GENE DELETION STUDIES REVEAL A CRITICAL ROLE FOR PROSTAMIDE/PROSTAGLANDIN F_{2α} SYNTHASE IN PROSTAMIDE F_{2α} FORMATION

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Running title: Prostamide/PGF_{2α} KO mice

Abbreviations

AKR	aldoketoreductase
C:	outflow facility
Fa:	aqueous flow
Fu:	uveoscleral outflow
IOP:	intraocular pressur
PG:	prostaglandin
PM/PGFS:	prostamide/prostaglandin F synthase
PGFS:	prostaglandin F synthase
TxA ₂ :	thromboxane A ₂

Abstract

Prostamide/prostaglandin F synthase (PM/PGFS) is an enzyme with very narrow substrate specificity and is dedicated to the biosynthesis of prostamide and prostaglandin F_{2α}. The importance of this enzyme,

relative to the aldoketoreductase (AKR) series, in providing functional tissue prostamide $F_{2\alpha}$ levels was determined by creating a line of PM/PGFS gene deleted mice. Deletion of the gene encoding PM/PGFS (fam213b) was accomplished by a two exon disruption. Prostamide $F_{2\alpha}$ levels in WT and PM/PGFS KO mice were determined by LC/MS/MS. Intraocular pressure was measured by tonometry and outflow facility was measured by the *iPerfusion* method in enucleated mouse eyes. Deletion of fam213b had no observed effect on behavior, appetite, or fertility. Intraocular pressure was significantly elevated by approximately 4 mmHg in PM/PGFS KO mice compared to littermate WT mice. No effect on pressure dependent outflow facility occurred, which is consistent with the typically reported prostanoid effect of increasing outflow through the unconventional pathway. The elevation of intraocular pressure caused by deletion of the gene encoding the PM/PGFS enzyme likely results from a diversion of the endoperoxide precursor pathway to provide increased levels of those prostanoids that raise intraocular pressure, namely prostaglandin D_2 (PGD₂), thromboxane A_2 (TxA₂). It follows that PM/PGFS may serve an important regulatory role in the eye by providing PGF_{2 α} and prostamide $F_{2\alpha}$ to constrain the influence of those prostanoids that raise intraocular pressure.

Keywords

- (a) Expertise key words: prostaglandins, eye, animal models, enzymology, gene expression
- (b) Free-form key words prostamide/prostaglandin F synthase, fam213b, prostamide $F_{2\alpha}$, prostaglandin $F_{2\alpha}$, intraocular pressure,

Introduction

The prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogs are established as first line therapies for treating glaucoma. The selective FP receptor agonists were designed by virtue of the need to attenuate the ocular side effects produced by the naturally occurring prostanoid $PGF_{2\alpha}$ (1-3). Similarly, bimatoprost is an analog of prostamide $F_{2\alpha}$ (4, 5). Both $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ are the products of an identical biosynthetic pathway, but differing with respect to the cyclo-oxygenase substrate. Thus, arachidonic acid is the primary precursor for $PGF_{2\alpha}$, whereas prostamide $F_{2\alpha}$ is biosynthesized from the naturally occurring mammalian endocannabinoid anandamide (6). Following oxidation of arachidonic acid or anandamide, the respective endoperoxide intermediates PGG_2/PGH_2 and prostamides G_2/H_2 , are converted to the active end-products by PGD, PGE, PGF, prostacyclin and thromboxane synthases (7,8). The first enzyme identified and named as a PGF synthase (PGFS) was an aldo-keto reductase (AKR1C3), a broad substrate specificity enzyme (9-13). More recently, a much more substrate specific enzyme and a member of the thioredoxin-like superfamily was discovered and was designated prostamide/prostaglandin F synthase (14,15), PM/PGFS, fam213. This enzyme facilitates a more restricted and targeted formation of $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ and thereby conferring tighter control of regulatory function(s). Gene deletion is a classical approach for discovering biological functions and the first results of these studies are presented herein, together with the methodology for creating prostamide/prostaglandin F synthase (fam213) knock-out mice. Given the importance of $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ in the treatment of glaucoma, these present studies focused on regulation of intraocular pressure.

Methods

Creation of prostamide/prostaglandin F synthase (fam213) knock-out mice.

