Prostaglandin D₂ inhibits wound-induced hair follicle neogenesis through the receptor, Gpr44

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Abstract

Prostaglandins (PGs) are key inflammatory mediators involved in wound healing and regulating hair growth; however, their role in skin regeneration after injury is unknown. Using wound-induced hair follicle neogenesis (WIHN) as a marker of skin regeneration, we hypothesized that PGD₂ decreases follicle neogenesis. PGE₂ and PGD₂ were elevated early and late respectively during wound healing. The levels of WIHN, lipocalin-type prostaglandin D₂ synthase (Ptgds) and its product PGD₂ each varied significantly among background strains of mice after wounding and all correlated such that the highest Ptgds and PGD₂ levels were associated with the lowest amount of regeneration. Additionally, an alternatively spliced transcript variant of Ptgds missing exon 3 correlated with high regeneration in mice. Exogenous application of PGD₂ decreased WIHN in wild type mice and PGD₂ receptor Gpr44 null mice showed increased WIHN compared to strain-matched control mice. Furthermore, Gpr44 null mice were resistant to PGD₂-induced inhibition of follicle neogenesis. In all, these findings demonstrate that PGD₂ inhibits hair follicle regeneration through the Gpr44 receptor and imply that inhibition of PGD₂ production or Gpr44 signaling will promote skin regeneration.
**Introduction**

Scar formation and tissue regeneration are opposite results of the wound healing process. While fibrosis is more common after skin injury, full skin regeneration results in complete replacement of adnexa and function. Examples of tissue regeneration in mammalian systems include annual regeneration in deer antlers, ear regeneration following tag removal in mice and rabbits, and regeneration of amputated digit tips and liver regeneration in both mice and humans (Han et al., 2005; Jia, ; Metcalfe et al., 2006; Price et al., 2005). In skin, Ito and Cotsarelis fully described and characterized de novo hair follicle neogenesis that is dependent on Wnt signaling after full-thickness wounding in mice. These regenerated hair follicles establish a stem cell population, express hair follicle-differentiation markers, produce a functional hair shaft, successfully transition through all phases of the hair cycle and include associated structures such as sebaceous glands (Ito et al., 2007). However, what triggers mammalian regeneration is not completely understood. Determining which factors regulate this process may reveal mechanisms and lead to specifically-designed therapies to enhance regeneration.

Prostaglandins (PGs) are lipid signaling molecules enzymatically derived from arachidonic acid that function in both an autocrine and paracrine manner to regulate broad functions. Prostaglandin-endoperoxidase synthase 2 (Ptgs2; prostaglandin G/H synthase, cyclooxygenase 2 (Cox2)) is a key enzyme in the prostaglandin biosynthesis pathway, converting arachidonic acid to prostaglandin H\(_2\), from which prostacyclin, thromboxane A2, PGD\(_2\), PGE\(_2\) and PGF\(_2\alpha\) are produced by specific synthase enzymes. Individual PGs often have opposing biological effects. For example, in the lung, PGE\(_2\) causes relaxation, while PGD\(_2\) causes contraction of bronchial muscle (L. S. Goodman, 1996).

Recent studies demonstrate that PGs regulate hair growth. Increases in prostaglandin levels within the epidermis through overexpression of Ptgs2 cause alopecia, sebaceous hyperplasia and may or may not cause a predisposition to squamous cell tumors (Bol et al., 2002; Muller-Decker et al., 1998; Neufang et al., 2001). Prostaglandin D\(_2\), E\(_2\) and F\(_2\alpha\), metabolism and signaling proteins are expressed in the hair follicle (Colombe et al., 2007; Garza et al., 2012). The FDA-approved PGF\(_2\alpha\) analog, bimatoprost, is used clinically to enhance hair growth of human eyelashes (Johnstone and Albert, 2002). PGE\(_2\) and PGF\(_2\alpha\) enhance hair growth in mice (Geng et al., 1992; Sasaki et al., 2005). In contrast, PGD\(_2\) inhibits hair growth in humans and mice (Garza et al., 2012), demonstrating the opposing functions of prostaglandins. PGD\(_2\) levels are significantly increased in bald scalp compared to haired scalp of patients with male pattern hair loss. Moreover, topically applied exogenous PGD\(_2\) inhibits the hair growth of mice and explants of human hair follicles through the PGD\(_2\)-GPR44 signaling pathway (Garza et al., 2012). These results imply a regulatory network in hair follicles wherein PGD\(_2\) inhibits, while PGE\(_2\)/F\(_2\alpha\) promotes hair follicle function.

