Analysis of PTEN stability in *Pggt1b* endometrial conditional knockout

Undergraduate Research Thesis

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by

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Abstract

The PTEN tumor suppressor protein is one of the most frequently mutated genes in human cancer. Due to an observation that loss of heterozygosity only occurs in 25% of lesions where PTEN expression is lost, a screen was performed in search of post-translational stabilizers and destabilizers of PTEN protein, and the gene Pggt1b was identified. Mice with a conditional knockout of Pggt1b in the endometrium were generated, and uteri were harvested and analyzed for pathology and PTEN expression. When PGGT1B was absent, slightly more hyperplasia was observed, along with slightly increased PTEN staining. However, these differences were not significant.

Background

Phosphatidylinositol (3,4,5)-triphosphate phosphase and tensin homologue (PTEN) is encoded by the Pten gene, which is among the most frequently mutated genes in advanced human cancers, including skin, prostate, and endometrial cancers. PTEN is a tumor suppressor and the most important negative regulator of the AKT pathway, which leads to cell survival, proliferation, and growth. In the AKT pathway, growth factors and other ligands bind to receptor-tyrosine kinases in the plasma membrane, and in response, the phosphoinositide-3 kinase (PI3K) phosphorylates the membrane-associated phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). AKT is recruited to the membrane when its PH domain binds PIP3, and it is phosphorylated and activated by PDK1 and mTORC. PTEN's principal tumor suppressor activity occurs at the plasma membrane, where it acts as a lipid phosphatase and opposes the activity of the AKT pathway by dephosphorylating PIP3 to PIP2. PTEN activity is regulated in part by phosphorylation of its C-terminal tail at several serine and threonine residues (S380/T382/T383). When PTEN is not phosphorylated here, it has a greater affinity for the plasma membrane and its lipid phosphatase activity is greater than the phosphorylated form. However, nonphosphorylated PTEN is also degraded more quickly. Phosphorylation at other sites can stabilize PTEN. Other post-translational modifications also regulate PTEN activity and stability. Acetylation of residues in the catalytic domain can negatively regulate PTEN activity, and ubiquitylation marks PTEN for nuclear import or targets it for degradation by the proteasome. Many of the proteins controlling these modifications are still unknown.

Screen for stabilizers and destabilizers of PTEN protein

Due to an observation that PTEN protein levels, but not mRNA levels, were altered in 75% of endometrial lesions in Pten+/Δ4-5 mice, a screen was conducted to find proteins that increase or decrease the stability of PTEN protein. Human mammary epithelial cells of the MCF10A cell line expressing Pten fused to the fluorescent marker mCherry (mCherry-Pten) were infected with an shRNA library in pGIPZ lentiviral vectors. Presence of the vector was selected for with puromycin. The cells were divided into three replicates and each replicate
was FACS sorted separately. The 10% of cells with either the most or the least mCherry-
PTEN were saved and cultured, before undergoing the same FACS sorting and being
separated according to the same cutoff value. Four rounds of FACS selection were
performed. The shRNA from the cells past the cutoff point was amplified using PCR, and the
identity of the shRNA of interest was determined using Illumina next-generation sequencing.
This resulted in a list of potential negative and positive regulators of PTEN stability at the
protein level. Protein geranylgeranyltransferase type I subunit β (PGGT1B) was among the
putative negative regulators of PTEN, suggesting that knocking out Pggt1b would increase
PTEN stability (Fig. 1A) (Bravo, V. unpublished data).

PGGT1B

PGGT1B is a geranylgeranyltransferase, an enzyme that regulates certain proteins by adding
a geranylgeranyl group to proteins with a CAAX motif in their carboxyl terminus.7,8 This
geranylgeranyl group is hydrophobic, and targets the modified protein to the plasma
membrane.7,8 Geranylgeranylation is a type of isoprenylation, a post-translational
modification that is important to many cell signaling pathways. For instance, the CAAX
proteins that are geranylgeranylated by PGGT1B include RHOA, RAC1, and CDC42, which are
associated with cytoskeleton regulation, cell motility, and cell division.8