The prostamide/prostaglandin F synthase (PM/PGFS) knock-out mouse was generated using the RENKA embryonic stem (ES) cell line, derived from C57BL/6N mice (16). To construct a PM/PGFS targeting vector, a 990 bp DNA fragment carrying exons 2 and 3, containing the enzyme active site of PM/PGFS, was amplified by PCR, and inserted into the targeting vector as described previously (17). In this clone, a DNA fragment of a PGK promoter-driven neo-poly (A) cassette was flanked by two Frt sites. These were flanked by two LoxP sites located 139 bp upstream of exon 2 and 298 bp downstream of exon 3. The 5' side included 5.7 kb of the PM/PGFS gene and the 3' side 6.5 kb, followed by an MC1 promoter-driven diphtheria toxin (DTA) gene (Fig. 1A). The targeting vector was introduced into RENKA ES cells by electroporation and recombinant clones identified by Southern blot hybridization (Fig. 1B). To produce germline chimeras, recombinant ES clones were microinjected into eight cell stage embryos of CD1 mice. To generate PM/PGFS knock-out mice, chimeric mice were crossed with TLCN-Cre mice, by which recombination was introduced throughout the whole body (18).

All procedures on living mice were carried out in compliance with the Imperial College Statement for Use of Animals in Research, under UK home Office project license 70/9064 and in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed in individually ventilated cages with access to food and water ad libitum, maintained at 21°C with a 12-hour light/dark cycle. Heterozygous mice were used for breeding and all pups genotyped prior to usage. Genotyping was carried out on DNA extracted from ear tissue following manufacturer's protocols (Express Extract, Kapa Biosystems, Cambridge, MA, USA). TaKaRa Ex Taq polymerase (RR001, Takara Biotech, Shiga, Japan) was used for PCR reactions, forward primer (CAACCAGTCTAACTAGGCT) and reverse primer (CATGTGACCTGAACCCCT) were cycled 30 times with an annealing temperature of 65°C to yield predicted products of 650 bp for knock-outs and 1500 bp for wild-types. PCR products were resolved by gel electrophoresis (1% agarose) in the presence of a DNA gel stain (SYBR Safe, Invitrogen, Carlsbad, CA). Bands were visualized on an imaging station (Biospectrum 500, UVP, Upland, CA).

Quantitative real-time PCR

PM/PGFS knock-out, male mice (n=13) and littermate wild type mice of both sexes (n=15), 10 to 15 weeks old, were humanely culled and harvested for eyes and approximately half were also harvested for inguinal, and gonadal white fat pads. Pairs of eyes were cleared of extraocular tissue, hemisected at the equator and lenses removed prior to homogenisation in TRIzol Reagent (ThermoFisher Scientific, Waltham, Massachusetts, USA) with a rotor-stat homogeniser (VDI 12, VWR, Leicestershire, UK). Non-ocular fat pads were placed in TRIzol immediately after dissection, shortly followed by homogenisation. Total RNA was extracted using PureLink RNA spin columns and DNase I treated following the manufacturer's protocol (ThermoFisher Scientific). RNA yield was determined using a NanoDrop ND-1000 spectrophotometer and Superscript VILO reverse transcriptase (ThermoFisher Scientific) was used to synthesise cDNA, following manufacturer's instructions. qPCR was carried out on 10 ng of starting RNA using SYBR select master mix (ThermoFisher Scientific). PM/PGFS primers (Table 1) were designed with AlleleID software (Premier Biosoft). cDNA was analysed in triplicate on the QuantStudio 6 Flex (applied Biosystems, ThermoFisher Scientific) Real-Time PCR system. GAPDH was used as the reference gene, RNA content was determined by the delta Ct method of comparative quantification (Ct [reference gene] - Ct [target gene]).

Gene	Forward Primer	Reverse Primer
PM/PGFS	TGATGAGAGCAAGCAAATCT	ACAGTACCTTGTCGCCAC
GAPDH	GCCTTCCGTGTTCTACC	CCTGGTCCTCAGTGTAGC

Table 1. Exon spanning gene expression primer sets for use with SYBR green qPCR. GAPDH was used as a housekeeping gene for relative mRNA abundance.

