vehicle and rapamycin-treated cKO. (D) active Caspase 3 IHC staining of the SVZ from vehicle or rapamycin-treated cKO mice. There were few active Caspase 3+ cells in either vehicle or rapamycin-treated cKO SVZ, and no difference was detected. Scale bar: 100 μ m. n=3 mice per genotype.

Figure 2-9. *Pten*^{cKO} SVZ neural progenitors were sensitive to irradiation and showed no change in apoptosis compared to control mice.
 (A) TUNEL (top panels) and active caspase 3 (bottom panels) IHC staining of the SVZ in matched coronal sections from mice injected with TM on P0-1 and analyzed at 18 days old. The endogenous levels of apoptosis were very low in the SVZ and no significant difference in apoptosis was detectable between untreated WT and cKO mice. (B) The same IHC staining as in panel A. Mice were irradiated with 4 Gy and tissue collected 5 hrs later. The progenitor cells in both WT and cKO SVZ underwent apoptosis after irradiation whereas the parenchyma surrounding the SVZ and the expanded part of the cKO SVZ were resistant to irradiation-induced apoptosis. Scale bar: 100 μ m.

***Pten*^{CKO} neuroblasts terminated tangential migration prematurely in the SVZ and RMS**

To determine if the ectopic neurons arose from proliferative cells in the SVZ that underwent premature termination of tangential migration along the SVZ-RMS-OB, we used BrdU birth dating. *Pten* deletion was induced by TM injection in newborn mice. Four days after TM induction, to allow sufficient time for *Pten* deletion and loss of Pten protein, BrdU was injected to label proliferating cells in the SVZ. As expected, the majority of labeled cells in control mice migrated into the GCL of the OB by 15 days after the BrdU pulse (Petreanu and Alvarez-Buylla 2002) (Figure 2-7A). In comparison, there was a significant accumulation of BrdU+ cells in the expanded SVZ and greatly reduced numbers of BrdU+ cells, and reduced granule cell density in the OB GCL in the *Pten*^{CKO} mice (Figure 2-7A,B,D). A high percentage of BrdU+ cells in the expanded SVZ showed neuronal morphology and expressed NeuN, indicating that the ectopic differentiated neurons arose from previously proliferating cells in the SVZ (Figure 2-7C). In control mice, there were few BrdU+ cells remaining in the SVZ, and none of them had neuronal morphology or expressed NeuN. They may correspond to slow-cycling neural stem cells or local proliferating glia (Figure 2-7C).

Inhibition of mTorc1 rescued the SVZ-RMS expansion in *Pten*^{CKO} brain

To determine if inhibition of a downstream effector in the aberrantly activated PI3K pathway could prevent the early termination of tangential migration and ectopic differentiation of *Pten*^{CKO} neuroblasts in the SVZ, we treated the mice with rapamycin, an inhibitor of mTorc1 activity. We previously showed that rapamycin required several days of administration to maximally block mTorc1 signaling in brain (Kwon et al. 2003). Therefore, we pre-treated the mice daily with rapamycin for three days, then induced the deletion of *Pten* with TM at P11-12 and continued rapamycin treatment until P31. Rapamycin treatment completely prevented the expansion of the SVZ in *Pten*^{CKO} mice (Figure 2-10A), although the SVZ remained *Pten*-null in rapamycin treated mice (Figure 2-10B). Rapamycin selectively blocked mTorc1 activity, as shown by lack of p-S6, a downstream indicator of mTorc1 activity (Figure 2-10C) without altering levels of p-Akt in the *Pten*-deficient SVZ (Figure 2-10D). Neither apoptosis nor proliferation was affected by rapamycin treatment (Figure 2-8).

Ex vivo time-lapse live imaging showed *Pten*^{CKO} neuroblasts had normal directional migration with increased speed

To understand how *Pten* loss affected the movement of tangentially migrating neuroblasts, we used time-lapse live imaging on acute brain slices. We transfected the expression construct *pCAG-Cre* into the SVZ by in vivo electroporation at age P2 and visualized transfected cells by expression of the EYFP Cre reporter in *R26R-EYFP;Pten*^{wt/wt} (wild-type) or *R26R-EYFP;Pten*^{loxP/loxP} (*Pten*^{CKO}) mice. Neuroblast migration was imaged with two-photon microscopy at the “elbow” of the RMS, at the

Figure 2-10. mTorc1 inhibition rescued the expansion of *Pten*^{cKO} SVZ.

Representative IHC staining for Dcx (A), Pten (B), pS6 (C), and pAkt (D) in the SVZ of matched coronal brain sections from vehicle or rapamycin treated WT or cKO mice. Mice were treated with rapamycin or vehicle from P8-P31, Cre activity was induced by TM injection at P11 and P12, and tissue was analyzed at P31. Rapamycin completely rescued the enlargement of cKO SVZ to restore the size and morphology seen in WT mice (n=4 in each group). The SVZ of rapamycin treated cKO mice remained Pten-null (B) and continued to show elevated pAkt (D), but showed suppression of pS6 (C), a downstream indicator of mTorc1 activity. Scale bar: 50 μ m.

region where the RMS curves from ventral towards rostral migration halfway between the SVZ and the OB (see Figure 2-11 C for the location) at age P19-20. The majority of EYFP+ cells in the wild-type RMS showed a bipolar morphology and migrated effectively. In contrast, there were two distinct populations of EYFP+ cells in the *Pten*^{CKO} RMS (Figure 2-12A). One population maintained bipolar morphology and migrated normally as in the wild-type control, while another population lost polarity and showed only local non-directional movement. Interestingly, the cells that had stopped directional migration showed active spontaneous membrane ruffling, which distinguished them from completely static cells seen at very low frequency in both the wild-type and *Pten*^{CKO} RMS (Supplementary movies A-1 and A-2). The average speed of all EYFP+ cells in the *Pten*^{CKO} RMS (mean: 0.0096 $\mu\text{m/s}$) was significantly slower than that of the wild-type (mean: 0.0143 $\mu\text{m/s}$) ($p < 10^{-19}$). However, the average speed of bipolar migrating neuroblasts in the *Pten*^{CKO} RMS (mean: 0.0173 $\mu\text{m/s}$) were slightly but significantly faster than the wild-type (mean: 0.0156 $\mu\text{m/s}$) ($p = 0.006$) (Figure 2-12B, C). The *Pten*^{CKO} bipolar migrating neuroblasts moved effectively in the appropriate caudal to rostral direction, as their endpoint speeds, a representation of directional movement, were slightly but significantly greater than the wild-type (mean 0.0145 $\mu\text{m/s}$ and 0.0131 $\mu\text{m/s}$, respectively) ($p = 0.047$). Accordingly, a substantial number of Pten-deficient neuroblasts were able to migrate to the OB (Figure 2-11). Both the EYFP+ bipolar and round non-polar cells in the RMS expressed Dcx, indicating that they were migrating neuroblasts or neuroblasts that stopped migration recently (Figure 2-13). These data indicate that Pten-deficient neuroblasts do not have an intrinsic defect that compromises migration.

Discussion

Pten function in neuronal migration and differentiation

Multiple conditional knock-out models have shown that *Pten* deletion during development results in incomplete neuronal migration causing disruption of the laminar structure in cerebral cortex and cerebellum. The accumulation of ectopic differentiated *Pten*^{CKO} neurons in the SVZ and RMS observed in the present study is consistent with defects in tangential migration, similar to the failure of radial glia-guided migration in the cerebral cortex and cerebellum observed in previous models (Backman et al. 2001; Groszer et al. 2001; Kwon et al. 2001; Marino et al. 2002; Yue et al. 2005). SVZ expansion associated with premature termination of tangential migration was also observed in mouse models knocking out PSA-NCAM (Tomasiewicz et al. 1993; Ono et al. 1994), serum response factor (Alberti et al. 2005), ApoER2/VLDL or Dab1 (Andrade et al. 2007), which might suggest an epistatic relationship between them and Pten.

PTEN and the PI3K-Akt-mTorc1 pathway have been implicated in cell migration regulation in multiple contexts. Mechanistically, this has been mostly clearly demonstrated in *Dictyostelium discoideum* where subcellular localization of Pten and PI3K to the trailing and leading edge of the cell, respectively, established a PIP3 gradient

Figure 2-11. EYFP+ cells in the *Pten*^{cKO} RMS and OB were Pten-null.

Pten^{flox/flox} (cKO) mice carrying a ROSA-lox-STOP-lox-EYFP allele were transfected with Cre by in vivo electroporation as described for Figure 2-12. EYFP and Dcx double IF labeling of the cKO RMS elbow from littermate mice electroporated at the same time as those used for in vivo time lapse imaging showed that both the arrested non-polar cells (arrowheads) and the elongated migrating cells (arrow) in the cKO RMS were positive for Dcx (overlay in the bottom right panel). Scale bar: 100 μ m.

Figure 2-12. Ex vivo time-lapse live imaging showed *Pten*^{cKO} neuroblasts had normal directional migration with faster speed.

