Molecular mechanisms of PI3K isoform dependence in embryonic growth

Sena Atıcı[1,2], Onur Çizmecioğlu[1]

[1]Department of Molecular Biology and Genetics, Bilkent University Faculty of Medicine, Ankara, Turkey
[2]Rudolf Virchow Center for Integrative and Translational Bioimaging, University of Würzburg, Würzburg, Germany

Abstract

Objective: The phosphoinositide 3-kinase (PI3K) pathway is an important signaling mechanism for cell proliferation and metabolism. Mutations that activate PIK3CA may make cells p110α dependent, but when phosphatase tensin homolog (PTEN) is lost, the p110β isoform of PI3Ks becomes more important. However, the exact mechanism underlying the prevalence of p110s remains unclear. In this study, our aim was to elucidate the processes behind PI3K isoform dependency in a cellular model of embryonic development.

Material and Methods: In order to understand PI3K isoform prevalence, mouse embryonic fibroblasts (MEFs) were used and p110β, PTEN and Rac1 activity was modulated using retroviral plasmids. Expression levels and cellular growth were assessed by performing immunoblots and crystal violet assays.

Results: The levels of PTEN had only a partial effect on the prevalence of PI3K isoforms in MEFs. The dependency on p110α diminished when PTEN was depleted. Of note, when PTEN expression was repressed, there was no full transition in dependency from one PI3K isoform to the other. Interestingly, the viability of PTEN-depleted MEFs became less dependent on p110α and more dependent on p110β when p110β was overexpressed. Nevertheless, the overexpression of p110β in conjunction with PTEN knock-downs did not result in a complete shift of isoforms in PI3Ks. Finally, we investigated Rac1 activation with a mutant allele and determined a more potent increase in p110β prominence in MEFs.

Conclusion: These findings suggest that multiple cellular parameters, including PTEN status, PI3K isoform levels, and Rac1 activity, combine to influence PI3K isoform prevalence, rather than a single determinant. (J Turk Ger Gynecol Assoc. 2024; 25: 159-66)

Keywords: PI3K isoform prominence, PTEN, Rac1, mouse embryonic fibroblasts

Received: 15 June, 2024 **Accepted:** 24 July, 2024

Introduction

The phosphoinositide 3-kinase (PI3K) pathway is a fundamental signaling cascade that regulates many physiological activities, such as cellular metabolism, growth, proliferation and survival. Dysregulation of PI3K signaling is implied in various diseases, ranging from cancer to metabolic disorders, making it a prime target for therapeutic intervention (1). However, the complexity of PI3K signaling arises from the existence of multiple isoforms with distinct functions and regulatory mechanisms. Understanding isoform selectivity within the PI3K pathway is paramount for unraveling its physiological roles and developing targeted therapies.

Class I PI3Ks are further categorized into class IA and class IB based on their regulatory subunits and activation mechanisms. Class IA PI3Ks are comprised of a catalytic subunit (p110α, p110β, and p110δ- encoded by *PIK3CA*, *PIK3CB* and *PIK3CD* respectively) and a regulatory subunit (p85α, p85β, p55γ, p55α, or p50α), whereas class IB PI3K consists of p110γ and regulatory subunits (p101 or p84) (2). Each isoform exhibits tissue-specific expression patterns and functional specificity, contributing to the complexity of PI3K signaling. PI3Ks possess lipid kinase activity and catalyze the phosphorylation of phosphatidylinositol lipids at the 3'-hydroxyl group (3). Phosphorylated phosphatidylinositols function as secondary messengers in cells, triggering the activation of the serine

Address for Correspondence: *Onur Çizmecioğlu*
e.mail: onur.cizmecioglu@bilkent.edu.tr ORCID: orcid.org/0000-0002-7608-6950
DOI: 10.4274/jtgga.galenos.2024.2024-6-7

Copyright© 2024 The Author. Published by Galenos Publishing House on behalf of Turkish-German Gynecological Association. This is an open access article under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 (CC BY-NC-ND) International License.

threonine kinase Akt, as well as other downstream proteins. Phosphatase tensin homolog (PTEN) is the primary inhibitor of PI3K (4).

The differential roles of class IA and class IB PI3K isoforms have been elucidated through a combination of genetic knockout studies, biochemical assays, and structural analyses. For instance, p110α is predominantly associated with growth factor signaling and oncogenic transformation, whereas p110β plays a crucial role in insulin signaling, cardiovascular function, and inflammatory responses (5). Similarly, p110δ and p110γ are primarily involved in immune cell function, making them attractive targets for immunomodulatory therapies (2). Moreover, genetic alterations in tumorigenesis can dictate the isoform prevalence in cancer. Cells bearing *PIK3CA* mutations can become dependent on p110α activity, whereas loss of PTEN function usually promote a p110β dependent state (6).

Isoform-specific inhibitors have emerged as promising candidates for precision medicine, enabling targeted therapies with reduced off-target effects (1). However, attaining isoform selectivity is difficult due to the structural similarity of PI3K isoforms and the interdependence of signaling networks. Computational modeling and structure-based drug design have aided in the creation of selective inhibitors of certain PI3K isoforms (7).

