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14. ABSTRACT The goal of this project is to test our hypothesis that cellular context in which initiating oncogenic event occurs may have a dominant role over specific oncogene function in determining the molecular phenotype of each tumor. To test this hypothesis, we originally proposed to transform neural stem cells (NSCs) and neural progenitor cells (NPCs) in vivo by expressing an activated form of <i>Notch1</i> (N1ICD) or deleting <i>XRCC2</i> . However, a reviewer suggested that we replace <i>Xrcc2</i> deletion with a more clinically relevant oncogene, and we chose to make new medulloblastoma models with oncogenic <i>PIK3CA</i> (PIK3CAH1047R). However, this change caused major delay in our progress since we have not been able to generate any tumors with PIK3CAH1047R expression. We successfully intercrossed PIK3CAH1047R (a frequent mutant allele of PIK3CA observed in human cancer) to <i>Sox2CreER</i> , <i>Atoh1-creER</i> , and <i>p53</i> <sup>-/-</sup> strains to generate PIK3CAH1047R; <i>Sox2-creER</i> ; <i>p53</i> <sup>-/-</sup> and PIK3CAH1047R; <i>Atoh1-CreER</i> ; <i>p53</i> <sup>-/-</sup> mice. We did not observe any medulloblastomas from these crosses before my laboratory moved to Houston Methodist Research Institute in Sept 2016. We had to restart the crosses here and while we could observe megacephaly in PIK3CAH2017R transgene-expressing brains, we did not observe any medulloblastomas. As a backup, we started generating a YAP-induced medulloblastoma models, and we successfully collected 5 samples from YAPS5A; <i>Atoh-CreER</i> medulloblastomas. When YAPS5A is expressed in NSC in developing brain, it caused major developmental defects and the embryos died in utero. We are now crossing YAPS5A to hGFAP-CreER mice to activate the transgene in postnatal NSCs. We will be able to perform the final tumor comparison analyses once we have these tumors.					
15. SUBJECT TERMS cancer stem cells, medulloblastoma, targeted therapy, therapy resistance, pediatric cancer, brain tumor, Notch1, PIK3CA, cell of origin, molecular subtypes, neural stem cells, neural progenitor cells, tumor initiation.					
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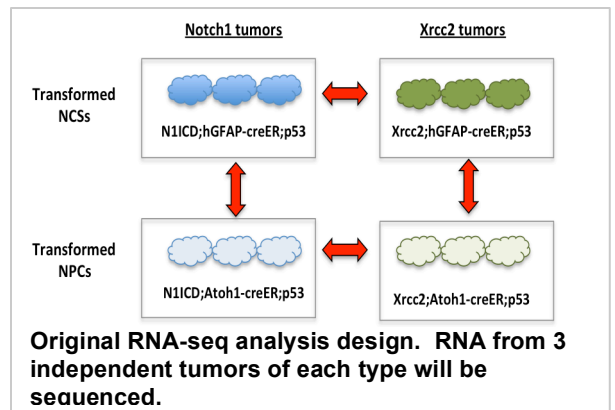
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## 1. INTRODUCTION:

The goal of this project is to test our **hypothesis that cellular context in which initiating oncogenic event occurs have a dominant role over specific oncogene function in determining the molecular phenotype of each tumor**. This hypothesis was based on our observations that certain oncogenes, such as *Id2* and constitutively active *Notch1* (N1ICD), induced DNA damage and apoptosis when activated in neural stem cells (NSCs) *in vivo* but had no observable effect when activated in neural progenitor cells (NPCs), immediate progenies of NSCs. These observations indicate that epigenetic changes that occur during NSC-to-NPC maturation can block oncogenes from functioning in NPCs. In other words, cellular context in which oncogene activation occurs may have a dominant role over some oncogene function.