(A) Representative static EYFP images corresponding to the time-lapse migration movies from the RMS (supplemental). A Cre expression construct was transfected by in vivo electroporation at P2 into the SVZ of WT or *Pten*^{flx/flx} (cKO) mice carrying a ROSA-lox-STOP-lox-EYFP allele. Acute brain slices were prepared at P19-20, and cells in which Cre-mediated recombination occurred were visualized by EYFP reporter expression using two-photon microscopy. The vast majority of WT neuroblasts had bipolar morphology and were migrating. There were two distinct populations of *Pten*^{cKO} (cKO) neuroblasts; cells with elongated bipolar morphology and normal migration (indicated by arrowheads), and cells with large rounded morphology that showed only local non-directional movement (indicated by arrows). Scale bar: 50 μm . (B and C) Quantification and histogram of average speeds for bipolar migrating EYFP+ cells (B) or all EYFP+ cells (C) from WT (red bars) and cKO RMS (green bars). $n=3$ mice per genotype.

Figure 2-13. The arrested EYFP+ cells in the *Pten*^{cKO} RMS were in the neuronal lineage.

Pten^{flox/flox} (cKO) mice carrying a ROSA-lox-STOP-lox-EYFP allele were transfected with Cre by in vivo electroporation as described for Figure 2-12. EYFP and Dcx double IF labeling of the cKO RMS elbow from littermate mice electroporated at the same time as those used for in vivo time lapse imaging showed that both the arrested non-polar cells (arrowheads) and the elongated migrating cells (arrow) in the cKO RMS were positive for Dcx (overlay in the bottom right panel). Scale bar: 100 μ m.

that was required for directional migration and chemotaxis (Funamoto et al. 2002; Iijima and Devreotes 2002). Deletion of the Akt homolog PkbA, rescued the cytokinesis and chemotaxis defects in *Pten* deficient Dictyostelium (Tang et al. 2011). In mammalian cells, the PI3K pathway can regulate cell motility and migration through Akt activation as well as through Akt-independent mechanisms such as RAC1/CDC42 signaling (Kolsch et al. 2008). In addition, overexpression of wild-type PTEN inhibited migration of human glioma cell lines in an in vitro wound healing assay, and this effect was dependent on the protein phosphatase activity of PTEN independent of PI3K signaling (Raftopoulou et al. 2004).

Unexpectedly, we found that *Pten*-null neuroblasts in the RMS had normal directional movement with faster speed using two-photon time-lapse microscopy of ex vivo slice cultures that maintain the physiological context for neuronal migration. Postnatal deletion of *Pten* divided the RMS neuroblasts into two distinct populations; one subset maintained their bipolar morphology and had normal directional migration and the other population lost polarity and stopped migration. This non-polar morphology, in association with increased frequency of spontaneous protrusions of the plasma membrane, was reminiscent of *Pten*-null Dictyostelium. However, the population of *Pten*^{CKO} neuroblasts that maintained bipolar morphology continued moving normally, demonstrating that *Pten* was not required for directional migration. This is consistent with the observation that some *Pten*-null cells reach the OB in *Pten*^{CKO} mice. A previous study in which *Pten* was deleted in Gfap-expressing neural stem cells also showed that *Pten*-deficient neurons were capable of reaching the olfactory bulb. This study also noted an expansion of Dcx+ cells in the SVZ and RMS, although they did not analyze expression of mature neuronal markers in these cells (Gregorian et al. 2009). Postnatal deletion of *Pten* in cerebellar granule neurons also showed that a subset of *Pten*-null granule cells migrated to the proper position in the internal granule layer, while many were ectopically located in the molecular layer and at the pial surface (Backman et al. 2001; Kwon et al. 2001). Failed migration of *Pten*-deficient cerebellar neurons has been suggested to be secondary to defects in the glia guiding their migration (Yue et al. 2005). However, the in vivo electroporation approach used in the present study targeted small numbers of cells and identified stationary Dcx+ neuroblasts without Cre-mediated deletion in other surrounding cell types. The results in the RMS are consistent with cell autonomous defects leading to ectopic positioning.

Normal directional migration of *Pten*-null neuroblasts is consistent with the finding that directional chemotaxis can still occur in Dictyostelium in the absence of a PIP3 gradient (Hoeller and Kay 2007). Additionally, several studies showed that *Pten* was not essential in directional migration and chemotaxis of mammalian leukocytes (Lacalle et al. 2004; Ferguson et al. 2007; Nishio et al. 2007; Subramanian et al. 2007; Heit et al. 2008). *Pten* deficient neutrophils showed an increased migration speed similar to the neuroblasts in the our study (Subramanian et al. 2007). Thus, there are likely context-dependent signals driving the effect of *Pten* and PI3K signaling on migration.

Pten-deficient neuroblasts demonstrate an “all or none” phenotype of normal directional migration or abnormal morphology associated with arrested migration. Taken

together, the data suggest that the ectopic localization of neurons is more likely attributed to premature differentiation and secondary loss of migration ability rather than intrinsic defects in migration. Indeed, the PI3K-Akt-mTorc1 pathway has been shown to play an important role in several crucial neuronal differentiation processes including neuronal polarity, axon guidance, dendrite arborization and spine morphogenesis (Campbell and Holt 2001; Jaworski et al. 2005; Kumar et al. 2005; Tavazoie et al. 2005; Chadborn et al. 2006; Kwon et al. 2006; Wildonger et al. 2008; Chow et al. 2009).

PI3K-Akt-mTorc1 pathway regulation in the SVZ-RMS-OB

The PI3K-Akt-mTorc1 pathway is dynamically regulated through the different developmental stages of neuronal lineage along the SVZ-RMS-OB. From birth in the SVZ to differentiation and integration into the local circuit of the OB, neural stem cells progress in phases to transit amplifying progenitors, migrating neuroblasts and ultimately terminally differentiated mature neurons. Accordingly, cell signaling pathways must be modulated to meet the varying requirements of these differentiation states or to induce transition between consecutive phases. In this study, we found that the PI3K-Akt-mTorc1 pathway is active in the transit amplifying cells and inactive in the migrating neuroblasts until activated again in the OB. Consistent with these findings, previous studies also found high expression of Egfr, a potent PI3K-Akt-mTorc1 pathway activating receptor, in the transit amplifying progenitors but not in the migrating neuroblasts in the SVZ. The abundant free ribosomes found in transit amplifying progenitors is consistent with activation of mTorc1 signaling (Doetsch et al. 2002; Kim et al. 2009; Kokovay et al. 2010).

Upregulation of the PI3K-Akt-mTorc1 pathway may act to drive proliferation of transit amplifying progenitors, while inactivation of PI3K-Akt-mTorc1 signaling through Pten expression may play a role in the transition to migrating neuroblasts which exit the cell cycle. In support of this theory, enhanced Egfr signaling in vivo expands the proliferative pool, arrests neuronal production (Doetsch et al. 2002; Aguirre et al. 2010) and negatively regulates migration in the SVZ (Kim et al. 2009). In our system, *Pten* deletion did not have a substantial effect on proliferation. The magnitude of PI3K signaling is a balance between the level of upstream activation of the pathway and the negative regulatory activity of Pten. In other studies, Pten loss has been shown to increase proliferation of neural stem cells in vitro, and during other developmental stages in the SVZ (Groszer et al. 2001; Gregorian et al. 2009). The proliferation consequences downstream of Pten loss are likely influenced by the extent of upstream activation of PI3K driven by the growth factor environment at different developmental stages.

In wild-type RMS, the PI3K-Akt-mTorc1 pathway is not activated in Dcx⁺ cells until they reach the OB, which correlates well with the time when they begin further differentiation. This suggests that the PI3K-Akt-mTorc1 pathway might be involved in the transition from a migratory neuroblast to a differentiating neuron in response to local environmental cues. In the absence of Pten, premature activation of PI3K signaling in neuroblasts in the RMS likely triggers precocious differentiation resulting in ectopic

positioning. Inhibition of mTorc1 activity effectively rescued ectopic differentiation of neuroblasts and prevented the expansion of the SVZ and RMS, further supporting premature differentiation as a primary cause instead of a defect in the intrinsic migratory machinery. mTorc1 modulates protein synthesis and cell growth and plays an important role in neuronal differentiation processes (Campbell and Holt 2001; Jaworski et al. 2005; Kumar et al. 2005; Tavazoie et al. 2005; Kwon et al. 2006; Wildonger et al. 2008; Chow et al. 2009). The rapamycin-mediated rescue of ectopic neurons in *Pten*^{CKO} brains occurred in the absence of proliferation effects. This could be explained by the recent finding that rapamycin potently inhibits S6K activity, but inefficiently and transiently inhibits 4EBP1 phosphorylation (Choo et al. 2008; Dowling et al. 2010). 4EBP1, independent of S6K, controls mTorc1-mediated cell proliferation (Dowling et al. 2010). Taken together, our results along with previous studies, suggest that proper temporal and spatial regulation of the PI3K-Akt-mTorc1 pathway plays an important role in regulating the appropriate timing and positioning for neuronal differentiation, but is not required for directional tangential neuronal migration.

