Development and characterisation of cell-based models of PTEN Hamartoma Tumour Syndrome

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Abstract

Germline mutations in the tumor suppressor gene PTEN result in a spectrum of multisystem disorders collectively referred to as PTEN hamartoma tumor syndrome (PHTS). Manifestations may include benign tumor-like growths (hamartomas), increased cancer risk, vascular malformations sand neurological comorbidities such as macrocephaly, autism spectrum disorder, intellectual dysfunction, and in some cases, epilepsy. Here we developed murine and human neuronal cell-based models of PHTS to support drug discovery efforts. Adenoviral PTEN shRNA transduction of primary mouse hippocampal neurons on the first day in vitro (DIV1) resulted in almost complete loss of PTEN protein expression by DIV10. By DIV14, knockdown of PTEN resulted in an increase in neurite outgrowth and branching, increased pAKT levels, and an increase in network burst duration as measured by multielectrode array (MEA). Similarly, knockdown of PTEN by ≥50% using an antisense oligonucleotide (ASO) approach in human iPSC-derived neurons led to increased pAKT levels and an increase in network firing rate. We utilized these models to screen tool compounds against PI3K pathway targets. Although the PI3Kα inhibitor alpelisib was able to normalise pAKT levels in human iPSC derived neurons, in murine neurons treatment with alpelisib resulted in increased pAKT in contrast to treatment with the PI3Kβ inhibitor GSK2636771, or the mTOR inhibitor everolimus, that both reduced pAKT. Given that dysregulation of PI3K signaling is a common feature of other disorders, including Fragile X syndrome and Rett syndrome, determining the mechanisms by which the PI3K pathway drives pathophysiology may inform treatment of diseases with overlapping manifestations. In sum, although cell-based models can be used to finely dissect signaling pathways underlying PTEN dysfunction in neurons, careful consideration of drug exposure time and response across model systems is required to support the identification of candidate drugs to address the neurological manifestations of PHTS.

Introduction

PTEN hamartoma tumour syndrome (PHTS) arises from the autosomal dominant inheritance of inactivating mutations in the PTEN tumour suppressor gene [1]. The currently accepted incidence is 1 in 200,000 although this figure may be as high as 2-4 in 10,000, primarily because the incidence of PTEN germline mutations in idiopathic ASD may be 2% [2] , suggesting that PHTS is underdiagnosed.

Loss of PTEN expression leads to an upregulation of PI3K-Akt-mTOR activity and the pathway has been implicated in the regulation of multiple aspects of neuronal growth and activity, astrocyte and microglial function (Figure 1). In animal models and in man, loss of PTEN expression in the CNS leads to overgrowth of cortical neurons and enhanced synaptic excitability.

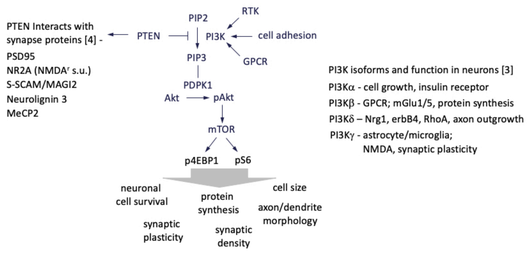

Figure 1. Schematic of PI3K signalling in neurons. PI3K generates phosphoinositol triphosphate (PIP3) that provides a membrane recruitment site for PTEN, PDPK1 and Akt. In turn, Akt activates mTOR leading to the activation of key regulators of translation and protein synthesis (4EBP1 and S6). Different isoforms of PI3K [3] have been associated with specific aspects of CNS function. PTEN, the negative regulator of PI3K, also interacts directly with various proteins [4] to further influence neuronal activity.

The PI3K signalling pathway is the most highly mutated pathway in cancer and has been the subject of intense drug discovery efforts for the past two decades. As a result, there exists a significant number of well-characterised small molecule inhibitors that provide tools to dissect the underlying biology and may represent candidates for the treatment of PHTS.