PGs are key inflammatory mediators involved in wound healing; however no study has examined their role in skin regeneration. We hypothesized that, given their presence during wound healing, PGs would impact wound-induced hair neogenesis (WIHN). With reports of PGE\(_2\) promoting regeneration (Goessling et al., 2009), we further hypothesized that PGD\(_2\) would inhibit regeneration. In this study, we characterize the fluctuations of prostaglandins...
throughout wound healing and demonstrate that levels of PGD$_2$ are inversely correlated with WIHN. Furthermore, we define an alternatively spliced transcript variant of lipocalin-type prostaglandin D$_2$ synthase (Ptgds, L-pgds) that correlates with the regeneration phenotype among several strains of mice. We demonstrate that PGD$_2$ inhibits hair follicle regeneration through the Gprotein coupled receptor Gpr44 (DP-2).

Results

PGD$_2$ and PGE$_2$ are expressed in reciprocal patterns during wound healing

PG levels during incisional wounding in mice using enzyme immunoassays have been previously published (Kapoor et al., 2007). In our study, we measured PGs during full-thickness excisional wounding in mice by mass spectrometry (Bell-Parikh et al., 2003). In addition to the absolute PG levels, the expression of PG synthase enzymes was assessed by qRT-PCR.

Anesthetized adult C57Bl/6J mice were wounded with a 1cm$^2$ full-thickness dorsal skin excision down to the level of skeletal muscle on post-natal day 21 (p21). The epidermis and dermis at the wound edge were sampled at various timepoints from pre-injury to 15 days postinjury. Histology revealed the emergence of an inflammatory infiltrate by post-wound day (PWD) 5. The healed wounds at PWD14 showed thickened epidermal and dermal layers compared to unwounded normal skin (Fig 1a). PGE$_2$ and PGF$_2\alpha$ levels increased early in wound healing while PGD$_2$ increased during the later stages of wound healing (PWD8 and onward) (absolute levels, Fig 1b; relative to baseline, 1c). Matching the relative levels of their products, mRNA levels of prostaglandin synthase enzyme for PGE$_2$ (Ptges) are significantly elevated during the early phases of wound healing while PGD$_2$ synthase (Ptgds) levels are elevated during the later stages of healing (Fig 1d).

PGD$_2$ levels inversely correlate with wound-induced hair neogenesis (WIHN)

To investigate whether PGD$_2$ inhibits hair follicle regeneration after wounding, we examined PGD$_2$ synthase and actual PGD$_2$ levels according to regeneration phenotype using three distinct background strains of mice: C57Bl/6J, FVB/N and Mixed (C57Bl/6J × FVB/N × SJL). Anesthetized adult mice (p21) were wounded with a 1cm$^2$ full-thickness dorsal wound as above. The wound was allowed to heal by contraction and re-epithelialization, which was complete approximately 12 days after wounding. The resulting visible scar was 2–4mm$^2$ in size. The numbers of regenerated hair follicles within the scar were detected by whole-mount keratin 17 immunohistochemistry on isolated epidermis between PWD 20–24. Hair follicles formed within a discrete area of the center of the scar (Fig 2a). Histology of K17-positive de novo hair follicles was confirmed with cross-sectional immunohistochemistry; adjacent sebaceous glands are also noted (Supp Fig 1).

Distinct levels of follicle regeneration were quantified among the different mouse strains, similar to published studies (Ito et al., 2007). C57BL/6J mice had the least amount of follicle regeneration (~5 follicles) while the Mixed strain background had a mean of 20 follicles, a 4-fold increase in follicle regeneration (Fig 2b). Next, we examined PGD$_2$ synthase (Ptgds) levels among strains. Prior to the appearance of regenerated hair follicles at
PWD20, mRNA levels of Ptgds were measured on ~PWD12 in the re-epithelialized skin. Ptgds levels negatively correlate with regeneration when strain backgrounds are compared. C57Bl/6J mice had the highest level of Ptgds mRNA expression and the lowest amount of follicle neogenesis (Fig 2c). Conversely, the Mixed strain had the lowest Ptgds mRNA expression and the highest amount of follicle neogenesis. Levels of regeneration and Ptgds were both intermediate in FVB.