Moreover, the regulation of PI3K signaling is intricately linked to crosstalk with other signaling pathways, such as Ras-MAPK, mTOR and Rac1 pathways (8). Crosstalk between PI3K isoforms and these pathways adds another layer of complexity to the regulation of cellular responses. The interplay between PI3K and Rac1, for example, orchestrates intricate cellular processes, ranging from cytoskeletal dynamics to cell migration and proliferation. Rac1, a small G protein, serves as an isoform-selective effector of PI3K, regulating p110β dependent cellular signaling (9).

The crosstalk between PI3K and Rac1 is bidirectional and elaborate (10). PI3K activation induces the recruitment and activation of Rac1 via its downstream effectors, such as guanine nucleotide exchange factors, leading to actin polymerization and cytoskeletal rearrangements essential for cell migration and invasion. Conversely, Rac1 activation particularly enhances p110β activity through feedback loops, amplifying downstream signaling pathways involved in cellular growth and survival (11,12). Moreover, recent studies have unveiled intricate regulatory mechanisms governing the PI3K-Rac1 interplay. For instance, scaffolding proteins like G protein-coupled receptor kinase-interacting proteins and PAK-interacting exchange factor (PIX) facilitate the spatial and temporal coordination between PI3K and Rac1, ensuring precise cellular responses to extracellular stimuli (13).

Carcinogenesis is linked to dysregulation of the interaction between PI3K isoforms and their effectors, wherein abnormal signaling may facilitate tumor cell invasion, metastasis, and resistance to treatment. Conversely, targeting this axis presents a promising therapeutic strategy for cancer treatment, as evidenced by the development of small molecule inhibitors targeting PI3K and Rac1 signaling pathways. Consequently, study of the determinants of PI3K isoform selectivity can inform us about how cellular conditions can dictate cell growth and proliferation.

Unraveling the complexities of this interplay holds significant promise for PI3K-dependent development of embryonic tissues as well as the development of novel therapeutic interventions targeting cancer and other diseases driven by dysregulated signaling pathways.

In this study, we investigated the molecular mechanisms underlying isoform selectivity in PI3K signaling, with a particular focus on the status of PTEN tumor suppressor, class IA PI3K isoforms and the small G protein, Rac1. We attempted to establish molecular genetic cellular models with distinct PIK3CA or PIK3CB dependences for cell proliferation and tried to switch this dependence by modulating PTEN function. Our results suggest that a single step modification of PTEN cannot account for a complete isoform switch. Our findings further implicate that activation of secondary factors e.g. Rac1 small G-protein activation can dictate the requirement of individual PI3K isoforms for the growth embryonic cells. Our findings, consequently, offer a more comprehensive understanding of the role that isoform selectivity plays in PI3K signaling and the implications of this phenomenon for translational medicine.

Material and Methods

Compounds and reagents

Crystal violet powder was supplemented from Sigma Aldrich. BYL719, KIN193, GDC001 and MK2206 were all purchased from SelleckChem.

Cell culture

The p110α flox/flox; p110β flox/flox mouse embryonic fibroblasts (MEFs) and HEK293 cells were cultured under standard growth conditions. This included high-glucose DMEM, L-glutamine, and 110 mg/L sodium pyruvate, along with 8% FBS (Biowest) and pen-strep solution (100 IU/mL and 100 mg/mL, Gibco). The cells were incubated in a humidified incubator at 37 °C. The cells used in the study were tested for mycoplasma and no contamination was detected. MEFs have been established from homozygotes of p110βflox/flox and p110αflox/flox mice that were embryonic day 13.5. Using the conventional 3T3 method, primary MEFs were immortalized, and polyclonal knock-outs were produced in accordance with previous publications (9). Adenovirus transductions encoding cre-recombinase (AdCre; Iowa Viral Vector Core, Iowa City,

Iowa, USA) was applied to floxed MEFs in order to produce knock-out cells, whereas AdLacZ was used as a control.

Plasmids and retroviral transductions

The pMX GFP Rac G12V plasmid was generously provided by Joan Brugge [Addgene plasmid # 14567, (14)]. Rac1(N17) was cloned from YFP-Rac1(N17) [a gift from Joel Swanson-Addgene plasmid # 11395, (15)] vector in pMX vector with BglII and HindIII compatible ligation. Two different shRNA constructs were used to deplete PTEN expression in MEFs (shPTEN#1: CCGGGCTAGAACTTATCAAACCCTTCTCGA GAAGGGTTTGATAAGTTCTAGCTTTT, shPTEN#2: CCGGCGACTTAGACTTGACCTAT ATCTCGAGATATAGGTCAAG TCTAAGTCGTTTTTG) using pLKO.1-puro plasmid (16). Stable MEF lines were produced via retro/lentiviral transductions. HEK293 cells were transfected with gag-pol/delta 8.3, vsv-g, and retro/lentiviral plasmids using lipofectamine 3000 (invitrogen) to produce retro/lentiviral particles. Retro/lentiviral particle-containing supernatants were collected at 48 and 72 hours post-transfection, combined, subjected to sterile filtration, and used for infections within 2-3 days.