Measurement of tissue prostamide F_{2α} levels by LC/MS/MS

PM/PGFS knock-out mice (n=5) and littermate wild type mice (n=5), 10 to 15 weeks old and of both sexes, were humanely culled and harvested for eyes and inguinal and gonadal white fat pads. For prostamideF_{2α}, tissues were dounce-homogenized and extracted with acetone containing an internal deuterated standard for prostamideF_{2α} quantification by isotope dilution ([²H]-4 prostamide F_{2α}). The lipid-containing organic phase was dried, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10, 70:30 and 50:50(v/v) chloroform/methanol. The 70:30 fraction was used for prostamideF_{2α} quantification by LC-MS-MS, using

an LC20AB coupled to a hybrid detector IT-TOF (Shimadzu Corporation, Kyoto, Japan) equipped with an ESI interface. LC analysis was performed in the isocratic mode using a DiscoveryHC18 column (15 cm, 62.1 mm, 5 mm) and methanol/water/acetic acid (53:47:0.05 by vol.) as mobile phase with a flow rate of 0.15 ml/min. Identification of prostamideF_{2a} was carried out using ESI ionization in the positive mode with nebulizing gas flow of 1.5 ml/min and curved desolvation line temperature of 250°C.

Intraocular pressure measurement

PM/PGFS knock-out (n=18) and littermate wild type (n=27) mice, aged 10 to 15 weeks old and of both sexes, received bilateral intraocular pressure measurement between the hours of 10:00 AM and 12:00 noon using a commercial rebound tonometer (TonoLab; Icare, Helsinki, Finland). Intraocular pressure was calculated as the mean of three consecutive tonometer readings as previously described (19). Statistical significance between groups was established using the unpaired Students T-test (mean ± 95% CI). Normal distribution was checked by the Kolmogorov-Smirnov test, equity of variances between groups was determined by Levene's test.

Measurement of outflow facility

PM/PGFS knock-out (n=18) and littermate wild type (n=22) mice, 10-15 weeks old and of either sex, were humanely culled by cervical dislocation and eyes enucleated immediately after death. Outflow facility was measured in individual eyes using the *iPerfusion* system, as previously described (20). Briefly, eyes were glued to a support platform submerged in PBS regulated at 35°C. Anterior chambers were cannulated with 33-gauge beveled needles (NanoFil, NF33BV-2, World Precision Instruments) attached to micro-manipulators and equilibrated for 30 minutes at 9 mmHg. The perfusate comprised 0.2 µm filtered DBG (PBS including divalent cations and 5.5 mM glucose). Six pressure steps equally spaced between 6 and 15 mmHg were carried out. For each step, steady state was considered once the slope of the ratio of flow rate to pressure was less than 0.1 nl/min/mmHg/min continuously for 1 minute. Steady state flow Q and pressure P were extracted by averaging over the last 4 minutes for each step. Pressure steps that failed to reach steady state were excluded from further analysis and eyes with 4 or fewer successful steps were omitted. Outflow facility was estimated by fitting a model of the form

$$Q = C_r \left(\frac{P}{P_r} \right)^\beta P$$

where C_r represents outflow facility at a reference pressure P_r (8 mmHg) and β characterizes the non-linearity of the Q - P relationship. Note that there is no term for flow at zero pressure, as this has been demonstrated to be zero (21).

Results

Transcription

Transcript for fam213b was undetectable by qPCR in homogenized anterior eye segments and excised inguinal fat pads of homozygous PM/PGFS knock-out mice (Fig. 2). In littermate wild type mice, mRNA was detected in both fat pad and anterior eye segment homogenates.

Tissue prostamide F_{2α} levels by LC/MS/MS detection

Prostamide F_{2α} could not be detected in inguinal fat taken from PM/PGFS knock-out mice, whereas inguinal fat taken from littermate wild type mice yielded low but consistently detectable amounts in all animals (table 2).. In gonadal white adipose tissue and whole eyes, prostamide F_{2α} was never detected in any of the PM/PGFS knock-out or littermate wild type mice. It should be noted that the diminutive mouse eye produces very low yields of ciliary body tissue, which would be further compromised by the immense dilution factor inherent by the presence of additional ocular structures in the anterior segment of the eye.