CHAPTER 3. CO-DELETION OF *PTEN* AND *TRP53* SYNERGIZES IN GENERATING MEDULLOBLASTOMA WITH A GROWTH PATTERN CENTERING AROUND BLOOD VESSELS

Introduction

Cells in the cerebellum arise from two germinal zones: the ventricular zone and the EGL. The ventricular zone is the germinal neuroepithelium during embryogenesis and generates deep cerebellar nuclei and Purkinje cells as well as other GABAergic neurons in the cerebellum (Goldowitz and Hamre 1998). *Ptf1a*, a bHLH transcription factor, is required for the GABAergic neuron specification in the ventricular zone and loss of function resulted in complete loss of cerebellar GABAergic neurons (Sellick et al. 2004; Hoshino et al. 2005).

At birth, the ventricular germinal neuroepithelium disappears and proliferative precursors in the rhombic lip emigrate along the pial surface and form the EGL (Carletti and Rossi 2008). The EGL proliferates most during early postnatal days and produces all the internal granule cells (Wang and Zoghbi 2001). Multiple cell signaling pathways, for instance, cyclinD2 (Huard et al. 1999), p27 (Miyazawa et al. 2000), *Shh* (Dahmane and Ruiz i Altaba 1999), N-myc (Knoepfler et al. 2002), PI3Ks (Kenney et al. 2004) and *Math1* (Ben-Arie et al. 1997) are crucial for cerebellar granule cell development among which the *Shh* signaling pathway is one of the best characterized. *Shh*, expressed by Purkinje cells, binds to its receptor, *Ptch1*, on granule cell precursors in the EGL, promotes proliferation and prevents their differentiation (Wallace 1999; Wechsler-Reya and Scott 1999). Unrestricted *Shh* signaling caused by *Ptch1* mutation leads to medulloblastoma in both human and mouse (Goodrich et al. 1997). *Pten* is also required for the normal development of the cerebellum. Deletion of *Pten* in the granule progenitors in the EGL lead to ectopic positioning and hypertrophy of cerebellar granule cells (Backman et al. 2001; Kwon et al. 2001; Marino et al. 2002).

Aside from the two well characterized germinal zones in the developing cerebellum, multipotent neural stem cells have been isolated from the cerebellum postnatally and are thought to be located in the white matter tracts (Lee et al. 2005) which might arise from embryonic ventricular zone and emigrate into the white matter tracts (Zhang and Goldman 1996). The identity and function of this pool of neural stem/progenitor cells are largely unknown.

Medulloblastoma is a collection of clinically and molecularly heterogeneous diseases and can be divided into several subtypes (Thompson et al. 2006; Kool et al. 2008; Northcott et al. 2010; Cho et al. 2011). The subtype of medulloblastoma harboring mutations in *Shh* pathway including *Ptch1* and *Sufu* is thought to originate from the EGL precursors and is supported by *Ptch1* knock-out mouse models (Schuller et al. 2008; Yang et al. 2008). The Wnt subtype of medulloblastoma is likely to develop from cells of the dorsal brainstem outside of cerebellum (Gibson et al. 2010). Cells of origin for other medulloblastoma subtypes are still unknown.

Cancer initiating cells, or cancer stem cells, are the subpopulation in the primary tumor that are capable of self-renewing, differentiating and regenerating its original tumor when transplanted in vivo. Cancer initiating cells have now been isolated from human cancers of the blood, breast, brain, skin, bone, and prostate (Ward and Dirks 2007). They are hypothesized to sustain tumor growth and be responsible for the relapse and metastasis. Cancer initiating cells could also be isolated from human medulloblastomas (Singh et al. 2003; Singh et al. 2004) and were shown to reside in a perivascular niche (Calabrese et al. 2007). The perivascular cell population were found to account for the re-growth of *Ptch1*-mutant medulloblastoma tumor after irradiation-induced apoptosis (Hambardzumyan et al. 2008). However, it is unknown whether there is a perivascular population of cells in the wild-type brain which is susceptible to oncogenic transformation and whether they could serve as cells of origin for medulloblastoma.

In addition to Shh and Wnt pathways, PI3K is another pathway aberrantly regulated in human medulloblastoma. Human medulloblastomas are found to over-express RTKs such as ERBB2 (Hernan et al. 2003) and PDGFRB (MacDonald et al. 2001; Gilbertson and Clifford 2003). Proliferation of human medulloblastoma cells are dependent on activation of PI3K/AKT signaling perhaps caused by reduction of PTEN expression (Hartmann et al. 2006). Recent whole genome sequencing of 88 human medulloblastomas found *PTEN* mutations occur together with either *PTCH1* or *TP53* mutations (Parsons et al. 2011) which suggests a cooperative effect between PI3K and *Ptch1* pathways. Mouse studies have also shown the synergistic effects of *Ptch1* loss with *Igf2*, PI3K or Akt in generating medulloblastomas (Hahn et al. 2000; Rao et al. 2004; Riobo et al. 2006). However, the exact cooperative effects need further investigation.

In the present study, we inactivated *Pten* in the neural stem/progenitor cells at birth using a Nestin (a marker for the neural stem/progenitor cells) promoter driven CreER transgenic mouse and revealed a novel perivascular proliferative niche in the cerebellum. Co-deletion of *Pten* and *Trp53* synergized in generating medulloblastoma with a growth pattern centering around blood vessels and extensive neuronal differentiation suggesting a potential new cell of origin for medulloblastoma.

Experimental Procedures

Mice

For Cre activity mapping, *Nestin-CreER^{T2}* transgenic mice were bred with *R26R-EYFP* (Srinivas et al. 2001) mice to generate *Nestin-CreER^{T2};R26R-EYFP* mice. Cre activities were detected by EYFP IF staining on frozen tissue sections. *Nestin-CreER^{T2};Pten^{loxP/loxP}* (*Pten^{cKO}*) mice were generated by crossing *Nestin-CreER^{T2}* mice to *Pten^{loxP}* mice (Suzuki et al. 2001), a gift from Tak Mak (University of Toronto, Toronto). *Nestin-CreER^{T2};Pten^{loxP/loxP};Trp53^{loxP/loxP}* (*Pten^{cKO};Trp53^{cKO}*) mice were generated by

breeding *Nestin-CreER^{T2};Pten^{loxP/loxP}* with *Trp53^{loxP}* mice (Jonkers et al. 2001). For all analyses including *Nestin-CreER^{T2}*, the transgene was hemizygous to avoid possible variation from transgene dosage. All procedures were reviewed and approved by the Animal Care and Use Committee at St. Jude Children's Research Hospital.

Genotyping polymerase chain reaction assays

PCR was used to genotype mice. Primers used were: *Cre*, forward primer: 5'AGCGATCGCTGCCAGGAT; reverse primer: 5'ACCAGCGTTTTTCGTTCTGCC; expected product: 157 base pairs. *Pten*, forward primer: 5'TTATCTGGATCAACTTTGGGCC; reverse primer: 5'TCCCACCAATGAACAAACAGT; expected products: 146 (wild-type) and 244 (loxP) base pairs. *Trp53*, forward primer: 5'CACAAAAACAGGTAAACCCAG; reverse primer: 5'AGCACATAGGAGGCAGAGAC; expected products: 288 (wild-type) and 370 (loxP) base pairs.

Induction of Cre activity

TM (Sigma) was dissolved at 5 mg/ml at 37°C in corn oil (Sigma) and sterilized by filtering and stored at 4°C in the dark for up to 10 days. Pups were injected daily with 3 mg/40g TM i.p. from P0-1. An equivalent volume of sterile filtered corn oil alone was used for vehicle injections. Two injections in the same mouse were separated by 24 hours.

Immunohistochemistry and immunofluorescence

Mice were anesthetized and perfused transcardially with 1x PBS followed by 4% PFA in PBS. Following dissection, tissues were post-fixed overnight in 4% PFA in PBS at 4°C, and then equilibrated in 25% sucrose in PBS for an additional 24 hours at 4°C. Tissues were embedded in TBS embedding media (Triangle Biomedical Sciences) on dry ice and cut into 12 µm thick cryosections. Tissue slides were equilibrated at room temperature for 20 minutes then washed three times in PBS prior to staining. For paraffin sections, mice were processed the same way as above. Following perfusion, tissues were post-fixed for an additional 24 hours in 4% PFA, embedded in paraffin, and cut into 5 µm sections. Primary antibodies used for immunostaining were against GFP (1:1000, Invitrogen A6455; 1:2000, Abcam #13970), Pten (1:100, Cell Signaling #9559), p-Akt S473 (1:50, Cell Signaling #9271), Gfap (1:200, Sigma G3893), Ki67 (1:5000, Novocastra NCL-Ki67p), CD34 (1:100, BD Pharmingen #553731), CD45 (1:400, BD Pharmingen #553076), CD133 (1:100, eBioscience #12-1331) and Nestin (1:100, Chemicon MAB353). For IHC, microwave antigen retrieval was performed for all antibodies and biotinylated secondary antibodies were used in conjunction with horseradish peroxidase-conjugated streptavidin (Elite ABC, Vector Laboratories). Color development was conducted with substrates NovaRed, DAB or VIP (Vector

Laboratories) and counterstaining with hematoxylin or methyl green (Vector Laboratories), respectively. For IF, Alexa Fluor 488, 647 (Invitrogen) and cyanine 3, 5 (Jackson ImmunoResearch) conjugated secondary antibodies were employed along with Vectashield mounting media containing DAPI (Vector Laboratories).