Next we measured the individual levels of PGD$_2$, PGE$_2$ and PGF$_{2\alpha}$ in re-epithelialized skin at PWD12 by mass spectrometry. The capacity to make PGD$_2$ corresponded to the degree of expression of Ptgds across the strains; PGD$_2$ levels were greatest within C57Bl/6J mice, intermediate within FVB/N and lowest in Mixed strain mice (Fig 2d). The levels of PGE$_2$ and PGF$_{2\alpha}$ did not display a similar pattern and the levels of PGF$_{2\alpha}$ in skin were 10-fold less than both PGD$_2$ and PGE$_2$ (Fig 2e, 2f). PGE$_2$ or PGF$_{2\alpha}$ did not correlate with regeneration ability across all strains. With no clear positive or negative correlation to the regeneration phenotype in the mouse strains, analysis was not continued for PGE$_2$ or PGF$_{2\alpha}$.

These results demonstrated that the PGD$_2$ pathway, but not the PGE$_2$ or PGF$_{2\alpha}$ pathway correlates with the degree of wound-induced hair neogenesis; regeneration ability was inversely related to PGD$_2$ levels.

Ptgds splice variant missing exon 3 correlates with low regeneration

Given that Ptgds mRNA expression and its product PGD$_2$ negatively correlated with the observed follicle regeneration among the strains of mice, we focused our investigation on understanding the location and expression of Ptgds. We localized Ptgds mRNA and protein within re-epithelialized skin (PWD12) from C57Bl/6J mice. For detection of RNA, we enzymatically separated epidermis from dermis and quantified Ptgds mRNA levels by qRT-PCR on each fraction. Ptgds was more abundant in epidermis than dermis (Fig 3a). Ptgds was detected by immunohistochemistry predominantly in the scar epidermis, within the cytoplasm of keratinocytes, rather than dermis (Fig 3b). There is precedence for Ptgds expression within epithelial tissues of the retina and gastric mucosa as well as in keratinocytes of the skin (Black et al., 2010; Garza et al., 2012; Hokari et al., 2009; Takeda et al., 2006). Consistent with the sporadic expression of Ptgds we detected in the dermis, fibroblasts can express Ptgds during injury and serve as a source of PGD$_2$ (Hokari et al., 2009).

Noting no discernable differences in the location of Ptgds protein between mouse strains and unable to detect changes in protein expression by immunoblotting, we investigated the possibility of alternative splicing of Ptgds as a possible explanation for lower levels of Ptgds mRNA and its resulting product, PGD$_2$. We investigated expression of Ptgds splice variants in re-epithelialized skin at PWD12 of C57Bl/6J, FVB/N and Mixed strain mice using Affymetrix exon probes followed by analysis with Partek Genome Studio software. We discovered that expression of exon 3 of the Ptgds gene varied significantly among our strains. Exon 3 expression was most abundant in C57Bl/6J and least expressed within Mixed strain mice. FVB/N mice demonstrated an intermediate level of exon 3 expression (Fig 3c). Thus, the expression of exon 3 was inversely correlated with the observed follicle regeneration in each strain (Fig 2a, 2b). Exons 1, 4 and 5 also had differential splicing
correlating to regeneration ability, but these differences were of much smaller magnitude; thus, we focused our further efforts on exon 3. These findings for exon 3 were confirmed with PCR using cDNA from PWD12 re-epithelialized skin in separate animals. PCR primers (Primer Set 1, Supp Fig 2) not in common with the Affymetrix probe were selected to border exon 3 (located within exon 2 and exon 4), thus detecting the presence or absence of exon 3 (Fig 3d). Two additional independent primer sets (Primer set 2 & 3; Supp Fig 2) were also used to validate these results. Sanger sequencing was performed on the PCR products (Primer Sets 1 and 2) and a portion of Ptgsd exon 3 is absent as noted by analysis using BLAST software (Fig 3f). Furthermore, in Mixed strain mice, additional smaller bands were detected by PCR with Primer Set 3 (Supp Fig 3). DNA sequencing confirmed the absence of Exon 2; thus, we identified a second splice variant of Ptgsd, (Supp Fig 3).

