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Genetic Deletion of Microsomal Prostaglandin E Synthase-1 Suppresses Mouse Mammary Tumor Growth and Angiogenesis

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Abstract

The cyclooxygenase/prostaglandin (COX/PG) signaling pathway is of central importance in inflammation and neoplasia. COX inhibitors are widely used for analgesia and also have demonstrated activity for cancer prophylaxis. However, cardiovascular toxicity associated with this drug class diminishes their clinical utility and motivates the development of safer approaches both for pain relief and cancer prevention. The terminal synthase microsomal PGE synthase-1 (mPGES-1) has attracted considerable attention as a potential target. Overexpression of mPGES-1 has been observed in both colorectal and breast cancers, and gene knockout and overexpression approaches have established a role for mPGES-1 in gastrointestinal carcinogenesis. Here we evaluate the contribution of mPGES-1 to mammary tumorigenesis using a gene knockout approach. Mice deficient in *mPGES-1* were crossed with a strain in which breast cancer is driven by overexpression of human epidermal growth factor receptor 2 (*HER2/neu*). Loss of mPGES-1 was associated with a substantial reduction in intramammary PGE_2 levels, aromatase activity, and angiogenesis in mammary glands from *HER2/neu* transgenic mice. Consistent with these findings, we observed a significant reduction in multiplicity of tumors ≥1mm in diameter, suggesting that mPGES-1 contributes to mammary tumor growth. Our data identify mPGES-1 as a potential anti-breast cancer target.

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Keywords

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1. Introduction

A wealth of evidence supports the inducible prostaglandin (PG) synthase prostaglandin-endoperoxide synthase 2, more commonly called cyclooxygenase-2 (COX-2), as a target for prevention of both breast and other cancers [1, 2]. COX-2 is overexpressed in numerous human cancers, including a significant proportion of breast neoplasias and the majority of colorectal cancers (CRC). Animal studies demonstrate convincing protective effects of both pharmacological inhibition and genetic ablation of COX-2 in numerous cancer models, most notably CRC models. We and others have shown that inhibiting or knocking out COX-2 suppresses mammary tumor formation in rodents [2–4]. Conversely, transgenic *COX-2* over-expression induces mammary tumor formation [5]. Protective effects of COX inhibition are supported by epidemiological observations of correlations between use of COX-inhibiting non-steroidal antiinflammatory drugs (NSAIDs) and reduced incidence of breast and colon cancers [1, 2]. Furthermore, clinical trials have established that selective COX-2 inhibitors, or COXibs, suppress formation and induce regression of colorectal adenomas [6–9]. Nevertheless, COXibs are not considered clinically useful for cancer prophylaxis in the general population due to associated cardiovascular toxicity, ironically identified in some of the same trials which demonstrated chemopreventive efficacy [10–12].

The cardiovascular toxicity of COX-2 inhibitors may be partially attributable to perturbation of the thromboxane:prostacyclin (TXA_2:PGI_2) ratio, since COX-2 inhibition causes selective depression of endothelial, COX-2-derived PGI_2 without reducing levels of COX-1-derived TXA_2 released from platelets [13]. Prothrombotic effects of COX-2 suppression have now been demonstrated in multiple animal models, and are phenocopied by deletion of prostacyclin receptors [14–17].

The adverse prothrombotic effects of COX-2 inhibition provide substantial impetus for developing alternative strategies for suppressing inflammation and neoplasia which leverage the efficacy of COX-2 inhibition while sidestepping the associated toxicity. Potential targets include the terminal synthases responsible for conversion of COX-2-generated PGH_2 to PGE_2, because PGE_2 is the prostanoid most strongly implicated in pain, inflammation and neoplasia. Three such enzymes have been identified with *in vitro* PGE_2 synthetic capacity: microsomal PGE_2 synthases (mPGES) 1 and 2, and a cytosolic PGES (cPGES/p23) [18, 19]. Gene knockout studies implicate mPGES-1 as playing a pivotal role in PGE_2 synthesis *in vivo* under several conditions [20–24]. Notably, peritoneal macrophages from *mPGES-1*-null mice are unable to produce PGE_2 in response to inflammatory challenge [22–24]. Similarly to *COX-2*, *mPGES-1* is constitutively expressed in a limited number of organs, is upregulated in response to various proinflammatory stimuli, and expression is suppressed by glucocorticoids [25, 26]. Strikingly, *mPGES-1* upregulation has been identified in numerous cancers, including those of the lung, head and neck, gastrointestinal tract, and breast [27–32]. Furthermore, genetic manipulation studies (overexpression, knockout and knockdown approaches) suggest that mPGES-1 may be a significant contributor to carcinogenesis [33–39], and thus potentially a viable alternative to COX-2 as an anti-neoplastic target.

In this study, we have used a genetic approach to evaluate the role of mPGES-1 in breast cancer, by crossing *mPGES-1*-deficient mice with a strain in which breast cancer is driven by *HER2/neu* overexpression. Loss of mPGES-1 was associated with a substantial reduction in intramammary PGE_2 levels, aromatase activity, and angiogenesis in mammary glands

from *HER2/neu* transgenic mice. Consistent with these findings, we observed a significant reduction in multiplicity of tumors greater than 1mm in diameter, suggesting that mPGES-1 contributes to mammary tumor growth.