In addition, we recently reported that cancer stem cells (CSCs) – a subset of cancer cells that have stem cell properties and tumor initiating ability- retain epigenetic memories of their cells of origin (Chow et al., 2014). We showed that CSCs derived from NSCs and NPCs depend on different mitogenic and survival pathways, even when they are transformed by the same oncogene *in vivo*. This finding has multiple implications: one of the most significant being that targeted therapies selected based on bulk tumor cell analysis may be ineffective in eradicating CSCs. We showed in two different SHH medulloblastoma mouse models that responsiveness of CSCs to SHH inhibitors varied greatly depending on the cell type in which tumor initiation occurred *in vivo*. If this novel discovery were generalizable, it would suggest that we will need to analyze CSCs (rare cells in the tumor) and not just the bulk tumor cells (current practice) to identify therapy combinations that will eradicate both CSCs and non-stem (bulk) tumor cells.



To directly test whether the cell-of-origin or the activated oncogene has more dominant role in determining molecular phenotypes of bulk tumor cells and CSCs, we proposed to generate and analyze spontaneous medulloblastomas by transforming NSCs and NPCs by expression of an activated form of *Notch1* (N1ICD) or oncogenic *PIK3CA* in the developing mouse cerebellum, using cell type- specific Cre drivers (*En2-Cre* for NSCs and *Atoh1-creER* for NPCs).

## 2. KEYWORDS:

cancer stem cells, medulloblastoma, targeted therapy, therapy resistance, pediatric cancer, brain tumor, Notch1, PIK3CA, cell of origin, molecular subtypes, neural stem cells, neural progenitor cells, tumor initiation.

## 3. ACCOMPLISHMENTS:

### Major goals of the project:

The stated goals of this project were to: 1) test the general applicability of our observation across multiple tumor models in which different oncogenic events initiate tumor formation and 2) test our hypothesis that cells in different stages of maturation in developing organs produce tumors with distinct molecular and cellular characteristics even when the initiating oncogenic event is the same.

To test the general applicability of our novel hypothesis, we proposed to transform NSCs and NPCs in the developing mouse cerebellum using cell stage- specific Cre drivers (*En2-Cre* or *GFAP-cre* for NSCs and *Atoh1-creER* or *Olig2-cre* for NPCs). We proposed to express activated *Notch1* (N1ICD) or an

oncogenic mutant form of PIK3CA in *p53*<sup>-/-</sup> brains. We proposed to analyze both bulk tumor cells and CSCs from each of these models and compare their molecular and cellular characteristics, including CSC culture behavior and AKT activation. We also proposed to compare molecular profiles of bulk tumors and CSCs of these tumors to determine whether the oncogene or the cellular context plays a more dominant role in driving the molecular phenotypes by unsupervised clustering analyses.

### What was accomplished:

During this period, we focused on generating new models of medulloblastoma by activating N1ICD and PIK3CA in cerebellar NSCs and NPCs in the developing mouse brain. However, since PIKCAH1047R expression could not produce full-blown tumors, we changed the model once again to YAP1 induced model (see below). We were able to collect tumor samples from 5 YAPS5A-induced medulloblastomas for analyses.

### N1ICD models:

We previously published that activated Notch1 (N1ICD) expression in the developing brain induces apoptosis due to DNA damage and p53 activation. When p53 is genetically deleted, ~40% of *N1ICD;GFAP-cre;p53*<sup>-/-</sup> mice developed spontaneous medulloblastomas (Natarajan et al., 2013). To generate medulloblastomas that arise from transformed NSCs, we intercrossed *N1ICD*, *En2-Cre*, and *p53* strains to generate *N1ICD;En2-cre;p53*<sup>-/-</sup> mice. To date, we have generated more than eight ***N1ICD;En2-cre;p53*<sup>-/-</sup> mice**; however, none of them formed medulloblastomas. The triple transgenic mice are viable, although they have shorter life span than wildtype mice. They appear to succumb to neurological defects.

To activate the same transgene in NPCs in the external granule layer (EGL), we intercrossed *N1ICD*, *Atoh1-CreER*, and *p53* strains to generate ***N1ICD;Atoh1-CreER;p53*<sup>-/-</sup>**. We activated the transgene in these mice by treating p3-5 pups with Tamoxifen. Unfortunately, we have not observed any medulloblastomas from these mice.