In summary, using Affymetrix probes followed by three independent sets of primers for traditional PCR with DNA sequencing, we corroborated our finding that C57Bl/6J mice have a higher proportion of non-spliced Ptgsd, while our Mixed strain has a higher proportion of Ptgsd missing exon 3 (Fig 3d, 3e, 3f). In summary, Ptgsd is preferentially spliced in strains of mice with higher levels of follicle regeneration.

**PGD2 inhibits wound-induced hair follicle neogenesis through Gpr44**

Finally, we directly investigated the effect of exogenous PGD2 on regeneration. First, we investigated whether PGD2 inhibits follicle neogenesis in wild type mice. Due to higher mean levels of follicle regeneration, Mixed strain mice were used for these experiments. After wounding, PGD2 was topically applied to healing wounds, beginning at PWD7 through two days post scab detachment. De novo hair follicles were assessed as in Fig 2a. Exogenous PGD2 decreased follicle regeneration in wild type mice compared to controls (Fig 4a).

More convincing evidence of PGD2 inhibition of follicle regeneration was sought using knockout mouse models. PGD2 activity is mediated via binding to two G protein coupled receptors: prostaglandin D2 receptor (Ptgdr, DP, DP-1) and Gpr44 (Crth2; DP-2) (Ricciotti and FitzGerald, 2011). The role of PGD2 signaling was investigated using individual PGD2 receptor knockout mice, Ptgdr−/− and Gpr44−/− as well as strain-matched (C57Bl/6J) control mice. Mice were wounded as above, with counting of de novo follicles on PWD20 as a measure of regenerative capacity. qRT-PCR determined that each of the two receptors was expressed at similar levels in wild type mice in normal skin as well as within healed scars. Previous studies demonstrated Ptgdr and Gpr44 expression within epidermal and follicular keratinocytes (Colombe et al., 2008). Mice lacking Ptgdr or Gpr44 receptors are phenotypically normal, with no observed skin, hair follicle or hair cycling defects. There was no difference in follicle regeneration after wounding between control mice and Ptgdr−/− mice. However, in mice lacking Gpr44, follicle regeneration was significantly increased (~2-fold) compared to wild type and Ptgdr−/− mice; mice that were heterozygous for Gpr44 had an intermediate level of regenerated follicles, but were not significantly different from wild type or knockout mice (Fig 4b).

We further examined the impact of PGD2 on mice that are heterozygous or null for Gpr44. Using additional cohorts of heterozygous and Gpr44−/− mice, we evaluated the effect of
PGD$_2$ on WIHN. PGD$_2$ was topically applied to healing wounds, beginning at PWD7 through two days post scab detachment. Exogenous PGD$_2$ did not significantly affect follicle regeneration in $Gpr44^{+/−}$ or $Gpr44^{-/-}$ mice compared to vehicle control (Fig 4c). As before, follicle regeneration with vehicle control was increased in null mice when compared to $Gpr44^{-/-}$, although not significantly.

The application vehicle for PGD$_2$ used in Figure 4c influenced follicle regeneration; vehicle increased the number of regenerated follicles in scars by an average of 9 follicles compared to non-vehicle treated mice ($n>10$; $p<0.05$) for $Gpr44$ null mice. Therefore, comparisons cannot be made between figure panels b and c. Also, results in Figure 4a cannot be compared to Figure 4b or 4c due to inherent variation in the strains.

Together, the ability of exogenous PGD$_2$ to inhibit WIHN in wild type mice, the increased level of WIHN in $Gpr44$ null mice, and the resistance to PGD$_2$ inhibition of WIHN in $Gpr44^{-/-}$ mice, collectively demonstrate that the mechanism for PGD$_2$ inhibition of WIHN occurs through $Gpr44$ activation.