Antibodies and western blotting

The antibodies were purchased from Cell Signaling Technology: anti-HA, anti-p110α, anti-p110β, anti-PTEN, anti-pAkt (Ser473), anti-pAkt (Thr308), anti-pS6 (Ser235/Ser236) and anti-pS6 (Ser240/Ser244). Santa Cruz was the source of the anti-Rac1 antibodies. The source of anti-β-actin antibodies was Sigma. We bought HRP linked secondary antibodies from Advansta. A standard western blotting approach was applied (17). The procedure involved scraping the cells into ice-cold PBS and lysing them in RIPA buffer (EcoTech) supplemented with 1 mM sodium orthovanadate (CST), 1 mM dithiothrietol (Bio-Rad), and protease/phosphatase inhibitor tablets (Roche) at 4 °C. After being electrophoresed with 10-12% SDS-PAGE, 15-20 μg of total protein were blotted onto nitrocellulose membranes (CVS). The membrane stripes were blocked for a duration of thirty minutes using 5% milk without fat in TBS. Subsequently, they were exposed to primary antibodies in a solution containing TBS, 5% milk, and 0.1% Tween-20 overnight at a temperature of 4 °C. Afterwards, the membranes were exposed to secondary antibodies, which were diluted in a buffer of 5% milk-TBS, for a duration of two hours at ambient temperature. Using an Amersham Imager 600, the Pierce ECL Western Blotting Substrate (ThermoFischer Scientific, US) electronic images of the blots were generated.

Cellular proliferation assays

The crystal violet experiments were conducted by inoculating 2×10^4 MEF cells in 6-well plates (SPL). The cells were washed with PBS and then treated with a solution of 10% acetic acid (Sigma) and 10% ethyl alcohol for 4 hours at ambient temperature. The staining procedure involved the use of 0.2% crystal violet (Sigma) and 10% ethyl alcohol for 30 minutes. Afterward, the wells were rinsed with distilled water and left to dry in the air. The crystal violet stain, which was associated with cells, was extracted using 2 mL per well of a 10% acetic acid solution. This extraction process was carried out for 20 minutes on a shaker. Spectrometric analyses were then performed at an optical density of 595 nm. The values were standardized to the baseline measurement (day 0) in each experiment. The figures shown represent the mean value obtained from three independent experiments.

Statistical analysis

The differential comparison between two groups was conducted using a two-tailed Student's t-test. T-tests were conducted with GraphPad Prism. Data are deemed statistically significant when the p-values are less than 0.05. The data is reported as the mean value plus/minus the standard deviation, based on three independent experiments, unless otherwise specified. The quantification of the band intensities (densitometry analysis) of western blot data was evaluated by ImageJ, according to the normalization of the band intensities of each protein to the band intensities of the loading controls.

Results

PI3K isoforms have diverse functions across different cell lines, and their reliance varies among cell types. We used MEFs in our studies as MEFs represent embyronic cellular models with a stable karyotype (18). The proliferation of cells in MEFs mostly relies on the p110α isoform in comparison to other isoforms of PI3K. When the drugs BYL719 (an inhibitor of p110α) and KIN193 (an inhibitor of p110β) were given, it was shown that MEFs were more responsive to the inhibition of p110α, which is consistent with earlier studies (Figure 1A). Cross cancer genomic investigations have shown that 30% of tumor samples harbor PTEN loss (19). Remarkably PTEN-loss induced carcinogenesis allows cancer cells to rely more heavily on the p110β rather than the p110α isoform (20). The current understanding of the molecular processes behind this occurrence remains incompletely understood. Hence, we aimed to examine the influence of PTEN deficiency on isoform reliance in MEFs. PTEN expression was eliminated using two distinct shRNA plasmids. PTEN knock-down efficiency was shown by the use of anti-PTEN immunoblots. Our findings indicate that the shPTEN #2 construct exhibits a greater effectiveness in reducing PTEN levels compared to shPTEN #1 (Figure 1B). Simultaneously, PTEN shRNA treatment resulted in an augmentation in Akt phosphorylation, as PTEN activity

inhibited the PI3K pathway. We investigated whether MEFs still rely on the p110α isoform in the setting of diminished PTEN expression. In order to find out if the loss of PTEN caused a change in isoform dependence, we subjected MEFs expressing shPTEN to treatment with small molecule inhibitors that specifically target distinct PI3K isoforms. The shPTEN-#2-MEF cells, which exhibited a superior suppression of PTEN, demonstrated increased resistance to elevated doses of p110α inhibitor in comparison to wild type (wt) and shPTEN-#1 MEF cells (Figure 1C). These data suggest that the degree to which MEF cells rely on p110α is influenced to some extent by the levels of PTEN. Nevertheless, the sensitivity to p110β inhibition remained unchanged, despite the downregulation of PTEN in these cell lines.

Furthermore, this finding was confirmed by biochemical analysis in a brief inhibitor treatment experiment, where we examined the levels of pAkt and pS6. The administration of BYL719 for 2 hours resulted in a near-complete elimination of pAkt or pS6 in both the control and PTEN repressed MEF lines. In contrast, KIN193 exhibited significantly less effectiveness in reducing Akt and S6 phosphorylations in both the control and PTEN repressed MEFs (Figure 1D). Cells however, retained their high sensitivity to pan-PI3K inhibition by GDC.