Array comparative genomic hybridization analysis

Genomic DNA was prepared by scraping tumor tissues from multiple deparaffinized sections, followed by Proteinase K-digestion and phenol-chloroform extraction. DNA quality was verified by gel electrophoresis. Control DNA was isolated from the spleens and livers of wild-type FVB mice. A total of 20 Pten; p53 double cKO tumors were analyzed using the Roswell Park Cancer Institute (RPCI) 6.5K mouse BAC array comparative genomic hybridization (aCGH).

Fluorescent in situ hybridization analysis

Fluorescent in situ hybridization (FISH) analysis was performed on paraffin sections by the St. Jude Cancer Center Core Cytogenetic Laboratory. Purified DNA from a BAC clone (RP24-70J8 cl.1) containing the murine *Ptch1* locus was labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals) by nick translation. The murine *Ptch1* clone is known to localize to mouse chromosome band 13B3 so a biotinylated mouse 13 control clone (RP24-386H10) was used as an internal control for the FISH assays. The *Ptch1* clone and the chromosome 13 control clone were combined with sheared mouse DNA and hybridized in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC to the deparaffinized and pepsin treated slides. Specific hybridization signals were detected by incubating the hybridized slides in fluorescein labeled anti-digoxigenin (Roche Molecular Biochemicals) and Texas red avidin (Vector Laboratories Inc). The cells were then stained with DAPI and analyzed. A total of 200 interphase cells were scored from each sample for the number of green (70J8) and red signals (386H10) per cell.

Quantitative real-time polymerase chain reaction assay

The same genomic DNA extracted for aCGH assay was used for real-time PCR analysis. The primer/probe set spanning *Ptch1* exon 2 was used, forward primer Ptch1ex2-SDS-F (5'-GGCTACTGGCCGGAAGC), reverse primer Ptch1ex2-SDS-R (5'-GAATGTAACAACCCAGTTTAAATAAGAGTCT) and Taqman probe Ptch1ex2-SDS-F (5'-CCGCTGTGGCTGAGAGCGAAGTTTC). *Gapdh* (located on mouse chromosome 6) was used as internal standards to normalize the data. Four nanograms of DNA from tumor samples or *Gapdh* DNA were amplified in TaqMan Fast Universal PCR Master Mix (Applied Biosystems) using a 7900HT Fast Real-Time PCR system (Applied Biosystems) (95°C for 20 seconds, then 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds). Standard curve was generated from three-fold serial dilutions of

Gapdh DNA. Quantitative real-time PCR for each sample was performed in triplicate and means were reported. The result of real-time PCR was analyzed with SDS ver2.3 software (Applied Biosystems).

***Ptch1* sequence analysis**

Ptch1 sequencing analysis was done as describe by Lee et al. (2006b). Total RNA was extracted from snap-frozen medulloblastoma samples using Trizol (Invitrogen). cDNA was synthesized from tumor RNA by reverse transcription using random hexamer primers and SuperScript II Reverse Transcriptase (Invitrogen). After cDNA synthesis, two rounds of PCR amplification were performed to obtain sufficient *Ptch1* products for sequencing. The first round of PCR was done with forward primer *Ptch1*-14 (5'-ACGCGCAATGTGGCAATGGAAGGC) and reverse primer *Ptch1*-R1 (5'-GAAGCGGCCGCTTCAGATTTTAATTACCC) to amplify a full-length *Ptch1*. Subsequently, the PCR products were further amplified with following five different sets of primers whose products overlapped each other and spanned a full-length of *Ptch1*. Set A: forward primer *Ptch1*-F (5'-ATGGCCTCGGCTGGTAACG) and reverse primer *Ptch1*-1 (5'-AAGGCCGGTCCATGTACCCATGGC); set B: forward primer *Ptch1*-2 (5'-GCTTAATCATTACACCTTTGGACTGC) and reverse primer *Ptch1*-6 (5'-AAAGGAGCATAGTGCTTCTCTGC); set C: forward primer *Ptch1*-5 (5'-TTGAGCCACAGGCCTACACAGAGC) and reverse primer *Ptch1*-8 (5'-GTCTGAGGTGTCTCGTAGGCCG), set D: forward primer *Ptch1*-7 (5'-TGGGAAACTGGGAGGATCATGC) and reverse primer *Ptch1*-10 (5'-GCTCAGGCGAAGGAGTGGGCAGTCG); and set E: forward primer *Ptch1*-9 (5'-GTGGAGTTCACCGTCCACGTGGC) and reverse primer *Ptch1*-R2 (5'-GAAGCGGCCGCTCAGTTGGAGCTGCTCCCCACGGC). The PCR products were sequenced by a routine Big Dye Terminator (v.3) Chemistry approach on Applied Biosystem 3700 DNA analyzer.

Results

***Nestin-CreER^{T2}* targets cerebellum at birth**

Nestin-CreER^{T2};R26R-LacZ or *Nestin-CreER^{T2};R26R-EYFP* mice were injected with TM to map the Cre activity at birth when brain is still undergoing active development. Consistent with the fact that developing brain has stronger expression of Nestin (Lendahl et al. 1990) and more common neural stem/progenitors, Cre activities in *Nestin-CreER^{T2}* lines were more robust in immature brain with strongest activities in the neural stem/progenitor cell niches including the SVZ and the EGL of cerebellum (Figure 2-2C). The bulk of cerebellum develops postnatally, so *Nestin-CreER^{T2}* could target the cerebellum including the EGL, IGL and white matter tracts very effectively with TM induction at birth (Figure 3-1). Littermate control with vehicle injection showed very low level of TM-independent leaky Cre activity (Figure 3-1).

Figure 3-1. *Nestin-CreER^{T2}* targets cerebellum at birth.

EYFP IF staining of the cerebellum from mice induced with vehicle or TM from P0-1. Tissue was harvested at P5. Strong EYFP signals were noticed in the EGL, IGL and white matter tract of cerebellum in the TM-induced mice whereas mice with vehicle induction had only very low level of EYFP. Scale bar: 100 μ m.

Deletion of *Pten* at birth resulted in disorganization and hypertrophy of cerebellum which mimics human Lhermitte-Duclos disease

We induced the deletion of *Pten* in the *Nestin-CreER^{T2};Pten^{loxP/loxP} (Pten^{CKO})* neonate mice to see the effect of broad *Pten* loss on a developing brain. The *Pten^{CKO}* mice did not show obvious abnormalities until 2-3 months of age when the animals started to exhibit lethargy, progressive enlargement of the skull and progressive neurological abnormalities including ataxia and seizure. One hundred percent of the mice (16/16) induced at birth were affected whereas none of the un-induced *Pten^{CKO}* mice or TM-treated wild-type littermates showed these deficits. Animals had to be euthanized due to severe ataxia and brain hypertrophy with a median survival of 153 days (Figure 3-2C). In addition to the dramatic expansion of the SVZ and RMS described in Chapter 2, brains from *Pten^{CKO}* mice induced with TM at birth showed profound hydrocephalus and dramatic increases in overall sizes especially in cerebellum (Figure 3-2A). The foliation was visible but the organization of cell layers was disrupted in the cerebellum. The molecular layer (ML) was dramatically thickened with many ectopic granule cells deficient of *Pten* staining. The majority of Purkinje cells were positive for *Pten* staining but the normal monolayer structure was not nicely formed. The IGL was thinner than wild-type control and composed of both *Pten* positive and negative granule cells (Figure 3-2B). The size of the *Pten*-deficient granule cells is larger than the *Pten* positive granule cells in aging mice (not shown). All these abnormalities mimic human Lhermitte-Duclos disease and are in agreement with previous findings but in a more severe manifestation (Backman et al. 2001; Kwon et al. 2001).