### PIK3CA models:

Because the reviewers had asked for (and DoD approved) replaced *Xrcc2*<sup>-/-</sup>-induced medulloblastoma model (proposed in the original submission) with PIK3CA-induced medulloblastoma model, we were behind schedule in terms of generating tumors. We first analyzed the effects of PIK3CA\* expression in different cellular compartments in the developing brain. As shown in Figure 1, expression of mutant PIK3CA\* in the developing embryo brain (by Nestin-Cre) induced severe dysplasia (Fig 1A, B), and ***PIK3CA\*;Nestin-cre*** mice died with hydrocephalus by weaning age. We validated elevated PIK3CA signaling in these brains by increased pAKT and pS6 expression in transgenic brains (Fig 1C, D). ***PIK3CA\**** expression in slightly more mature neuroepithelium (by GFAP-Cre) induced milder dysplasia with prominent rosette formation in the neuroepithelium in ***PIK3CA\*;hGFAP-cre*** brains (Fig

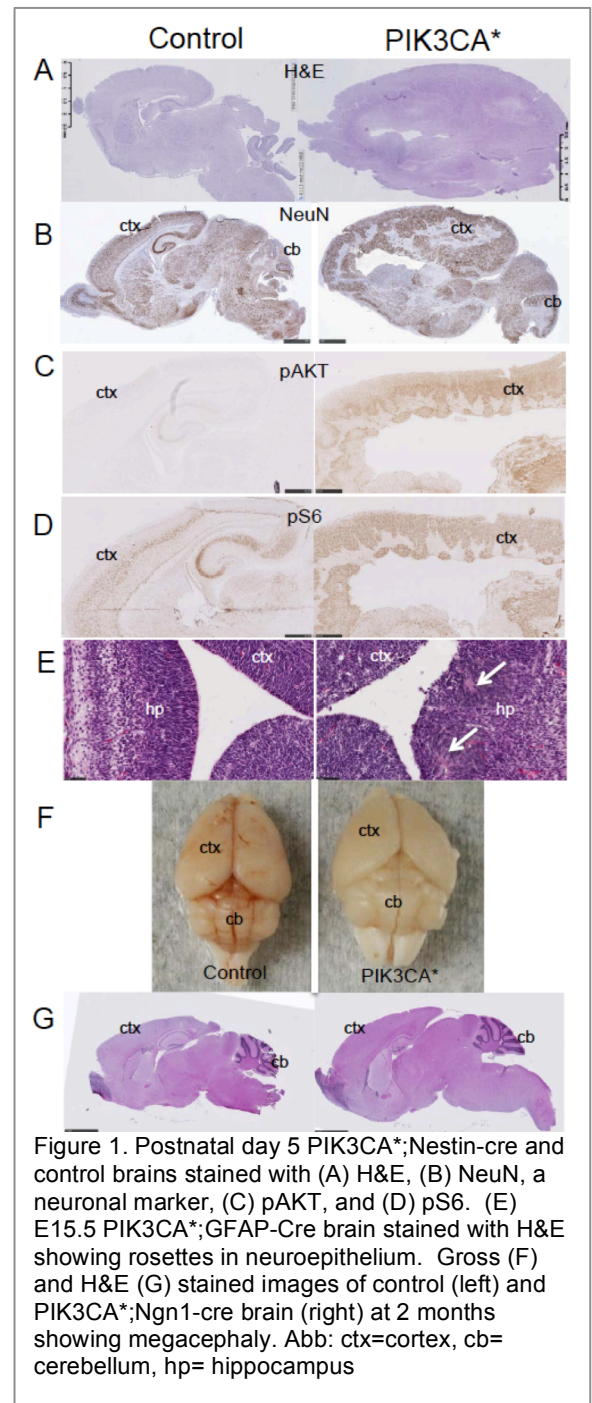


Figure 1. Postnatal day 5 *PIK3CA*\*;*Nestin-cre* and control brains stained with (A) H&E, (B) NeuN, a neuronal marker, (C) pAKT, and (D) pS6. (E) E15.5 *PIK3CA*\*;*GFAP-Cre* brain stained with H&E showing rosettes in neuroepithelium. Gross (F) and H&E (G) stained images of control (left) and *PIK3CA*\*;*Ngn1-cre* brain (right) at 2 months showing megacephaly. Abb: ctx=cortex, cb= cerebellum, hp= hippocampus