Human malignancies often have PI3Ks mutated or amplified. Patients may have *PIK3CA* activating mutations, however this does not apply to the remaining PI3K isoforms (21). The proliferative capacity of p110β, δ, and γ isoforms is enhanced when they are excessively expressed as unaltered proteins. To

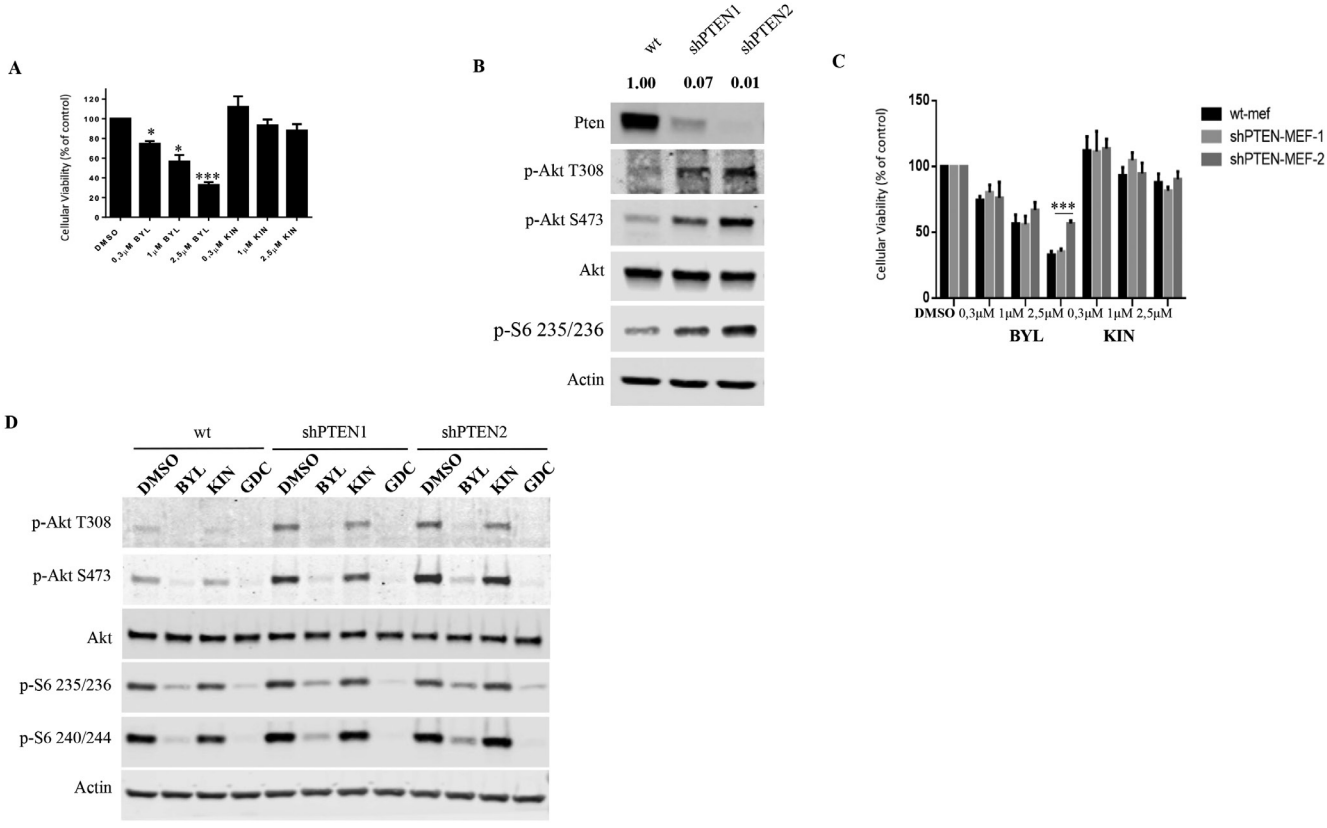

Figure 1. Molecular characterization of PI3K isoform prevalence in MEFs. (A) MEFs were treated with varying doses of p110α and p110β specific inhibitors. Crystal violet assays were performed to determine the cellular proliferative capacity. (B) PTEN was knocked-down in MEFs using shRNA constructs. Knock-down efficiency was determined in an immunoblot using anti-PTEN antibodies. pAkt and pS6 antibodies were used to assess the level of PI3K activation whereas anti-actin were used as loading control. (C) PTEN knocked-down MEFs were treated with varying doses of p110α and p110β specific inhibitors. Crystal violet assays were performed to determine the cellular proliferative capacity. (D) Control or PTEN knocked down MEFs were treated either with DMSO or isoform specific PI3K inhibitors, BYL-719 (BYL) and KIN-193 (KIN), specific for p110α and p110β or GDC, a pan-PI3K inhibitor respectively. The efficacy of PI3K signaling in these cells was assessed using immunoblots for p-Akt (T308 and S473) and p-S6 (S235/236). Actin served as the control for loading. *p<0.05, ***p<0.005, (the error bars in the graphs represent the standard deviation, and the experiments were conducted independently three times)