TISSUE	Wild type mice	Knock out mice
Ocular anterior segment	0	0
Inguinal white adipose tissue	1.11 ± 0.56	0
Gonadal white adipose tissue	0	0

Table 2. Prostamide F_{2α} levels (pmol/g tissue) mean values ± SEM in ocular anterior segment tissues, inguinal white adipose tissue, and gonadal white adipose tissue. No detectable prostamide F_{2α} is designated 0. N=5.

Ocular effects

Intraocular pressure values obtained from PM/PGFS knock-out and littermate wild type mice are compared in figure 3. Intraocular pressure was significantly elevated by 3.6 [2.9, 4.3] mmHg ($P<0.0001$, $N=45$, mean [95% confidence interval]) in response to deletion of the PM/PGFS enzyme (Fig. 3A). Outflow facility, as measured in enucleated mouse eyes using the iPerfusion system, was 4.9 [4, 6] nl/min/mmHg ($N=18$) for PM/PGFS knock-out mice and 5.6 [5, 7] nl/min/mmHg ($N=22$) for littermate wild-type mice (Figure 3B). The facility at a physiological pressure drop, C_r , was compared between PM/PGFS knock-out and littermate wild type mouse eyes using an unpaired version of the weighted t -test of the log-transformed data as described previously (Sherwood *et al.*, 2016). Outflow facility was insignificantly different between the two groups (15% [-12, 51%] $P=0.31$, Fig. 3B).

Discussion

Two distinct families of enzymes are known to be involved in the biosynthesis of $\text{PGF}_{2\alpha}$ and prostamide $\text{F}_{2\alpha}$; the aldoketoreductase family of reductases, notably AKR1C3 (8-13) and PM/PGFS, which is a member of the thioredoxin-like superfamily (14, 15). To date the relative biosynthetic and regulatory importance of these two enzymes in living tissues has remained unclear. By studying prostamide $\text{F}_{2\alpha}$ tissue levels in inguinal fat, it became clear that deletion of the gene that encodes PM/PGFS prevents the biosynthesis of prostamide $\text{F}_{2\alpha}$. Further, a functional study on intraocular pressure revealed a distinct increase in PM/PGFS knock-out mice, thereby revealing a regulatory function for PM/PGFS for the first time.

$\text{PGF}_{2\alpha}$ and prostamide $\text{F}_{2\alpha}$ are known to produce profound and long-acting ocular hypotony (1, 22). Despite these pronounced attributes, no evidence has emerged that $\text{PGF}_{2\alpha}$ and prostamide $\text{F}_{2\alpha}$ are involved in the physiological regulation of intraocular pressure. Deletion of the gene that encodes both the FP receptor and the heterodimeric prostamide $\text{F}_{2\alpha}$ receptor (23, 24) essentially abolishes ocular hypotensive response to prostanoid FP receptor agonist prodrugs and bimatoprost (25). Moreover, in FP receptor knock-out mice, there is no effect on baseline intraocular pressure or the diurnal increase in intraocular pressure that occurs in the early dark phase, 6:00pm – midnight (26). In line with this result in FP knock-out mice is the common knowledge that clinical use of cyclo-oxygenase inhibitors does not result in any meaningful change in intraocular pressure. This is despite the fact that all prostanoids are found endogenously present in the eye (27-29). It seems that under most circumstances prostanoids tending to either increase or decrease intraocular pressure are held in balance and that this balance is not altered, but rather nullified, by global inhibition of prostanoid biosynthesis. It was, therefore, initially considered unexpected that deletion of an enzyme involved in the biosynthesis of $\text{PGF}_{2\alpha}$ and prostamide $\text{F}_{2\alpha}$ would significantly elevate resting intraocular pressure.