Preliminary data

Pggt1b expression in different tissues

Real-time quantitative PCR was used to determine the expression levels of Pggt1b in wild-
type mice. The expression of the housekeeping genes that are normally used for qPCR
normalization has been shown to vary significantly based on the tissue in which they are
being expressed.9–11 Therefore, when measuring the expression of one gene across multiple
organs, it is more accurate to normalize qPCR data to the arithmetic mean of a group of
reference genes.9–12 A group of five reference genes was chosen for normalization, including
ActB, Cltc, Gapdh, Tbp, and 18sRNA. The data were normalized to a weighted average of all
five reference genes, where the genes whose expression varied the least between organs
were given the most weight. qPCR was performed for thirteen organs in two female wild-type
FVB mice (Fig. 1B). The expression of Pggt1b was highest in the adrenal gland, which
suggested that deleting Pggt1b in the adrenal gland may give a more noticeable phenotype
than deleting it in another tissue. The expression of Pggt1b in the uterus was similar to its
expression in other organs. However, because of our lab’s interest in the endometrium, we
decided to explore whether PGGT1B regulates PTEN stability in murine endometrium. The
endometrium is the tissue lining the uterus, and is composed of both the luminal epithelium
and the stroma found between the luminal epithelium and the uterine muscle, but does not
include the muscle. Pggt1bflx/flx mice were obtained from the Bergo lab in Goteborg, Sweden,
and crossed with mice expressing Sprr2f-cre, an endometrium-specific cre expressed under
the Sprr2f promoter in the endometrial epithelium.8
Confirmation of the conditional deletion of *Pggt1b*

A protocol for the isolation of the endometrium in mice was used in order to specifically analyze the endometrium using immunoblot or qPCR. To test the method, the purity of the endometrial cells was determined by plating an aliquot of cells on a glass coverslip and staining for CK-8, an epithelial marker that should be present in the endometrium but not in stromal fibroblasts that could be contaminating the sample. DAPI was used as a nuclear stain. As expected, none of the fibroblast control cells expressed CK-8. The endometrial cells were reasonably pure, because only a few cells didn't express CK-8 (Fig. 2A). These cells were probably fibroblasts from the stroma that were released during the trypsin treatment. To confirm the conditional deletion of *Pggt1b* in the endometrium of sexually mature mice, qPCR was performed in the endometrium of a *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} mouse to show the removal of exon 6, in comparison with the endometrium of a *Pggt1b*\textsuperscript{flx/flx} mouse. Endometrial cells were cultured for one day after isolation to remove any contaminating blood. One primer set was designed with the forward primer in exon 6, so that the reaction would fail in the presence of cre (Fig. 2B). However, the fold change for the presence of exon 6 compared to total *Pggt1b* was approximately 0.45 in the *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} mouse, much higher than expected (Fig. 2C). Another primer set was designed to give different product sizes in the *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} endometrium compared to the *Pggt1b*\textsuperscript{flx/flx} endometrium (Fig. 2B). However, in the *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} endometrium, the melt curve for these reactions showed two distinct peaks, indicating that two different products were present (Fig. 2D). This suggests that either the cre is less than 100% effective, or that the endometrium cells were contaminated by other epithelial cells. To further confirm the conditional knockout, immunohistochemistry using an antibody against PGGT1B was performed in the uterus of one *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} mouse and one *Pggt1b*\textsuperscript{flx/flx} mouse (Fig. 2E). PGGT1B staining is clearly visible in the oviducts of both mice, which were used as positive controls. PGGT1B staining is present in the endometrium of the *Pggt1b*\textsuperscript{flx/flx} mouse, and is almost entirely missing from the endometrium of the *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} mouse. The cre was determined to be effective and specific. *Pggt1b*\textsuperscript{flx/flx} mouse embryonic fibroblasts (MEFs) were isolated and established via serial passaging. *Pggt1b*\textsuperscript{flx/flx} MEFs were infected with an empty pBABE-puro vector or a pBABE-puro vector containing cre. After puromycin selection, protein was extracted, and an immunoblot against PGGT1B was performed to confirm PGGT1B knockdown (Fig. 2F). There was a significant reduction in PGGT1B protein in the *Pggt1b*\textsuperscript{flx/flx}:cre\textsuperscript{+} cells.