***Pten* inactivation at birth revealed novel ectopic perivascular proliferative niches in the cerebellum**

In addition to the cerebellar disorganization and hypertrophy, we identified multiple perivascular proliferative niches in the cerebellum (20/24 mice) when deleting *Pten* in neonatal neural stem/progenitor cells at birth, which have never been observed before in previous *Pten^{CKO}* mouse models (Backman et al. 2001; Kwon et al. 2001; Fraser et al. 2004). The perivascular hyperplastic lesions were observed as early as 3 months of age but were not found in 30-day-old mice (data not shown). These hyperplastic lesions grew surrounding CD34+ blood vessels and had high percentage of Ki67+ cells (Figure 3-3). The perivascular proliferative lesions varied in their sizes from as few as several cells to hundreds of cells. They were distributed throughout cerebellum but were more frequently found in anterior and lateral cerebellar lobules and in areas around the pia between lobules (data not shown). They are not infiltrating inflammatory cells (CD45-) but have a progenitor-like appearance. They tend to express Nestin and CD133 which mark neural stem/progenitor cells or brain tumor initiating cells (Lendahl et al. 1990; Singh et al. 2003; Singh et al. 2004; Coskun et al. 2008) as well as Gfap that labels neural stem cells in the SVZ (Doetsch et al. 1999)(Figure 3-3). These perivascular precursor-like cells are *Pten* null and p-Akt positive indicating that they derived from cells that underwent Cre-mediated recombination (Figure 3-3). However, these perivascular

Figure 3-2. Deletion of *Pten* at birth resulted in disorganization and hypertrophy of cerebellum.

Representative H&E (A) and Pten (B) IHC staining of the cerebella from mice induced with TM at birth. (A) H&E staining of the wild-type (WT) and *Pten*^{CKO} (cKO) brains showed dramatic hypertrophy of the *Pten*^{CKO} brain. (B) Pten IHC staining of the WT and cKO brains showed disrupted organization of the cerebellum and ectopic Pten-deficient granule cells in the molecular layer in the *Pten*^{CKO} brain. Scale bar: 200 μ m. (C) Kaplan-Meier survival curves shows *Pten*^{CKO} mice had a median morbidity age of 153 days due to macrocephaly and hydrocephaly (brown curve).

Figure 3-3. Pten inactivation at birth reveals novel perivascular proliferative niches in the cerebellum.

IHC staining as indicated of the cerebella from mice induced with TM at birth. The perivascular lesion growing surrounding CD34+ blood vessels has a high percentage of Ki67+ cells. The perivascular cells are negative for CD45, a marker for leukocytes, but have progenitor-like appearance. They tend to express Nestin, CD133 and Gfap indicating their neural stem/progenitor identity. These perivascular precursor-like cells are Pten null and positive for p-Akt indicating they are derived from cells that underwent re-mediated recombination. Scale bar: CD45, Pten and p-Akt=100 μ m, others=50 μ m.

hyperplastic lesions were never found to develop into full-blown tumors in mice up to 10 months of age.

Co-deletion of *Pten* and *Trp53* at birth resulted in medulloblastoma with unusual vascularization

Since *TP53* is one of the most frequent mutations in several common primary human brain tumors (Frank et al. 2004; Parsons et al. 2008; TCGA 2008; Parsons et al. 2011), we asked whether or not loss of *Pten* during early postnatal development would cooperate with *Trp53* deficiency in generating full-blown cerebellar malignancy. *Nestin-CreER^{T2};Pten^{loxP/loxP}* mice were bred to *Trp53^{loxP}* mice to generate *Nestin-CreER^{T2};Pten^{loxP/loxP};Trp53^{loxP/loxP}* (dKO) mice and induced at birth. All of the dKO mice (26/26) developed cerebellar tumors which resemble human medulloblastoma with a median tumor onset of 63 days of age and *Nestin-CreER^{T2};Pten^{loxP/+};Trp53^{loxP/loxP}* mice (22/25) succumbed to the same tumor with a one-month delay in onset compared to dKO mice (Figure 3-4D and data not shown). All the dKO medulloblastomas manifested as highly cellular neoplasms of classic medulloblastoma pathology composed of small blue cells with two striking features, high vascularization and perivascular clustering of tumor cells, which distinguish this tumor model from other existing mouse medulloblastoma models (Figure 3-4A and B). *Pten* IHC staining showed that the vast majority of the tumor bulk was devoid of *Pten* with only endothelial cells retaining *Pten* (Figure 3-4C). The majority of the *Nestin-CreER^{T2};Trp53^{loxP/loxP}* mice have to be euthanized for peripheral tumors probably due to leaky Cre activity outside of CNS (Figure 3-4D). Interestingly, the *Nestin-CreER^{T2};Pten^{loxP/loxP};Trp53^{loxP/+}* mice (5/8) developed glioma like malignancy in the cerebellum instead of medulloblastoma by 5-6 months old (see Appendix). Besides cerebellum, the dKO and *Nestin-CreER^{T2};Pten^{loxP/loxP};Trp53^{loxP/+}* mice also developed glioma like tumors in the forebrain (18/26, 5/8 respectively) and brain stem (5/26, 8/8) (see Appendix).

***Pten*;Trp53 double knock-out medulloblastoma showed a growth pattern centering around blood vessels**

The dKO medulloblastomas were highly proliferative and showed predominant neuronal differentiation by IHC staining which confirms their identity as primitive neuroectodermal tumors (Figure 3-5). Most interestingly, these highly vascularized medulloblastomas often exhibited a growth pattern centering around blood vessels. The tumor cells showed a mature neuronal immunophenotype except those around blood vessels which expressed primitive markers including Nestin (Figure 3-5). At the edges of the tumors that were invading the non-tumor brain parenchyma, the Nestin⁺ and Ki67⁺ proliferative cells were often located immediately around blood vessels, which are reminiscent of the perivascular proliferative niches in the *Pten^{CKO}* cerebellum (Figure 3-3 and Figure 3-5). These data suggest that the dKO medulloblastomas might initiate from the perivascular proliferative niches in the *Pten^{CKO}* cerebellum.

Figure 3-4. Co-deletion of *Pten* and *Trp53* at birth resulted in medulloblastoma with unusual vascularization.

(A and B) Representative H&E staining of medulloblastoma generated by co-deletion of *Pten* and *Trp53* at birth shows pathology of classic medulloblastoma and high vascularization. (C) Pten IHC staining shows *Pten*^{CKO};*Trp53*^{CKO} dKO medulloblastoma cells are devoid of Pten except for the blood vessel. (D) Kaplan-Meier survival curves show *Pten*^{CKO};*Trp53*^{CKO} dKO mice with a median tumor onset of 63 days of age (red curve), *Pten*^{CKO} mice with a median morbidity age of 153 days of age (brown curve) and *NestinCreER*^{T2};*Trp53*^{loxP/loxP} (*Trp53*^{CKO}) with a median morbidity age of 135 days (green curve). *Pten*^{CKO} mice were euthanized due to macrocephaly and hydrocephaly. The majority of *Trp53*^{CKO} were euthanized because of peripheral tumors. All genotypes were induced with TM at birth. Scale bar: B and C=50 μ m.

Figure 3-5. *Pten;Trp53* double knockout medulloblastoma showed a growth pattern centering around blood vessels.

IHC staining as indicated of the medulloblastomas from dKO mice induced with TM at birth. Tuj1+ and NeuN+ IHC show the dKO medulloblastomas exhibited a neuronal immunophenotype except those cells around blood vessels. Nestin+ and Ki67+ IHC staining of the edges of the medulloblastomas show proliferative pregenitor cells were often located immediately around blood vessels that were invading the non-tumor brain parenchyma. Scale bar: 50 μ m.

***Pten;Trp53* double knock-out medulloblastoma spontaneously lost chromosome 7, 13 and 16**

Tumorigenesis is considered a multi-step process involving initiation, promotion and progression. To understand what other secondary genetic alterations in addition to the initiating *Pten* and *Trp53* mutations might be involved in the medulloblastoma formation, we did aCGH comparing genomic DNA from the tumors and reference genomic DNA from liver and spleen. We identified 3 most recurrent and consistent genomic abnormalities in tumor samples. All of the 18 medulloblastomas showed loss of chromosome 13 on which *Ptch1* is located (Figure 3-6). In addition, 5 of the tumors had focal homozygous deletion of the BAC probe encompassing *Ptch1*. Thirteen and eighteen out of the 18 medulloblastomas also showed loss of the entire or part of chromosome 7 and 16 respectively (Figure 3-6). No recurrent focal homozygous deletion on chromosome 7 or 16 could be found. Interestingly, two of the cerebellar non-medulloblastoma tumors included in this aCGH analysis displayed a completely different landscape of genomic alterations than the medulloblastoma samples and failed to show loss of chromosomes 7 or 13 (Figure 3-6).

Spontaneous deletion and mutation of *Ptch1* occurred in *Pten;Trp53* double knock-out medulloblastomas

To further investigate whether *Ptch1* was the target gene of the genomic alteration on chromosome 13, we performed FISH analysis and detected loss of at least one allele of *Ptch1* in two medulloblastoma samples used for aCGH analysis (Figure 3-7A). Real-time PCR assay on the medulloblastoma genomic DNA further confirmed the copy number loss of *Ptch1* in both tumor samples (Figure 3-7B). We also performed RT-PCR and direct sequencing to analyze *Ptch1* mRNA and found mutations of *Ptch1* in another two medulloblastomas samples used for aCGH analysis (Figure 3-7C). These data support the hypothesis that *Ptch1* is the tumor suppressor gene on chromosome 13 that was selectively inactivated in dKO medulloblastoma by loss of heterozygosity.