These findings are difficult to explain by the involvement of an aldo-ketoreductase, despite being eminently capable of substituting for absent PM/PGFS by producing $\text{PGF}_{2\alpha}$ and prostamide $\text{F}_{2\alpha}$, which are both ocular hypotensive agents. Specifically, one member of the aldo-keto reductase family has been extensively characterized and is designated prostaglandin F Synthase (PGFS) and AKR1C3. In terms of prostanoid formation, it is a dual function enzyme that converts the endoperoxide intermediate PGH_2 to $\text{PGF}_{2\alpha}$ and reduces PGD_2 to $11\beta\text{-PGF}_{2\alpha}$ (9, 10), a stereoisomer almost as potent as $\text{PGF}_{2\alpha}$ (30). Unlike $\text{PGF}_{2\alpha}$, the activity of prostamide $\text{F}_{2\alpha}$ is greatly reduced by re-arrangement of the 11-OH group to the β configuration (30). PGFS (AKR1C3) is not expressed in the eye (15). There are, however, alternative enzymes in the aldo-ketoreductase superfamily (31) which could possess $\text{PGF}_{2\alpha}$ and prostamide $\text{F}_{2\alpha}$ synthase activities. AKR1C1, 2, 3, and 4 share more than 86% sequence identity (32). Nevertheless, the marked increase in intraocular pressure in PM/PGFS knock-out mice, about 20%, suggests that the loss of

PM/PGFS is not compensated for by functional expression of AKR1C1-4 in the eye. Although it was not possible to detect prostamide $F_{2\alpha}$ in ocular anterior segment tissue, it was reliably detected from inguinal fat from wild type mice but not from PM/PGFS knockout mice. It appears that prostamide/PGF synthase is the primary source of $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ since (1) prostamide $F_{2\alpha}$ levels in inguinal fat were not detected in PM/PGFS knock-out mice, and (2) a marked functional effect on intraocular pressure was observed in PM/PGFS knock-out mice.

The absence of $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ synthesizing enzymes from the ocular anterior segment and a resultant increase in intraocular pressure, would suggest that these prostanoids are involved in its negative regulation. Moreover, the increase in intraocular pressure recorded in PM/PGFS knock-out mice was substantial and similar to that associated with peak levels of diurnal intraocular pressure in mice (26). This notion would, however, be flawed since disruption of the gene encoding FP and the prostamide $F_{2\alpha}$ -sensitive receptors does not alter intraocular pressure (25,26). A more satisfactory explanation would be the diversion of the endoperoxide precursor PGH_2 to alternative PG synthases. This would likely be PGD and thromboxane (Tx) synthases, since their products PGD_2 and thromboxane A_2 (TxA_2) have been shown to markedly increase intraocular pressure (33, 34). PGD_2 has been reported to produce a biphasic effect on intraocular pressure, with an initial ocular marked hypertensive phase (33). TxA_2 is structurally highly unstable but its selective TP receptor analog U-46619 exclusively produces ocular hypertension that increases over time (34). No effect on pressure-dependent outflow facility was observed in PM/PGFS knock-out mice, suggesting a possible effect on the uveoscleral pathway, aqueous humor secretion, or both in the ocular hypertension produced by deleting the PM/PGFS gene in mice.

In addition to producing decreased intraocular pressure, an important role for prostamide $F_{2\alpha}$ has been discovered in centrally mediated pain (35) and adipogenesis (36). To date, however, the role of the enzyme PM/PGFS has not been directly studied in regulatory and disease processes. This is the first reported study on PM/PGFS using knock-out mice. The evidence that emerged suggests that this enzyme regulates intraocular pressure not by producing ocular hypotensive $PGF_{2\alpha}$ and prostamide $F_{2\alpha}$ but rather by diverting PGH_2 conversion to excessive production of PGD_2 and TxA_2 , both of which cause ocular hypertension. In this series of experiments, the much more substrate specific enzyme PM/PGFS was the important enzyme for prostamide $F_{2\alpha}$ biosynthesis and not AKR1C3.

Data Availability

The data described in this manuscript is located at the following institutions.

Dept. of Bioengineering, Imperial College London, London, United Kingdom

Département de Médecine, Faculté de Médecine, Université Laval, Quebec, Canada

Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy

Dept. of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan

Dept. of Nutrition, Koshien University, Takarazuka, Hyogo, Japan.

Acknowledgements

Y Inoue was a postdoctoral fellow, financially supported by Allergan, who greatly contributed to the creation of a PM/PGFS knock-out mouse. She sadly passed away at a young age after completion of this research.

We gratefully thank Rie Natsume (Brain Research Institute, Niigata University) for her technical assistance.

These studies were financially supported by grants from JSPS KAKENHI and Allergan Inc..