**Preliminary pathology and immunoblots**

*Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} mice were bred with *Pten*\textsuperscript{+/Δ4-5} mice, and the uteri of 14 mice of various genotypes were harvested (Fig. 3A). H&E-stained sections were used to analyze the uterine pathology (Fig. 3B). Although the sample size was small, the *Pggt1b*\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} mice in both a *Pten*\textsuperscript{+/Δ4-5} and a *Pten*\textsuperscript{+/+} background appeared to have a greater amount of hyperplasia and carcinoma than the *Pggt1b*\textsuperscript{flx/flx} mice (Fig. 3C). In *Pggt1b*\textsuperscript{flx/flx} MEFs infected
with a pBABE-puro vector or a p-BABE-puro-cre vector, an immunoblot against PTEN showed
the appearance of an unknown band above the PTEN band in the \textit{Pggt1b}^{flx/flx:cre^+} cells only
(Fig. 3D).

**Hypothesis**

If PGGT1B is a negative regulator of PTEN, then conditionally knocking out \textit{Pggt1b} in MEFs
should increase the stability of PTEN protein. PTEN immunohistochemistry should also show
increased staining in the endometrium of mice where \textit{Pggt1b} is conditionally knocked out. \textit{Pggt1b}^{flx/flx:Pten^+/+} and \textit{Pggt1b}^{flx/flx:Pten^+Δ4-Δ5} mice would be expected to have an greater
number of endometrial lesions than \textit{Pggt1b}^{flx/flx:Pten^+/+:Sprr2f-cre^+} or \textit{Pggt1b}^{flx/flx:Pten^+Δ4-Δ5:Sprr2f-cre^+} mice. However, the preliminary pathology data do not support this prediction,
because increased hyperplasia and carcinoma were seen in \textit{Sprr2f-cre^+} mice.

**Experiments**

**Pathology and PTEN staining**

A second cohort of mice was harvested to increase the sample size to \textit{n}=40, and the uterine
pathology of the new animals was analyzed with H&E staining (Fig. 4A). In a \textit{Pten^+/+}
background, the \textit{Pggt1b}^{flx/flx:Sprr2f-cre^+} mice have slightly more hyperplasia than the \textit{Pggt1b}^{flx/flx} mice, and in a \textit{Pten^+Δ4-Δ5} background, the \textit{Pggt1b}^{flx/flx:Sprr2f-cre^+} mice have
slightly more carcinoma than the \textit{Pggt1b}^{flx/flx} mice. However, it was difficult to compare
possible hyperplasia between mice because the characteristics of the endometrium change
with the estrous cycle. A subset of the uteri was stained for PTEN via IHC (Fig. 4B). The PTEN
staining seemed variable, but it was difficult to decide whether levels were really changing
between genotypes. It was also not known whether estrous cycle stage affected \textit{Pten}
expression. The estrous cycle refers to the cycle of physiological changes in the female
reproductive tract that are caused by changes in reproductive hormones, and is the
equivalent of the menstrual cycle in humans. Therefore, the PTEN staining was quantified in
the cytoplasm of endometrial cells using a Vectra microscope and inForm software. An H-
score was calculated to reflect the intensity of the staining in each picture taken (Fig. 4C). An
unpaired t test used to determine whether PTEN levels were significantly higher in the \textit{Pggt1b}^{flx/flx:Pten^+/+:Sprr2f-cre^+} mice than in the \textit{Pggt1b}^{flx/flx:Pten^+/+} mice yielded a p-
value=0.222, which is not significant. The unpaired t test to compare PTEN levels in
\textit{Pggt1b}^{flx/flx:Pten^+Δ4-Δ5:Sprr2f-cre^+} versus \textit{Pggt1b}^{flx/flx:Pten^+Δ4-Δ5} uteri gave p=0.898, which is
not significant. There was not a significant difference in PTEN levels between mice of the
same genotype that were harvested in different estrous cycle stages. These data suggest
that loss of \textit{Pggt1b} does not affect PTEN protein stability in either \textit{Pten^+/+} or \textit{Pten^+Δ4-Δ5}
backgrounds.
Estrous cycle staging

To permit better classification of hyperplasia and other proliferative phenotypes, and to be sure that estrous cycle stage isn’t a confounding factor in measuring PTEN levels, mice were staged for estrous cycle and classified as either proestrus, estrus, metestrus, or diestrus based on the cytology of vaginal smears (Fig. 4D). Mice were harvested at diestrus, the stage during which the endometrium is the least proliferative, and the pathology of these animals will be analyzed soon.