MEFs: Mouse embryonic fibroblasts, PTEN: Phosphatase tensin homolog

investigate whether increasing the levels of p110β-wt in MEFs leads to a change in the PI3K preponderance in favor of the p110β isoform, ectopic p110β-wt expression was performed (Figure 2A). Proliferation experiments were conducted using 1-2 μM of BYL719 and KIN193. No alterations in susceptibility or resistance to BYL719 and KIN193 treatment were seen when p110β was highly expressed in MEFs (Figure 2B, C). MEFs still exhibited a greater reliance on p110α and a lesser reliance on p110β, irrespective of increased p110β expression. The positive controls utilized in this experiment were GDC0941 at a concentration of 1 μM and MK2206 at a concentration of 2 μM. GDC0941 is an inhibitor that targets all isoforms of PI3K, whereas MK2206 is an inhibitor that specifically targets all isoforms of Akt (Figure 2C). Due to the more thorough inhibition of the PI3K pathway under these settings, we found a reduced rate of cell growth in those cell lines.

Next, our objective was to investigate whether MEFs still rely on PI3K activity in general upon reduced PTEN expression. We utilized genetically modified α,β+/+ MEF cells that had LoxP sites in the first exons of *PIK3CA* and *PIK3CB*. AdCre were used to excise out the floxed sequences concurrently, resulting in the generation of double knock-outs for the p110α and p110β proteins (17). Using the crystal violet test, we demonstrated that cells treated with AdCre exhibited significantly lower viability compared to untreated control cells (Figure 2D). The results indicated that PTEN knock-down did not reverse the growth abnormalities caused by the knockdown of ubiquitously expressed class IA PI3K isoforms. Cells continue to need class IA PI3Ks for proliferation even in the absence of PTEN.

To establish if PI3K isoform predominance was governed by multiple genetic components, we overexpressed p110β under the conditions of PTEN repression. We transfected p110β-wt in shPTEN-MEFs and tested proliferation in the presence of isoform-specific inhibitors. Combining PTEN knock-down with p110β overexpression reduces p110α reliance even further. Despite this, these cells did not exhibit a greater degree of dependence on p110β at intermediate doses of the inhibitor (Figure 2E).

When greater amounts of BYL719 (2 μM) and KIN193 (5 μM) were administered, the overexpression of p110β resulted in a partial resistance to the treatment of p110α inhibitor (BYL). In addition, the overexpression of p110β-wt significantly enhanced the susceptibility of MEFs to the p110β inhibition (KIN) (Figure 2F). Furthermore, we noticed a tendency towards resistance in the GDC0941 treatment group, though it lacked statistical significance. Based on literature, GDC0941 demonstrates only