Discussion

In this study, we used an inducible *Nestin* promoter driven Cre and deleted *Pten* in a wide-spectrum of the progenitors in cerebellum at birth when the brain is still undergoing active development. We recapitulated the phenotypes of neuronal hypertrophy and ectopic positioning found in previous studies and confirmed that *Pten* regulates neuronal size and positioning (Backman et al. 2001; Kwon et al. 2001; Fraser et al. 2004). The more severe phenotypes of cerebellar disorganization and hypertrophy compared to previous studies might be because of the more potent and ubiquitous Cre activity in neural stem/progenitor cells driven by the *Nestin* promoter than by the *Gfap* promoter used previously. An unexpected finding in this study is that we revealed a novel perivascular proliferative progenitor niche in the cerebellum by deletion of *Pten* at birth which has never been found in any other studies. This suggests the presence of an

Figure 3-6. *Pten;Trp53* double knockout medulloblastoma spontaneously lost chromosome 7, 13 and 16.

(A) aCGH heat map of cerebellar tumors from *Pten;Trp53* dKO mice induced with TM at birth. Each column represents one tumor sample. Numbers on the right indicate the chromosomes. The green color indicates copy number loss whereas red indicates copy number gain. All medulloblastomas (the two samples indicated by arrows were from non-medulloblastoma samples) had loss of chromosome 7, 13 and 16 as highlighted in red. (B) aCGH analysis of chromosome 13 from one representative medulloblastoma sample shows hemizygous deletion of whole chromosome 13 and homozygous deletion at one BAC probe containing *Ptch1* as indicated by an arrow.

Figure 3-7. Spontaneous deletion and mutation of *Ptch1* in *Pten;Trp53* double knockout medulloblastoma.

(A) FISH analysis of a representative *Pten;Trp53* dKO medulloblastoma sample. Red dot is a BAC probe located on one end of chromosome 13 outside of *Ptch1* and green dot is a BAC probe containing *Ptch1*. (B) Quantitative real-time PCR analysis of *Ptch1* on genomic DNA from dKO medulloblastomas. (C) Sequencing of cDNA synthesized from *Ptch1* mRNA of 2 dKO medulloblastomas. Red boxes indicate the mutations by comparing reference wild-type sequence (Ref.) with medulloblastoma sequences (Tumor).

unknown perivascular germinal niche in the developing cerebellum that is normally regulated by Pten but regresses upon completion of development, because we could not see the niche anymore when deleting Pten in adulthood (data not shown). Alternatively, Pten deletion may have perturbed normal development and resulted in a perivascular germinal niche that does not normally exist. Our study is consistent with the previous study that identified neural stem cells in the murine postnatal cerebellum (Lee et al. 2005) and suggests that the neural stem cells in the cerebellum are probably localized in a vascular niche. This is consistent with the finding that the SVZ neural stem cells also reside in a vascular niche (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008). We tried to grow neurospheres from both wild-type and *Pten*^{CKO} P21 cerebella but did not get consistent results (data not shown). Our study also underlines the importance of Pten regulation in the neural stem/progenitor cells in the cerebellum indicating Pten is required for silencing or withdrawing the neural stem cells upon completion of development under physiological conditions. Our results are consistent with the finding that Pten negatively regulate proliferation and self-renewal of embryonic neural stem cells (Groszer et al. 2001; Groszer et al. 2006) and is novel in that Pten loss caused spatially and temporally ectopic perivascular progenitor niches.

The cerebellar perivascular hyperplasia, however, did not show progressive expansion up to 10 months of age and was never found to be transformed into full-blown tumor masses. This is consistent with our finding in Chapter 2 that Pten deletion is not sufficient to initiate tumorigenesis in neural stem/progenitor cells in the fore-brain and is in contrast to Pten function in other stem cells. For instance, in hemopoietic stem cells, inactivation of Pten causes short-term expansion, but long-term decline and eventual leukemogenesis (Yilmaz et al. 2006; Zhang et al. 2006) indicating a tissue-specific stem cell regulation by Pten. Activation of oncogenic signals such as c-Myc, E1A and Ras could not only promote proliferation and growth, but also induce cell cycle arrest and cell death (Askew et al. 1991; Lowe and Ruley 1993; Serrano et al. 1997) constraining tumorigenesis. Pten deficiency, a strong oncogenic stress, has been shown to induce p53, p16^{Ink4a} or p19^{Arf} and cause cellular senescence in prostate and hematopoietic cells (Chen et al. 2005; Lee et al. 2011). This might account for the limited cerebellar perivascular hyperplasia observed in this study. So we deleted *Trp53*, another common mutated tumor suppressor in human brain tumors in addition to deletion of Pten, which indeed resulted in medulloblastoma. This corresponds to recent large-scale human medulloblastoma sequencing data showing that *PTEN* and *TP53* mutations occurred simultaneously (Parsons et al. 2011). Therefore inactivation of *PTEN* and *TP53* might also synergize in generating human medulloblastoma.

The *Pten* and *Trp53* dKO medulloblastoma in this study has very unique features that distinguish it from other mouse medulloblastoma models (Wetmore et al. 2000; Lee and McKinnon 2002; Zindy et al. 2003). Firstly, our medulloblastoma is highly vascularized manifesting as high-density of capillaries, extensive arborization and glomeruloid structures. This difference could be explained by the Pten inactivation in our medulloblastoma model and consequent PI3K pathway activation which have been shown to increase tumor angiogenesis through induction of VEGF or HIF1 in various tumor types (Giri and Ittmann 1999; Jiang et al. 2000; Zhong et al. 2000; Wen et al.

2001). In supporting this idea, one recent study demonstrates that loss of one copy of *Pten* promoted tumorigenesis and increased angiogenesis in a mouse medulloblastoma driven by over-expression of *SmoA1* (Castellino et al. 2010). In addition, the potential perivascular origin of intrinsically angiogenic cells for this medulloblastoma might also account for its extensive vascularization. Secondly, our medulloblastoma is special in that it grows exclusively around blood vessels and show strong neuronal differentiation of cells not adjacent to blood vessels. This illustrates a lineage hierarchy of cancer cells in which medulloblastoma cancer stem/progenitor cells maintain themselves in a perivascular niche and start to differentiate when migrating out from the niche. This is consistent with a previous study showing the perivascular Nestin-expressing tumor cells were responsible for the recurrence of *Ptch1* and *Pten* dKO medulloblastoma after irradiation (Hambardzumyan et al. 2008). The extensive neuronal differentiation of our *Pten* null medulloblastoma in contrast to the medulloblastoma models with intact *Pten* which show less neuronal differentiation underscores the role of *Pten* inactivation and PI3K activation in promoting neuronal lineage differentiation of medulloblastoma cells. This is supported by two other medulloblastoma models with extensive nodularity of neuronal differentiation in which *Pten* is inactivated (Hambardzumyan et al. 2008; Castellino et al. 2010).

Human medulloblastoma is a heterogeneous disease that can be classified into several molecular subgroups based on genomic and expression profiles, one of which is enriched for mutations in sonic hedgehog (SHH) pathway (Thompson et al. 2006; Kool et al. 2008; Northcott et al. 2010; Cho et al. 2011). Mouse models of spontaneous medulloblastoma seem to over-represent the SHH subgroup of human disease in that the majority of mouse medulloblastomas harbor *Ptch1* mutation (Wetmore et al. 2000; Zindy et al. 2007; Frappart et al. 2009) especially when involving *Trp53* mutation. Our *Pten* and *Trp53* dKO medulloblastoma also spontaneously developed *Ptch1* mutations as well as other genomic abnormalities. One hundred percent penetrance of medulloblastoma and *Ptch1* mutation indicates strong cooperation between PI3K, P53 and SHH pathways and potent selection pressure for *Ptch1* inactivation in murine medulloblastoma genesis. Our tumor model is also very relevant to human disease given the recent sequencing data show *PTEN* mutation occurs exclusively together with either *TP53* or *PTCH1* mutations in all the medulloblastoma samples harboring *PTEN* mutation (Parsons et al. 2011). Although the subtype of SHH medulloblastomas are shown to be able to arise from the EGL (Schuller et al. 2008; Yang et al. 2008), our study suggests a potential new cell of origin for this subgroup.

CHAPTER 4. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Pten and Postnatal Brain Development

Pten and postnatal neurogenesis

Postnatal brain development involves neurogenesis and gliogenesis as well as other processes such as angiogenesis. Postnatal neurogenesis mainly happens in three brain regions: the SVZ-RMS-OB, cerebellum and dentate gyrus. The present study showed that loss of Pten cause ectopic neuronal differentiation in both the SVZ-RMS-OB and cerebellum suggesting a unified Pten function in neurogenesis in different compartments of brain. However, Pten loss also showed region-specific phenotypes: the expansion is more dramatic on ventral and dorsal parts of the SVZ but less severe in the middle part which might be explained by the heterogeneity of the neural stem cells in the SVZ (Merkle et al. 2007).