Immunoblot

\( \text{Pggt1b}^{\text{flx/flx}} \) MEFs were again infected with pBABE-puro and pBABE-puro-cre vectors, to try to replicate the unknown band that appeared in the PTEN immunoblot after the addition of cre in the first experiment (Fig. 5A). PGGT1B was significantly reduced in the \( \text{Pggt1b}^{\text{flx/flx}:\text{cre}^+} \) MEFs, but there was no difference in the amount of PTEN protein between the \( \text{Pggt1b}^{\text{flx/flx}:\text{cre}^+} \) and \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs, and the shifted band that was previously seen did not appear. \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs stopped dividing after the addition of cre, making it difficult to obtain enough cells for immunoblots. This may be because the loss of PGGT1B means that RAS family proteins, which control cellular processes like proliferation, differentiation, and apoptosis, are no longer isoprenylated and targeted to the membrane. MEFs are dependent on RAS activity for division.\(^{13}\) In another attempt to replicate the unknown band, \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs were infected again with pBABE-puro and pBABE-puro-cre, and \( \text{Pggt1b}^{\text{flx/flx}:\text{cre}^+} \) cells were harvested at 7, 8, and 9 days after the first infection (1, 2, and 3 days after the end of puromycin selection). Immunoblot against PGGT1B, PTEN, and GAPDH was performed (Fig. 5B). PGGT1B protein was significantly reduced in all of the \( \text{Pggt1b}^{\text{flx/flx}:\text{cre}^+} \) MEFs. There was no change in PTEN protein levels between \( \text{Pggt1b}^{\text{flx/flx}:\text{cre}^+} \) and \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs, and the unknown band was not observed.

Discussion

RAS small GTPases have been shown to bind the PI3K catalytic subunit p110 to activate PI3K, and RHO family small GTPases probably act together to activate PI3K via a positive feedback loop.\(^{14}\) These RHO family small GTPases include CDC42 and RAC1, which are known to be geranylgeranylated by PGGT1B.\(^8\) RHOA, RAC1, and CDC42 have been shown to accumulate in their GTP-bound active form in macrophages missing \( \text{Pggt1b} \), possibly because geranylgeranylation is necessary for RHO proteins to bind to RHO-GAPs.\(^{15}\) Therefore, the absence of PGGT1B could cause activation of PI3K and increased AKT activity, and increased AKT activity is associated with decreased PTEN activity. PTEN is phosphorylated at the C-terminus by casein kinase 2 (CK2), and this phosphorylation decreases PTEN lipid phosphatase activity at the same time as it prevents ubiquitination and degradation of PTEN.\(^{16}\) PTEN is more stable when it is less active. This could explain the higher PTEN levels found in some \( \text{Pggt1b}^{\text{flx/flx}:\text{Spr2f-cre}^+} \) endometrial samples, despite the higher occurrence of hyperplasia and carcinoma in these mice. PGGT1B is already being
considered as a target for drug therapies in cancer, because PGGT1B deficiency has been shown to decrease the development of tumors in mice that have K-RAS-induced lung cancer. However, the data from these experiments suggest that mice lacking \textit{Pggt1b} in the endometrium are more likely to display hyperplasia and carcinoma phenotypes than control mice. This means that any geranylgeranyltransferase inhibitor drugs used to treat cancer may cause significant side effects that should be considered before therapy.