**Discussion**

Wound healing usually results in inadequate tissue repair by scarring or fibrosis. In some cases, however, tissue regeneration can occur. Our understanding of the control of wound scarring versus tissue regeneration is incomplete. The contribution of inflammatory mediators, including prostaglandins, during the wound healing process is well-established. PG functions during tissue regeneration are less studied, but it is known that PGE$_2$ can stimulate liver regeneration (Goessling et al., 2009). Given the demonstration of increased hair growth by PGF$_2α$, and PGE$_2$ (Geng et al., 1992; Sasaki et al., 2005) as well as decreased hair growth by PGD$_2$ (Garza et al., 2012), we hypothesized that these prostaglandins may be important in regulating hair neogenesis in WIHN. Normal hair growth is regulated by transition between catagen, anagen and telogen phases of the hair cycle. During anagen, the hair follicle partially regenerates, suggesting that pathways which control the hair regeneration cycle may also control hair follicle neogenesis. In this manuscript, we demonstrated that PGD$_2$ correlates with decreased follicle regeneration after wounding, that PGD$_2$ has the capacity to inhibit follicle regeneration and that the mechanism of this inhibition is through the $Gpr44$ receptor.

One previously unreported discovery is that $Ptgds$ is alternatively spliced, such that partial removal of exon 3 correlates with higher levels of regeneration. The absence of exon 3 likely affects the functionality of the final product. The structure of $Ptgds$ is that of the typical lipocalin $β$-barrel comprised of eight anti-parallel $β$-barrel strands, three $α$ helical regions and a C-terminal $β$-strand. $Ptgds$ is the only enzyme within the lipocalin family of proteins; it catalyzes the formation of PGD$_2$ from PGH$_2$. Like other lipocalin family members, $Ptgds$ also functions as a transport protein for lipophilic compounds like retinoids, gangliosides, bilirubin and $β$-amyloid peptides (Akerstrom et al., 2000). Exon 3 of $Ptgds$ encodes amino acids 86 through 110 of the protein sequence, forming the D and E strands (Shimamoto et al., 2007). The E strand, together with the F strand, forms the flexible E-F loop responsible for the open/closed formations of the calyx. Based on our sequence results,
20 amino acids are removed from exon 3, including Leu96 and Cys89. Leu96 in exon 3, with its bulky side chain, along with other nearby hydrophobic amino acids, acts to divide the central cavity into two compartments: one for binding and converting PGH2 to PGD2, while the other compartment binds lipophilic compounds, such as retinoids (Kumasaka et al., 2009). Cys89 in exon 3 forms a disulfide bridge with Cys186, which is important in stabilizing the protein structure. In all, exon 3 encodes amino acids involved in central cavity division, open/closed conformation formation and protein stability.

We also identified a splice variant of Ptgds that completely lacks exon 2 (Supp Fig 3). Four key serine, threonine and cysteine moieties are located within exon 2 and mutations within these residues dramatically decrease Ptgds enzymatic activity (Shimamoto et al., 2007). Alternative splicing of Ptgds, such that portions of exon 2 or exon 3 are absent may result in decreased functionality of Ptgds and contribute to the lower level of PGD2 found in the strains expressing spliced Ptgds. In support of this hypothesis, an alternative splice variant of cyclooxygenase 1 (Cox-1) lacking 37 amino acids in exon 9, results in no detectable PGH2 product (Schneider et al., 2005). Conversely, splice variants of receptor and enzymes can exert dominant negative effects over their complete signaling and catalytically active forms (Stamm et al., 2005).

We also found that during wound healing, the capacity of tissue to generate PGE2 and PGD2 is separated over time, thus providing evidence consistent with the distinct functions of prostaglandins. These results are similar to those observed after incisional wound healing in DBA/1 mice (Kapoor et al., 2007). In C57Bl/6J and DBA/1 strains and models of wound healing, PGE2 is the more abundant product during the early phases of wound healing. Elevated levels of PGE2, a potent immune activator, are consistent with progressing inflammation (Sakata et al.). Whereas, at later stages when inflammation is resolving, PGE2 levels taper off and PGD2 becomes the predominant prostaglandin.

Our final previously unreported finding was that the mechanism of PGD2 inhibition of wound-induced hair neogenesis is through the Gpr44 receptor. Gpr44 is expressed on immune cells including eosinophils, neutrophils, mast cells, basophils, a subpopulation of memory Th2 cells and monocytes (Nagata et al., 1999a; Nagata et al., 1999b). It mediates the chemotaxis of these pro-inflammatory cells during allergic inflammation (Hirai et al., 2001). Our results show that in the absence of Gpr44, WIHN is increased in our experimental wound model, which suggests that follicle regeneration is possible in the absence of this pro-inflammatory milieu of cells. Likewise, WIHN is suppressed in wild type mice in the presence of PGD2, arguing that the presence of pro-inflammatory mediators inhibits regeneration.