Neural stem cells in the SVZ reside in a niche with highly organized architecture in which they contact the ventricle with apical processes and blood vessels with basal processes (Mirzadeh et al. 2008; Shen et al. 2008; Tavazoie et al. 2008). In the expanded *Pten^{ckO}* SVZ, this specialized organization seems to be disrupted in that the proliferative progenitor cells are intermingled with differentiated neurons and sometimes located on the edge of the expansion away from the ventricle. Surprisingly, this altered architecture did not interfere with neurogenesis, given that no substantial changes in proliferation or apoptosis and no signs of an exhausted pool of neural stem cell were detected. In-depth analysis of the *Pten^{ckO}* SVZ by immunostaining or electron microscopy could provide specific insights into the relationship of neural stem cells to the ventricle and blood vessels in an altered niche.

The present study shows deletion of Pten in neuroblasts caused ectopic differentiation without disrupting directional migration. This suggests the migration defect of Pten null neuroblasts is secondary to a premature differentiation in which programmed differentiation was accelerated and neuroblasts lost their migration capability before they reached their normal destination. Future experiments need to be done looking for direct evidence of precocious differentiation, for instance, by comparing wild-type and *Pten^{ckO}* neuroblasts at several timepoints after acute Pten deletion to see whether the differentiation program in *Pten^{ckO}* cells is expedited by examining temporal differentiation markers. Our rapamycin treatment experiment showed that the premature differentiation of *Pten^{ckO}* is dependent on mTorc1. Future study needs to address whether terminal differentiation of wild-type neuroblasts is also dependent on mTorc1 by knocking out mTorc1 components. We could not address this by rapamycin treatment because rapamycin differentially inhibits downstream signaling of mTorc1 (Choo et al. 2008; Dowling et al. 2010) and has inconsistent penetration and inhibition in different compartments of brain which often results in incomplete inhibition. However, one

potential pitfall is that complete abolishment of mTorc1 activity might be incompatible with cell survival.

One interesting phenomenon is that ectopic differentiated Pten-null neurons were distributed throughout the SVZ-RMS-OB indicating that *Pten*^{CKO} neuroblasts terminated their migration at different places. The possible reasons for this could be firstly that neuroblasts are born and start their migration at different regions of the SVZ. Secondly, deletion of Pten may have a stochastic effect on the termination of neuroblast migration. So it is possible mTorc1 signaling is not activated yet in the *Pten*^{CKO} neuroblasts maintaining directional migration at the timepoint of observation because the activation of mTorc1 pathway is determined by Pten status as well as other unknown factors. For instance, engagement of ligands (external cues) with tyrosine kinase receptors on the membrane is likely needed in the *Pten*^{CKO} neuroblast for full activation of PI3K-Akt-mTorc1 pathway but might not be readily available to every neuroblast at a given timepoint. This might actually be a reflection of how neuroblasts terminate their migration naturally because in the wild-type brain migrating neuroblasts could stop anywhere throughout the whole OB layers. Future experiments could examine the status of mTorc1 signaling in the *Pten*^{CKO} neuroblasts that still maintain directional migration by means of, for instance, double labeling of EYFP and p-S6. Thirdly, migrating neuroblasts are a heterogeneous population (Merkle et al. 2007) that might respond to loss of Pten differentially. This is not likely the reason, however, because Pten null neurons were found to be in all of the OB layers that the neuroblasts normally integrate into. Since the neuroblasts give rise to multiple types of interneurons in the OB (Ihrie and Alvarez-Buylla 2008), specific markers including TH, calbindin and calretinin for these interneurons could be used to examine if any of the interneuron subtype is more susceptible to Pten loss.

Pten and postnatal gliogenesis

Unlike neurogenesis which mainly happens during the embryonic stage, gliogenesis including astrogliogenesis and oligodendrocytogenesis peaks postnatally (Sauvageot and Stiles 2002). Loss of Pten resulted in hypertrophy and hyper-proliferation of astrocytes when using a Cre deleting Pten in both neurons and astrocytes during embryonic development (Fraser et al. 2004). This effect also contributed to the expansion of the SVZ and hypertrophy of the whole brain observed in this study. In the expanded SVZ of *Pten*^{CKO} but not wild-type mice, there were many hypertrophic astrocytes intermingled with retained Dcx+ neuronal precursors and differentiated NeuN+ mature neurons (see Appendix). There were also a huge number of hypertrophic astrocytes throughout the brain when Pten was deleted with the *Nestin-CreER*^{T2} transgene at birth (data not shown). However, astrocyte hypertrophy or hyper-proliferation could not be detected when Pten was deleted in the developmentally mature brain (Chow et al. 2011). The discrepancy in these studies is likely because Pten was deleted at different developmental stages or in different types of cells indicating cell-type-specific or stage-specific Pten functions in astrocytes. Future study needs to find out whether the hypertrophic astrocytes in the *Pten*^{CKO} SVZ are differentiated from Pten null neural

stem/precursor cells or whether they are reactive astrocytes due to a non-cell-autonomous effect of Pten loss. It is not clear if there is a specific cell context in which loss of Pten will cause a cell-autonomous hypertrophy and hyper-proliferation of astrocytes. Preliminary data show that there were far fewer hypertrophic astrocytes and very few EYFP+ astrocytes in the SVZ when Pten was deleted by Cre electroporation compared to by the inducible *Nestin-CreER^{T2}* transgene. This suggests the astrocytosis in the *Pten^{cko}* SVZ is likely a non-cell-autonomous effect (data not shown).

Remaining questions on Pten and development

Other questions raised in this study are what is the relationship between cell migration and differentiation? Are these two processes inter-linked or independent? Our study suggests that migration and differentiation are coordinated with each other. However, to what extent are they inter-connected? Does interruption of one process necessarily affect the other process? How is the differentiation affected in the neuroblasts in which only the migration machinery, for instance, mutations in PAR6 α , LIS1, NUDEL, CDK5 and DCX, is interrupted.

Why is there a discrepancy regarding whether Pten is required for directional migration in both Dictyostelium and mammalian cells? Is the discrepancy due to a cell-context dependent effect of Pten on directional migration or simply technical issues?

What are the cues that tell the migrating neuroblasts to stop and differentiate in the OB? One of the candidates is reelin, a critical extracellular protein regulating neuronal positioning (D'Arcangelo et al. 1995). Reelin had been shown to be required for the detachment of migrating neuroblasts from the RMS into the OB (Hack et al. 2002) and be able to activate PI3K-Akt-mTor pathway regulating dendritic growth (Jossin and Goffinet 2007). Disruption of ApoER2/VLDL, the receptors for reelin and Dab1, the downstream adapter protein (Andrade et al. 2007) showed a similar SVZ expansion associated with premature termination of tangential migration.

Pten and Brain Tumorigenesis

Cooperativity between Pten and other tumor suppressors

Neural stem/progenitor cells are one of the largest proliferative populations in the brain throughout life and are hypothesized as cells of origin for brain tumors (Sanai et al. 2005). In this study, deletion of Pten in the neural stem/progenitor cells in the SVZ of adult mice resulted in a dramatic expansion of the SVZ and RMS mainly due to early termination and ectopic differentiation with no progressive hyperplasia or malignant tumor formation. Similarly, the deletion of Pten in neural stem/progenitor cells in the developing postnatal brain only caused a very restricted hyper-proliferation around blood vessels in the cerebellum but not full-blown malignant tumor. These data indicate that

Pten loss of function alone is not sufficient for malignant transformation of the neural stem/progenitor cells in the absence of other gene mutations. This is consistent with patient data that although *PTEN* mutation is commonly found in 15-40% of GBMs, it rarely occurs in the low-grade gliomas (Ohgaki and Kleihues 2009). This could be due to the cell cycle arrest, senescence or apoptosis induced by Pten loss sensed by cells as an oncogenic stress similar to Ras and c-Myc activation mutations. Future studies need to address whether Pten deletion can indeed induce p53, p16^{Ink4a} or p19^{Arf} in the neural stem/progenitor cells whose tumor suppression had been shown to restrain tumor formation in other cell types (Chen et al. 2005; Lee et al. 2011). Nevertheless, we are able to generate high-grade astrocytomas arising from the SVZ when co-deleting *Pten* and *Trp53* in adult brain (see Appendix). In the developing brain, we could generate high-grade gliomas as well as medulloblastomas in *Nestin-CreER^{T2};Pten^{loxP/loxP};Trp53^{loxP/loxP}* mice, or highly infiltrative gliomas in *Nestin-CreER^{T2};Pten^{loxP/loxP};Trp53^{loxP/wt}* mice (see Appendix). However, co-deletion of *Pten* and *Cdkn2a*, another commonly mutated gene locus in human gliomas encoding p16^{Ink4a} and p19^{Arf}, is not effective in generating any brain tumor in either mature or developing brains (data not shown). This indicates different cooperativities between Pten and other tumor suppressor pathways and tissue-specific cooperativity between Pten and p16^{Ink4a} or p19^{Arf} pathways.