Several additional experiments should be performed. First, the pathology of the uteri from the mice that have been staged and harvested at diestrus will be analyzed. Considering the previous results, it is expected that there will be slightly more hyperplasia and carcinoma in the mice that are \textit{Pggt1b}^{flx/flx}:\textit{Sprr2f-cre} compared to \textit{Pggt1b}^{flx/flx} mice. An inducible cre system for infection of MEFs has been developed. This will enable the generation of large numbers of \textit{Pggt1b}^{flx/flx}:\textit{Sprr2f-cre} MEFs, which was previously expensive because they didn’t divide after infection with \textit{pBP-cre}. Cre was cloned into the vector backbone pCW57.1. This vector contains rtTA as well as a TRE promotor, and is an all-in-one tet on system. \textit{Pggt1b}^{flx/flx} MEFs will be infected with either pCW57.1-cre or pCW57.1 alone, and cre expression will be induced by adding doxycycline. These MEFs will be used for a more extensive time course experiment to determine whether the shifted band that was observed in the preliminary immunoblot is dependent on how much time has elapsed after \textit{Pggt1b} conditional knockout. MEFs will be harvested at 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours after doxycycline addition, and immunoblots for PGGT1B, PTEN, and GAPDH will be performed. RAC1 is a member of the RHO family of small GTPases and has a role in lamellipodia formation and cell motility. RHOA and CDC42 are also known to function in cell motility during chemotaxis. RHOA is localized in the back of chemotactic neutrophils, while CDC42 localizes to the leading edge of the cell. Loss of PGGT1B increases the active forms of RHOA, RAC1, and CDC42, so the lesions observed in the endometrium could be due to disorganization and dysregulation of cell motility. The inducible cre system will also be used to grow enough MEFs for use in an assay to measure RAC1 activation. If the GTP-bound form of RAC1 is more prevalent in \textit{Pggt1b}^{flx/flx}:\textit{cre} MEFs than \textit{Pggt1b}^{flx/flx} MEFs, it could suggest that the GTP-bound forms of RHOA and CDC42 are also more prevalent. RHOA-associated kinase (ROCK) is known to phosphorylate and activate PTEN. If RHOA is more activated when PGGT1B is missing, then PTEN may be more phosphorylated as a result. The unknown band seen in the PTEN immunoblot for \textit{Pggt1b}^{flx/flx}:\textit{cre} MEFs may be a result of this phosphorylation. In this case, treating the protein extract with a phosphatase should remove the band. Finally, in future experiments involving any tissues that could be influenced by female reproductive hormones, it would be well advised to stage the mice and harvest them during one or two predetermined stages only.
**Materials and methods**

**Estrous cycle staging**

Vaginal lavage was performed using approximately 20 µL of sterile PBS. Cells were spread on a slide and stained with standard Dip Quick cytology stain. Smears were classified as proestrus, estrus, metestrus, or diestrus based on the relative amounts of nucleated epithelial cells, cornified epithelial cells, and leukocytes observed.

**Endometrium isolation**

Sexually mature female mice were euthanized and perfused with heparinized saline at 6 mL/min. The uterus was extracted and chopped into small cross-sectional pieces about 2 mm wide. The uterus pieces were incubated in 1% trypsin in Ca²⁺- and Mg²⁺-free HBSS at 37 °C for one hour. The trypsin was removed and neutralized, and the cells were pelleted and washed with HBSS. Cells were either plated in DMEM with 10% FBS, or processed immediately. An aliquot of cells was plated on a cover slip and stained for CK-8 and DAPI by immunofluorescence to determine purity.

**Mouse embryonic fibroblast isolation and establishment**

A pregnant mouse was euthanized and embryos were extracted 13.5 days after a plug was seen. The head and organ spot of each embryo was removed, and embryos were partially homogenized with a sterile razor blade. The tissue was incubated in 0.25% trypsin at 37 °C for 40 minutes in total. Cells were pelleted, resuspended, and plated in DMEM with 10% FBS. 600,000 cells were plated on a 100 mm dish every 3 days for 17 passages.

**Cell culture and transfection**

All cells were maintained in DMEM with 10% FBS. Phoenix Eco cells were plated at a density of 500,000 per well of a 6-well plate, and transfected with either pBABE-puro or pBABE-puro-cre using jetPEI Polyplus transfection reagent. Viruses were harvested and applied to Pggt1b^{flx/flx} MEFs, and Polybrene was used to increase the transduction efficiency. pCW57.1 was a gift from David Root (Addgene plasmid #41393). Cre was amplified from the pBABE-puro-cre vector and recombined into pCW57.1 using the In-Fusion cloning system.

**Genotyping**

PCR genotyping was performed using DNA from tail biopsies. Pggt1b was genotyped using forward primer CCTGAATGCAGATCTGTGGA and reverse primer CCTATGAAAGCAGCACGAGA. The Pggt1b^{+} and Pggt1b^{flx} alleles yielded 280 bp and 360 bp products, respectively. Sprr2f-cre^{+} was genotyped using forward primer GGTACACACGTCCTGGAAT and reverse primer TTCCCATTCTAACAACAGG to yield a 165 bp product. To verify the presence of DNA in the reaction, an internal control was used with forward primer CTAGGCCACAGAATTGAAAGATCT and reverse primer GTAGGTGGAAATTCTAGCATCATCC, to yield a 320 bp product. Pten was
genotyped using forward primer GAATGCCATTACCTAGTAAAGCAAGG, forward primer GGGTTACACTAAACTAACGAGTCC, and reverse primer GAATGATAATAGTACCTACTTCAG. The \( \text{Pten}^+ \) allele gave a 220 bp band and the \( \text{Pten}^{\Delta 4-5} \) allele gave a 280 bp band.