References

1. Giuffr, G. 1985. The effects of prostaglandin $F_{2\alpha}$ in the human eye. *Graefes Arch. Clin. Exp. Ophthalmol.* 222: 139-141.
2. Stjernschantz, J. 2001. $PGF_{2\alpha}$ -isopropyl ester to latanoprost: a review of the development of xalatan. *Invest. Ophthalmol. Vis. Sci.* 42: 1134-1145.
3. Hellberg, M.R., Sallee, V.L, McLaughlin, M.A., Sharif, N.A., Desantis,L., Dean, T.R. Zinke, P.W. 2001. Preclinical efficacy of travoprost, a potent and selective FP prostaglandin receptor agonist. *J. Ocul. Pharmacol. Ther.* 17: 421-432.
4. Woodward, D.F., Krauss, A.H., Wang, J.W., Protzman, C.E., Nieves, A.L., Liang, Y., Donde, Y., Burk, R.M., Landsverk, K., Struble, C. .2007. Identification of an antagonist that selectively blocks the activity of prostamides (prostaglandin-ethanolamides) in the feline iris. *Br J Pharmacol.* 150: 342-352.
5. Woodward, D.F., Wang, J.W., Poloso, N.J. 2013. Recent progress in prostaglandin $F_{2\alpha}$ ethanolamide (prostamide $F_{2\alpha}$) research and therapeutics. *Pharmacol. Rev.* 65: 1135-1147.
6. Yang, W., Ni, J., Woodward, D.F., Tang-Liu, D.D.-S., Ling, K.-H.J. 2005. Enzymatic formation of prostamide $F_{2\alpha}$ from anandamide involves a newly identified intermediate metabolite, prostamide $F_{2\alpha}$. *J. Lipid Res.* 46: 2745-2751.
7. Kozak, K.R., Crews, B.C., Morrow, J.D., Wang, L.-H., Ma, Y.H., Weinander,, R., Jakobsson, P.-J., Marnett, L.J. 2002. Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J. Biol. Chem.* 277: 44877-44865.
8. Koda, N., Tsutsui, Y., Niwa, H., Ito, S., Woodward, D.F., Watanabe, K. 2004. Synthesis of prostaglandin F ethanolamide by prostaglandin F synthase and identification of bimatoprost as a potent inhibitor of the enzyme: new enzyme assay method using LC/ESI/MS. *Arch. Biochem. Biophys.* 424: 128-136.
9. Watanabe, K., Yoshida, R., Shimizu, T., Hayaishi, O. 1985. Enzymatic formation of prostaglandin F_2 alpha from prostaglandins prostaglandins H_2 and D_2 . Purification and properties of prostaglandin F synthetase from bovine lung. *J. Biol. Chem.* 260: 7035-7041.
10. Watanabe, K., Iguchi, Y., Iguchi, S., Arai, Y., Hayaishi, O., Roberts, L.J. 2nd. 1986. Stereospecific conversion of prostaglandin D_2 to (5Z, 13E)-(15S)-9 alpha-11beta, 15-trihydroxyprosta-5,13-dien-1-oic acid (9 alpha,11 beta-prostaglandin F_2) and of prostaglandin H_2 to prostaglandin F_2 alpha by bovine lung prostaglandin F synthase. *Proc. Natl. Acad. Sci. USA.* 83: 1583-1587.

