Investigation of the role of the PTEN proximal genes in $NRAS^{Q61}$ and $BRAF^{V600E}$ driven melanoma in vitro

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I Abstract

English

Cutaneous melanoma remains the leading cause of mortality of skin cancer, with 75% of all deaths associated with skin cancers, and possessing a 5-year survival rates. Moreover, cutaneous melanoma is the most diagnosed cancer among young adults between 25 to 29 years old. Considering the genetic heterogeneous nature of cutaneous melanoma, uncovering the genetic underpinnings for initiation and progression of malignant melanoma is crucial toward the improvement of targeting strategies. $BRAF^{V600E}$ and $NRAS^{Q61}$ has been identified as the two most common oncogenic mutations in melanoma. $BRAF^{V600E}$ mutations has been identified as the earliest and the most prevalent melanoma mutations. $BRAF^{V600E}$ mutations stimulates cellular proliferation and survival through sustaining the activation of the MAPK pathway. However, BRAF hyperactivation alone is insufficient for the full malignancy conversion in melanoma due to oncogenic induced senescence. This is consistent with the observation of BRAFV600E mutations in moles. Progression to malignancy in $BRAF^{V600E}$ melanoma is accompanied by silencing of one or more tumor suppressor genes such as PTEN. PTEN negatively regulates the PI3K-AKT pathway, which promotes cellular survival and proliferation in parallel to the MAPK pathway. Moreover, it has been illustrated that $BRAF^{V600E}$ cooperates with PTEN loss to induce malignant melanoma. NRAS mutations are the second most common oncogenic mutations in melanoma, associated with aggressive tumors and shorter survival in early and late stage melanoma. Unlike $BRAF^{V600E}$ mutations, $NRAS^{Q61}$ mutations stimulate cellular survival and proliferation through the

hyperactivation of the MAPK pathway and the PI3K-AKT pathway. The dual activation aspect of mutant NRAS to both MAPK and PI3K-AKT signaling, provides a rationale for the dual inhibition of both signaling cascades to restrain tumor growth in NRAS mutant melanomas. In addition, there are studies suggesting the that concurrent inhibition of the PI3K-AKT pathway and MAPK pathway is a promising effective therapeutic target for human cancers harbouring RAS mutations. By implementing an shRNA-mediated knockdown approach, this research aims to investigate which of proteins upstream and downstream of PTEN in the PI3K-AKT pathway (PTEN proximal proteins, PI3K, PDK1, AKT family members), mediate the malignancy of $NRAS^{Q61}$ and $BRAF^{V600E}$ melanomas *in vitro*. Thus, the main objective of this research is to identify potential druggable contributors to melanoma development and drug resistance. Toward this goal, I have used a unique inducible shRNA lentiviral expression system to effectively and specifically knockdown PTEN proximal genes, to assess their role in melanoma malignancy. I investigated the effect of PTEN proximal genes knock down in $NRAS^{Q61}$ and $BRAF^{V600E}$ driven melanoma cell lines on proliferation, viability and progression through cell cycle. Using this *in vitro* model, I determined that the PTEN proximal gene knockdown decreased proliferation in melanomas derived from $BRAF^{V600E}$ and more significantly in NRAS ^{Q61}. More importantly, illustrate that the knockdown of the PTEN proximal genes uniquely causes be cell cycle arrest in NRAS ^{Q61R} melanoma cell line.

Française

Le mélanome cutané est la première cause de mortalité du cancer de la peau, avec 75% des décès associés à celui-ci et ayant un taux de survie de 5 ans. De plus, le mélanome cutané est le cancer le plus diagnostiqué chez les jeunes adultes de 25 à 29 ans. Compte tenu de l'hétérogénéité génétique du mélanome cutané, la découverte des fondements génétiques impliqués dans l'initiation et la progression du mélanome malin est cruciale et permet d'avoir des stratégies ciblées dans sa prise en charge. BRAFV600E et NRASQ61 ont été identifiés comme étant les deux mutations d'oncogènes les plus courantes dans le mélanome. Les mutations BRAFV600E sont des mutations qui apparaissent plus précocement et sont les plus fréquentes. BRAFV600E stimule la prolifération cellulaire et la survie en maintenant l'activation de la voie MAPK. Cependant, l'hyper-activation de BRAF seule est insuffisante pour la transformation maligne en mélanome en raison de la sénescence induite par cet oncogène. Ceci est cohérent avec l'observation des mutations BRAFV600E au niveau des grains de beauté. La progression vers une tumeur maligne dans le mélanome BRAFV600E s'accompagne de la désactivation d'un ou de plusieurs gènes suppresseurs de tumeurs tel que PTEN. PTEN régule négativement la voie PI3K-AKT, favorisant la survie et la prolifération cellulaires parallèlement à la voie MAPK. De plus, il a été montré que BRAFV600E coopère avec la perte de PTEN pour induire le mélanome malin. La mutation NRAS, deuxième mutation la plus courantes dans le mélanome, est associée à des tumeurs agressives et à une survie plus courte dans le mélanome de stade précoce et avancé. Contrairement aux mutations BRAFV600E, les mutations NRASQ61 stimulent la survie et la prolifération cellulaire grâce à l'hyper-activation de la voie MAPK et de la voie PI3K-AKT. L'aspect de double activation de la

mutation NRAS, à la fois de la voie de signalisation MAPK et de la voie PI3K-AKT, fournit une justification pour la double inhibition des deux cascades de signalisation pour freiner la croissance tumorale dans les mélanomes où NRAS est muté. De surcroit, certaines études suggèrent que l'inhibition concomitante de la voie PI3K-AKT et de la voie MAPK est une cible thérapeutique efficace prometteuse pour les cancers humains porteurs de mutations RAS. Mon projet vise à identifier les protéines en amont et en aval de PTEN dans la voie de signalisation PI3K-AKT (protéines proximales PTEN, PI3K, PDK1, membres de la famille AKT) impliquées dans la malignité des mélanomes NRASQ61 et BRAFV600E, en utilisant une approche de knockdown médiée par shRNA. L'objectif principal de cette recherche est d'identifier les contributeurs au développement du mélanome et à la résistance aux médicaments et qui seraient de potentiels cibles médicamenteuse. Pour cela, j'ai utilisé un système d'expression lentiviral de shRNA unique inductible pour éliminer efficacement et spécifiquement les gènes proximaux PTEN, afin d'évaluer leur rôle dans la malignité du mélanome. J'ai étudié les effets du knockdown des gènes proximaux PTEN dans les lignées cellulaires du mélanome induites par NRASQ61 et BRAFV600E, en particulier sur la prolifération, la viabilité et la progression à travers le cycle cellulaire. En utilisant ce modèle in vitro, j'ai déterminé que le knockdown du gène proximal PTEN diminuait la prolifération dans les mélanomes dérivés de BRAFV600E, et plus significativement dans NRAS Q61. Pour finir, j'ai démontré que le knockdown des gènes proximaux PTEN provoque l'arrêt du cycle cellulaire uniquement dans la lignée cellulaire du mélanome NRAS Q61R.

II Acknowledgments

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III Author contributions

This thesis was written entirely by myself (Naima Mohamed). The work described herein is the research efforts for my graduate degree and was performed by me unless otherwise stated. I would like to recognise Angeline De Bruyns for her efforts in triaging the shRNAs using the luciferase assay and selecting the shRNAs that targets PI3K-AKT pathway genes. She also worked in optimizing and testing the lentiviral delivery system, which was crucial for my work. Her contribution to this project has been illustrated in Figures (3.1-3). Also, I must recognise Kyle Lewis for generating the lentiviral vectors capable of targeting multiple PTEN proximal gene simultaneously. Finally, I would to recognise the efforts of Sam Garnett, who made the pTRIPz plasmid Gateway and making it compatible to create the pTREG vector, I used extensively during the course of my work.

1. Introduction

1.1 introduction to Melanoma

Melanoma is the cancer of melanocytes, which are the pigment-producing cells found predominantly in the skin and eyes. Melanocytes are neural-crest derived and reside just above the basement membrane of the epidermis [1]. Their primary function is to produce melanin after stimulation by the MSH (melanocyte stimulating hormone), which is secreted by keratinocytes in response to UV signals [2]. This paracrine signaling will eventually lead to the deposition of melanin at the apical surface of keratinocyte nuclei, producing a protective shield against UV damage [2].

Histological classification divide melanoma derived within epithelia into four groups:1- Cutaneous melanoma: lentigo and desmoplastic melanomas (from areas on the head and neck with high ultraviolet exposure); 2- mucosal melanomas (anorectal, vaginal, nasal, gastrointestinal tract); 3- Acral melanomas (arising from lesions in palms, nails and soles). Also, melanoma can arise from areas outside of epidermis like in the eye (Uveal melanoma) [2]. Most melanoma cases are cutaneous with 91.2% prevalence and the non-cutaneous melanoma are relatively rare including the uveal (5.2%) and mucosal (1.3%) [3].

According to the World Health Organization, globally cutaneous melanoma has been increasing over the past decades, with the highest prevalence in Australia, New Zealand, North America, in northern and western Europe [4]. The average age of a patient diagnosed with melanoma is 63, however, melanoma is the most diagnosed cancer among the young adults between 25 to 29-year-olds in the United States [5]. In comparison to other skin cancers such as basal cell carcinoma and squamous cell carcinoma,

cutaneous melanoma is much less prevalent with 132,000 cases occurring globally each year comparing to 2-3 million cases in non-melanoma skin cancers [6]. Nevertheless, while the diagnosis and treatment of non-melanoma skin cancer increased by 77% by 2014, the melanoma deaths is predicted to decrease by 5.3% in 2020 [7,8]. Mortality associated with non-melanoma skin cancer is rare comparing to malignant melanoma, which accounts for 75% of all deaths associated with skin cancer [9]. In fact, cutaneous melanoma is recognised for being the leading cause of mortality of skin cancers because of its highly tendency to metastasize and resistance to therapeutic treatment [10]. The surgical resection of regional melanoma (spreading nearby the skin or lymph nodes) has a 65% of 5-year relative survival rate[11]. This number drops to an abysmal 25% when the melanoma metastasizes to distant organs [11]. Furthermore, melanoma is a genetic heterogeneous disease, associated with one of the greatest burdens of somatic genetic mutations along with lung tumors in comparison to other human tumors, containing ~200 nonsynonymous mutation [12,13,14]. The rising incidence and the poor prognosis motivated researchers to understand the biology of melanoma and its genetic heterogeneity toward improving the design of the targeting strategies.

Histological studies suggest that melanoma-genesis is multistep process. Melanoma progression is characterised by acquiring additional genetic mutations that promote a survival, proliferative or/and invasive advantage [2,15,16]. Malignant melanoma often arises from acquired or congenital nevi (or moles) without dysplasia (benign) or dysplastic nevi [15]. Nevi develop from an uncontrollable proliferation of melanocytes as a result of an oncogenic mutation[15,16]. Nevi can be categorized into three groups based on their location: junctional (melanocytes restricted to the epidermis), intradermal (melanocytes

confined at the dermis) and compound (at both an epidermis and dermis) nevi [2]. Interestingly, melanocytic nevi can remain growth arrested for the lifetime of the individual due to a phenomenon known as oncogene induced senescence (OIS)[17-21]. OIS is mediated by the engagement of tumor suppressive mechanisms by two key players, p53 and retinoblastoma protein (RB) [22-26]. P53, is a transcription factor [27]that, in addition to many other genes, induces expression of its transcriptional target *CDKN1A*, which encodes p21CIP1, a cyclin dependent kinase (CDKs) inhibitor, leading to cell cycle arrest [28,26]. Activated hyperphosphorylated RB binds to the E2F-family transcription factors to repress their transcriptional targets and inhibit cell cycle progression[29].

The best-known locus associated with cellular senescence and melanoma susceptibility is *CDKN2A* [30-33]. This locus encodes two distinct proteins that function as tumor suppressors: $p14^{ARF}$ and $p16^{INK4A}$. Both proteins negatively regulate the cell cycle by modulating p53 and pRB levels and/or activity. INK4A directly inhibits cyclin dependent kinases CDK4 and CDK6 mediated phosphorylation of pRB, preventing the progression from G1 to S phase in the cell cycle [34]. ARF sequesters double minute 2 homolog (HDM2), a ubiquitin ligase targeting p53, thereby preventing p53 proteasomal degradation. Hence, elevated ARF levels prohibit cell cycle progression by stabilizing tumor suppressor p53 [35,36].

Melanoma can arise from benign and or dysplastic nevi in what appears to be a stepwise manner, progressing from nevi to the radial growth phase (RGP) melanoma, which is an intra-epidermal lesion with a local micro invasion of the dermis [1]. Fortunately, surgical resection tends to be curative to RGPs [37,38]. When melanoma cells are capable to progress to the vertical growth phase (VGP) by breaking through the basement

membrane to the dermis and the subcutaneous tissue, they acquire a metastatic potential [16,1]. However, not all melanomas pass through each of these individual phases as RGP and VGP; they can develop directly from nevi or melanocytes directly and progress to metastatic melanoma. Nonetheless, the most crucial stage in melanoma is the transition to VGP as melanocytes become capable of anchorage-independent growth [39,40]. Surgical resection of early staged melanoma has a 98% of 5-year survival rate and this number drops significantly to 16% when it metastasizes to distant organs[2].

Understanding of the biology underlying melanoma's initiation and progression requires the understanding of its genetics. Mutational analysis has identified high-risk genes and environmental factors that would increase susceptibility to melanoma. All of which will be addressed down below.

1.2 Genetics and Environmental factors involved in Melanoma susceptibility

Melanoma etiology is intricate, and it involves genetic and environmental risk factors. The main environmental risk factor associated with melanoma genesis is the exposure to ultraviolet radiation (UVR) [41,42]. In general, UVR from the sun or tanning beds could cause DNA damage by forming pyrimidine dimers, genetic mutations, photoproducts and oxidative stress that facilitate carcinogenesis [43-45]. In particular, a crucial genetic-environmental interaction that contributes to melanoma genesis is the role of polymorphisms in the melanocortin receptor 1 (*MC1R*) gene in sensitivity to the UV [46,47]. MC1R is stimulated in response to sunlight Exposure. Exposure to the sunlight, stimulates p53 to activate the expression of POMC in keratinocytes, a **p**recursor protein **o**f alpha-melanocyte-stimulating hormone(α-MSH). The binding of the α-MSH to the MC1R

induces the melanin production in melanocytes. The deposition of melanin in keratinocytes protects the skin from DNA damage caused by the UV, by forming a UV protective shield around the nuclie[48].

Different degrees of pigmentations in skin colour and hair, and in response to the UV radiation is mediated by the germline polymorphisms in the *MC1R* gene [49]. For instance, *MC1R* is remarkably polymorphic in whites with particular alleles associated with melanoma susceptibility [47,49]. The increased polymorphism in Caucasian populations, with a number of MC1R alleles that contribute to light-skin, red/blonde hair, inability to tan in whites and minimal ability to produce the UV protective shield around the nuclei, follows an augmented risk of melanoma development[15,50-53]. Moreover, this phenotype has been associated with the number and severity of melanoma tumours in familial and sporadic cases at a much higher rate in populations with lighter skin color than those with darker skin color [2,15,46,47,54].

Another factor involved in melanoma susceptibility is the family history of melanoma, as 5 –10 % of melanoma cases occur in familial setting [55], suggesting genetic underpinnings are responsible. A major high-risk melanoma susceptibility gene is *CDKN2A*, as inactivating mutations of the *CDKN2A* gene occur in 20% - 40% of cases in melanoma-prone families [56]. To a much lesser extent than *CDKN2A*, cyclin dependent kinase 4 (CDK4) locus has been identified as a high-risk melanoma susceptibility gene [57-61]. These latter mutations are interesting in that they encode a version of CDK4 that is resistant to inhibition by INK4A [57]. Recently, genes (tert pot1, shelterin) that are involved in telomere maintenance has been associated with melanoma susceptibility. The

germline mutations in genes that play a role in telomere maintenance contribute to around 1% of familial melanoma [62-66].

Another pathway that controls progression through the cell cycle, and is frequently mutant in sporadic melanoma and other cancers, is the MAPK (**m**itogen **a**ctivated **p**rotein **k**inase) signaling pathway. The MAPK signaling pathway transmits extracellular signals to cytoplasmic and nuclear effectors that act to mediate cell survival, proliferation, differentiation and progression through the cell cycle[67-69]. The relevance of the most common MAPK pathway mutations in melanoma will be discussed below.