2. Materials and methods

2.1. Materials

Enzyme-linked immunoassay (ELISA) kits for PGE_2 analysis were purchased from Cayman Chemicals. Lowry protein assay kits were obtained from Sigma. 1β-[^{3}H]-androstenedione was from Perkin-Elmer Life Science. RNeasy mini kits were purchased from Qiagen. MuLV reverse transcriptase, RNase inhibitor, oligo $(dT)_{16}$, and SYBR green PCR master mix were obtained from Applied Biosystems. Real-time PCR primers were synthesized by Sigma-Aldrich. All other chemicals were obtained from Fisher Scientific or Sigma-Aldrich.

2.2. Mouse Experimental Procedure

All mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Rockefeller University or the New York Blood Center. Both facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and operate in accordance with Federal (PHS Policy on the Human Care and Use of Animals, Guide for the Use and Care of Laboratory Animals, Animal Welfare Act), State and local laws and regulations. Mice were provided food and water *ad libitum*. The previously reported *mPGES-1* −/− strain [23] was obtained from Pfizer on a hybrid DBA/1:lacJ strain background, and crossed with FVB/J wildtype mice at The Jackson Laboratory using targeted speed congenics to introduce the targeted *mPGES-1* allele onto an FVB background. Previous characterization of this strain confirmed the lack of mPGES-1 protein in *mPGES-1*-null mice [23]. MMTV/NDL mice express a mutationally activated *HER2/neu* allele (NDL, Neu Deletion mutant) that induces mammary hyperplasia and tumorigenesis [40]. MMTV/NDL and FVB-*mPGES-1* +/− mice were interbred to generate MMTV/NDL, *mPGES-1* +/− progeny, which were backcrossed with FVB-*mPGES-1* +/− mice to generate females of the required test genotypes: MMTV/NDL, *mPGES-1* +/+; MMTV/NDL, *mPGES-1* +/−; and MMTV/NDL, *mPGES-1* −/−. Genotypes were determined by PCR analysis of tail-tip-derived genomic DNA as previously described [4, 23]. Virgin test females were sacrificed at 20 weeks of age, and tissues were harvested as described below. All test animals were overtly healthy at sacrifice, and animal weights were not different between the three groups. Weights at sacrifice (mean±SD) of test animals were: MMTV/NDL, *mPGES-1* +/+, 25.0±2.4g; MMTV/NDL, *mPGES-1* +/−, 24.9±2.1g; MMTV/NDL, *mPGES-1* −/−, 24.4±1.9g.

2.3. Mammary Tissue Harvesting and Analysis

Abdominal (#4) mammary glands (MGs) were either snap-frozen in liquid nitrogen and stored at −80°C, or stained with carmine alum and mammary wholemounts prepared as previously described [4]. Tumor multiplicity was scored in carmine alum-stained mammary wholemounts in a blinded manner by two independent investigators using an eyepiece graticule on a dissecting microscope, and a threshold diameter of 0.25mm. To evaluate the effect of *mPGES-1* deficiency on tumor size, tumor multiplicity was rescored using 1.0mm as the threshold diameter. Axillary (#3) MGs were fixed in formalin and embedded in paraffin for histopathological analysis.

Anti-CD31 immunohistochemistry (IHC) was performed using monoclonal rat anti-mouse CD31 antibody (Pharmingen) on 5μm sections of formalin-fixed, paraffin-embedded MGs, and sections were counterstained with methyl green as previously described [4]. One slide was examined from each animal, and several microscopic fields were examined on each

slide. The number of CD31-positive blood vessels associated with a ductal or lobular unit was scored in each field, and a mean value was generated for each animal.

PGE_2 in snap-frozen MGs was assayed using an ELISA kit as previously described [41]. Aromatase activity in microsomes prepared from snap-frozen MGs was assayed by measuring tritiated water release from 1β-[^{3}H]-androstenedione, as previously described [42].

Vascular endothelial growth factor A (*VEGF-A*) expression was assayed by quantitative reverse transcriptase-coupled PCR (RT-PCR) of cDNA prepared from snap-frozen MGs. Primers used were: forward, 5′-GAA AGG GAA AGG GTC AAA AA-3′; reverse, 5′-CAC ATC TGC AAG TAC GTT CG-3′. *VEGF-A* transcript levels were normalized to GAPDH transcript level for each sample. Relative fold induction was determined using the ddC_T (relative quantification) analysis protocol.

2.4. Statistical Analysis

Tumor multiplicity data—The mean tumor counts from the two investigators among the three groups (MMTV/NDL, *mPGES-1* +/+; MMTV/NDL, *mPGES-1* +/−; and MMTV/NDL, *mPGES-1* −/−) were compared using ANOVA followed by a t-test for pair-wise comparisons. *P* values from the pair-wise comparisons were adjusted for multiple comparisons using Tukey's method.