11. Watanabe, K., Fujii, Y., Nakayama, K., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S., Hayaishi, O. 1988. Structural similarity of bovine lung prostaglandin F synthase to lens epsilon-crystallin of the European common frog. *Proc. Natl. Acad. Sci. USA*. 85: 11-15.
12. Watanabe, K., Fujii, Y., Ohkbubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S., Hayaishi, O, 1991. Expression of bovine lung prostaglandin F synthase in Escherichia coli. *Biochem. Biophys. Res. Comm.* 181: 272-278.
13. Chen, L.Y., Watanabe, K., Hayaishi, O. 1992. Purification and characterization of prostaglandin F synthase from bovine liver. *Arch. Biochem. Biophys.* 296: 17-26.
14. Moriuchi, H., Koda, N., Okuda-Ashitaka, E., Daiyasu, H., Ogasawara, K., Toh, H., Ito, S., Woodward, D.F., Watanabe, K. 2008. Molecular characterization of a novel type of prostamide/prostaglandin F synthase, belonging to the thioredoxin-like superfamily. *J. Biol. Chem.* 283: 792-801.
15. Watanabe, K., Woodward, D.F. 2015. Prostamide F_{2α} biosynthesizing enzymes. In *The Endocannabinoidome: The World of Endocannabinoids and Related Mediators*. V Di Marzo and JW Wang, editors. Elsevier, Amsterdam, Holland, 101-110.
16. Mishina, M., Sakimura, K. 2007. Conditional gene targeting on the pure C57BL/6 genetic background. *Neurosci. Res.* 58:105–112.
17. Akashi, K., Kakizaki, T., Kamiya, H., Fukaya, M., Yamasaki, M., Abe, M., Natsume, R., Watanabe, M., Sakimura, K. 2009. NMDA receptor GluN2B (GluR epsilon 2/NR2B) subunit is crucial for channel function, postsynaptic macromolecular organization, and actin cytoskeleton at hippocampal CA3 synapses. *J. Neurosci.* 29: 10869–10882.
18. Nakamura, K., Manabe, T., Watanabe, M., Mamiya, T., Ichikawa, R., Kiyama, Y., Sanbo, M., Yagi, T., Inoue, Y., Nabeshima, T., Mori, H., Mishina, M. 2001. Enhancement of hippocampal LTP, reference memory and sensorimotor gating in mutant mice lacking a telencephalon-specific cell adhesion molecule *Euro. J. Neurosci.* 13: 179–189.
19. Overby, D.R., Bertrand, J., Tektas, O.-Y., Boussommier-Callej, A., Schicht, M., Ethier, C.R., Woodward, D.F., Stamer, W.D., Lütjen-Drecoll, E. 2014. Ultrastructural changes associated with dexamethasone-induced ocular hypertension in mice. *Invest. Ophthalmol. Vis. Sci.* 55: 4922–4933.
20. Madekurozwa, M., Reina-Torres, E., Overby, D.R., Sherwood, J.M. 2017. Direct measurement of pressure-independent aqueous humour flow using iPerfusion. *Exp. Eye Res.* 162:129–138.
21. Sherwood, J.M., Reina-Torres, E., Bertrand, J.A., Rowe, B., Overby, D.R. 2016. Measurement of Outflow Facility Using iPerfusion. *PloS one*. United States, 11(3), p. e0150694. doi: 10.1371/journal.pone.0150694.
22. Woodward DF, Chen J, Kharlamb A et al. 2005. The formation, metabolism, and ocular

- hypotensive activity of the prostamides and related substances. *In Focus on Glaucoma Research*. SM Reece editor. Nova Science, New York, USA 217-231.
23. Liang, Y., Woodward, D.F., Guzman, V.M., Chang, W.W., Evinger, A.J., Pablo, J.V. 2008. Identification and pharmacological characterization of the prostaglandin FP and FP receptor variant complexes. *Br. J. Pharmacol.* 154: 1079-1093.
24. Sugimoto, Y., Hasumoto, K., Namba, T., Irie, A., Negishi, M., Kakizuka, A., Narumiya, S., Ichikawa, A.. 1994. Cloning and expression of a cDNA for mouse prostaglandin F receptor. *J. Biol. Chem.* 269: 1356-1560.
25. Ota, T., Aihara, M., Narumiya, S., Araie M. 2005. The effects of prostaglandin analogues on IOP in prostanoid FP-deficient mice. *Invest. Ophthalmol. Vis. Sci.* 46: 4159-4163.
26. Crowston, J.G., Morris, C.A., Lindsey, J.D., Weinreb, R.N. 2007. Prostaglandin FP receptors do not contribute to 24-hour intraocular pressure variation in mice. *Invest. Ophthalmol. Vis. Sci.* 48: 2095-2098.
27. Cryan, L.M., Fitzgerald, D.J., O'Brien, C. 2009. Ocular prostaglandin production and morphology in mice lacking a single isoform of cyclooxygenase. *Prostaglandins Leukot. Essent. Fatty Acids.* 81:401-409.
28. Eom, Y., Lee D.Y., Kang, B.-R., Heo J.-H., Shin, K.-H. Kim H. M., Song, J. S. 2014. Comparison of aqueous levels of inflammatory mediators between toxic anterior segment syndrome and endotoxin-induced uveitis animal models. *Invest. Ophthalmol. Vis. Sci.* 55:6704-6710.
29. Urquhart, P., Wang, J.W., Woodward, D.F., Nicolaou, A. 2015. Identification of prostamides, fatty acyl ethanolamines and their biosynthetic precursors in rabbit cornea. *J. Lipid Res.* 56: 1419-1433.
30. Matias, I., Chen, J., De Petrocellis, L., Bisogno, T., Ligresti, A., Fezza, F., Krauss, A.H.-P., Shi, L., Protzman, C.E., Li, C., Liang, Y., Nieves, A.L., Kedzie, K.M., Burk, R.M., Di Marzo, V., Woodward, D.F. 2004. Prostaglandin ethanolamides (prostamides); in vitro pharmacology and metabolism. *J. Pharmacol. Exp. Ther.* 309: 745-757.
31. Penning, T.M. 2015. The aldo-keto reductases (AKRs): overview. *Chem. Biol. Interact.* 234: 236-246.
32. Penning, T.M., Chen, M., Yi, J. 2015. Promiscuity and diversity in 3-ketosteroid reductases. *J. Steroid Biochem. Mol. Biol.* 151: 93-101.
33. Nakajima, M., Goh, Y., Azuma, I., Hayaishi, O. 1991, Effects of prostaglandin D2 and its analogue, BW 245C, on intraocular pressure in humans. *Graefes Arch. Clin. Exp. Ophthalmol.* 229: 411-415.