1.3 MAPK signaling pathway in melanoma

The MAPK pathway consist of Raf/MEK/ERK signaling cascade. This pathway is activated by Ras (HRAS, KRAS and NRAS in humans), which is a GTPase protein localized in the plasma membrane [70]. RAF (ARAF, BRAF, CRAF in humans), MEK 1/2 and ERK 1/2 are serine-threonine kinases [71]. This pathway regulates cell survival, differentiation and proliferation in response to extracellular signals [72,73]. The MAPK pathway can be stimulated by receptor tyrosine kinases (RTK), cytokines and heterotrimeric G-protein-coupled receptors (GPCR) activated by extracellular growth factors [74]. This stimulation will facilitate the switch of the membrane-bound GTPase protein RAS from the GDP bound to GTP bound state, changing it to its active conformation. The exchange of GDP for GTP is catalyzed by a family of guanine nucleotide exchange factors (GEFs) such as SOS [74,75]. Normally, RTK autophosphorylation and dimerization facilitates the recruitment of adapter proteins that bind to the activated receptor such as Grb2 (growth factor receptor-bound protein2). Grb2

also binds to SOS simultaneously, which causes SOS to bind Ras and catalyzes the GDP to GTP exchange on Ras [76]. The activity of RAS is also negatively regulated by stimulating its intrinsic GTPase activity by the GTPase activating proteins (GAP) [75,77].

GTP-bound RAS serves as a docking site for RAF kinases to the plasma membrane. Indeed, GTP-bound RAS transduces extracellular signaling by its association with a spectrum of downstream effectors including RAF and PI3Kα (discussed in the following section) [78-80]. RAS effectors contain a RAS binding domain (RB), which is essentially a prerequisite to their interaction with the GTP-bound form of RAS and their activation [78,81].

In case of RAF kinases, this interaction causes conformational changes and phosphorylation events to activate the kinase activity. Subsequently, RAF kinases recruit and phosphorylate MEK 1/2 kinases which consequently phosphorylate ERK 1/2 kinases [68,69,71]. Activated ERK phosphorylate a large number of substrates, including transcription factors that regulate the cellular response to the extracellular signal[72,73,82].

The MAPK pathway is a crucial activator of melanoma cell proliferation as ERK is activated in 90% of melanomas[82]. A major breakthrough in understanding melanoma occurred in 2002 when activating mutations in *BRAF* were identified in 59% of melanomas [83,84]. The vast majority of these mutations occur at a single base mutation, altering a T to A at the 1799 position of the BRAF gene and this results in the substitution of a glutamic acid for valine at the 600 codon [83,84,85] ($BRAF^{V600E}$). This mutation renders a 400 to 600-fold increase in BRAF activity comparing to the wild type function[69,86,87]. In addition to melanoma BRAF, and in particular BRAFV600E mutations have been identified in

number of cancers such as thyroid cancer, colorectal cancers, ovarian, breast and lung cancers.[84,88,89].

The hyperactivation of BRAF activity will stimulate proliferation, survival, and promotes angiogenesis and metastatic spread of melanoma [90,91] .Interestingly, this high frequency of *BRAF* mutation in malignant melanoma does not seem to be directly a result of ultraviolet light induced mutation, as the T to A change at the 1799 position is distinct from the CC to TT or C to T changes associated with pyrimidine dimer formation after ultraviolet exposure[92]. Moreover, considering the oncogenic role of BRAFV600E in melanoma, interestingly, the expression of oncogenic BRAF alone is insufficient for full malignant conversion. This is consistent with the observation of the BRAFV600E mutation is in up to 80% of benign nevi(moles)[93]. As mentioned before, nevi rarely progress to melanoma because they remain growth arrested and engaged with OIS [86,93-96]. Indeed, oncogenic BRAF expression in melanocytes is not sufficient for a complete progression to metastatic melanoma [*94,96*] It has been illustrated in vitro and in vivo (mouse models), that $BRAF^{V600E}$ sustained mutation induces cell cycle arrest, which is demonstrated by the p16 INK4a and senescence-associated β-galactosidase (SA-β-Gal) activity (senescence markers) [94-96]. This has been further demonstrated with proliferation markers (BrdU) and cell viability staining [*94,95*].

The second most prevalent MAPK signaling pathway mutations are in the *NRAS* gene and are observed in 15-20% of melanoma tumors [97]. Indeed, it has been illustrated *in vivo* that when oncogenic HRAS ($HRAS^{V12G}$) or NRAS ($NRAS^{Q61K}$) expression is induced in a mouse model null for INK4a, melanoma genesis and maintenance become upregulated[98,99]. Although they share functional and structural similarities, mutations in

NRAS, *KRAS* and *HRAS* are known to be present 20%, 2% and 1% in all melanomas[100]. There is poor understanding of why NRAS mutations are more frequent in melanoma, however, there is suggestions that NRAS could be overexpressed in melanocytes relative to the other isoforms or it activates different signaling pathways than the other isoforms[101,77].

The most common oncogenic mutation reported for NRAS alters codon 61 (encoding Q61R or Q61K) [23]. Biochemical studies shew that Q61 mutations cause a deficient intrinsic GTPase activity in RAS, reduced sensitivity to GAPs, and an increased intrinsic nucleotide exchange rate. These modifications will lock RAS into its activated GTP associated conformation. Other less frequent NRAS mutations at G12/G13, render NRAS insensitive to the binding by the GAPs [77,102,103].

In contrast with the BRAF mutants, NRAS mutants are rarely present in benign melanocytic nevi with exception to the congenital nevi, which do not possess *BRAF* mutations [104]. Additionally, patients harboring NRAS mutations tend to be older (>55 years) than patients with BRAF mutations. Moreover, NRAS mutations are found more frequently in areas with chronic exposure to the UV, whereas BRAF-mutant melanomas are more common in intermittently sun-exposed skin [105,106]. However, NRAS mutations in melanoma do not present a classic UVR-induced signature, which, as prior mentioned, are characterized by C to T transitions associated with pyrimidine dimer formation[107.]

Simultaneous presence of BRAF and NRAS mutations in the same tumours are rare [108]. More importantly, BRAF and NRAS activating mutations has been demonstrated to be mutually exclusive at the single cell level [109]. This suggests that the existence of both BRAF and NRAS activating mutation does not confer an advantage for melanoma

initiation and progression or are selected against in tumorigenesis [109-112]. This is consistent with the concept of synthetic lethality relationship, which suggests that double mutants are synthetic lethal if a mutation of either gene is compatible with viability, but simultaneous mutation could deliver signals impairing cellular fitness[113]. Furthermore, recently, the simultaneous appearance of activating mutations in RAS and RAF were demonstrated to be disadvantageous for tumorigenesis because they cause senescence. The expression of $BRAF^{V600E}$ and $KRAS^{G12D}$ in a mouse model presented a reduced a tumor formation and higher levels of senescence markers, such as β-galactosidase staining and expression of $p14^{ARF}$ and $p16^{INK4A}$ [114].

In addition to activating MAPK pathway, RAS proteins activate the phosphoinositide 3-kinase (PI3K)/ AKT pathway that functions in parallel to MAPK pathway in regulating proliferation and survival along with other crucial cellular process such as metabolism, motility and angiogenesis. The activation of RAS causes the activation of the P110, the catalytic subunit of PI3K [115,116]. The PI3K-AKT pathway will be discussed in detail in the following section.

Progression to malignant melanoma is generally/often accompanied by the silencing of one or more of tumor suppressors such as CDKN2A and P53 [117]. Loss of *CDKN2A* expression is found in almost 20% of sporadic cases of melanoma, and mutations in *TP53* are less frequent (13%) [118,119]. Mutations in *TP53* and *CDKN2A* are mutually exclusive [119]. This could be also correlated with the concept of synthetic lethality as loss of ARF and P53 simultaneously is potentially selected against in tumorgenesis[30,120,121].

Another tumor suppressor commonly silenced in melanoma is PTEN. PTEN negatively regulate PI3K/AKT pathway, which is heavily implicated in human cancer including melanoma [122-124] and will be discussed in detail in the next section.

1.4 PI3K-AKT pathway in melanoma

Another critical cell signaling pathway that determines multiple cellular processes, notably survival and proliferation is the PI3K-AKT pathway. Activated PI3Ks (phosphatidylinositol 3-kinase) phosphorylate the membrane bound phosphatidylinositol-4,5-biphosphate (PI-4,5-P_2) at the inositol ring to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3) [125]. Elevated levels of membrane bound PIP_3 leads to the recruitment of proteins containing pleckstrin homology (PH) domain to the membrane. Like the MAPK pathway, the catalytic subunit of class I PI3Ks (P110α, β, δ, γ encoded by *PI3KCA*, *PI3KCB*, *PIK3CD*, and *PIK3CG*) can be activated by growth factors induced receptors including RTKs, GPCRs and, as mentioned before, by RAS signaling [125-127].

Of particular relevance to melanoma, serine-threonine protein kinases AKT/PKB (Protein Kinase B) ,which has three isoforms (AKT1/PKB β, AKT2 PKB α, AKT 3/PKB γ), and PDK1 (Phosphoinositide- dependent kinase 1) are recruited to the membrane via their plekstrin homology (PH) domain and sequentially activated [128-129]. To become fully activated, AKT must be phosphorylated at its Thr308 residue [130,131,132], which resides in the catalytic domain, by PDK1, and at its Ser473 residue (regulatory domain) by mTORC2 (mechanistic target of rapamycin complex 2) [130,131,132]. Through its serine-threonine kinase activity, AKT phosphorylates a large number of proteins that regulate proliferation, survival, angiogenesis, metabolism and migration [133,134].

AKTs are well-established in regulating cell survival, in part, by regulating apoptosis through directly phosphorylating and inhibiting pro-apoptotic proteins such as BAD, a BCL-2. Furthermore, AKT regulates the expression of pro-apoptotic proteins. AKT phosphorylates Fork head Box O transcription (FOXO) factors in their nuclear localization sequence, which will lead to their retention in the cytosol. This phosphorylation will prevent FOXO from inducing the transcription of their pro-apoptotic targets such as BIM and PUMA[135,136]. Also, AKT phosphorylates Mdm2, causing it to localize to the nucleus and negatively regulate P53 [137].This will antagonize the P53-mediated induction of the expression of downstream effectors regulating cell cycle arrest or apoptosis such as *P21* or *NOXA* and *PUMA* [137,138]. Essentially through its inhibitory phosphorylation, AKT also promotes cell proliferation by antagonizing the P53- mediated induction of cell cycle arrest promoting targets such as p21[137,138], and by antagonizing FOXO's activation of its cell cycle arrest transcriptional targets such as p21 and p27[135-140].

Moreover, AKT's role enhances cell growth by activating mTOR (mammalian target of rapamycin) complex 1, which activates S6K1, a kinase that phosphorylates several downstream effectors to promote mRNA translation [141,142,143].

Significantly, several pro-apoptotic AKT effectors have been found to be downregulated such as BAD [144,145], PUMA and NOXA [146,147,148] in melanoma and associated with melanoma's poor prognosis.

PTEN is both a lipid phosphatase and a dual-specificity protein phosphatase that functions as a tumour suppressor [149-153]. PTEN antagonizes PI3Kinase activity by dephosphorylating the phosphatidylinositol-3,4,5- triphosphate (PIP_3) at the 3' position and converting it back into PIP_2 [154-156]. The mapping of the homozygous deletions on

human chromosome 10q23 from tumor samples, which is recognized as a deletion hotspot for tumor suppression [157-160], led to the isolation of *PTEN* as a tumor suppressor candidate [157,158]. More importantly, PTEN mutations found in tumors has been demonstrated to ablate its phosphatase activity to disrupt its function as a tumor suppressor[157].

Perhaps the most evident manifestation of PTEN's tumor suppressor function is the identification of PTEN germline mutations in patients with Cowden syndrome. Patients with Cowden syndrome develop multiple benign tumors (hamartomas) and are at high risk for developing thyroid, uterus, brain, and renal cancer [161]. It is interesting to note that PTEN loss of function has been detected in 10%-30% of melanomas, due to frameshift mutations, deletions or epigenetic mechanisms[137,138,142]

Activating mutations in PI3K are relatively common in colon and breast cancer [165,166] yet, PI3K mutations are rarely found in melanoma. Indeed, analyses of melanoma cell lines identified that *PIK3C* mutations are less than 3% of the samples [167,168]. In contrast to normal melanocytes, AKT3 is overexpressed in 60% of melanoma cases and hyper-activated in 43% of melanomas[169,170]. Moreover, studies have identified AKT3 as the most hyperactivated or overexpressed AKT isoform in melanoma [171,172]. This is consistent with studies that demonstrated AKT3 contribution to melanoma progression[173]. Interestingly, *PTEN* mutations and deletions are mutually exclusive with NRAS mutations in melanoma[174,175]. On contrary, many of PTEN loss mutations appeared to be concurrent with BRAF activating mutations [175,176]. In fact, mutational activation of $BRAF^{V600E}$ in mice coupled with PTEN gene silencing elicits development of metastatic melanoma [*20*]. Furthermore, the combinational treatment of rapamycin (mTORC1 inhibitor) and

PD325901(MEK inhibitor) to melanoma mouse models noted a shrinkage of melanomas [20]. Taken together, these data reinforce the importance of the PI3K-AKT signaling in melanoma progression and is consistent with the finding that PTEN functional loss is common in late-stage melanoma [176,177]. The role of the PI3K-AKT pathway in mediating the resistance to BRAFv600e and NRAS targeted the therapy is discussed down below.

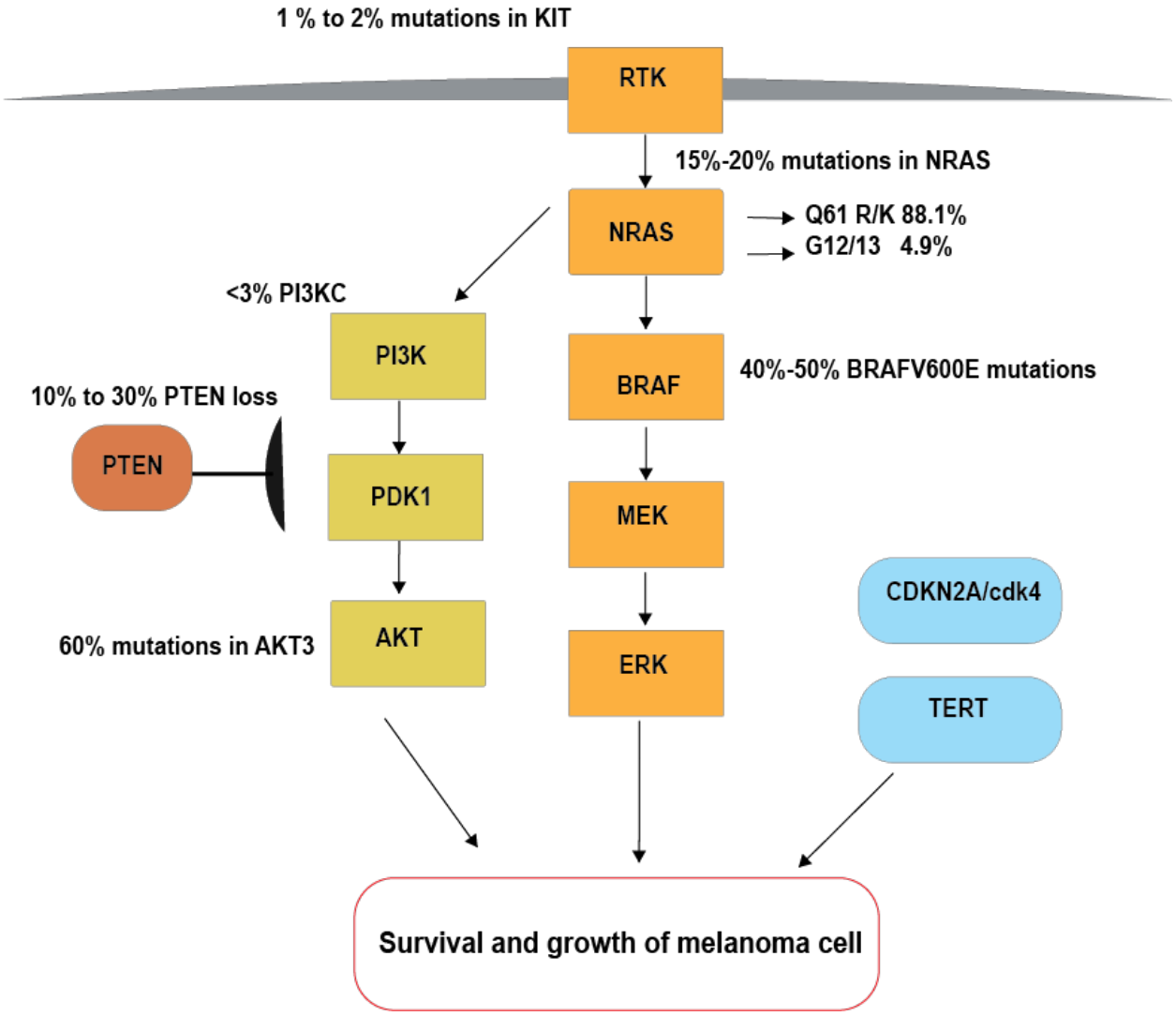

Figure1.1. schematic of relevant cell signaling pathways and frequency of mutations in cutaneous melanoma