Unified function of Pten in neurogenesis and medulloblastoma genesis?

One striking similarity between *Pten^{CKO}* neuroblast in the SVZ and *Pten;Trp53* dKO medulloblastoma cell is that both of them had extensive neuronal differentiation. If we envision medulloblastoma genesis as a perturbed uncontrollable neurogenesis, this suggests Pten might have a similar role in normal neurogenesis and medulloblastoma genesis, that is to negatively regulate self-renewal and proliferation of neural or cancer stem cells and to keep the committed neuronal progenitors, either normal or cancerous, in an undifferentiated status. If this is true, does Pten have a similar role in the genesis of other brain tumor?

Why is the perivascular niche only seen in the cerebellum?

Even though the *Nestin-CreER^{T2}* targeted neural stem/progenitor cells throughout the developing brain, we only observed perivascular proliferative lesions in the cerebellum when deleting Pten at birth. There are multiple possibilities to explain this finding. Firstly, cerebellum lags behind other parts of brain during development which might provide a permissive environment for maintaining the perivascular niche. Secondly, there might be more primitive stem/progenitors only present during early development which had receded in regions other than cerebellum by birth. Supporting this idea, we saw less or no perivascular proliferative lesions when deleting Pten at later postnatal days. Thirdly, it is possible *Nestin-CreER^{T2}* target certain cell types only in the cerebellum. Future study needs to carefully compare the difference in the cell types targeted by Cre in different parts of the brain.

Cells of origin of brain tumors

Different brain tumors might arise from different cells of origin. The Shh subtype of medulloblastoma might arise from the granule cell precursors of the cerebellar EGL whereas the Wnt subtype of medulloblastoma might develop from cells of dorsal brain stem (Gilbertson and Ellison 2008; Gibson et al. 2010). Although gliomas are hypothesized to originate from neural stem cells or glial progenitors, gliomas can arise from within or outside of proliferative niches in patients and mouse models (CBTRUS 2010; Chow et al. 2011). In this study, tumors developed throughout the brain including the OB, cortex, SVZ, thalamus, hypothalamus, brain stem and cerebellum etc. This indicates that besides neural stem/progenitors in the germinal niches such as the SVZ, SGZ and EGL, cells outside of the proliferative niches could also serve as cells of origin. The other possibilities include NG2 cells (the oligodendrocyte progenitors present in both developing and mature brain)(Trotter et al. 2009) and cells associated with blood vessels. Because these two cell types are found to be the most common proliferative cells outside of germinal niches (Dawson et al. 2003) if we think the proliferative cells are more susceptible to malignant transformation. Future study could introduce common genetic lesions found in patients into the specific cell types to determine their potential as cells of origin for brain tumors. In this study, we also found that the same genetic mutations could initiate different brain tumors, medulloblastoma or gliomas, developing from different brain regions, indicating that cells of origin also determine the histological types of brain tumors.

Future directions for *Pten;Trp53* double knock-out medulloblastoma

Since the *Pten;Trp53* double knock-out medulloblastoma exhibit a growth pattern centering around blood vessels, it will be interesting to determine whether they initiated from certain cells around blood vessels by checking early timepoints when the tumor just starts to grow. If so, what is the exact cell associated with the blood vessels that give rise to the medulloblastoma? Why do the tumor cells adjacent to the blood vessels show a primitive and less neuronal differentiation? What is the status of Shh pathway in the tumor cells immediately adjacent to the blood vessels compared to those moving away from the blood vessels? How does this medulloblastoma compare to human medulloblastoma harboring both *PTEN* and *TP53* mutations, and to other existing mouse medulloblastoma models?

Closing summary

Overall, our study on Pten function in the context of both development and tumorigenesis show Pten regulates appropriate positioning of terminal neuronal differentiation by keeping neuroblasts in an undifferentiated state. Undifferentiated neuroblasts can still maintain the directional migration without Pten. Pten is required for preventing ectopic perivascular proliferative niche and medulloblastoma formation.

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APPENDIX. SUPPLEMENTARY DATA

Figure A-1. Increased number and size of astrocytes in the expanded *Pten*^{cKO} SVZ. Representative Gfap IHC staining of the SVZ from matched coronal brain sections of *Nestin-CerER*^{T2};*Pten*^{loxP/loxP} (cKO) mice injected with TM on P30-32 and analyzed at 6 months of age shows increase number and size of astrocytes in the expanded cKO SVZ. Bottom panels are the dorsal part of the SVZ. Scale bar: top panels=200 μ m, bottom panels=50 μ m.

Figure A-2. Deletion of *Pten* synergizes with *Trp53* mutations to drive high-grade gliomas in adult brain arising from the SVZ.

*Nestin-CerER^{T2}; Pten^{loxP/loxP}; Trp53^{loxP/loxP} (*Pten^{cKO}; Trp53^{cKO}*), *Nestin-CerER^{T2}; Trp53^{loxP/loxP} (*Trp53^{cKO}*)* and *Nestin-CerER^{T2}; Pten^{loxP/loxP} (*Pten^{cKO}*)* mice were induced with TM on P28-30. (A) Top panels: H&E staining of representative high-grade gliomas arising from the SVZ. Middle and bottom panels: representative H&E, Gfap, Pten and p-Akt IHC staining of the high-grade gliomas from the SVZ show the tumor is Gfap+, Pten- and p-Akt+. Scale bar: 50 μ m. (B) Kaplan-Meier survival curves show *Pten^{cKO}; Trp53^{cKO}* mice with a median tumor onset of 180 days of age (red curve,) and *Trp53^{cKO}* with a median morbidity age of 395 days (green curve). The *Pten^{cKO}; Trp53^{cKO}* mice developed high-grade gliomas with 50% of penetrance as well as peripheral tumors. The majority of *Trp53^{cKO}* were euthanized because of peripheral tumors.*

Figure A-3. *Nestin-CreER^{T2};Pten^{cKO};Trp53^{cHET}* mice developed highly infiltrative gliomas throughout brain.
Nestin-CerER^{T2}; Pten^{loxP/loxP};Trp53^{loxP/loxP} (*Pten^{cKO};Trp53^{cKO}*), *Nestin-CerER^{T2};Pten^{loxP/loxP}; Trp53^{loxP/wt}* (*Pten^{cKO};Trp53^{cHET}*) and *Nestin-CerER^{T2}; Pten^{loxP/loxP}* (*Pten^{cKO}*) were induced with TM on P0-1. (A) Kaplan-Meier survival curves show *Pten^{cKO};Trp53^{cHET}* mice with a median survival of 142 days of age (green curve) compared to 63 days for *Pten^{cKO};Trp53^{cKO}* mice (red curve which developed medulloblastomas) and 153 days for *Pten^{cKO}* mice (brown curve which were euthanized due to macrocephaly and hydrocephaly) (see also Chapter 3). (B) *Pten^{cKO};Trp53^{cHET}* mice developed highly infiltrative gliomas throughout brain. White boxes indicate the regions of interest shown in Figure A-4 with high magnification. 1: brain stem; 2: corpus callosum and cortex; 3: thalamus; 4: cerebellum; 5: SVZ.

Figure A-4. Highly infiltrative gliomas developed in *Nestin-CreER^{T2}*; *Pten^{CKO}*; *Trp53^{CHET}* mice.

Representative H&E and Ki67 IHC staining of infiltrative gliomas developed in the regions shown in Figure A-3 from *Nestin-CreER^{T2}*; *Pten^{loxP/loxP}*; *Trp53^{loxP/wt}* (*Pten^{CKO}*; *Trp53^{CHET}*) mice induced with TM on P0-1. Scale bar: 50 μ m.

Supplementary movie A-1. WT mice.

Supplementary movie A-2. *Pten*^{cKO} mice.

A Cre expression construct was transfected by in vivo electroporation at P2 into the SVZ of WT or *Pten*^{flax/flax} (cKO) mice carrying a ROSA-lox-STOP-lox-EYFP allele. Acute brain slices were prepared at P19-20, and cells in which Cre-mediated recombination occurred were visualized by EYFP reporter expression using two-photon microscopy. Images were captured every 5 minutes for 2.5 hours, and are presented in a movie of 4 seconds. Movies show the z-stack projections of 20 consecutive optical planes of section.

VITA

Guo Zhu was born in Hunan Province, China in 1978. He graduated from the Xiangya School of Medicine at the Central South University with a Bachelor of Medicine in Medicine in 2002 and a Master of Science in Pathology and Pathophysiology in 2005. After moved to the United States in 2005, he enrolled in the Integrated Program in Biomedical Sciences at the University of Tennessee Health Science Center for graduate study. In June 2006, he joined Dr. Suzanne Baker's laboratory at the St. Jude Children's Research Hospital and conducted research in Pten function in the brain development and tumorigenesis. He is expected to receive his Doctor of Philosophy degree from the University of Tennessee in May 2011.