While these data suggest that Gpr44 normally inhibits WIHN through recruitment of inflammatory cells, alternative interpretations are possible. Gpr44 null mice show features of both enhanced and decreased airway inflammation (Chevalier et al., 2005; Shiraishi et al., 2008). Conflicting data in the literature demonstrates that PGD2 both enhances and reduces allergic responses, with the Gpr44 receptor playing a critical role (Arimura et al., 2001; Hammad et al., 2007; Matsuoka et al., 2000; Matsushima et al., ; Satoh et al., 2006;
Shiraishi et al., 2008; Trivedi et al., 2006; Yamamoto et al.). It is therefore also possible that PGD$_2$’s actions through Gpr44 may inhibit pro-regenerative immune factors.

A motivation for this study is the ability of PGD$_2$ to inhibit hair lengthening (Garza et al., 2012). Here we demonstrate that PGD$_2$ also inhibits hair regeneration after wounding. Thus, PGD$_2$ and Gpr44 inhibition of the hair follicle occurs in multiple contexts and may be exploited in future therapies. Pharmaceutical companies are already focused on the development of Gpr44-selective antagonists for the treatment of asthma with at least nine other known Gpr44 antagonists in Phase II clinical trials (Jones et al., 2009; Norman, 2010; Pettipher and Whittaker). In addition to previous studies suggesting that Gpr44 antagonists may be beneficial in androgenetic alopecia, our results suggest that formulations of Gpr44 antagonists may decrease scarring during wound healing. A specific example is ramatroban, an orally-active, dual Gpr44 and thromboxane A2 receptor antagonist, which is approved in Japan for the treatment of allergic rhinitis in humans (Sugimoto et al., 2003). Future studies could examine the effect of ramatroban in stimulating hair follicle neogenesis.

Materials and Methods

Animals

All animal protocols are approved by the Johns Hopkins University Animal Care and Use Committee. C57Bl/6J, FVB/N and Mixed strain (C57Bl/6J × FVB/N × SJL/J) animals were obtained from The Jackson Laboratory and George Cotsarelis (UPENN). Ptgdr$^{-/-}$, Gpr44$^{+/+}$, Ptgs$^{-/-}$ knockout mice were obtained from original sources as previously described (Garza et al, 2012). Heterozygous mice for Gpr44 were bred by crossing WT and Gpr44$^{+/+}$ animals; genotype was verified by PCR with the following primers: 5’-CTC-GCC-GGA-CAC-GCT-GAACTT-GT-3’, 5’-TGG-GGT-CAA-ACT-CAG-CTC-CTC-ACG-3’ and 5’-GCG-GCG-GCT-AACAAG-TCG-GAT-AG-3’. All animal colonies were maintained within animal facilities with standard humidity, 12 hour light/dark cycle and laboratory diet ad libitum. Both male and female mice were used in our experiments and gender did not impact our results.

Wound Induced Hair Neogenesis (WIHN) Assay

A 1 cm$^2$ full-thickness wound on the backs of 21-day old male and female mice was performed as previously described (Ito et al., 2007). Scars were harvested 8–12 days after the scab detached from the wound (PWD20-24). This time point represents approximately 16 days after contraction has ended and approximately 14 days after re-epithelialization has occurred. The dermis and epidermis were separated using overnight EDTA treatment and stained for keratin 17 (Abcam Inc, Cambridge MA) on whole mount epidermis to identify regenerated hair follicles. Numbers of regenerated follicles were quantified in the re-epithelialized skin as published (Ito et al., 2007).

Mass Spectrometry

Baseline tissue, a 1-cm$^2$ piece of full-thickness skin, was taken from each animal at wounding. Wound edges from 1 hour, 1 day, 3 days and 5 days post-wounding and re-epithelialized tissue (healed scar) at PWD12, PWD14 and PWD16 were collected for.
analysis. All samples were collected in acetone, frozen in liquid nitrogen and stored at
−80°C. Prostaglandins were isolated from samples by tissue homogenization in acetone for
90 seconds. Samples were centrifuged at 13,500 rpm at 4°C for 10 minutes and the resulting
supernatant was assessed for PGD₂, PGE₂ and PGF₂α levels by mass spectrometry as
described (Garza et al., 2012).