1.5 Therapeutics and mechanisms of resistance in BRAF/NRAS mutant melanoma

The prevalence of activating *BRAF* and *NRAS* mutations in melanoma led to testing the effect of small molecule inhibitors of the MAPK pathway. The discovery that almost 50% of melanoma patients harboured the BRAFV600E mutation, provided the impetus to develop pharmacological kinase inhibitors that preferentially bind and inhibit V600-mutant over wildtype BRAF or, much less common BRAF mutations [84,177-180]. Two such inhibitors are vemurafenib (also known as PLX4032) and dabrafenib (also known as GSK2118436)[177,178]. Multiple studies demonstrated that these inhibitors induced apoptosis in BRAF-mutant melanoma cell lines and regression in xenograft models [21,182]. Treatment of previously untreated BRAF mutant melanoma patients with vemurafenib provided much improved response rates (~ 48% patients had tumour shrinkage) over dacarbazine, a DNA damaging agent used as the standard of care (5% patients had tumour reduction) [180,183]. Clinical trials comparing the efficacies of vemurafenib treatment to those of dacarbazine treatment (the standard of care for late stage melanoma patients) demonstrate improved progression-free survival (PFS) (the time between the treatment initiation and tumour growth or patient's death) of 5.3 months compared to 1.6 months for dacarbazine [183]. This resulted in the FDA-approval for Vemurafenib for metastatic BRAF melanoma in 2011[179,21,183-185]. Similarly, dabrafenib treatment improved progression-free survival over dacarbazine treatment (5.1 months versus 2.7 months), and when used in combination with vemurafenib achieved 76% response rate in BRAF-mutant melanoma patients [177,178]. This was a breakthrough in metastatic melanoma targeted treatment since before 2011, dacarbazine and interleukin-2 were implemented and approved by the FDA,

despite lacking a significant influence on the overall survival of patients [180,186,187]. Furthermore, BRAF inhibitors provided an improved side effect profile [188,189].

Despite the initial promise of use of BRAF mutant inhibitors in metastatic melanoma, enthusiasm has been restrained due to *de novo* or secondary drug resistance. Patients with BRAFV600E mutations achieve a degree of tumor shrinkage with vemurafenib and dabrafenib treatment. However, the degree of reduction varies and only 10% of patients achieve a complete tumor regression. Furthermore, the PFS of clinical responses to these inhibitors is generally limited to 6 to 7 months, with the majority of patients relapsing within 12 months [49,55]. Pathway analysis of the patients with BRAFV600E mutation on the vemurafenib phase I trial demonstrated that tumor regression was not achieved unless at least 60% reduction of phosphorylated ERK was achieved [184]. This limited response, indicates that the resistance may be due to mechanisms that sustain ERK signaling to become insensitive to RAF inhibitors, or reduce the oncogenic dependency of the tumor for MAPK signaling by switching dependence to an alternative survival pathway [190-193].

Several mechanisms conferring drug resistance exist including the acquisition of mutations that sustain the activation of ERK such as increased signaling by MAPK - activating RTKs, CRAF amplification, acquisition of activating NRAS mutations (signaling through CRAF) or MEK mutations [190,192,194-200]. Many of ERK dependent resistance mechanisms could be overcame by the dual BRAF and MEK inhibition since It is generally thought that inhibiting both the oncogene and its downstream effector simultaneously decreases the chance of developed resistance. Indeed, currently, the single-agent BRAF inhibition therapeutic approach in treating advanced melanoma has shifted to become

combinational with MEK inhibitors, and it has been shown to be effective with PFS of 2 years in patient with BRAFV600E mutations[202-204].

Nevertheless, *In vitro* studies have shown that a subset of the BRAFV600E-melanoma cell lines resistant to PLX4032, exhibit distinct resistance mechanisms that are not ERK dependent and insensitive to MAKP inhibitors [190,191,201]. The mechanism of resistance to BRAF inhibitor has been associated with overexpression of receptor tyrosine kinases, like PDGFR β or IGF1R[190,201]. Additionally, *in vitro* studies illustrate that although ERK remains activated in many BRAF-inhibitor- resistant melanoma cell lines, abrogating of ERK activation using MEK inhibition did not have considerable effects on viability suggesting that an additional pathway is promoting the survival of these cells [191].

Exposing BRAF-inhibitor resistant melanoma cell lines to IGF1R inhibitors caused a suppression in AKT activation but not an inhibition on MAPK activation[191]. This observation was followed by the confirmation that a concurrent inhibition for the IGF1R/AKT pathway and MAPK pathway significantly induces cell death [191]. Moreover, statistical analysis illustrated a durable activation of P70S60K and S6 in the BRAF-inhibitor resistant compared to the sensitive cell lines[193]. Following that notion, combined triple treatment of Rapamycin (mTOR inhibitor), PX-866 (PI3K inhibitor) and PLX4720 (BRAF inhibitor) resulted in a notable death in the BRAF mutant resistant cell lines [193].

Thus, this data indicates that resistance to BRAF inhibition is associated with enhanced PI3K-AKT pathway as a protective survival mechanism and provides a rationale for combinational targeting to MAPK and PI3K-AKT pathway in melanoma treatment.

Interesting, one out of five cases of melanomas that relapsed after PLX4032 treatment were correlated with overexpression of phosphorylated AKT with/or homozygous loss of PTEN [191]. Moreover, PTEN loss/mutations in patient samples with BRAFV600E was associated with a trend of shorter PFS after treatment with Dabrafenib (8 months vs 1 month) [205]. The role of PTEN as tumor suppressor in regulating resistance to BRAF inhibitors has been demonstrated after a comparison between BRAF mutant melanoma cell lines lacking PTEN expression ($PTEN^-$) and BRAF mutant melanoma cell lines expressing ($PTEN^+$), after treatment with BRAF inhibitors [206]. This study indicated that the re-introduction of PTEN into ($PTEN^-$) cells notably increased the expression of BIM and apoptosis in response to BRAF inhibitor treatment. More importantly, the knockdown of PTEN in ($PTEN^+$), led to the inhibition of BIM expression which was shown to be induced BRAF inhibitor treatment [206]. Furthermore, the co-targeting of MAPK pathway and PI3k of in ($PTEN^-$) -BRAF mutant melanoma cell lines caused with an increase of apoptosis induction [206,207]. Specifically, the resistance to apoptosis after BRAF is inhibited or knocked down is mediated by AKT3 over expression in melanoma cell lines [206,207]. These studies propose that concurrent inhibition of the PI3K-AKT pathway could overcome the resistance to BRAF inhibitors. Taken together, this illuminates a promising addition to the combinational therapeutic approach that would combat the development of resistance after BRAF inhibition in BRAF-mutant melanoma patients.

NRAS mutations are the second most common oncogenic alteration in melanoma (15%-20%) and represent a significant clinical challenge since they are associated with more aggressive tumors and a shorter survival in early and late stage melanoma [105,208]. While PLX4032 decreases the activity of MEK/ERK 1/2 in BRAFV600E/K melanoma cell

lines, it increases the activity of MEK/ERK 1/2 pathway and proliferation of NRAS-mutant melanoma cell lines[209,210]. One possible explanation to this paradoxical effect is through the activation of CRAF by the heterodimerization with BRAF that is bound to BRAF inhibitor[209-212]. Identifying an effective therapeutic inhibitor for mutant *RAS* remains appealing, since *RAS* mutations are found in many aggressive malignancies. Earlier strategies were focused in targeting the farnesylation of NRAS [213]. Farnesylation is a critical RAS post-translational modification, that is the covalent attachment of a farnesyl pyrophosphate to the cysteine residue in the CAAX motif of RAS (C cysteine, A denotes aliphatic, aliphatic, any amino acid), catalyzed by farnesyltransferase(FT). This post-translational modification creates a lipid hydrophobic domain that is required for RAS localization to the membranes [213]. FT inhibitors has been developed to obstruct farnesylation and reduce the translocation of RAS to the membrane and therefore reduce its ability to mediate the activation of its downstream effectors [213]. FT inhibitors has shown a promising clinical efficiency in treating patients with acute myelogenous leukemia[214,215]. However, no significant clinical response or anti-tumor activity was reported in NRAS mutant melanoma patients, although a significant inhibition of RAS-downstream effectors like ERK and AKT was achieved[213,215-217]. It has been shown that treatment of FTI inhibitors to NRAS results in lipidation by alternative modification proteins geranylgeranyltransfersases (GTTases), thus bypassing dependency on FTases [88,218,220]. Moreover, targeting both FTases and GTTases suffers from limiting cytotoxicity effect, since these modifications target several cellular proteins other than RAS (>100) [220,221]. Thus, these inhibitors will lead to prohibitive off target effect, limiting their clinical effectiveness.

Another promising approach is designing optimal GTP analogs and inhibitors that inhibits RAS function by targeting the SOS-mediated nucleotide exchange of Ras [217,222,223]. However, the designed compounds elicit off-target effects by hindering the normal RAS function more than the oncogenic RAS [217,222,223]. A better understanding of the interaction between the designed compounds with oncogenic RAS is required to implement the inhibition of RAS-nucleotide exchange as a therapeutic approach [222,223]. Most recently a subset of RAS mutations, specifically those Gly to Cys mutations (G12C), have been targeted with small molecules, that reduce their sensitivity to GEFs [224-226].That said these inhibitors will target only a small subset of NRAS mutation in melanoma (<4.9%)[227].

Currently, the therapeutic approach of targeting downstream kinases of the pathway such as MEK 1/2, has been implemented in NRAS melanomas treatment. As mentioned before, BRAF inhibitors paradoxically promote proliferation in NRAS mutant malignancy, which switched the focus on only MEK inhibitors to inhibit MAK signaling in NRAS mutant melanoma. Binimetinib, an MEK 1/2 inhibitor, is the first agent to show promising activity in NRAS melanoma, with 20% partial response and 3.7 months of PFS [228]. Nevertheless, this partial response demonstrates that MEK inhibitors insolation are insufficient in NRAS mutant melanomas treatment but does provide encouragement to develop MEK inhibitor-based combinational therapy. Two particular pathways implemented in such combinational approach are CDK4/Rb and PI3K/AKT. The observation of the mutations of cell cycle genes such as *CDKN2A* in melanoma at high frequency suggested CDK4 as co-target with MEK in NRAS mutant melanomas [229]. A combined pharmacological inhibition using trametinib (MEK inhibitor) and CDK4

palbociclib (CDK4 inhibitor) in vivo led to tumor regression and motivated the application of this combinational therapeutic approach for the treatment of NRAS-mutant melanomas [229]. Promising, but limited, outcomes were achieved in phase I/II clinical trials that combine Binimetinib and LEE011 (CDK4 inhibitor) treatment with partial response of 33% [230]. Until recently, there was a paucity in developing NRAS-mutation targeting strategies for the NRAS-mutant melanoma genetic cohort. Understanding pathways that contribute to this limited response is a long sought and an elusive goal and remained a therapeutic challenge for NRAS mutant melanomas. The dual activation aspect of mutant NRAS to both MAPK and PI3K-AKT signaling, provides a rationale for the dual inhibition of both signaling cascades to restrain tumor growth in NRAS mutant melanomas. In vitro and in vivo models are required to provide insights into the role of the PI3K-AKT pathway in NRAS melanoma, and whether it could possibly contribute to a sustained critical response in threat of NRAS-mutant melanoma. This will integrate with the data suggesting that concurrent inhibition of the PI3K-AKT pathway and MAPK pathway is a promising effective therapeutic target for human cancer harbouring RAS mutations [231,232].

1.6 Research Objectives:

Following the notion that the activation of the PI3K-AKT pathway is an attractive target to overcome melanoma malignancy and drug resistance, an experimental study should be designed to particularly build on current BRAF/NRAS mutant melanoma models that address the PI3K-AKT as a rational therapeutic target. More importantly, this study must illustrate the extent of the oncogenic dependency of melanoma upon specific PI3K and

AKT isoforms along with PDK1(referred to as PTEN proximal genes), and how they contribute to the resistance developed to MEK/BRAF inhibitors.

Hence, the goal of my project is to investigate the genetic role of PTEN proximal genes in melanoma cell lines to educate efforts geared toward effectively targeting the PI3K-AKT pathway in melanoma for a sustainable therapeutic response. This will be accomplished by systematically ablating the expression of PI3K-AKT pathway signaling molecules proximal to PTEN in vitro via a short hair pain RNAs (shRNAs) approach (Figure 1.1). The shRNAs will be stably integrated using a lentiviral delivery system.

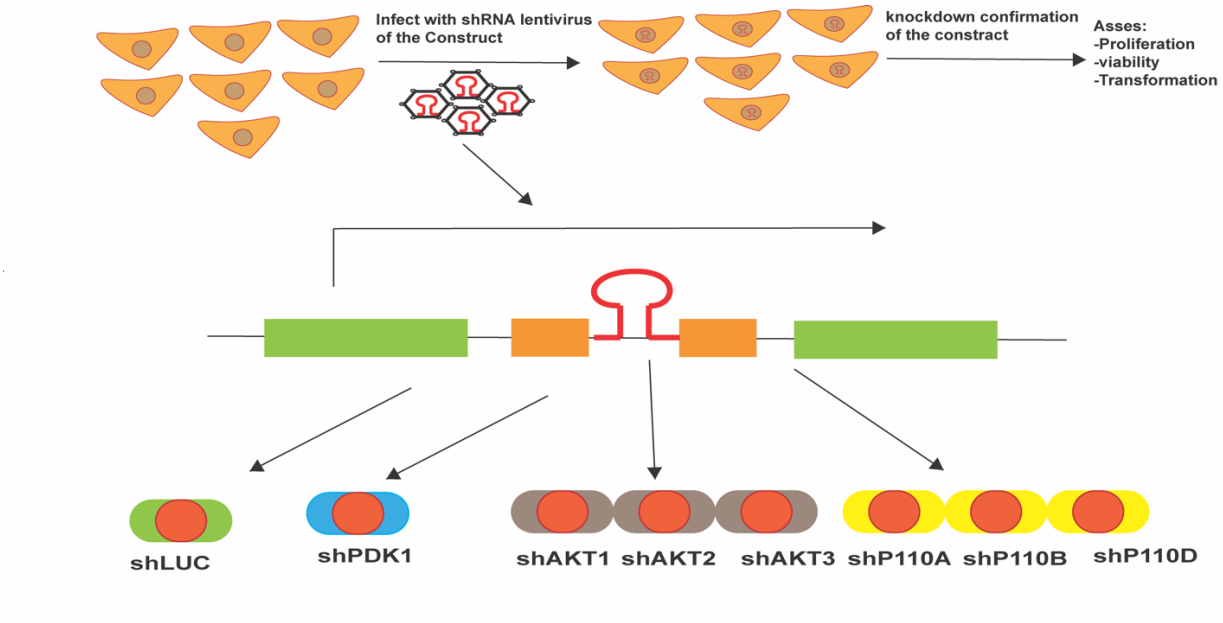

Figure 1.2. Experimental Approach. Melanoma cell lines are infected with lentiviral shRNA vectors containing shRNAs targeting the PTEN proximal signalling molecules in the PI3K-AKT pathway. The vector has shRNAs of multiple isoforms for targeting AKT and PI3K. Cells that have stably incorporated the lentiviral insert are selected and used for subsequent experiments to test the effect of shRNA-mediated target knockdown on the malignant phenotype of melanoma cells *in vitro* (figure adapted from A. DeBruyns 2015)

At the commencement of this project, there was preclinical inhibitors for multiple components of the PI3K-AKT pathway like PI3K inhibitors, dual PI3K/mTOR inhibitors, AKT inhibitors and mTORC1 inhibitors [233,234]. Each of those inhibitors was countered with

several challenges such as overlapping specificity. This is a critical challenge, since experimental data for different tumor types, indicated that the different mutations that boost the activity of the PI3K-AKT pathway, result in functional dependency on a specific range of effectors and thus sensitivity to a range of therapeutic agents over the other. For instance, some cancer cells with PTEN loss are dependent upon P100B than P110A for growth and survival [235,236]. Moreover, it has been previously illustrated that in many melanomas, PI3K-AKT signaling is mostly mediated by the AKT3, which represents a potential target [172]. Substantially, there is a great level of homology between the AKT isoforms and the PI3K isoforms, hence making isoform-specific inhibitors is challenging. Also, the overlapping downstream effectors makes it difficult to determine the efficacy of the isoform-specific pharmacological inhibition, given they're functionally similar. Implementing an shRNA-based approach would overcome this challenge by allowing assaying for a sufficient knockdown by detecting levels of proteins and mRNA. This will facilitate the efficiency evaluation of the construct inhibition process and the functional efficacy of the knockdown.