PTEN reduction in mouse primary neurons leads to increased neurite outgrowth and pAkt levels

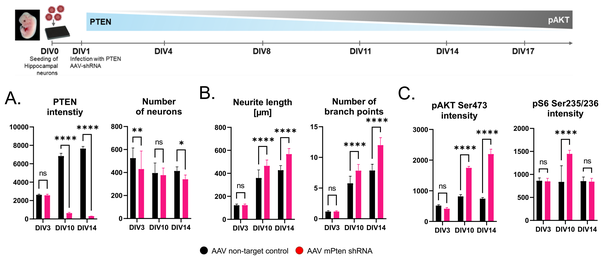

Figure 2. Hippocampal neurons were isolated from embryonic day 16 mouse embryos. Twenty-four hours after plating cells were transduced with an adeno-associated virus expressing a shRNA targeting PTEN, fixed at different time points and stained for image analysis. PTEN KD resulted in complete PTEN loss robustly at DIV10 (A) accompanied by (B) increase of neurite length and branching, and (C) PI3K pathway activation as measured by increased pAKT (Ser473) levels. An increase in pS6 (Ser235/236) intensities showed a different time profile. Neurite outgrowth was assessed with antibodies against Tuj1 combined with MAP2 and DAPI staining for neuronal detection. Mean ± SD are shown (Two-Way ANOVA, *p<0.05, **p<0.01, ****p<0.0001).

PTEN reduction in human iPSC-derived neurons leads to increased pAkt levels

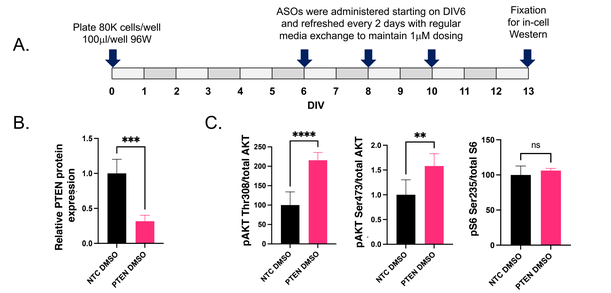

Figure 3. Antisense oligonucleotide (ASO)-mediated PTEN knockdown in human iPSC-derived neuron cultures over time (A) resulted in (B) a decrease in PTEN protein, (C) an increase in levels of pAKT at Thr308 and Ser473, but not pS6 Ser235. PTEN DMSO, treated with a PTEN-targeting ASO; NTC, non-targeting control ASO-treated. Bars are mean ± SD (T-Test; **p<0.01, ***p<0.001, ****p<0.0001).

References

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PTEN reduction in mouse primary neurons leads to increased synaptic activity

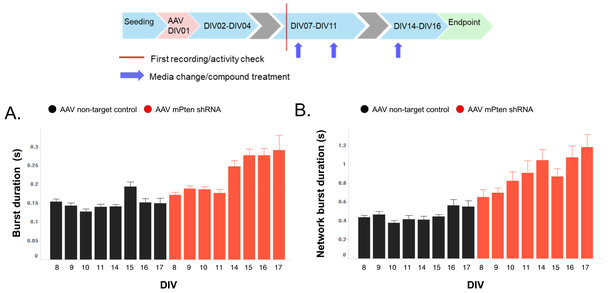

Figure 4. Mouse hippocampal neurons were plated into 48-well microelectrode array plates. Twenty-four hours after plating, neurons were transduced with an adeno-associated virus expressing a shRNA targeting PTEN. Synaptic activity was monitored over a 17-day period DIV8-17 as shown. Analysis of synaptic activity revealed that PTEN loss in primary hippocampal neurons resulted in an increase in burst duration (A) and network burst duration (B). Mean ± SEM are shown.

PTEN reduction in human iPSC-derived neurons leads to hyperexcitability

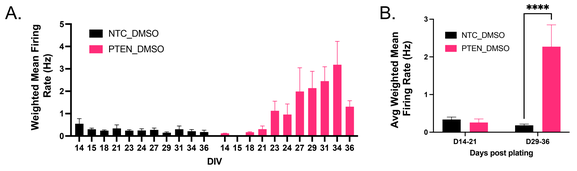

Figure 5. Antisense oligonucleotide (ASO)-mediated PTEN knockdown in human iPSC-derived neuron cultures resulted in (A) an increased weighted mean firing rate, beginning after 23 DIV. (B) Further, the average weighted mean firing rate of iPSC-derived neurons treated with a PTEN-targeting ASO was >4-fold higher than non-targeting control (NTC) ASO-treated neurons between DIV 29-36. Bars are mean ± SD (T-test; ****p<0.0001).