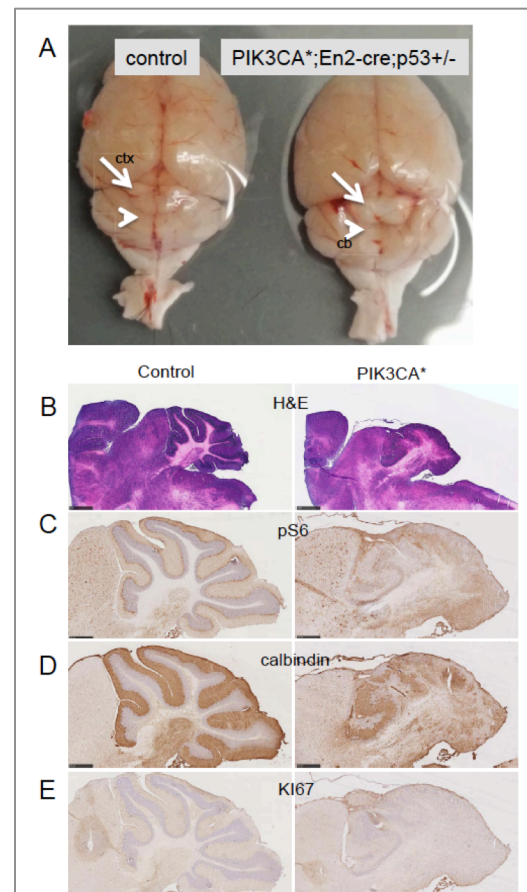
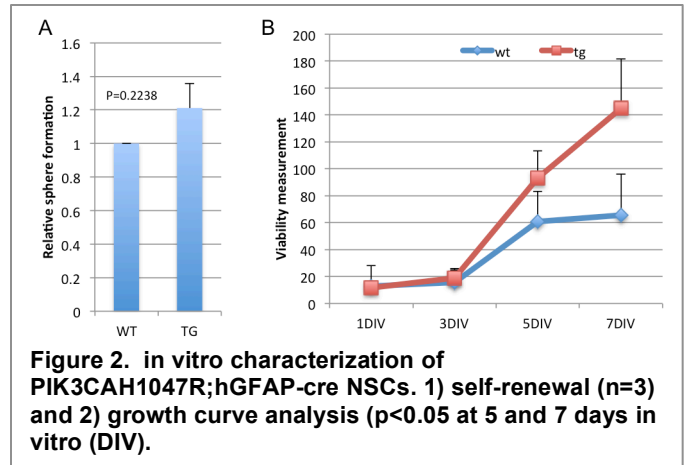


1E), but still resulted in hydrocephalus and lethality by weaning age. Interestingly, *PIK3CA*\* expression in committed neural progenitors (by *Ngn1-cre*) did not result in dysplasia although the *PIK3CA*\*;*Ngn1-cre* brains are megacephalic, Fig 1F, G). These mice also died around 2 months of age, likely from seizures. These analyses showed that the *PIK3CA*\* transgenic model we use is functional and that oncogenic *PIK3CA* expression in the developing brain affects proliferation and differentiation, as anticipated.

To circumvent early lethality associated with *PIK3CA*\* expression, we made two modifications to our approach. First, we used a milder and more clinically relevant allele of *PIK3CA*, *PIK3CAH1047R*, to activate the PI3K pathway. Since *PIK3CA*\* is a strong allele of *PIK3CA* (truncation mutation), we tested a common point mutation in *PIK3CA* gene in human tumors, *PIK3CA* H1047R. Unfortunately, *PIK3CAH1047R*;*Nestin-cre* mice die soon after birth. They are born with obvious megacephaly (not shown). To directly test whether *PIK3CAH1047R* expression increased self-renewal and proliferation of NSCs, we isolated NSCs from E15.5 transgenic (*PIK3CAH1047R*;*hGFAP-cre*) and control littermates. We performed self-renewal (Fig. 2A) and growth curve analyses (Fig. 2B). While *PIK3CAH1047R* expression did not increase self-renewal significantly, it did increase overall proliferation significantly. In addition, we observed that transgenic NSCs were larger in size, consistent with anticipated increase in mTOR signaling (not shown).