Herein I describe knocking down the following PTEN proximal proteins: the class I PI3Ks catalytic subunit isoforms (p110- α, p110- β, P110 δ), PDK1, and AKT isoforms (AKT1, AKT2, AKT3) in melanoma cell lines. I sought to target the PTEN proximal genes, since kinases tend to be more feasible to be targeted pharmacologically. Moreover, I illustrate assaying the expression of the proximal PTEN proteins and examining the effect of the knockdown on viability, proliferation and transformation on melanoma cell lines.

2. Materials and Methods:

2.1 Culture of Mammalian cell lines

All the cell lines were grown in humidified incubator at 37°C and 5% CO_2. HEK 293T (human embryonic kidney) and A375 melanoma cell lines were cultured in DMEM (Wisent) with 10% heat inactivated Fetal Bovine Serum (FBS) (Wisent) and 1% penicillin/streptomycin (PS) (Wisent). Sbcl2, WM9 and WM793 melanoma cell lines were cultured in Tu2% medium (80% MCDB153 (Sigma), 20% Leibovitz's L-15 (Wisent), 5µg/ml insulin (Bovine (Sigma-Aldrich & cat# i6634)), 1.68 mM $CaCl_2$) with 2% of heat inactivated FBS and 1% PS. MM485 was cultured in RPMI (Wisent) with 10% FBS and 1% PS. Sk-mel-2 was cultured in EMEM (Wisent) with 10% heat inactivated FBS and 1% PS.

All the cell lines were maintained in 100mm treated dishes with 10ml of media or in 150 mm tissue culture dishes with 20 ml of media and sub-cultured every 4-5 days when appropriate. When trypsinizing the cells, 0.05% (w/v) trypsin-EDTA solution (Wisent) was used for the 293T cells, the A375 cells and the MM485, and the 0.25% Trypsin-EDTA (w/v) was used for the other melanoma cell lines.

Table 1. Melanoma cell lines and their mutational status

Cell line	Type of lesion	Site	Reported mutations
WM9	metastasis	LN axilla	BRAF(V600E,) PTEN hem. del.
Sbcl2	primary RGP	-	NRAS(Q61R)
A-375	metastasis	-	BRAF(V600E), (PTEN WT)
Sk-mel-2	metastasis	thigh	NRAS(Q61R) (PTEN WT)
MM485	metastasis	LN	NRAS(Q61R), CDKN2A(W110Stop)

2.2 Transfections

24 hours before transfection, 8×10^6 of 293T cells were seeded per 150 mm dish and, 5×10^6 of 293T cells were seeded per 100 mm dish. In a 100mm dish, 16 µg of plasmid DNA (5.2 µg of PAX2 plasmid, 2.8 µg pCI-VSVG plasmid, 8 µg lentiviral vector) diluted in 550 µl of Opti-MEM (Invitrogen #11058-021) was used along with 42.6µl of PEI (polyethyleneimine, 1mg/ml). For 150mm dishes, 36 µg of plasmid DNA (11.7 µg of PAX2 plasmid, 6.3 µg pCI-VSVG plasmid, 18 µg lentiviral vector) in 1200 µl Opti-MEM was used along with 95.9 µl of PEI. The transfection mix was incubated at room temperature for 30 min the added dropwise to cultured 293T dishes after a media change. The lentiviral vectors used are Table1.

2.3 shRNAs in the Lentiviral vectors used

Mentioned below are the shRNAs inserted in lentiviral vectors and used to achieve knockdown for PTEN proximal genes.

Table 2. ShRNAs in the Lentiviral vectors used to target PTEN proximal genes (AKT, PDK1, shPI3KC) and the luciferase control. The number indicates shRNA used after testing for successful target knockdown (Figure 3.2)

Target	shRNA#	Generated by
Luc	-	A.DeBruyns 2015[181]
AKT (AKT1, AKT2, AKT3)	Three shRNAs for three target isoforms arranged in the same vector. For AKT1: #1 or #2 For AKT 2: #7 or #11 ForAKT3: # 13 or #16	K Lewis 2016 [241]
PDK1	#18	A.DeBruyns 2015[181]
shPI3KC	Three shRNAs for three target isoforms arranged in the same vector. For PIK3CA: #30 or #32 For PIK3CB: #34 or #36 For PIK3CD: # 39 or #41	K. Lewis 2015 [241]

2.4 Lentivirus Titration-TurboRFP Positive colonies

This titration was performed as explained in pTRIPz technical manual with minor modifications[237]. On Day 1, 5×10^4 293T cells were seeded per well in 24-well dish (6

wells for each viral preparation) in 500µl/well of regular media. The dish was pre-coated with poly-DL-lysine as per product information (Sigma #P9011). On Day 2, the media was changed with 225µl / containing 8 µg/ml polybrene. Virus stocks were thawed and 100µl of 5-fold dilutions of each were prepared in a 96 well plate, with dilutions ranging from 5^{-1} to 5^{-5} fold. Then 25 µl from each viral dilution was transferred to the 24 well destination plate containing 293T cells and last well was left as uninfected control with 25 µl of regular growth media added. Cultures were incubated for 24 hours at 37 ºC. Then gently, 1ml of media (DMEM with serum) containing doxycycline (1µg/ml) was added to each well and incubated for 72 hours. Turbo RFP expressing colonies of cells were counted under a fluorescence microscope. Each multi-cell colony was counted as 1 transduced cell, as the cells will be dividing over 72hours culture period. The following formula was used to calculate the viral titer = # of TurboRFP positive colonies counted × dilution factors ×40=#TU/ml (transducing unit/ml). Then an average of the calculated titers from all the countable wells was calculated to get the final viral titer.

2.5 Lentivirus Titration- Puromycin-Resistant colonies

To titer virus by the puromycin selection, on Day 1, 1.5 $\times 10^5$ 293T cells were seeded to each well of poly-DL-lysine coated 6-well plates. On Day 2, 2ml 5-fold serial dilutions of virus stocks were prepared in DMEM (with serum) containing 8µg/ml of polybrene [ranging from 5^{-2} to 5^{-6}]. After removing the media from the cells in the 6-well plate, 1 ml of each serial dilution was added on the cells, leaving one well to have just polybrene-DMEM as uninfected control. On day 3, the media was changed with fresh DMEM (with serum). On day 4, media was replaced by DMEM containing puromycin at 4µg/ml and

incubated for 7-9 days to allow puromycin-resistant colonies to grow, with replacing the media every 2-4 days with puromycin containing media. Cells were washed in 1X PBS, fixed with zinc formalin fixative (Sigma, Z2902) for 1 hour, and then stained with 0.1% crystal violet for 30 minutes, followed by two rinses with 1X PBS. Then plates were left to dry overnight, and crystal violet stained colonies were counted in the following day. Colonies were counted using cell counter plugin in the image J software. The lentiviral titer was calculated using the following formula: colony number per well by the dilution factor (TU/ml). An average of calculated titers from all the countable was calculated to get the final viral titer.

2.6 Lentivirus infection

Infections were done at specific MOIs for cytotoxicity issues. The number of TUs required were calculated as: MOI X #cells to be infected. Infection were performed at 37ºC overnight with polybrene concentrations 4-8 µg/ml as above. The next day, the media was changed and replaced with fresh regular growth media. Two days post infection, infected cells were selected by adding puromycin to the regular growth media at 2-4µg/ml for at least 4 days.

2.7 Doxycycline Treatment

The expression of the shRNAs in the pTRIPz -shRNA cell lines were induced by the treatment of doxycycline 1µg/ml. Every two days, the doxycycline. Containing media was changed during the induction period.

2.8 Colony formation assay of cells

Cells were seeded from 100-500 cells/well in 6-well plates in a triplicate experimental design (3 wells doxycycline treated and 3 wells untreated controls). The doxycycline treatment (1µg/ml) was done in the following day. Cells were incubated at 37ºC for 7-10 days until cells in the control wells form sufficiently large colonies. Cells were fixed, stained with 0.1% crystal violet and counted as above.

2.9 Proliferation assays

The melanoma cell lines were seeded in 12-well dishes in triplicate (10000 cells/well or 25 000 cells/well), such that there is a plate for each day. The following day, media was changed with a doxycycline containing media (1µg/ml) and media (±doxycycline) was changed every other day. For cell counting assay, cells were trypsinized and counted using a hemocytometer at the indicated time.

For IncuCyte® live-cell analysis assays, the cell lines were seeded in a triplicate experimental design in 96 well plate at 500 to 1000 cells per well, and the doxycycline treatment was applied on the next day. The IncuCyte® ZOOM 2016 software captured images every 4hours to calculate the % confluency of well over a period of 7 days [238].

2.10 Cell lysis and protein quantification

PLC lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerol (v/v), 1% Triton X-100 (v/v), 1 mM EGTA, 1.5 mM MgCl 2 , 10 mM NaF, and 10 mM Na 4 P 2 O 7 , Aprotinin, Leupeptin, and Pepstatin at 1 µg/mL, 1 mM PMSF, 1 mM orthovanadate) was used to

lyse cells. A375-derived cells were washed with cold PBS twice, and incubated for 20 minutes on ice in the presence of 500 µl of lysis buffer. The dish was occasionally tapped to loosen the cells from the plate and a cell scraper was used to collect the cells in the lysis buffer into microtube. The Sbcl2 cell lines were trypsinized and collected in 15ml tubes then rinsed once with 1XPBS. After aspirating the PBS, the pellets were resuspended with 100µl lysis buffer, and then rocked at 4ºC for 30 minutes. Lysates were cleared by centrifugation at 16100g at 4ºC. The supernatant was collected in another tube and stored in the -80 freezer until protein quantification assay. The protein concentration assay was done using the BCA protein assay kit (#CA82601-004).

2.11 Immunoblotting assay

The protein samples were prepared in 1X Laemmli buffer (67 mM Tris pH 6.8, 10% v/v glycerol, 1.25% w/v SDS, 0.0025% w/v bromophenol blue and 2.5% v/v 2-mercaptoethanol) at 45-60µg of protein per well for loading on to the gel. Then protein samples were boiled at 100 ºC for 5 minutes before loading. The protein sample were run in 10% acrylamide SDS-PAGE, and then transferred onto a PVDF membrane in transfer buffer (25 mM Tris base, 192 mM Glycine) containing 20% methanol. Transfers were run at 400mA for 2 hours. The membranes were blocked using 5% milk-TBS-T (TBS-T: 50 mM Tris base, 150 mM NaCl and 0.05% Tween-20) for 1 hour at room temperature. Following that, blots were incubated in primary antibody overnight at 4 ºC. The primary antibodies were diluted in 5% BSA-TBS-T or 5% milk-TBS-T. The primary antibodies used, and their dilutions were:

PI3Kinase p110α (Cell Signalling #4249, 1:500), PI3Kinase p110β (Cell Signalling #3011, 1:500), PI3Kinase p110δ (Santa Cruz #sc-7176, 1:1000), PDK1 (Cell Signalling #3062 1:1000), AKT1 (Cell Signalling #2938, 1:1000), AKT2 (Cell Signalling #3063, 1:1000), AKT3 (Cell Signalling #8018, 1:1000), α-Tubulin (Sigma #T5168, 1:8000), and GADPH (cell signalling #2118, 1:1000).

The next day, membranes were washed with TBS-T three times for 5 minutes at room temperature then incubated in horseradish peroxidase (HRP)- linked secondary antibody at a dilution of 1:3000 to 1:10000 in 5% milk-TBS-T for 1 hour. The secondary antibodies used were either anti-rabbit (GE Healthcare #NA934) or anti-mouse (GE Healthcare #NA931). Following the incubation, the blots were washed with TBS-T three times for 5 minutes at room temperature and detected on X-Ray film using Amersham ECL Western Blotting Detection Reagent (GE Healthcare #RPN2106).

2.12 Microscopy

Cellular fluorescence was observed using a Leica DM IL LED inverted microscope with X-cite series 120 Q UV source. Photos were captured using the QICAM Fast 1394 camera attachment (Q IMAGING) and filter sets from CHROMA.

2.13 BrdU cycle analysis and Flow cytometry

Melanoma cell lines were left untreated or were treated with doxycycline (1μg/ml) for 5-8 days. Plates were seeded to become sub confluent just before the addition of BrdU (control untreated and doxycycline treated), the media is replaced with a fresh media containing 10μM BrdU, and the plate is incubated for 4-6 hours. Then the media was

removed and transferred to a 15 ml falcon tube, and the plates were washed with 1XPBS and collected by trypsinization and added to the same falcon tube. The tubes were spun at 1500rpm for 5 minutes at 4ºC, washed with cold 1XPBS and spun at 1500rpm for 5 minutes. The pellets were resuspended in 100µl cold 1XPBS then fixed by slowly adding 1ml of 70% EtOH. Then cells were stored at -20 up to 7 days until used.

Cells were pelleted by centrifugation at 1500 rpm for 10 min at 10 ºC and washed with 1XPBS. The DNA was denatured by slowly adding 1ml 2M HCl while vertexing and incubation at for 30 minutes at room temperature. Then, the cells were pelleted, and acid neutralized twice by resuspending in 1ml 0.1M Sodium Borate pH 8.5. Cells were pelleted, resuspended with PBS-T, 0.5% BSA (1xPBS, 0.5% BSA, 0.2% Tween-20), then pelleted, resuspended with 20µl of FITC Mouse Anti-BrdU (BD Pharmingen, 51-33284X) and incubated for 20 minutes in the dark at room temperature. After adding 1ml 1XPBS, cells were pelleted and resuspended in 1ml PI staining solution (25µg/ml propidium iodide, 0.2mg/ml RNAseA, 40mM sodium citrate, 1% 51 triton-x 100). Flow cytometry was done using the BD FACSCalibur (BD Biosciences) and analysis FACS Diva Software.

3. Results

3.1 ShRNA triaging using a luciferase reporter system

Initial efforts of this project were dedicated toward selecting successful shRNA candidates that could effectively and specifically knockdown each of the PTEN proximal gene targets, and this was approached by the previous graduate student Angeline de Bruyns. Isoform-specific shRNAs were screened for efficacy and specificity using a dual luciferase reporter system. The dual luciferase reporter system is a method developed to efficiently triage shRNAs using the psiCHECK luciferase reporter system [239]. This system uses a plasmid expressing two transcripts independently, one encoding Renilla luciferase and one encoding Firefly luciferase (Figure 3.1A). cDNA target sequences are cloned downstream of the Renilla luciferase stop codons and upstream of a polyadenylation (polyA) sequence. Therefore, Renilla luciferase and the target cDNA are transcribed as one mRNA transcript. For efficient mRNA translation, a lariat structure must be formed between the 5'-cap and the polyA-tail of the transcript [240,162]. Therefore, the shRNA-induced cleavage of the target cDNA sequence prevents efficient translation of the Renilla luciferase upstream, which can be quantified. Firefly luciferase activity is used as a transfection control, as the plasmid contains a firefly luciferase gene, regulated by a different promoter and thus expressed independently of the Renilla luciferase gene.