34. Woodward DF, Lawrence RA, Fairbairn CE, Shan T, Williams LS. Intraocular pressure effects of selective prostanoid receptor agonists involve different receptor subtypes according to radioligand binding studies. *J Lipid Med.* 1993; 6: 545-553.
35. Gatta, L., Piscitelli, F., Giordano, C., Boccella, S., Lichtman, A., , Maione, S., , Di Marzo, V. 2012. Discovery of prostamide F2 α and its role in inflammatory pain and dorsal horn nociceptive neuron hyperexcitability. *Plos ONE.* 2012; 7: e3111, doi; 10.1371/journal.pone.003111.
36. Silvestri, C., Martella, A., Poloso, N.J., Piscitelli, F., Capasso, R., Izzo, A., Woodward, D.F., Di Marzo, V. 2013. Anandamide-derived prostamide F2 α negatively regulates adipogenesis. *J. Biol. Chem.* 288: 23307-23321.

Legends to Figures

Figure 1. (A) A schematic representation of PM/PGFS mRNA, genomic allele, targeting vector, targeted PM/PGFS floxed allele and knock-out allele. Numbered boxes of genomic allele represent the exon sequences of PM/PGFS. Frt sites are indicated by open half-circles. neo, PGK-promoter-neo-pA cassette; DTA, diphtheria toxin cassette; A, Afl II; E, EcoR I. Shadowed boxes indicate 5'- and 3'- Southern blot probes. **(B)** Southern blot analysis of EcoR I- or Afl II- digested genomic DNA prepared from the wild-type (WT), PM/PGFS-floxed (neo) and PM/PGFS knock-out mice.

Figure 2. Gene expression analysis of the PM/PGFS by qPCR. PM/PGFS mRNA was undetectable in homogenized anterior segments and inguinal fat pads of PM/PGFS knock-out mice. Similar levels of PM/PGFS mRNA were found in the homogenised anterior segments and inguinal fat pads of littermate wild type mice. Samples were run in triplicate, $dCt = Ct [\text{reference gene}] - Ct [\text{target gene}]$, with GAPDH as the reference gene, Ct values greater than 35 cycles were excluded. Circles represent individual samples ($\circ$) and red crosses ($\times$) show sample means with error bars as two standard deviations.

Figure 3. (A) Intraocular pressure in PM/PGFS knock-out mice, 16 [15, 17] mmHg (N=18), was significantly greater than littermate wild type mice, 13 [12, 13] mmHg (N=27) ($P < 0.0001$). However outflow facility **(B)** was not significantly different, 4.9 [4, 6] vs 5.6 [5, 7] nl/min/mmHg respectively (15% [-12, 51], mean, 95% CI; $P = 0.3$, N=22).

X

X