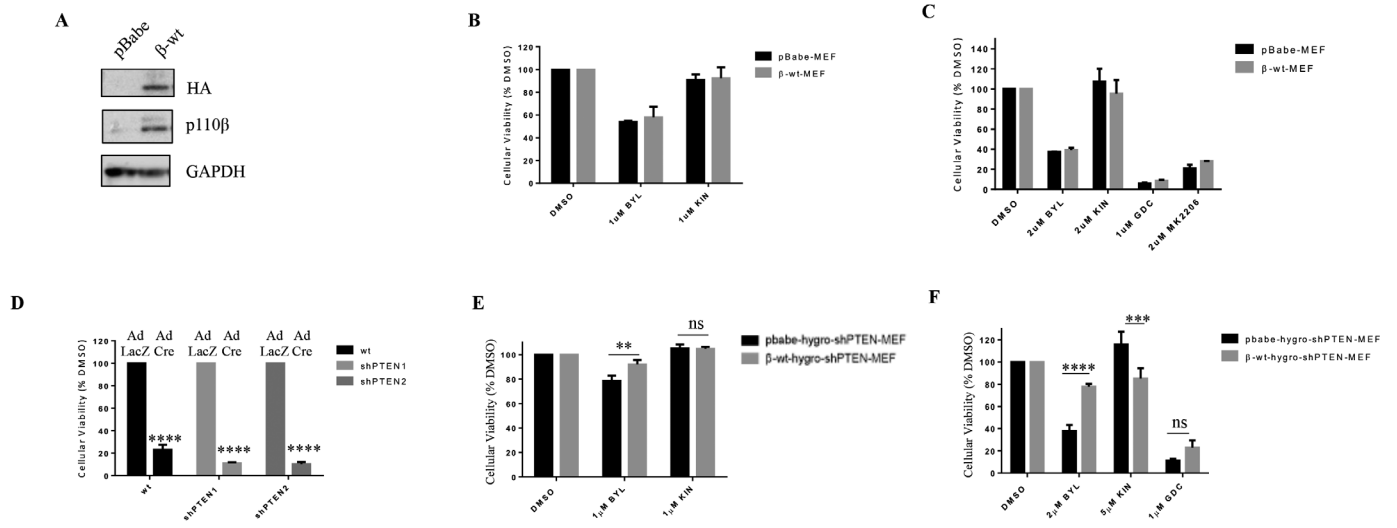

Figure 2. PTEN loss in combination with an increase in PIK3CB expression promote an elevated dependence on p110β. (A) Overexpression of p110β is achieved through retroviral expression of PIK3CB in MEFs. (B) Isoform specific PI3K inhbitors were applied to wt or p110β overexpressing MEFs, crystal violet assays were performed. (C) An increased amount of isoform specific PI3K inhbitors as well as pan-PI3K (GDC) and pan-AKT (MK2206) inhibitors were applied to wt or p110β overexpressing MEFs, crystal violet assays were performed. (D) The shPTEN1 and 2 MEFs were subjected to two rounds of AdCre treatments, and their proliferation rates were assessed using the crystal violet test. As a negative control for PI3K knockdown, LacZ expressing adenoviruses were used. The spectrometric quantification of crystal violet staining data was performed for MEFs treated with adenovirus. (E) The cellular viability of p110β overexpressing shPTEN-MEFs treated with BYL719 and KIN193 was measured. (F) Crystal violet assays for shPTEN MEFs treated with higher concentrations of BYL719 and KIN193 were quantified. **p<0.01, ***p<0.005, ****p<0.0001, ns: Not significant, p>0.05) (the error bars in the graphs represent the standard deviation, and the experiments were conducted independently three times)

MEFs: Mouse embryonic fibroblasts, PTEN: Phosphatase tensin homolog

moderate effectiveness against the p110β and γ isoforms in comparison to the p110α and δ isoforms (22). As GDC0941 is less effective at stopping p110β in p110β overexpressing MEFs, the cells in turn might have changed their dependence on p110β, enabling them to proliferate in the face of p110α blockade.

These findings indicate that increasing the expression of p110β only, is not enough to make MEFs more reliant on p110β. Similarly, the inhibition of PTEN alone did not demonstrate sufficient effectiveness in making cells more reliant on p110β. However, when p110β is overexpressed and PTEN is abolished, cells become less dependent to p110α and more vulnerable to p110β inhibition. Nevertheless, even when these modifications are combined, they do not appear to cause a full transition from reliance on p110α to p110β. Many parallel signaling pathways might cooperate with PI3K for sustaining cell proliferation. Rac1 is a small G protein that has the ability to control a wide range of processes, such as the activation of transcription factors, cell growth, cell transformation, programmed cell death, and the organism's innate immunity (10).

In order to understand the interaction of PI3K with Rac1, we decided to utilize a constitutively active version of Rac1 (Rac1 V12) as well an inactive mutant (Rac1 N17). These mutations either inhibit or promote GTPase activity of the enzyme regulating its function. We established stable MEF lines which express these Rac1 mutants and have not detected noticeable changes in the expression level of either p110α or p110β isoforms, or in phosphorylation of core PI3K pathway components, Akt and S6 (Figure 3A). As Rac1 is implicated in p110β dependent regulation of cell growth, we wished to understand the PI3K isoform dependency in the presence of these mutant Rac1 versions. Although, KIN treatments lead to similar growth profiles for control (13% inhibition) or Rac1 N17 (9.5% inhibition) expressing MEFs in comparison to DMSO, we saw an increased dependency on p110β upon expression of an activated Rac1 allele (53% growth inhibition) (Figure 3B). Interestingly, p110α prominence in these MEFs did not change upon expression of either Rac1 version. These results suggest that MEFs expressing an activated Rac1 allele become more reliant upon p110β for their growth in comparison to wild type MEFs.

Discussion

The PI3K signaling pathway triggers a sequence of highly controlled biochemical reactions at the plasma membrane that are necessary for the initiation and progression of the cell cycle. There are several variants of PI3Ks in eukaryotic cells. The specific roles of these diverse PI3K isoforms in cell cycle progression, as well as their activation patterns at different phases of the cell cycle, are not fully understood. Furthermore, changes in the PI3K pathway are identified in a diverse array of cancer types. PTEN is a frequently mutated gene that is usually shown to be deleted in multiple kinds of cancers, such as breast, prostate, and ovarian cancer. When PTEN is not present, p110β becomes the predominant isoform among class IA PI3Ks. Nevertheless, the precise mechanism behind the change in isoform dependency in a PTEN-null setting remains unclear. Pan-PI3K inhibitors were employed to treat cancers that are promoted by deregulated PI3K, however, these inhibitors exhibit significant adverse effects (23). Instead, inhibitors specifically targeting the p110β isoform, such as AZD8186 and KIN193, were explored for the treatment of PTEN-null cancer types. Treatments with standalone p110β inhibition led to temporary suppression of the PI3K pathway, followed by the development of resistance to inhibition in the cells. Research has indicated that tumor cells may develop a dependence on other types of PI3K when treated with inhibitors. Along these lines, it was shown that when PTEN-null cells were treated