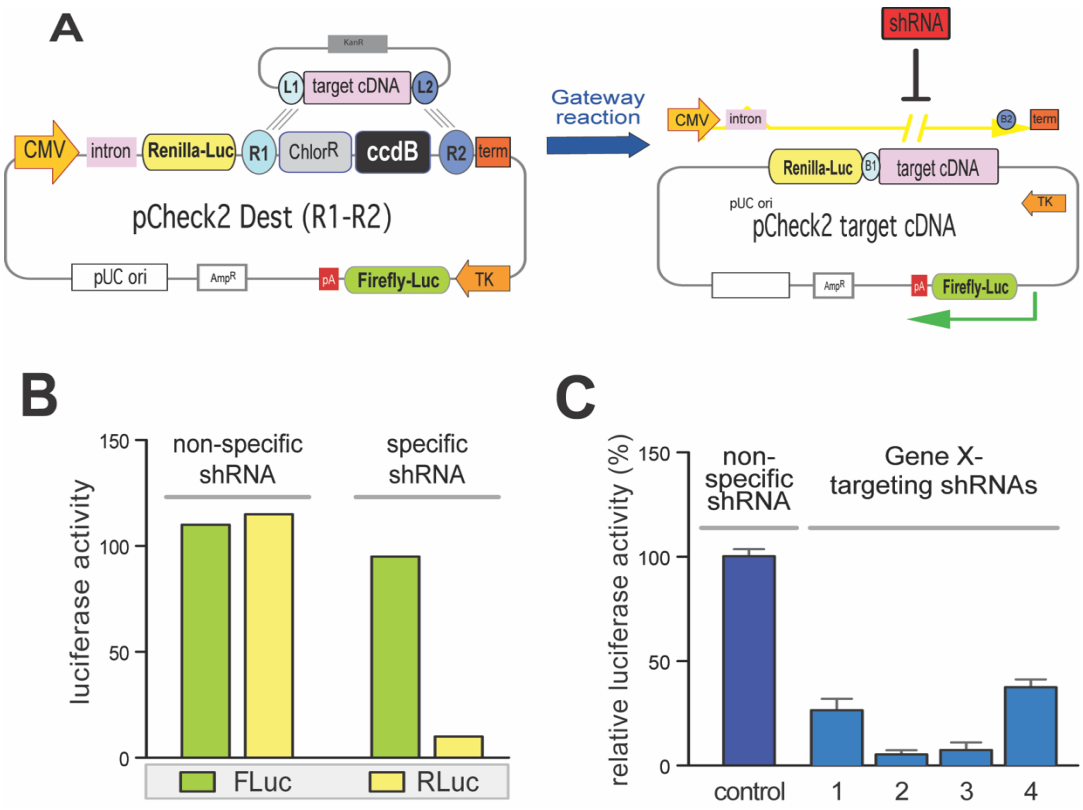

Figure 3.1: Rapid Triage of novel ShRNA using dual luciferase reporter system. (A) Target cDNA are cloned into pCheck2 Dest (between R1and R2) through gateway recombination. The resulting plasmid produces CMV-driven transcript (yellow arrow) encoding Renilla luciferase and the non-translated target cDNA, and Thymidine kinase(TK)-driven transcript (green arrow) encoding Firefly luciferase used as an internal control for the dual luciferase system.(B) Two different shRNAs are compared by normalizing for transfection efficiency via the activity of the Firefly luciferase and for specific knockdown by the Renilla luciferase activity. (C) The data of shRNAs targeting the same gene is normalized for luciferase activity observed for non-specific shRNA. (Figure taken from A. DeBruyns 2015) [181]

Because firefly and Renilla luciferases use different substrates and reaction conditions for bioluminescence, the relative levels of Renilla over firefly luciferase can be used as a surrogate for shRNA mediated targeting of the target cDNA. Also, the psiCHECK plasmid is Gateway-system compatible,allowing for the rapid generation of plasmids having PTEN Proximal genes of interest. Hence, after the generation of the pTREG-shRNA vectors their efficacy was rapidly assessed, and successful shRNAs was selected.

HEK 293-T cells were co-infected with the psiCheck2 reporter plasmid and an shRNA expression plasmid (shTest). The shTest plasmid contains the shRNA cloned into an miRNA-30 cassette. The advantage of embedding the shRNA sequence in a miRNA-30 cassette is that the shRNA can be stably expressed from any RNA polymerase II

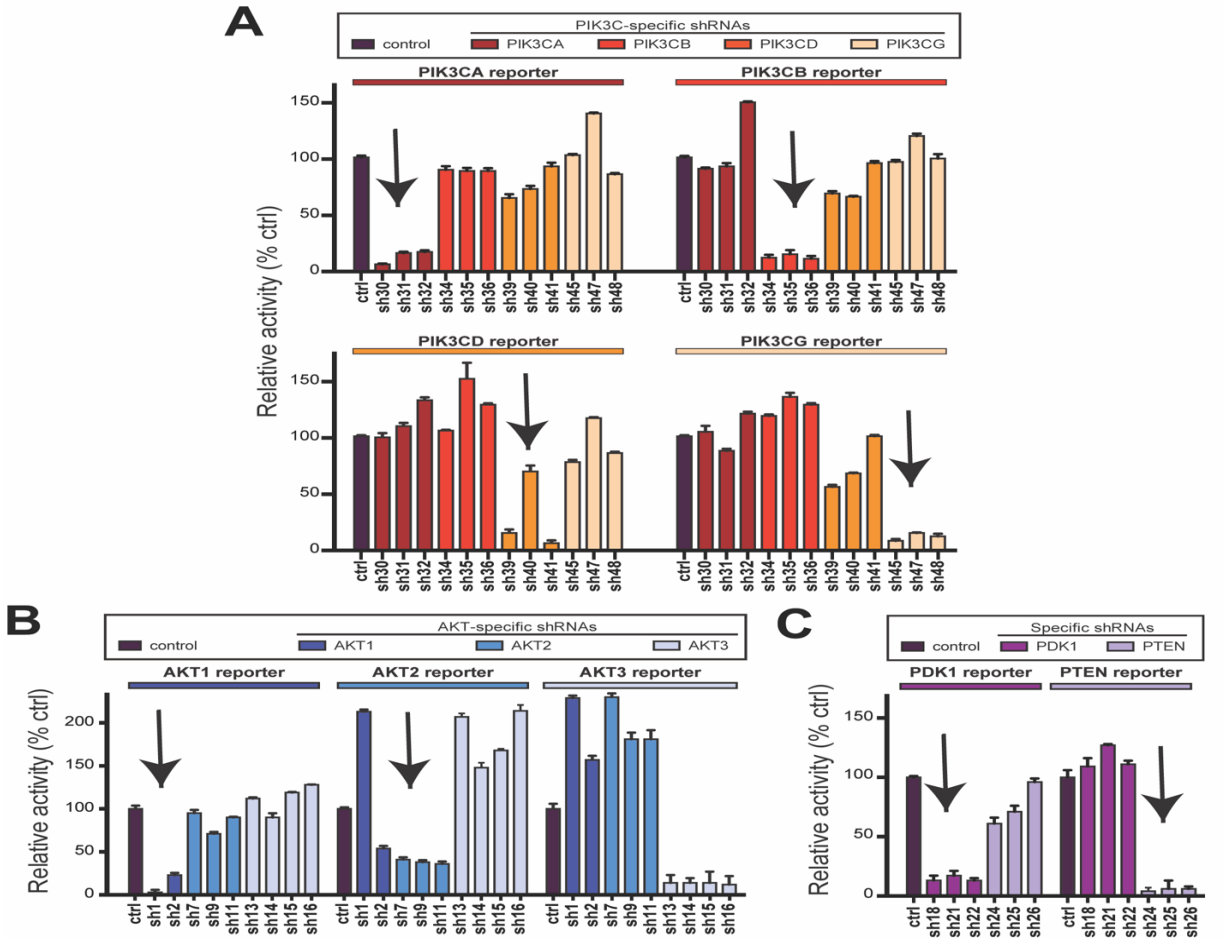

Figure 3.2. The results of the Luciferase assay triaging for shRNAs targeting PTEN proximal genes. To test for specific knockdown, HEK 293-T cells were co-infected with the shTest plasmid psiCheck2 plasmid contain cDNA of targets. Colors of the bars for the relative activity represent the specific reporter each shRNA is intended to target with arrows to indicate group of successful shRNA candidates for each target. (A)Luciferase assay readout of PI3K shRNA specificity. (B) Luciferase assay readout of AKT shRNA. (C) Luciferase assay readout of PTEN and PDK1 shRNA specificity. Error bars represent relative standard error. (Figure modified from A. DeBruyns,2015, arrows indicate selected shRNAs that were used in this study)

promoter, permitting expression in a constitutive, inducible, or tissue specific manner[163,164]. Additionally, the miRNA is placed in the same transcriptional unit as eCFP (enhanced cyan fluorescent protein) to permit visual tracking of the expressed shRNA. The preliminary rounds of luciferase assay triaging identified the effective shRNA candidates at targeting their mRNA transcripts. The most effective shRNAs were selected for a second round of luciferase assays to test for specificity within similar groups of targets. For example, AKT shRNAs were cross tested against all of the AKT target reporter plasmids. In a like manner, the PI3K shRNAs were tested against all of the PI3K Target reporter plasmids. Thus, the luciferase reporter assays allowed identification of successful shRNA candidates, specific for each PTEN proximal gene of interest (Figure 3.2)[181].

3.2 PTEN proximal genes Knockdown in Melanoma cell lines

3.2.1 Inducible shRNA delivery system

After the identification of shRNA candidates that could efficiently target each PTEN proximal gene of interest, two types of lentiviral vectors with the targeting shRNAs were constructed using Gateway recombination technology. One lentiviral vector allows constitutive expression of the shRNA (pLEG lentiviral vector), and the other permits inducible expression of the shRNA (pTREG lentiviral vector)[181]. Since the PI3K-AKT pathway regulates cell viability, the knockdown of pathway components is predicted to induce apoptosis [123,133]. Using constitutively expressed shRNAs was unideal to use for studying genes in the PTEN proximal genes since, as supported by previous research, the PI3K-AKT pathway mediates drug resistance by decreasing susceptibility to

apoptosis[191,193,231,232]. Using drug-inducible knockdown vectors (pTREG) can provide a means to control shRNA expression and to more easily monitor lethality of PTEN proximal gene knockdown. pTREG is derived from a pTRIPz-derived vector and harbours a Tet-On inducible shRNA cassette. In this vector, the shRNA expression is controlled by the reverse tetracycline transactivator (rtTA) binding to the TetO operator sequence of the Tet-responsive element (TRE) and this binding requires the presence of tetracycline or tetracycline analogue, like doxycycline [239,219]. Moreover, pTREG encodes for a selectable marker (puromycin resistance) and fluorophore (TurboRFP), with the latter being on the same transcript as the miR30-embeded shRNA. Thus, permitting the ability to visually monitor shRNA expression. Assessment of the pTREG lentiviral expression system using one shRNA to AKT2 (AKT2, shRNA #11) confirmed inducible knockdown of the target [181]. Briefly, AKT2 protein knockdown using constitutive or inducible vectors was compared directly (Figure 3.3B) using a luciferase assay (transducing simultaneously with the psiCHECK plasmid) or a western blot assay[181]. Both lentiviral expression systems produced suitable knockdown of the target specifically. As expected the constitutive shRNA produced slightly greater knockdown, however the inducible system produced increased knockdown with longer doxycycline treatment.

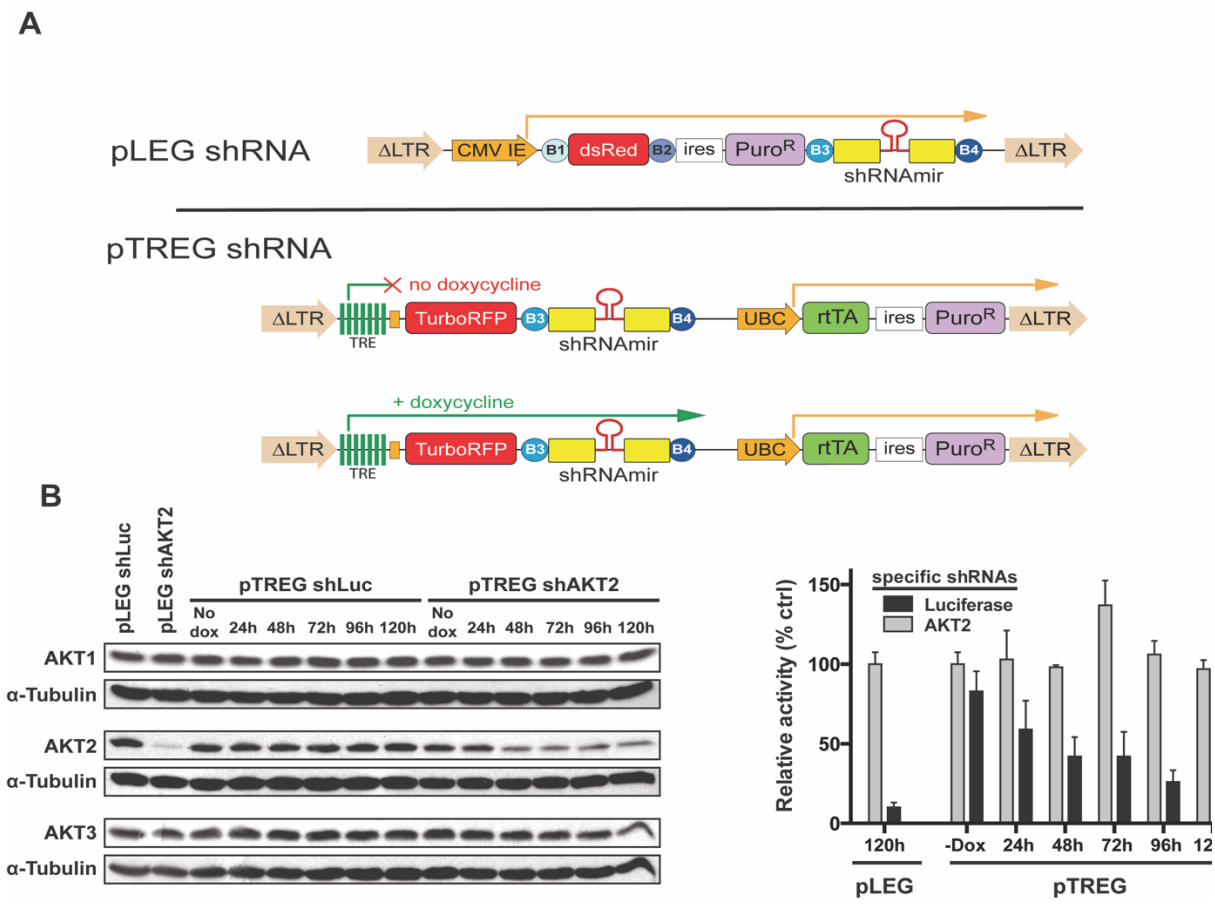

Figure 3.3. pLEG and pTREG shRNA-mediated knockdown of targeted genes. After identifying successful shRNA candidates, two lentiviral vectors of the targeting shRNAs were constructed using gateway recombination technology, pLEG and pTREG. (A) Both lentiviral vectors have puromycin selection markers. The pLEG shRNA allows constitutive expression of the shRNA driven by the CMV promoter. pTREG is pTRIPz-derived and allows a doxycycline-regulated expression of the shRNA under control of the Tet-responsive element (TRE). (B) Comparison between the AKT2 protein knockdown in a constitutive or inducible manner using a luciferase assay or a western blot assay. (Figure taken from A. DeBruyns 2015) [181]

3.2.2 Infecting the melanoma cell lines and knockdown confirmation

Armed with specific shRNAs, I set out to determine the contribution of the PTEN proximal genes to melanoma cell viability and proliferation. At the onset of my project, I had at my disposal four pTREG-shRNA lentiviral vectors, which are: shLUC, shPDK1 and two vectors that simultaneously targeted PIK3C and AKT family members: shPIK3C (a vector that simultaneously targeted PIK3C (A, B, and D)) and shAKT (a vector that simultaneously targeted (1,2,3)) (Table.2). Simultaneous knockdown of all AKT or PI3K isoforms was chosen to readily identify which gene/isoform was required for melanoma cell viability or proliferation. p110-γ was not detected in the melanoma cell lines (or even 293T cell) tested[181] and as a result PIK3CG-shRNAs were excluded from this study.