**Quantitative real-time PCR (qRT-PCR)**

The levels of gene expression were accessed by qRT-PCR in a parallel time course to that of
mass spectrometry. For mRNA analysis, samples were collected and stored in RNA Later™
(Sigma, St Louis MO) at −20°C. Early time points contained wound-edge only (~1–2mm
border) and time points after re-epithelialization consisted of the “scar area” only. Samples
were homogenized using a tissue grinder, processed with RNasy Fibrous Tissue kit
(Qiagen, Valencia, CA) and transcribed to cDNA (High Capacity RNA to cDNA; Applied
Biosystems, Carlsbad, CA). qRT-PCR was performed on samples (50ng cDNA) for genes of
interest using inventoried TaqMan gene expression assays from Applied Biosystems.
Differences in gene expression were assessed by comparative ΔΔCₚ values with fold change
calculations.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed paraffin-embedded mouse skin
sections using the avidin-biotin complex method and AEC development (ABC kit and AEC
Substrate kit for Peroxidase; Vector Laboratories). Baseline and ~PWD12 healed scars were
subjected to deparaffinization, rehydration and antigen retrieval with TRILOGY buffer (Cell
Marque) prior to immunohistochemistry. Sections were incubated overnight with rabbit
polyclonal Ptgds antibody (LifeSpan Biosciences Inc. Seattle, WA). For Supplemental
Figure 1, Cytokeratin 17 (Abcam, Cambridge, MA) was used. Negative controls were
prepared with purified rabbit IgG antibody (Invitrogen, Camarillo, CA). Sections were
counterstained with hematoxylin using standard procedures.

**Ptgds splice variant analysis**

Splice variant analysis among mouse strains within *Ptgds* gene were identified and analyzed
by Partek Genome Studio software (Partek INC., Saint Louis MO, USA). Splice variant
expression within the third exon was confirmed by standard PCR and agarose gel
electrophoresis. Primers are as follows: sense 5’ AGGGCCATGACACAGTGCAGC and
antisense 3’ GAGGGTGCCGATCGGAAAGTC (Primer Set 1) which identifies a 371 base
pair amplicon for the complete exon 3 compared to a 300 base pair amplicon if exon 3 is
affected. These initial PCR results were confirmed by two additional primer sets: Primer Set
2 with amplicons 371 and 300 respectively, 5’
AGGGCCATGACACAGTGCAGCCTTCACTTTCT and 3’
GAGGGTGCCGATCGGGAAGTCCTGCTGGGCTTGGG; and Primer Set 3-5’
AAGACAAGTTTCTGGGGCGCTG and 3’-GTGGATGCTGCCCAGTGAGG (amplicons
of ~240 versus ~180 with exon 3 splicing). See Supplemental Figure 3 for all primer
locations. cDNA was subjected to PCR with PCR MasterMix (Promega, Madison WI) and
Veriti Thermal Cycler (Applied Biosystems INC, Carlsbad CA). Relative quantification of PCR results was assessed by Image J software.

**Topical PGD2 treatment**

PGD2 (Cayman Chemical, Ann Arbor, MI), was reconstituted in ethanol at a concentration of 50mg/mL. 10µg PGD2 in ethanol/5% polyethylene glycol/2% glycerol was applied daily beginning on PWD7 and continued daily until two days post scab detachment. Ethanol/polyethylene glycol/glycerol alone was applied in parallel for vehicle control. Regenerated hair follicles were assessed by K17 IHC at PWD20-24.