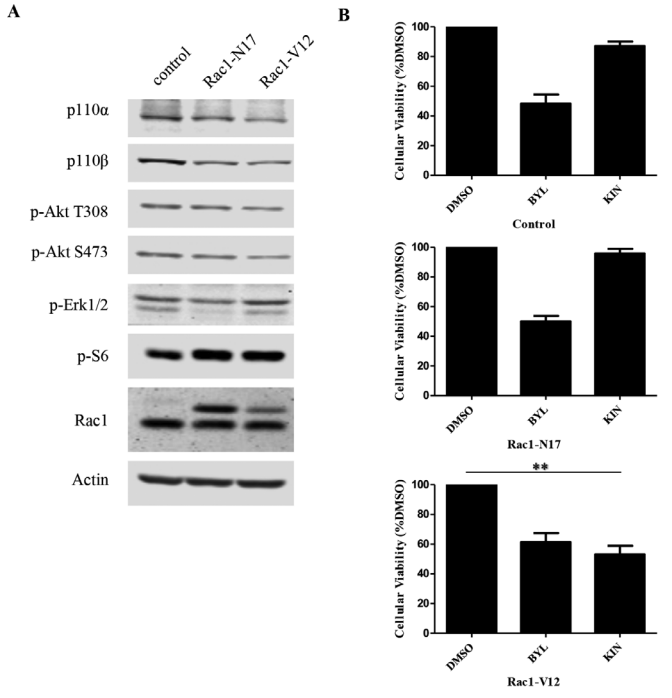

Figure 3. Activated Rac1 expression tilts the balance towards increased p110β prominence. (A) MEFs expressing mutant Rac1 versions were analyzed for expression of the depicted proteins and phosphor-proteins in immunoblots. Actin served as the control for loading. (B) Either empty vector control or mutant Rac1 expressing MEFs were treated with moderate doses of p110α and p110β specific inhibitors (BYL, KIN). Crystal violet assays were performed to determine the cellular proliferative capacity. **p<0.01, (the error bars in the graphs represent the standard deviation, and the experiments were conducted independently three times)

MEFs: Mouse embryonic fibroblasts

with p110β monotherapy, the p110α isoform was activated by IGFR or other RTKs (24). Discovering the molecular connection between PTEN loss and p110β dependency might improve existing treatment options and result in more efficient suppression of potential feedback pathways. The present study used immortalized MEFs, primarily due to their untransformed characteristics, low mutational burden and the presence of wild type PTEN expression. Our research aimed to determine whether cells require p110α and p110β for their cellular proliferation, regardless of the presence or lack of wild type PTEN. Firstly, our findings indicated that even when PTEN was efficiently suppressed, MEFs still required the activity of the ubiquitous class IA isoforms for cell growth. Under shPTEN treatment settings, we examined the dependence on the *PIK3CA* and *PIK3CB* genes. While PTEN suppression did not result in a shift in single isoform dependency in MEFs, there was a reduction in the effectiveness of p110α.

We overexpressed p110β in MEFs to see if it causes a more potent p110β dependency. However, we observed no shift in isoform reliance from p110α to p110β. Next, we coupled p110β overexpression and PTEN suppression. The combination resulted in a partial tolerance to p110α inhibitors and increased susceptibility to p110β inhibitors. GDC0941, a pan-PI3K inhibitor, became less effective against p110β overexpressed MEFs in the absence of PTEN. MEFs with insufficient PTEN relied more on the p110β isoform due to GDC0941's low selectivity for it. Overexpressing p110β alone did not increase cellular dependence on p110β. Different isoforms of PI3Ks engage with distinct small GTPases to regulate their activity. For example, the Ras protein binds specifically with the p110α isoform, while Rac1 preferentially activates the p110β isoform (11). In order to understand whether Rac1 collaborated with p110β and improved its potency in MEF growth, we exogenously expressed an activated Rac1 allele (V12) in MEFs and monitored their proliferative potential in response to isoform specific PI3K inhibition. Our data indicated that MEFs expressing an activated Rac1 allele became significantly more dependent on p110β. These findings corroborate prior reports describing a signaling node composed of GPCRs, trimeric G-proteins, Rac1 and p110β possibly establishing a feedback loop, enhancing the potency of the p110β generated signal (9,11,12).

Study limitations

We did not create a triple compound molecular genetic model that included all the genetic modifications. To achieve a more complete isoform transition, an activated Rac1 expression may need to be added to the existing combination in the MEFs in future studies.

Conclusion

We observed that alterations in PTEN and p110β expression in untransformed MEFs had a limited impact on the prevalence of PI3K isoforms. The inhibition of PTEN led to an overall activation of the PI3K pathway, but caused a decreased dependence on p110α. Our data suggest that no single genomic alteration would likely determine PI3K isoform dependency. A combination of several genetics elements, such as PTEN status, p110 expression levels and Rac1 activation status, have a profound effect on the relative effectiveness of the individual PI3K isoforms. These factors together shape the context-dependent reliance on p110α and p110β, making it particularly critical in certain pathological conditions, such as cancers with PTEN loss, where it supports key signaling pathways driving tumor growth and survival.