Lentiviral toxicity and inadequate target knockdown were previously encountered when using the pTREG-shRNA system in melanoma cell lines [181]. Thus, I started my project by determining the suitable viral titer to efficiently infect the melanoma cell lines and produce sufficient knockdown of PTEN proximal genes. Lentiviruses were titrated using RFP expression as a surrogate following a 72hour induction with doxycycline treatment. This method is both efficient and, in comparison to tittering using drug selection, rapid [237]. After calculating the lentivirus titers, individual melanoma cell lines were infected with a range of MOI. The MOI that lead to a minimal cytotoxicity and highest selection after using puromycin treatment was determined. Typically, I found that MOI of 2 IU/cell balanced low toxicity with sufficient knockdown of targets.

To furtherelucidate the underpinnings of the PI3K-AKT pathway in melanoma, I focused on melanoma cell lines derived from the two most common mutations, BRAFV600E and NRAS Q61R, found in two cutaneous melanoma cell lines Sbcl2 (NRAS

Q61R and BRAF Wild type) and A-375 (BRAFV600E, PTEN Wild type) [29]. I started this project with the desire of producing an effective knockdown with the pTREG inducible system on multiple melanoma cell lines derived from the common cutaneous melanoma mutations (NRAS Q61R and BRAFV600E) (Table.1). Unfortunately, I encountered challenges during the lentiviral infection of the melanoma cell lines, such as cytotoxicity or slow proliferation rate following the infection. I performed an experiment to investigate the effect of the lentiviral infections on the melanoma cell lines using a lentiviral construct that expresses CFP immediately upon integration into the recipient cell's genome. This allowed me to comprehend if the cytotoxicity is because of unsuccessful infection (or construct integration) in the melanoma cells, leading to negative puromycin selection. Figure (3.4) shows pictures of the melanoma cell lines 72 hr post infection and confirms the cytotoxic effect of infecting melanoma cell lines and the lack of successful CFP expression. Sbcl-2 cells had the least cytotoxic effect and showed successful CFP expression. Following that notion, the Sbcl-2 cells were infected with MOI of 2 IU/cell, selecting few colonies to grow for up to 4 weeks because of the slow proliferation rate post infection.

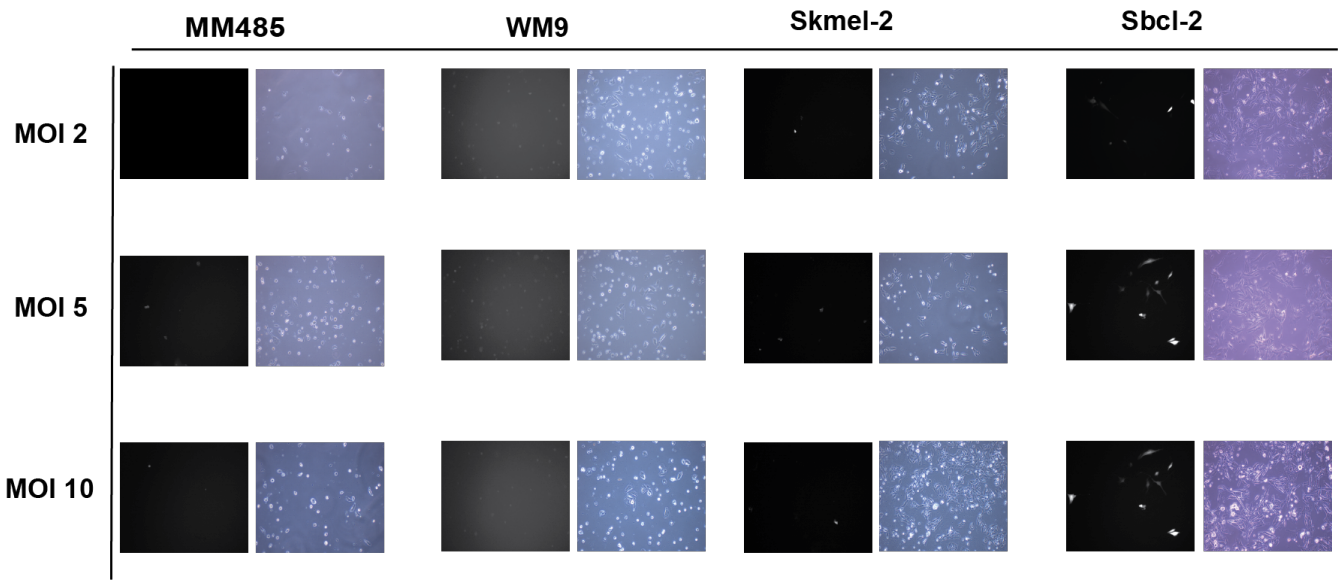

Figure 3.4. CFP expression in infected melanoma cell lines. Fluorescence microscopy images at 10x magnification of multiple melanoma cell lines infected with CFP lentivirus at different multiplicity of infections, 3 days post infection.

A375 and Sbcl2 stably expressing Dox-inducible pTREG vectors targeting PDK1, PIK3C(A,B,D), AKT(1,2,3) or luciferase as a control (shLuc) were treated with doxycycline for a minimum five days, after which lysates were derived andanalyzed by western blot (Figure 3.5). The knockdown more attainable for some isoforms by showing either a complete knockdown or reduction in expression. For instance, P110D was the only isoform of the PI3K to be knocked down in the A375. On the other hand, Sbcl-2 cells

showed a reduction in the expression of the three isoforms of P110. A complete knock

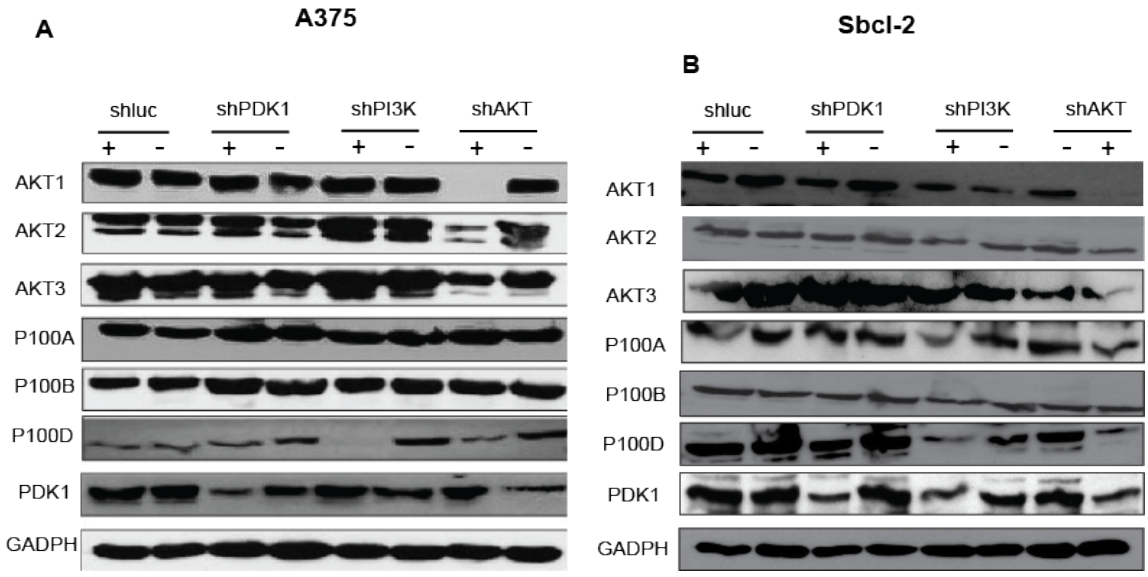

Figure 3.5. **Western blot analysis of the pTREG inducible system.** a) Western blot analysis of the PTEN Proximal protein expression in A375 (B) Western blot analysis of the PTEN Proximal protein expression in Sbcl-2

down of AKT1 was shown in both Sbcl-2 and A375 derived cell lines, and differentially reduced levels of AKT2 and AKT3 in both cell lines, with a remarkable reduction of AKT2 in A375 and a notable reduction of AKT3 in Sbcl-2. Additionally, (Figure 3.5) illustrates a detectable reduction in the expression of PDK1 in both cell lines. The western blots show that the knockdown of each PTEN proximal genes, whether in a specific isoform or in multiple isoforms, is achievable in melanoma cell lines using pTREG-shRNA infection.

The Turbo RFP expression, regulated in a doxycycline inducible manner, was seen by fluorescence microscopy, visually confirming the shRNA expression before

proceeding with western blotting (Figure 3.6). Essentially, (Figure 3.6) reveals higher RFP intensity in A375 derived cell lines than Sbcl-2 derived cell lines, like due to faster proliferation and a tendency to clump.

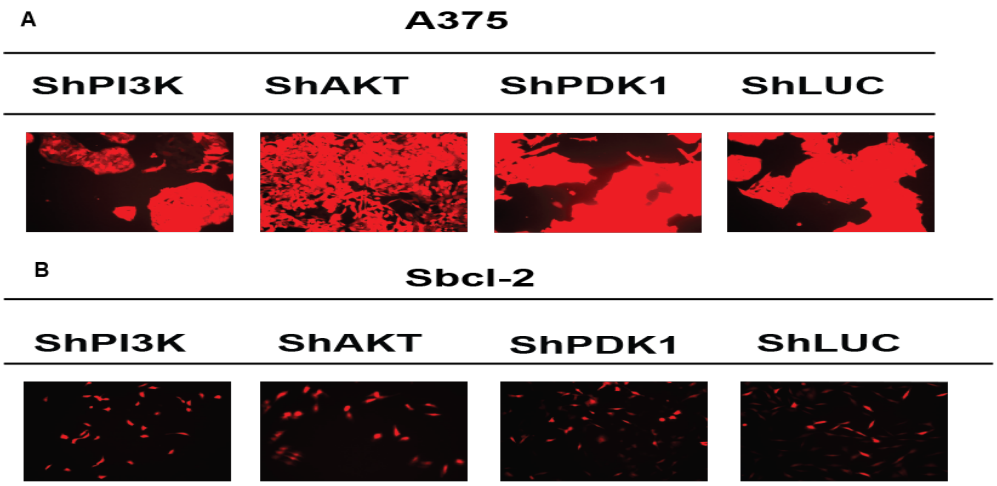

Figure 3.6. RFP expression analysis of the pTREG inducible system.

A) pictures of the A375-derived cell lines at 5th day of doxycycline treatment.

B) Pictures of the Sbcl-2-derived cell lines at 5th day of doxycycline treatment.

Together, these data illustrate success in ablating the expression of PTEN proximal genes using PTREG-shRNA lentiviruses in two melanoma cell lines: A375 and Sbcl-2.

3.2.3 Proliferation and viability assays of pTREG-ShRNA derived cell lines

The two potential phenotypes that I sought to investigate, after the validation of the knockdown, were proliferation and viability. Proliferation assays were quantified by relative cell number or confluency per well. The shRNA-cell lines derived from Sbcl-2 and A375 were seeded at 10000 – 25000 cells/well and treated the next day with doxycycline over 5-7 days. To determine the dynamics of cell proliferation I made use of Incucyte™ technology, which allows one to assess various cell parameters, in near real time over the course of 7 days. Here, images were taken every four hours and cell confluency was measured as a surrogate of cell growth and proliferation. shLUC control cells were included to assess any effects due to shRNA expression or doxycycline toxicity [48,70,81]. In addition, each cell population was compared to an untreated control to examine the effect of the knockdown of the PTEN proximal protein.

Analysis of confluency, cell count, and the images taken demonstrate a reduction in the proliferation of the A375 derived cell lines in response to shRNA induction (Figures 3.7, 3.8). Importantly, no toxicity was observed in control cells (shLuc-cells) at the concentration of doxycycline used and decreased cell number or cell confluency starts to be noticeable after 3 days.

On the other hand, the Sbcl2-doxy cell lines demonstrated a decrease in proliferation comparing to the Sbcl2-non doxy that is leveling off (Figure 3.10). This decline in growth pattern is produced in a similar pattern between the cell counting data (Figure 3.11) and the cell confluency measurements.

Additionally, pictures comparing doxycycline treated and non-treated cells suggested a slowdown in proliferation more than cell death, which can be observed under

the microscope by the rounding and the shrinkage in the cell's morphology followed by detachment (Figure 3.12), or by using trypan blue in counting (data not shown).

To determine whether these shorter-term proliferation reductions correlate with differences in colony forming ability, I performed colony formation assays. Melanoma cell lines were seeded at low density (500-1000 cells/100mm dish), treated with doxycycline for two weeks to induce shRNA expression, fixed, crystal violet stained and quantified for colony formation.

Interestingly, few colonies were observed in the doxycycline treated A375 and Sbcl2, in stark contrast with the non-treated doxycycline wells (Figure 3.13). However, the Sbcl2 colonies were too small and not clearly stained using crystal violet, since they have narrower-appearing morphology

Taken together, these results demonstrate that the knockdown of PTEN proximal genes negatively affects the proliferation of two melanoma cell lines with different genetic status, but moreso in the Sbcl2 line.

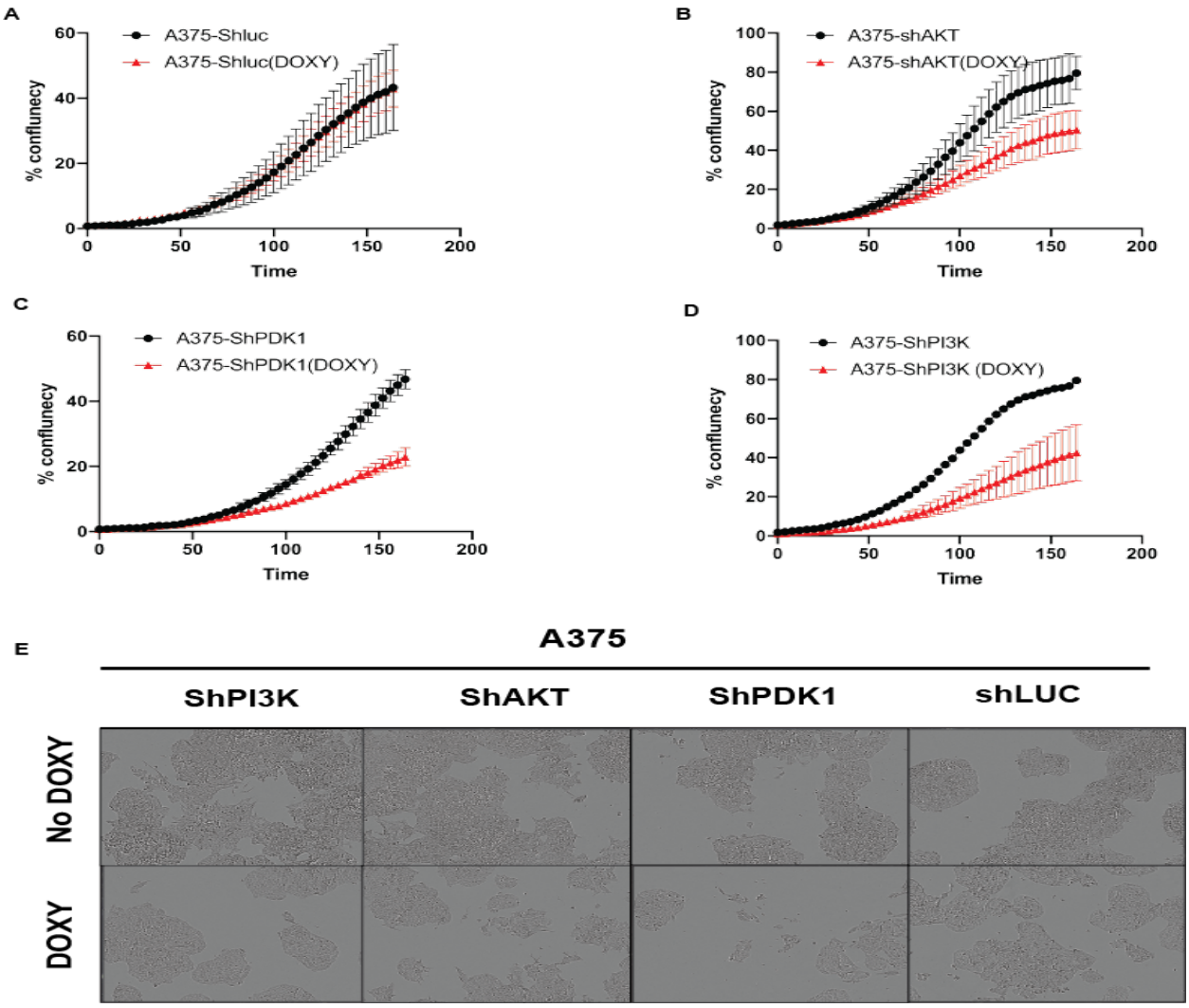

Figure3.7. Confluency analysis using the incucyte for A375-pTREG derived cell lines. (A) Confluency curves of A375-shluc cell lines -/+ doxycycline. (B) Confluency curves of A375-shAKT cell lines -/+ doxycycline. (C) Confluency curves of A375-shPDK1 cell lines -/+ doxycycline. (D) Confluency curves of A375-shPI3K cell lines -/+ doxycycline. The data is representative of 2 experiments in triplicates, and confluency measurement for 164 hours. (E) Incucyte pictures of A375-pTREG derived cell lines -/+ doxycycline after 144 hours, under 10X magnification.