**Real-time quantitative PCR**

RNA was extracted from cells or flash-frozen tissues using Trizol, and cDNA was synthesized. The wild-type expression of \( \text{Pggt1b} \) was determined using the forward primer GTGGGCATGGATATGAAGAAAG and the reverse primer CACCCAGAAGGATAGCAGGTGT. The normalization genes used in this experiment were ActB (forward primer GCTTTTGCAGCTCCTTCGTT; reverse primer CAGGTCAGGATACCTCTCTTGCT), Cltc (forward primer GGAACTGCGGAAGATTTGGAAG; reverse primer GCGAGGTGAACAGGAGGATAG), Gapdh (forward primer CAGGGCAAGTGGAGATTG; reverse primer CGGAGATGATGACCCTTTTGG), Tbp (forward primer TGACTCCTATGACCCCTACTCC; reverse primer TGGTCTCTGACCTGCTCTGT), and 18sRNA (forward primer CGCAGCTAGGAAATAATGGAATAG; reverse primer CAGTCGCCATGTTATGG). The population standard deviation was calculated for each normalization gene across all of the organs. The raw weight was designated as \( 1/(\text{standard deviation}) \). The weight was designated as \( (\text{raw weight}/\Sigma(\text{raw weight}))n \). The \( n \)th root of \( \Sigma(\text{average of triplicate})(\text{weight}) \) was used as the Ct reference value to calculate the \( \Delta \text{Ct} \). The fold change was calculated as \( 2^{\Delta \text{Ct}} \).

**Immunoblot**

The PGGT1B antibody used in this project was purchased from Abcam; the PTEN antibody was purchased from Cell Signaling Technology; and the GAPDH antibody was purchased from Santa Cruz Biotechnology. All fluorescent secondary antibodies were purchased from LI-COR Biosciences, and all blots were read on a LI-COR fluorescent imager.

**PTEN quantification**

The Vectra 3.0 microscope was used to take 40x images of each cross-section of uterus stained for PTEN. The endometrium in each section was manually selected, and the inForm software was used to partition the cells into nuclei and cytoplasm and quantify the PTEN stain in the cytoplasm into 10 bins. An H-score was calculated for each image by taking H-score=\([0(\% \text{ cells in bin } 1)]+(1(\% \text{ cells in bin } 2))+\ldots+(9(\% \text{ cells in bin } 10))]\times100\), giving a number between 1 and 900 that reflected the intensity of PTEN staining in each image. Between 20 and 120 images were quantified for each mouse, depending on the size and quality of the uterus cross sections. The H-scores of each image were graphed as box-and-whisker plots for each slide.
Figure 1. Preliminary experiments. A) Scheme for the PTEN stability screen. Figure Verónica Bravo. B) qPCR for *Pggt1b* expression levels in thirteen organs of interest. Data were normalized to a weighted average of five reference genes.
Figure 2