Ethics Committee Approval: *Not applicable.*

Informed Consent: *Not applicable.*

Author Contributions: *Concept: O.Ç.; Design: S.A., O.Ç.; Data Collection or Processing: S.A., O.Ç.; Analysis or Interpretation: S.A., O.Ç.; Literature Search: O.Ç.; Writing: O.Ç.*

Conflict of Interest: *No conflict of interest is declared by the authors.*

Financial Disclosure: *The authors declared that this study received no financial support.*

References

1. Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. Nat Rev Cancer. 2015; 15: 7-24.
2. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002; 296: 1655-7.
3. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. Biochim Biophys Acta. 1998; 1436: 127-50.
4. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov. 2009; 8: 627-44.
5. Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, et al. Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. Nature. 2008; 454: 776-9.
6. Vanhaesebroeck B, Whitehead MA, Piñeiro R. Molecules in medicine mini-review: isoforms of PI3K in biology and disease. J Mol Med (Berl). 2016; 94: 5-11.
7. Zhu J, Sun D, Li X, Jia L, Cai Y, Chen Y, et al. Developing new PI3Kγ inhibitors by combining pharmacophore modeling, molecular dynamic simulation, molecular docking, fragment-based drug design, and virtual screening. Comput Biol Chem. 2023; 104: 107879.
8. Ciruelos Gil EM. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. Cancer Treat Rev. 2014; 40: 862-71.
9. Cizmecioglu O, Ni J, Xie S, Zhao JJ, Roberts TM. Rac1-mediated membrane raft localization of PI3K/p110β is required for its activation by GPCRs or PTEN loss. Elife. 2016; 5: 17635.

10. Wertheimer E, Gutierrez-Uzquiza A, Rosemblit C, Lopez-Haber C, Sosa MS, Kazanietz MG. Rac signaling in breast cancer: A tale of GEFs and GAPs. Cell Signal. 2012; 24: 353-62.
11. Fritsch R, de Krijger I, Fritsch K, George R, Reason B, Kumar MS, et al. RAS and RHO Families of GTPases Directly Regulate Distinct Phosphoinositide 3-Kinase Isoforms. Cell. 2013; 153: 1050-63.
12. Yuzugullu H, Baitsch L, Von T, Steiner A, Tong H, Ni J, et al. A PI3K p110β-Rac signalling loop mediates Pten-loss-induced perturbation of haematopoiesis and leukaemogenesis.Nat Commun. 2015; 6: 8501.
13. Rathor N, Chung HK, Wang SR, Qian M, Turner DJ, Wang JY, et al. β-PIX plays an important role in regulation of intestinal epithelial restitution by interacting with GIT1 and Rac1 after wounding. Am J Physiol Gastrointest Liver Physiol. 2018; 314: 399-407.
14. Hall AB, Gakidis MA, Glogauer M, Wilsbacher JL, Gao S, Swat W, et al. Requirements for Vav guanine nucleotide exchange factors and Rho GTPases in FcgammaR- and complement-mediated phagocytosis. Immunity. 2006; 24: 305-16.
15. Hoppe AD, Swanson JA. Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. Mol Biol Cell. 2004; 15: 3509-19.
16. Kaysudu I, Gungul TB, Atici S, Yilmaz S, Bayram E, Guven G, et al. Cholesterol biogenesis is a PTEN-dependent actionable node for the treatment of endocrine therapy-refractory cancers. Cancer Sci. 2023; 114: 4365-75.
17. Demir M, Cizmecioglu O. ZAP70 Activation Compensates for Loss of Class IA PI3K Isoforms Through Activation of the JAK-STAT3 Pathway. Cancer Diagn Progn. 2022; 2: 391-404.
18. Meek RL, Bowman PD, Daniel CW. Establishment of mouse embryo cells in vitro. Relationship of DNA synthesis, senescence and malignant transformation. Exp Cell Res. 1977; 107: 277-84.
19. Millis SZ, Ikeda S, Reddy S, Gatalica Z, Kurzrock R. Landscape of Phosphatidylinositol-3-Kinase Pathway Alterations Across 19 784 Diverse Solid Tumors. JAMA Oncol. 2016; 2: 1565-73.
20. Wee S, Wiederschain D, Maira SM, Loo A, Miller C, deBeaumont R, et al. PTEN-deficient cancers depend on PIK3CB. Proc Natl Acad Sci U S A. 2008; 105: 13057-62.
21. Kang S, Denley A, Vanhaesebroeck B, Vogt PK. Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase. Proc Natl Acad Sci U S A. 2006; 103: 1289-94.
22. Sarker D, Ang JE, Baird R, Kristeleit R, Shah K, Moreno V, et al. First-in-human phase I study of pictilisib (GDC-0941), a potent pan-class I phosphatidylinositol-3-kinase (PI3K) Inhibitor, in patients with advanced solid tumors. Clin Cancer Res. 2015; 21: 77-86.
23. Bauer TM, Patel MR, Infante JR. Targeting PI3 kinase in cancer. Pharmacol Ther. 2015; 146: 53-60.
24. Schwartz S, Wongvipat J, Trigwell CB, Hancox U, Carver BS, Rodrik-Outmezguine V, et al. Feedback suppression of PI3Kα signaling in PTEN-mutated tumors is relieved by selective inhibition of PI3Kβ. Cancer Cell. 2015; 27: 109-22.