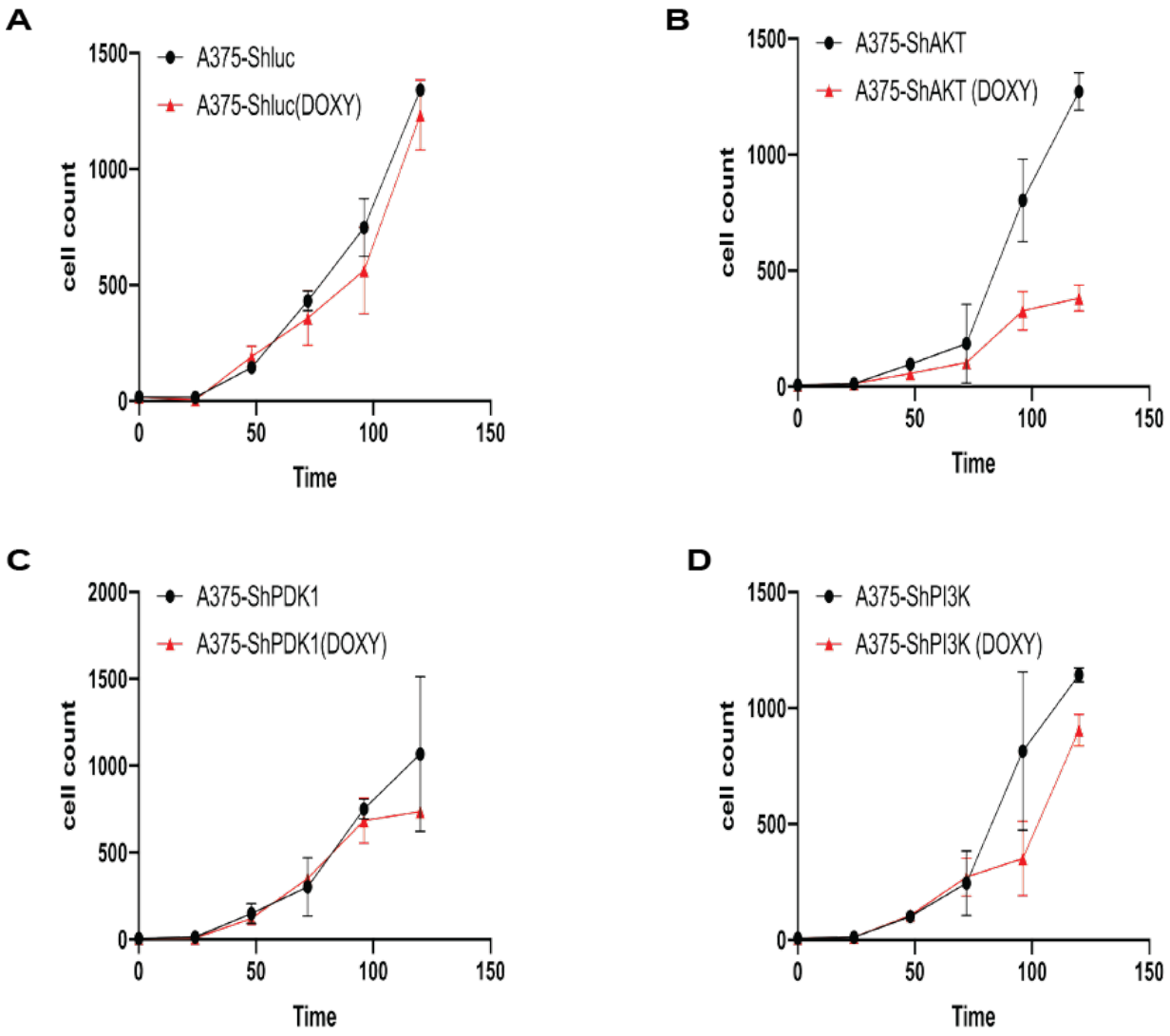

Figure 3.8. proliferation assay using cell counting for A375-pTREG derived cell lines. (A) proliferation curves of A375-shluc cell lines -/+ doxycycline. (B) proliferation curves of A375-shAKT cell lines -/+ doxycycline. (C) proliferation curves of A375-shPDK1 cell lines -/+ doxycycline. (D) proliferation curves of A375-shPI3K cell lines -/+ doxycycline. This data is representative of 1 experiment in triplicates, and cell counting measurements for 120 hours.

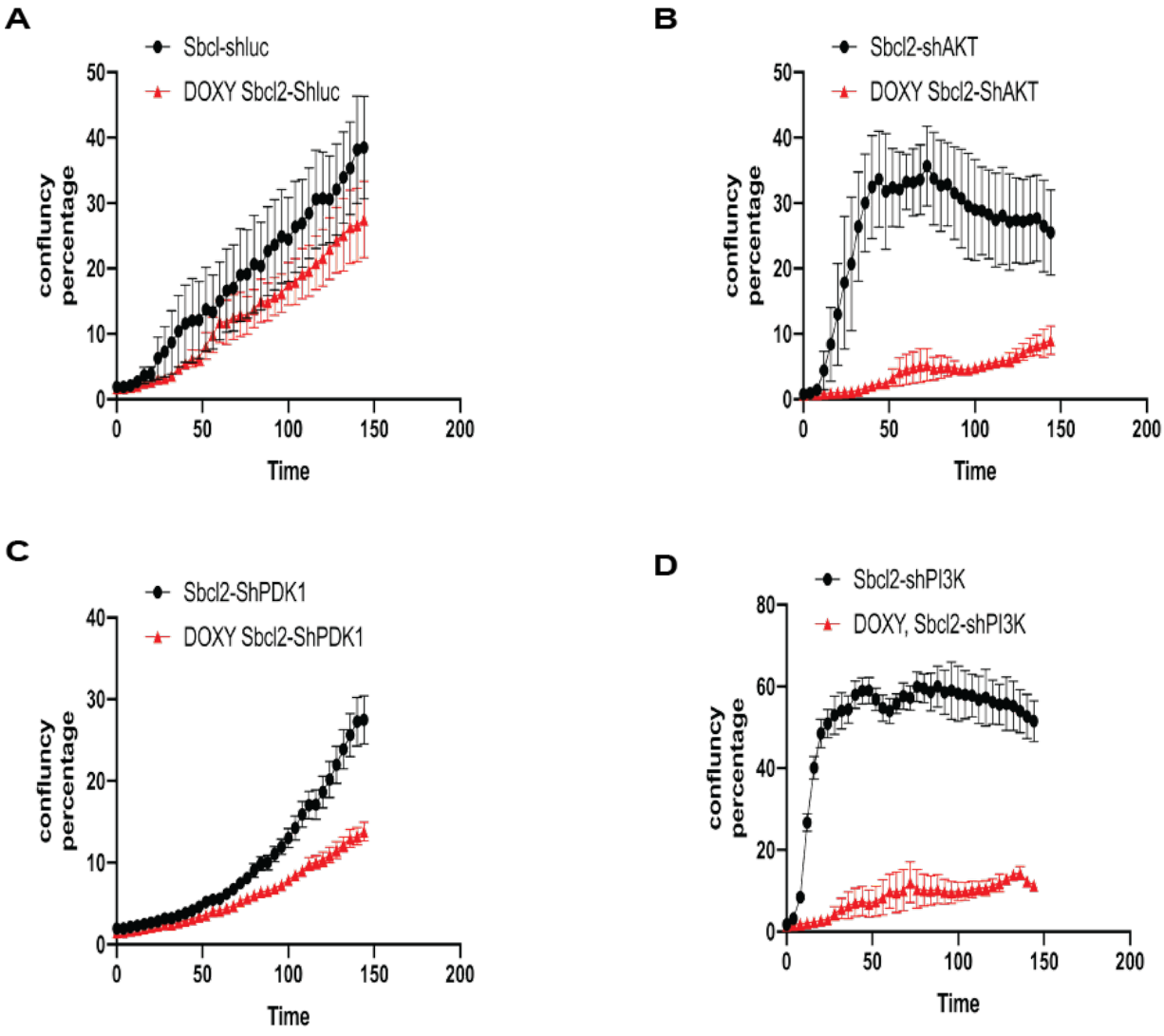

Figure 3.10. Confluency analysis using the incucyte for Sbcl2-pTREG derived cell lines. (A) proliferation curves of Sbcl2-shluc cell lines -/+ doxycycline. (B) proliferation curves of Sbcl2-shAKT cell lines -/+ doxycycline. (C) proliferation curves of Sbcl2-shPDK1 cell lines -/+ doxycycline. (D) proliferation curves of Sbcl2-shPI3K cell lines -/+ doxycycline. The data is representative of 1 experiment in triplicates, and confluency measurement for 144 hours.

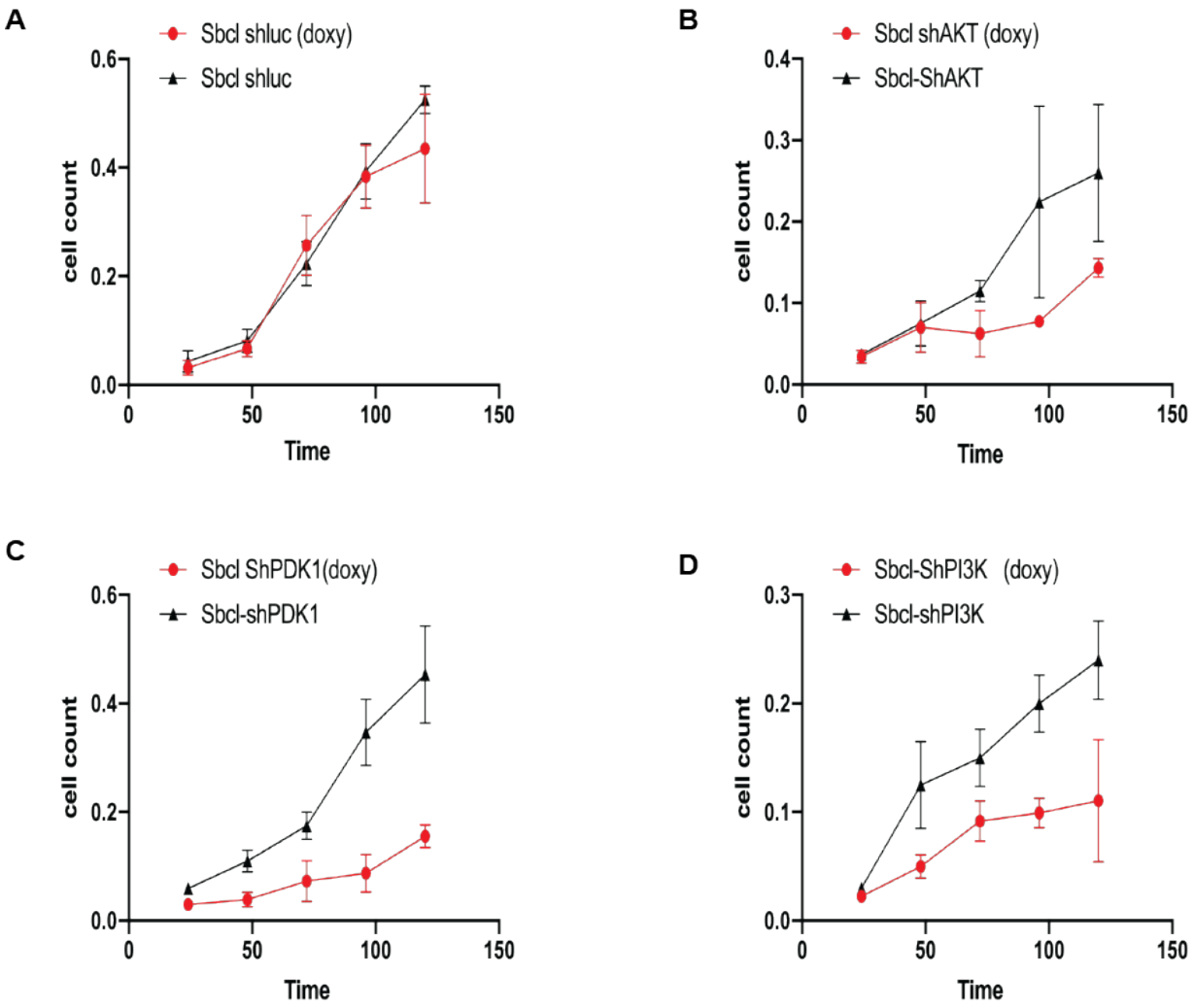

Figure 3.11. Proliferation assay using the cell counting for Sbcl2-pTREG derived cell lines. (A) proliferation curves of Sbcl2-shluc cell lines -/+ doxycycline. (B) proliferation curves of Sbcl2-shAKT cell lines -/+ doxycycline. (C) proliferation curves of Sbcl2-shPDK1 cell lines -/+ doxycycline. (D) proliferation curves of Sbcl2-shPI3K cell lines -/+ doxycycline. This data is representative of 1 experiment in triplicates, and cell counting measurements for 5 days.

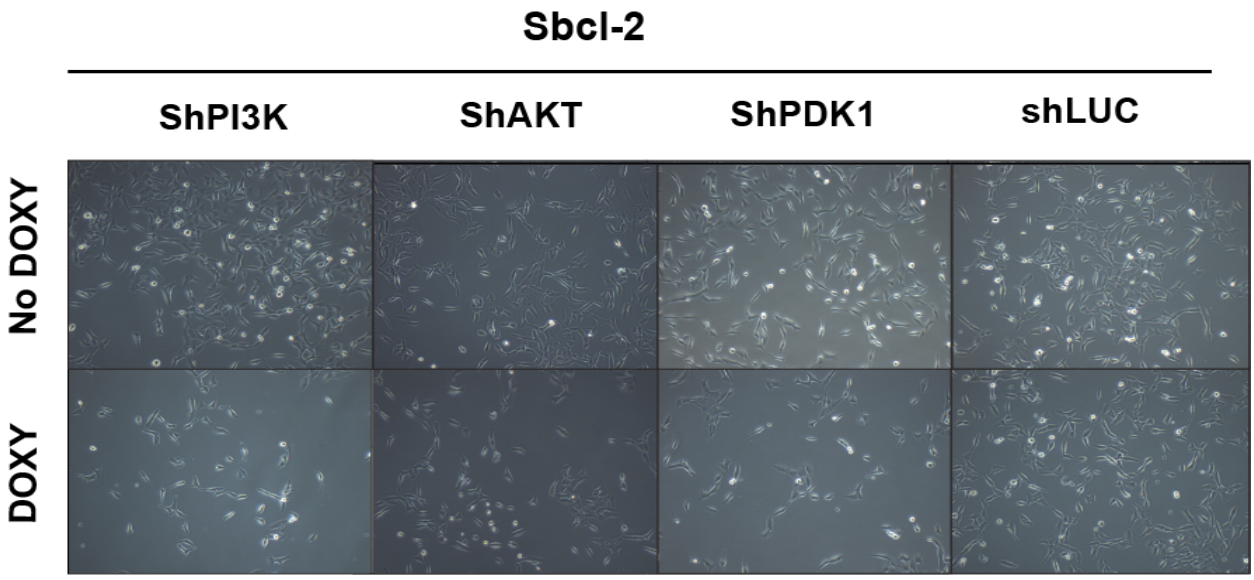

Figure 3.12. Pictures of Sbcl2-pTREG derived cell lines after induction. Pictures of Sbcl2-pTREG derived cell lines -/+ doxycycline after 96 hours.