**Statistical Analysis**

Each experiment was repeated with at least 3 independent litters of animals. Numbers of independent animals are noted for each experiment. Data was analyzed using paired t-test or ANOVA single factor. Statistical significance was considered at p < 0.05.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PGD2 and PGE2 are expressed in reciprocal patterns during wound healing
(a–e) Full excision skin wounding to the depth of skeletal muscle was performed on
C57Bl/6J mice. On the listed days, tissue from the wound edge was sampled for
measurement (a) Hematoxylin and eosin histology of normal (baseline), 6-hour wound edge,
PWD5 and 2 days after scab detached (PWD14); wound edges shown with dashed line
(yellow). Scale bar = 100µm (b–c) Levels of PGD2, PGE2 and PGF2α measured by mass
spectrometry, depicted in either (b) absolute ng/g tissue or as (c) fold change in ng/g tissue.
relative to baseline; n = 3; p < 0.05. (d) mRNA expression of Ptgsd1 and Ptges1 as determined by qRT-PCR. n = 3; p < 0.05.
Figure 2. Prostaglandin D₂ levels negatively correlate with hair follicle regeneration
(a–e) Full excision skin wounding as described in Fig 1. was performed on C57Bl/6J, FVB/N and Mixed strain mice. (a) Hair follicle regeneration was assessed by K17 immunohistochemistry on isolated epidermis at PWD 20–24. Representative images are shown: scar outlined in blue, WIHN area within red. Scale bar: 100µm. (b) Quantification of regenerated follicles within scars by strain; n ≥ 33, p < 0.05. (c) qRT-PCR of Ptgds mRNA levels (shown as fold change) in PWD12 re-epithelialized skin; n = 3, p < 0.05. (d–f) Mass
spectrometry measurement of individual prostaglandins in re-epithelialized skin at PWD12. 
(d) PGD$_2$; (e) PGE$_2$; and (f) PGF$_{2\alpha}$; n > 3; p ≤0.05.
Figure 3. Splicing of Exon 3 in Ptgsd positively correlates with hair follicle regeneration

(a–e) Newly re-epithelialized skin (~PWD12) after WIHN was excised then examined with qRT-PCR and immunohistochemistry. (a) C57Bl/6 skin was enzymatically separated into epidermis and dermis for qRT-PCR of Ptgsd mRNA, n = 5–6 samples; p < 0.05. (b) Immunohistochemistry on C57Bl/6 skin with Ptgsd polyclonal rabbit antibody and isotype control antibody. Total magnification is 100X with insets at 200X. Scale bar = 100µm. (c) mRNA isolated from the C57Bl/6J (red), FVB/N (green) and Mixed (blue) background strains was measured by Affymetrix® exon probes. Ptgsd exon map with relative abundance levels of each exon graphed below according to strain, (d) PCR verification of Ptgsd splice variant expression in C57Bl/6 and Mixed strains. PCR Primer Set 1 generates amplicon size of 371bp in the presence of exon 3 and 300bp with splicing of exon 3, and are independent probes from those used in (c). Representative gel showing 4 samples of each strain shown. (e) Relative quantification of splice variant expression in mouse strains. A ratio of “whole exon 3” to “spliced exon 3” was used for analysis; n=9, p < 0.05. (f) Sanger sequencing of the PCR products (Primer Sets 1 and 2) confirms alternative splicing of Ptgsd exon 3 (yellow highlight) with the missing portion of exon 3 in red.
Figure 4. PGD$_2$ inhibits WIHN in wild type mice but not in Gpr44 null mice, which otherwise have increased hair follicle regeneration

(a) Full excision wounding as described in Fig 1. was performed on Mixed strain mice with topical application of PGD$_2$ beginning on PWD7. Topical application of PGD$_2$ to wild type mice decreased WIHN; n = 7–9, p = 0.06. (b) Individual prostaglandin receptor null (Ptgdr KO and Gpr44 KO), Gpr44$^{+/−}$ and WT (C57BL/6J) mice were subjected to the WIHN assay and regenerated follicles were detected by K17 immunohistochemistry. Gpr44 KO mice had a 2-fold increase in regenerated follicles compared to wild type and Ptgdr KO mice; n = 21–
31 mice/genotype, p < 0.05. \textit{Gpr44}\textsuperscript{+/−} mice demonstrated an intermediate level of regeneration. (c) WIHN assay was performed on \textit{Gpr44}\textsuperscript{+/−} and \textit{Gpr44} KO mice with topical application of PGD\textsubscript{2} beginning at PWD7. Topical application of PGD\textsubscript{2} to both \textit{Gpr44}\textsuperscript{+/−} and \textit{Gpr44} KO mice had no effect on WIHN; n = 8–12. Similar to 4b, \textit{Gpr44} KO mice demonstrate increased WIHN compared to \textit{Gpr44}\textsuperscript{+/−} mice.