To circumvent this early developmental defect, we switched our Cre driver to *Sox2-CreER*. In these mice, Cre is activated in *Sox2*+ cells only when treated with Tamoxifen. We generated *PIK3CAH1047R*;*Sox2CreER* mice and treated them with Tamoxifen at p3-p5. We aged these mice to test whether they develop spontaneous tumors, but again did not observe any tumors.

To generate new medulloblastoma models induced by *PIK3CA*\* expression, we directed *PIK3CA*\* expression in the developing cerebellum by mating *PIK3CA*\* mice to the *En2-Cre* driver. *En2-Cre* is active in mid/hind brain neuroepithelium from very early on (E9.0 onwards). *PIK3CA*\*;*En2-cre* mice are viable (>240 days) but they have hypoplastic vermis and hyperplastic superior colliculus (Fig 3A), suggesting that the effects of *PIK3CA*\* expression is cell context-specific. Furthermore, cerebellar hemispheres were disorganized (Fig 3B), and marker analyses for activated PI3K pathway (pS6, Fig 3C), purkinje neurons (calbindin, Fig 3D), and proliferation (Ki67, Fig 3E) suggest that aberrant elevation of *PIK3CA* signaling affects cell proliferation/survival, differentiation and migration. Together, these results indicate that *PIK3CA*\* expression in early cerebellar stem cells may result in oncogene-induced apoptosis or senescence at an early age. To test whether *PIK3CA*\* expression induced p-53 dependent apoptosis and whether blocking this process induces tumor formation, we



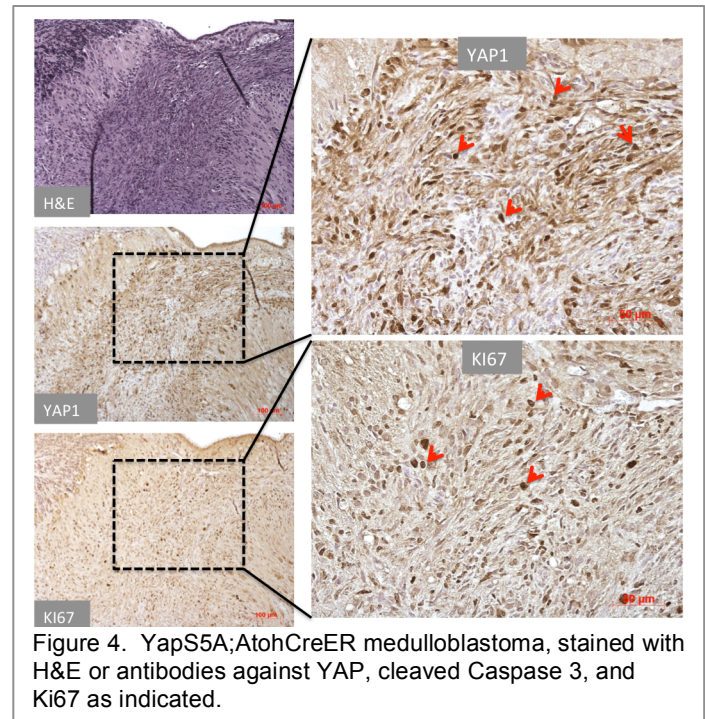
generated **PIK3CA<sup>\*</sup>;En2-cre;p53<sup>-/-</sup>**. Again, these mice are viable and no tumor formation was observed before we had to sacrifice the mice to move to Houston.

We are currently analyzing PIK3CAH1047R expression in cerebellar NPCs, using *Atoh1-CreER* inducible driver in EGL progenitor cells. We will determine whether embryonic and postnatal day EGL progenitor cells respond similarly as NCSs to PIK3CAH1047R expression and whether deleting the p53 tumor suppressor gene function will result in spontaneous medulloblastoma formation.