Conclusions

- Reduced PTEN protein expression in murine and human iPSC-derived neurons resulted in similar biochemical and cellular responses, suggesting a degree of consistency between the model systems.
- PTEN knockdown increased neurite length and branching in mouse primary neurons and increased levels on pAkt in both mouse and human iPSC derived neurons.
- PTEN knockdown increased synaptic activity in both mouse primary neurons and human iPSC derived neurons; although it is unknown if the reduced levels of PTEN achieved experimentally provide a direct comparison with PTEN loss in PHTS neurons.
- Phenotypic and signaling responses in models of mouse and human PTEN loss are similar; point of intervention, exposure time and measured outputs should be considered when comparing data between models.
- Charaterisation of morphology, functional and biochemical properties are critical for interpretation of response to PI3K pathway inhibitors.

PI3Kα inhibition (alpelisib) failed to normalise PTEN-KD-induced neurite length and synaptic activity and increased pAkt levels in mouse neuronal model

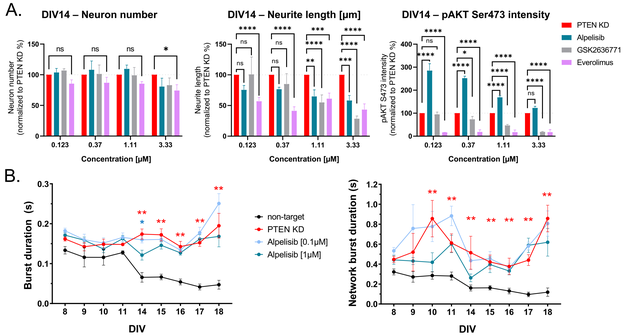

Figure 6. Compound treatment in two PTEN-KD mouse hippocampal neuron models shown in Figure 2 (A, neurite outgrowth) and Figure 4 (B, synaptic activity). In (A) cells were treated on DIV10 and fixed for staining on DIV14. Mean ± SD are shown (two-way ANOVA, *p<0.05,**p<0.01,***p<0.001,****p<0.0001). Data normalised to PTEN-KD DMSO (100%). In (B) cells were treated on DIV7, DIV10, and DIV14 and monitored until DIV18. Mean ± SEM are shown; Stepdown Bonferroni adjustment comparing PTEN-KD vs non-target (**, p<0.01) or Alpelisib-treated PTEN-KD vs PTEN-KD (*, p<0.05).

PI3Kα inhibition normalised PTEN-KD-induced increases in pAKT and pS6 in human iPSC-derived neurons; hyperexcitability was not reversed by pAKT inhibition.

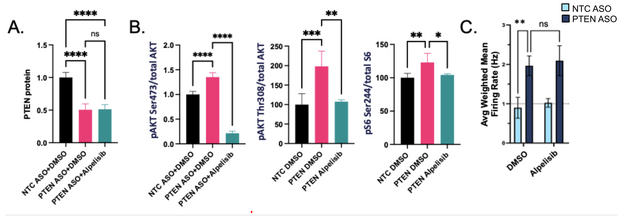

Figure 7. (A) Antisense oligonucleotide (ASO)-mediated PTEN knockdown in human iPSC-derived neuron cultures resulted in a decrease in PTEN protein. The PI3Kα inhibitor, alpelisib (100nM), had no effect on PTEN protein levels (A) but was able to reduce (B) the PTEN knockdown-induced increase in at Thr308 and Ser473 pAKT and Ser244 pS6 levels. (C) Notably, the increase in average weighted mean firing rate of iPSC-derived neurons treated with a PTEN-targeting ASO was not impacted by 2 hr treatment with the PI3Kα inhibitor, alpelisib. The hyperexcitability changes induced by PTEN KD may manifest in long-term changes (e.g., expression of channels) that are not reversed by acute pAKT inhibition. Bars are mean ± SD (ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Acknowledgements

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