A

Endometrium 20x

Endometrium 10x

Fibroblasts 10x

B

C

Fold change in exon 6

D

Melt Curve
Figure 2. Confirmation of knockout of *Pggt1b*. A) CK-8 and DAPI staining in isolated murine endometrial cells to determine purity. CK-8 is an epithelial marker. Fibroblasts were used as a negative control. Arrows indicate cells in the endometrium that are positive for DAPI but not CK-8. These cells may represent fibroblast contamination from the stroma. B) qPCR primer scheme. C) qPCR to show the absence of exon 6 in the endometrium of a *Pggt1b*\textsuperscript{flx/flx}:*Sprr2f-cre*\textsuperscript{+} mouse. The reaction uses primers 2F+2R, and results are normalized to total *Pggt1b* using the primers 1F+1R. The high fold change in the *Sprr2f-cre*\textsuperscript{+} endometrium could indicate contamination or a less functional cre. D) The melt curve for the reaction with primers 3F+2R shows two peaks in the *Pggt1b*\textsuperscript{flx/flx}:*Sprr2f-cre*\textsuperscript{+} endometrium, indicating the presence of the intact *Pggt1b*\textsuperscript{flx/flx} gene, which could indicate contamination by other epithelial cells, or that the cre is not as functional as expected. Orange, *Pggt1b*\textsuperscript{flx/flx}; yellow, *Pggt1b*\textsuperscript{flx/flx}:*Sprr2f-cre*\textsuperscript{+}. E) Confirmation of the conditional deletion of *Pggt1b* in the endometrium via IHC for PGGT1B. The oviducts are used as a positive control. The staining in the luminal epithelium of the uterus is nearly absent in *Pggt1b*\textsuperscript{flx/flx}:*Sprr2f-cre*\textsuperscript{+} mice. F) Immunoblot of extracts from *Pggt1b*\textsuperscript{flx/flx} MEFs infected with pBP and pBP-cre, with antibodies against GAPDH and PGGT1B.
Figure 3. Preliminary pathology of Pgt1b\textsuperscript{flx/flx}:Pten\textsuperscript{+/+}:Sprr2f-cre\textsuperscript{+} and Pgt1b\textsuperscript{flx/flx}:Pten\textsuperscript{Δ4-5/+}:Sprr2f-cre\textsuperscript{+} mice. A) Genotyping PCR reactions for i) Pgt1b, ii) Sprr2f-cre, iii) Pten. B) Examples of uterine lesions in Pgt1b\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+} and Pgt1b\textsuperscript{flx/flx}:Sprr2f-cre\textsuperscript{+}:Pten\textsuperscript{Δ4-5/+} mice. C) Histogram representing pathology of the first cohort of Pgt1b\textsuperscript{flx/flx}:Pten\textsuperscript{+/+}:Sprr2f-cre\textsuperscript{+} and Pgt1b\textsuperscript{flx/flx}:Pten\textsuperscript{Δ4-5/+}:Sprr2f-cre\textsuperscript{+} mice. CIS, carcinoma in situ. n = 14. D) Immunoblot of extracts from Pgt1b\textsuperscript{flx/flx} MEFs infected with pBP and pBP-cre, with antibodies against PTEN and GAPDH.
Figure 4

A

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- carcinoma
- CIS
- hyperplasia
- unremarkable

B

- unremarkable
- hyperplasia
- carcinoma in situ
- carcinoma
Figure 4. Pathology of \( \text{Pggt1b}^{\text{flx/flx}}:\text{Sprr2f-cre}^+ \) and \( \text{Pggt1b}^{\text{flx/flx}}:\text{Sprr2f-cre}^+:\text{Pten}^{\Delta4-5/+} \) mice. A) Histogram representing pathology of the first and second cohort of \( \text{Pggt1b}^{\text{flx/flx}}:\text{Sprr2f-cre}^+ \) and \( \text{Pggt1b}^{\text{flx/flx}}:\text{Sprr2f-cre}^+:\text{Pten}^{\Delta4-5/+} \) mice together. CIS, carcinoma in situ. \( n = 40 \). B) Examples of PTEN staining in the endometrium. C) Quantification of PTEN staining in the endometrium. The H-scores for every image of a slide were graphed together as a box-and-whisker plot that quantified the intensity of the PTEN stain in the cytoplasm. The red line represents \( \text{Pggt1b}^{\text{flx/flx}}:\text{Pten}^{+/+} \) mice; yellow is \( \text{Pggt1b}^{\text{flx/flx}}:\text{Pten}^{+/+}:\text{Sprr2f-cre}^+ \); green is \( \text{Pggt1b}^{\text{flx/flx}}:\text{Pten}^{+/+}/\Delta4-5 \); blue is \( \text{Pggt1b}^{\text{flx/flx}}:\text{Pten}^{+/+}/\Delta4-5:\text{Sprr2f-cre}^+ \). D) Representative pictures of vaginal cytology showing the proestrus, estrus, metestrus, and diestrus stages (figure Cecilia Cuitíño).
Figure 5. \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs. A) \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs were infected with pBP and pBP-cre vectors. Immunoblots were performed against PTEN, PGGT1B, and GAPDH. There was no difference in PTEN protein levels, and the larger band seen previously in the \( \text{Pggt1b}^{\text{flx/flx}:\text{cre}^+} \) cells was not observed. B) \( \text{Pggt1b}^{\text{flx/flx}} \) MEFs infected with pBP and pBP-cre vectors were harvested 7, 8, or 9 days post-infection. Immunoblots were performed against PTEN, PGGT1B, and GAPDH. There was no difference in PTEN protein levels, and the larger band was not observed.
References


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