#### **Alternative models of medulloblastoma:**

Despite our continued efforts to produce PIK3CA-\* induced medulloblastomas, so far, we have not observed any localized tumors in the cerebellum. Therefore, we are tested another oncogene: YAP1.

YAP is a transcriptional effector of the Hippo pathway and its expression is elevated in SHH subgroup of human medulloblastomas. Interestingly, it is amplified in a subset of SHH medulloblastomas, and it has been shown to be an oncogene in other tissues. To test whether YAP1 can induce medulloblastomas on its own, we expressed YapS5A (constitutively active form of YAP) in the developing cerebellum by treating **YapS5A;AtohCreER** mice with Tamoxifen at p3-p5. These mice developed medulloblastomas between p19 and p36 (Fig 4). So far, we have collected 5 samples from this model to analyze or this study. We are now trying to generate YAP1-induced medulloblastomas using NSC-activated Cre drivers (using my startup funding at HMRI).



**Training opportunities:** N/A

**Results dissemination:** We are currently preparing a manuscript describing a new model of megalencephaly induced by PIK3CA. Human genomics analyses strongly implicated PIK3/AKT pathway activation in patients with megalencephaly and our model is a faithful model for this neurodevelopmental condition.

#### **4. IMPACT:**

**Impact of the principal and other disciplines:** Nothing to report

**Impact on technology transfer:** Nothing to report

**Impact on Society:** While we were not able to generate a new brain tumor model, we were able to generate a new mouse model of megalencephaly using PIK3CA strains. This will be a valuable resource for those studying this syndrome and knowledge gained from our analyses will reveal new insights into early stages of tumorigenesis in the brain and the relationship between megalencephaly and brain tumors.

#### **5. CHANGES/PROBLEMS:**

**Problems or delays:**

This project was delayed due to three main problems. One, we observed higher than anticipated incidence of sarcoma formation from mice in p53+/- or p53-/- backgrounds. We had to sacrifice triple transgenic mice before they could form brain tumors; hence, we are behind schedule in terms of collecting spontaneous medulloblastomas. To bypass this limitation, we started crossing floxed-p53 mice to N1ICD and PIK3CA\* mice so that we can delete p53 only in cells that are also expressing N1ICD or PIK3CA\* oncogenes in the brain. The second reason for the delay is that the reviewers had asked us to change the second oncogenic event (*Xrcc2* deletion) to a more clinically-relevant genetic event (we chose PIK3CA mutation). This change was approved pre-award by DoD. However, since this is a new model, we had to do more model characterization than anticipated, which caused some delay. We continue to mate these mice to generate transgenic mice; however, if the tumor incidence rate is low or the latency is too long, we will analyze YapS5A-induced tumors. Finally, we moved my laboratory from The Jackson Laboratory to Houston Methodist Research Institute in 2016. This caused major disruptions in laboratory operation, particularly in mouse room work.

**Changes with significant impact on expenditure:** Nothing to report

**Changes to human subjects, animals, or agents:** We changed the mouse model *Xrcc2* in the original proposal to PIK3CA and now YAP1 for reasons explained above.

**6. PRODUCTS:** Nothing to report



## 7. PARTICIPATION & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name	Kyuson Yun
Project Role	Principal Investigator
Researcher Identifier (NIH Commons ID)	KYUSONYUN
Nearest person month worked	2
Contribution to Project	overall supervision, experimental design and analysis.
Funding Support	N/A

Name	Ryota Nakada
Project Role	Co-Op Associate
Researcher Identifier	N/A
Nearest person month worked	2
Contribution to Project	PCR genotyping to set up appropriate mating and histological analysis of mutant brains.
Funding Support	N/A

Has there been a change in the active other support of the PD/PIs or senior/key personnel since the last reporting period?

N/A

What other organizations were involved as partners?

Nothing to report

## 8. SPECIAL REPORTING REQUIREMENTS:

None

## 9. APPENDICES:

N/A