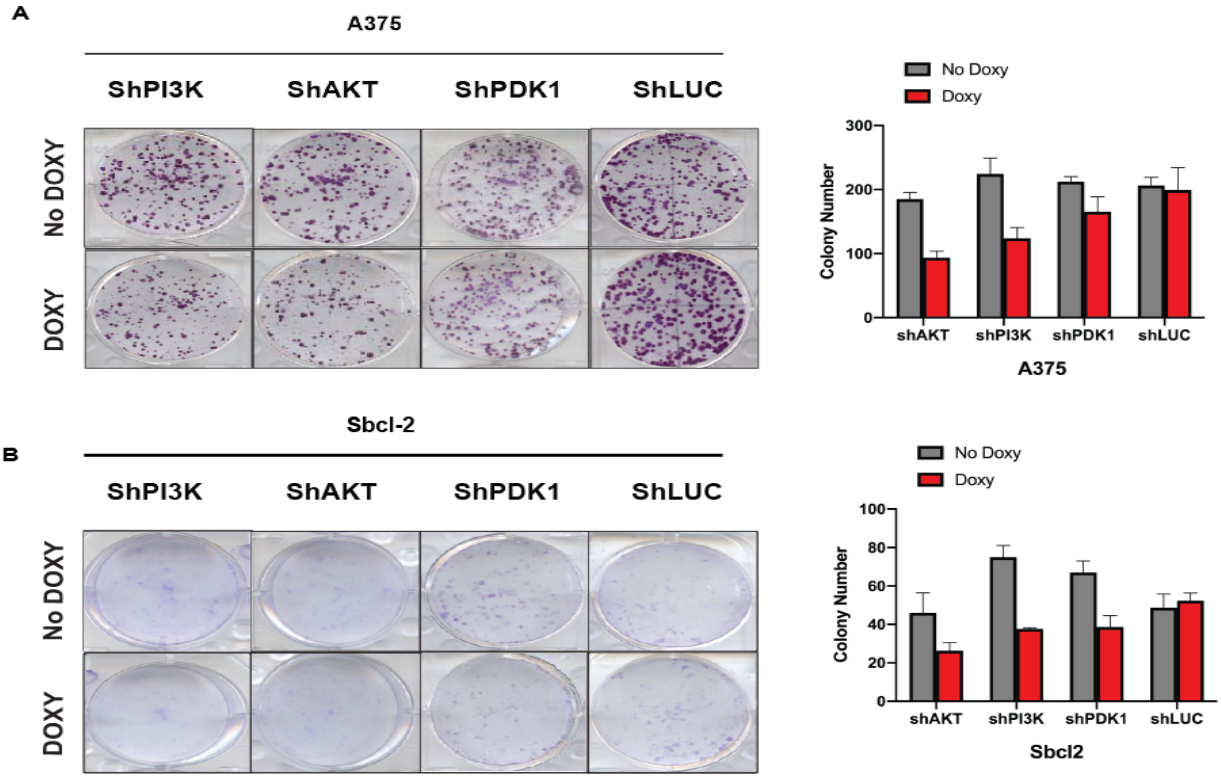

Figure 3.13. Colony formation assay for pTREG-derived cell lines. (A) Representative images and quantification of A375-pTREG derived cell lines cultured in -/+ doxycycline for over 7 days. (B) Representative images and quantification of Sbcl2-pTREG derived cell lines cultured in -/+ doxycycline for over 10 days. The data is representative of one experiment in triplicates.

3.2.4 Cell cycle analysis of pTREG-ShRNA derived cell lines

Inhibition of PTEN proximal gene expression resulted in a decrease in cell proliferation in two melanoma cell lines. Thus, I assessed the effect on the cell cycle using BrdU incorporation and DNA content analysis via flow cytometry. This procedure can resolve the cell cycle phases in a given cell population into G_0/G_1, S, and G_2/M by measuring BrdU incorporation. As expected, doxycycline and control shRNA expression had no effect on cells by themselves. Additionally, similar cell cycle profiles were seen in untreated cells harboring inducible shRNAs to PTEN proximal genes. When shRNAs were induced, the S-phase population of the Sbcl2 -pTREG derived cells was decreased comparing to their non-treated version (Figure 3.15). This decrease was more prevalent in Sbcl2 cells expressing shPI3K and ShAKT. This reduction in S-phase was coupled with the increase in the G1-phase population of doxycycline-treated cell lines. Thus, these results suggest that the knock down of PTEN proximal genes in Sbcl-2 can cause a G1 cell cycle arrest.

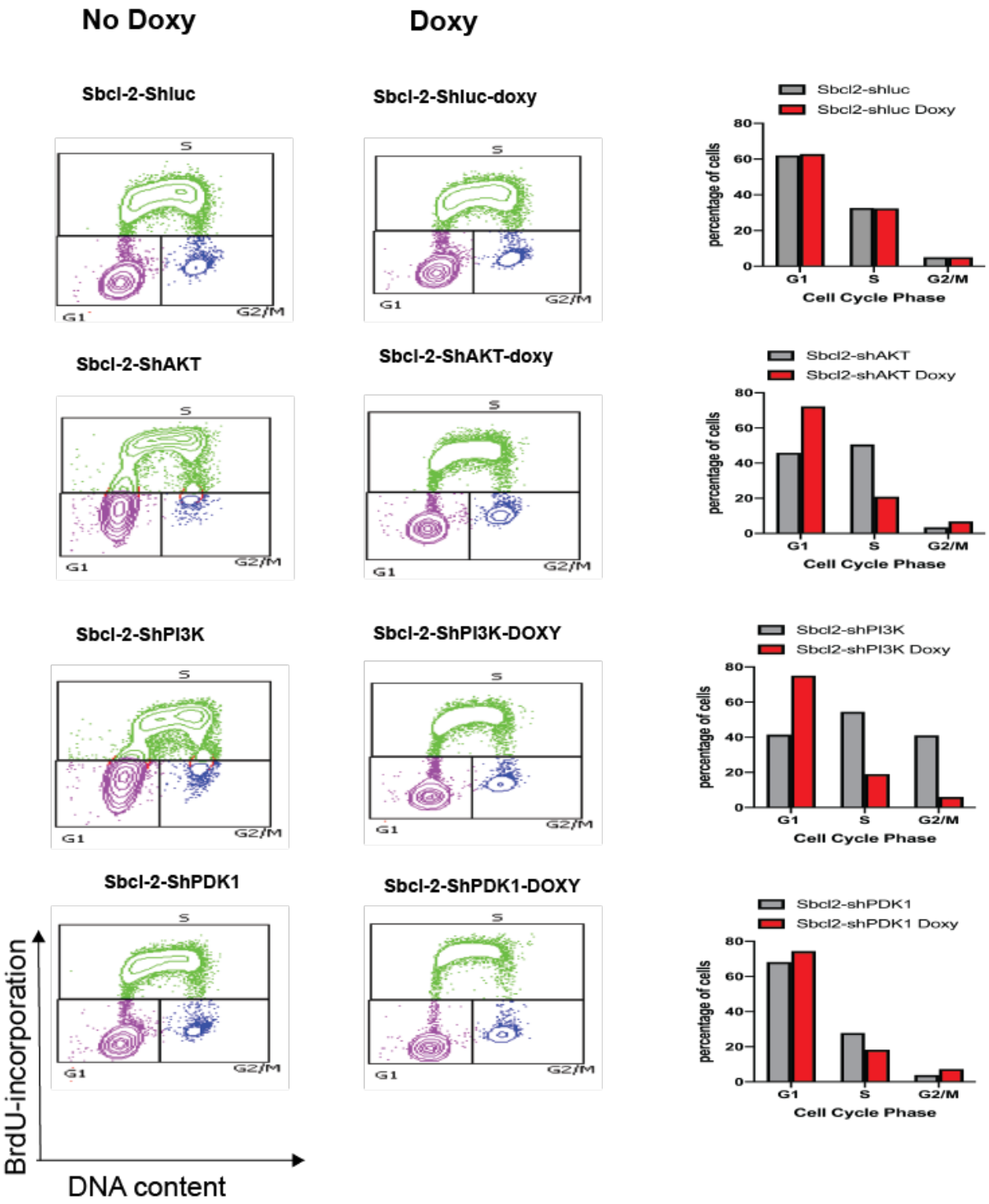

Figure 3.14. Effect of the pTREG-mediated Knockdown of PTEN proximal genes on cell cycle progression of Sbcl-2 cells. BrdU cell cycle analysis of Sbcl2-pTREG derived cell lines treated with doxycycline for 7 days. The cells were pulsed with BrdU along with the untreated cell lines for 5 hours. Boxes denote G_0/G_1, S-Phase and G2/M. This data is representative of one experiment.

4. Discussion

The work described here illustrates the application of pTREG lentiviral vector to knock down PTEN proximal genes in melanoma cell lines. This method successfully produced an inducible knockdown in two melanoma cell lines with different genetic profiles, A375 (NRAS wildtype, BRAFV600E, PTEN Wild type) and Sbcl-2 (NRAS Q61R, BRAF wildtype, PTEN Wild type). We previously used a unique and rapid shRNA triaging method that consist of a dual-luciferase reporter assay to identify individual shRNAs that would specifically knockdown intended targets in vitro. The Dankort lab's pTREG-system allowed the co-expression of three shRNAs from a single doxycycline inducible cassette, to knockdown all expressed members of PTEN proximal gene families (PIK3C, PDK, AKT) in melanoma cell lines. I optimized infections to reduce cytotoxicity of pTREG-lentiviruses by producing lentiviruses in a serum-free medium, altering the MOI, and using optimal doxycycline doses. These optimizations reduced infection associated toxicities that our lab had observed in the past. Instead, the knockdown in melanoma cell lines could be enhanced by prolonging the doxycycline treatment period and changing media frequently (every 2 days).

After implementing these changes, this study answered fundamental questions surroundingoncogenic dependency on the PI3K-AKT pathway in melanoma in vitro. This was examined using the inducible knockdown system that permits timed induction of shRNA expression. Moreover, the non-treated shRNA infected cell lines served as a suitable control for the experiments. These results suggested oncogenic dependency on the PI3K-AKT pathway in melanoma derived from NRAS Q61R or BRAFV600E. This was

evident from a decline in cell growth and proliferation after the induction of the knockdown of the PTEN proximal genes. Furthermore, this phenotype is supported by repeated presentations in confluency and cell count growth curves. In particular, the decline in cell growth was remarkable in the doxycycline induced pTREG-Sbcl-2 cell lines.

Since the PI3K-AKT pathway phosphorylates downstream effectors that regulate survival and proliferation, the reduction in cell growth could be cell cycle arrest, apoptosis, or both. It is important to note that morphologically I observed very few cells showing signs of apoptosis (rounded, non-refractile, or floating cells were not seen in increased numbers upon shRNA induction). Moreover, cell counting after staining with trypan blue did not suggest cell death. This study suggests that this decrease in cell number is most likely a result of indication of a cell cycle arrest in the G1-phase. Significantly, this phenotype was prevalent after the knockdown of all the studied PTEN proximal genes in Sbcl-2. This melanoma cell line represents a subset of melanoma patients with NRAS Q61mutation, which is 88.1% of NRAS melanoma patients [227]. Indeed, the existence of NRAS Q61R mutation causes the overactivation of MAPK pathway and the PI3K-AKT pathways by hyperactivated RAS. The occurrence of the cell cycle arrest after knocking down the PTEN proximal genes suggests a proliferation dependency that is more biased toward the PI3K-AKT pathway.

Overall, this work contributes to the growing body of research around the role of the PI3K-AKT pathway in melanoma. Furthermore, these results pinpoint potential targets for NRAS mutant derived melanoma, and it rationalizes engaging the PTEN proximal protein inhibitors with current MEK inhibitors therapeutic approaches.

5. Future directions

Now that the resultant phenotype knocking down PTEN proximal genes has been pinpointed to be cell cycle arrest in Sbcl-2 cells, it must be interrogated whether this cycle arrest is reversible, or whether it is senescence. In other words, the next question to examine is if this phenotype will perpetuate after the withdrawal of the doxycycline treatment. This could be examined preliminarily by growth curves that compare the proliferation of the doxycycline treated Sbcl-2-shRNA cell lines after a media change with regular media, with the same cell line with maintained doxycycline treatment. Additionally, the expression of the negative cell cycle regulators such as $p15^{ink4b}$ and $p21^{cip1}$ could be examined as one of the hallmarks of senescence. Likewise, the possibility of apoptosis appearing as a later phenotype must be interrogated after a prolonged induction period. Longer term viability assays, coupled with biochemical markers of apoptosis (caspase 3 cleavage, DNA fragmentation, TUNEL staining, vital dye loss) can be conducted to determine if this is the case. These results will be necessary to more fully explore the biological phenotype of inhibition in this system. Additionally, it would be interesting to determine whether particular isoforms of the genes targeted here are responsible or individual PTEN proximal genes are required for cell cycle progression. This can be addressed through expression of single shRNAs or pairwise combination. The use of specific pharmacological inhibitors could also determine isoform requirements, although this requires isoform specific inhibition. Finally, in-depth transcriptional analysis will be informative in determining specific downstream effecters of the PTEN proximal genes that are essential for the cell cycle arrest in Sbcl2. This could provide more thorough

understanding of which stream of the AKT-regulated pathways is more likely to be affected by the induced knockdown.

My results raised further questions regarding how PTEN proximal genes could be leveraged therapeutically. For instance, future work could be geared toward determining how PTEN proximal genes contribute to acquired resistance after targeting MAPK pathway in melanoma. This could be examined by targeting the PTEN proximal genes concurrently with BRAF and/or MEK inhibitors in NRAS mutant melanoma which are resistant to BRAF and MEK inhibition, starting with Sbcl-2. These cell lines can be selected after chronic treatment with MEK or BRAF inhibitors [78].Hence, with the knockdown of each PTEN proximal gene in melanoma cell line resistant to BRAF and MEK inhibition, it will be determined which one of these proteins knock down could sensitize the melanoma cell line to the MAPK inhibition. This will improve our understanding to the contribution of the PTEN proximal genes to development of acquired resistance in melanoma after MAPK inhibition and determining which combination of MAPK and PI3K-AKT targeting drugs will be prioritized for clinical testing.

6. Conclusion:

Overall, the work presented in this thesis sought to further our understanding of the role of the PI3K-AKT pathway in $BRAF^{V600E}$ or $NRAS^{Q61R}$ - driven melanoma. In particular, a unique pTREG shRNA lentiviral system has been introduced into melanoma cell lines to target PTEN proximal genes and determine the effect of the knockdown on their proliferation and survival. In this work, I started with the previously identified shRNA plasmids that has been tested to be successful at effectively and specifically targeting PTEN proximal genes in the PI3K-AKT pathway using a dual luciferase reporter system. This has been further verified by constitutive expression of the shRNAs using the pLEG-shRNA lentiviral plasmid. After optimizing conditions for the induction of the pTREG shRNA expression, like the doxycycline treatment and the appropriate virus titer, I validated that the successful shRNA pTREG candidates at my disposal were functional in ablating their intended PTEN proximal genes in two melanoma cell lines, Sbcl-2 and A375. Furthermore, I identified that the knockdown of the PTEN proximal genes causes a decrease in the proliferation of both cell lines, which are derived from $BRAF^{V600E}$ or $NRAS^{Q61R}$. More importantly, this phenotype was interpreted uniquely to be cell cycle arrest in Sbcl-2. This has been validated using cell cycle flow cytometry analysis, since there was increased cell population in the G^1 phase in comparison to the little cell population in the S phase. Since Sbcl-2 presents a population of melanoma patients with a common NRAS activating mutation, my findings are encouraging to continue exploration of the potential of the targeting the PI3K-AKT pathway in NRAS mutant melanoma patients. This would aid in understanding the limited response and therapeutic

challenges experienced with the current MEK inhibitors implemented in NRAS melanomas treatment.

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