PGE_2 levels, aromatase activity, and gene expression—The non-parametric Wilcoxon rank-sum test was used to compare PGE_2 levels, aromatase activity, and *VEGF* expression levels, between MGs from MMTV/NDL, *mPGES-1* +/+ and MMTV/NDL, *mPGES-1* −/− mice.

Microvessel density—Average CD31-positive blood vessel counts in multiple microscopic fields per mouse MG sample were compared between MMTV/NDL, *mPGES-1* +/+ and MMTV/NDL, *mPGES-1* −/− mice using a Student t-test.

3. Results

The goal of this study was to evaluate the contribution of the terminal PGE_2 synthase mPGES-1 to mammary tumorigenesis. Despite the identification of multiple proteins with *in vitro* PGE_2 synthase activity, mPGES-1 has emerged as a key determinant of PGE_2 synthesis under numerous conditions, such as in response to inflammatory stimuli (e.g. lipopolysaccharide) as well as in lactating murine mammary gland [22–24, 43]. Furthermore, numerous studies using overexpression and genetic ablation approaches have implicated mPGES-1 in experimental tumorigenesis, particularly in gastrointestinal cancer models [33–35, 37, 39]. Here we used a genetic approach to evaluate the potential contribution of mPGES-1 to breast cancer. Specifically, we determined the consequences of knocking out *mPGES-1* in MMTV/NDL mice, a model of *HER2/neu*-overexpressing breast cancer that we previously employed to demonstrate that Cox-2 contributes to HER2/neu-induced mammary tumorigenesis [4]. The MMTV/NDL strain expresses a mammary-targeted, mutationally activated *HER2/neu* transgene, and exhibits mammary hyperplasia which progresses through mammary intraepithelial neoplastic (MIN) lesions resembling human ductal carcinoma *in situ* (DCIS) to invasive breast cancers, which ultimately metastasize to the lung [40]. Multiple MIN lesions develop in each mammary gland in virgin females by 20 weeks of age [4]. Thus, we used the MMTV/NDL strain as a useful quantitative model of intraepithelial neoplasia analogous to mutant *Apc* strains frequently used to study intestinal neoplasia.

3.1. Effect of mPGES-1 deletion on HER2/neu-induced mammary tumorigenesis

Mice with targeted deletion of *mPGES-1* were obtained on a DBA/1lacJ background, and the mutant *mPGES-1* allele was introgressed onto an FVB background to negate potential confounding effects due to mixed strain backgrounds. Subsequently, MMTV/NDL mice were interbred with *mPGES-1*-deficient mice to generate test females of three genotypes in which to compare mammary tumor multiplicity: MMTV/NDL, *mPGES-1* +/+; MMTV/NDL, *mPGES-1* +/−; and MMTV/NDL, *mPGES-1* −/−. Tumor multiplicity was analyzed in carmine alum-stained #4 abdominal mammary gland wholemounts harvested from 20-week old virgin females of all three genotypes, using two size thresholds (0.25mm and 1.0mm diameter). Tumor multiplicity was similar in all three cohorts when we scored all tumors of ≥ 0.25mm diameter (Table 1). Interestingly however, *mPGES-1* deficiency was associated with a reduction in the number of tumors of ≥ 1.0mm in diameter: tumor number was significantly reduced in both *mPGES-1* heterozygous and null animals relative to those carrying two wildtype *mPGES-1* alleles (Table 1; P=0.008 and 0.025, respectively). These data suggest that mPGES-1 contributes to mammary tumor growth. Given the similar findings in MMTV/NDL mice that were *mPGES-1* heterozygous and nullizygous, we focused exclusively on comparisons of *mPGES-1* wildtype and null tissues for subsequent mechanistic analyses.

As anticipated, mammary PGE_2 levels were substantially reduced by genetic ablation of *mPGES-1*. Median PGE_2 levels of 18.0 ng/mg protein were detected in MGs from MMTV/NDL, *mPGES-1* +/+ mice (Figure 1). Deletion of *mPGES-1* caused an approximately 60% reduction in intramammary PGE_2 levels (MMTV/NDL, *mPGES-1* −/−, median=7.4 ng/mg protein; P=0.002).

3.2. Microvessel density and VEGF expression are reduced by genetic ablation of mPGES-1

Our subsequent studies focused on identifying potential mechanisms by which mPGES-1 might regulate HER2/neu-dependent mammary tumor growth. COX enzymes and COX-derived PGE_2 are strongly implicated in angiogenesis [44, 45]. Notably, we previously reported a profound reduction in vascularization both of MIN lesions and normal-appearing mammary gland in MMTV/NDL mice lacking functional Cox-2 [4]. Furthermore, transgenic *COX-2* overexpression in mouse MG drives extensive vascular development [46]. Importantly, angiogenesis is considered to be an obligate step in tumor growth. Based on the observed reduction in intramammary PGE_2 levels and corresponding suppression of tumor growth in MMTV/NDL mice lacking functional mPGES-1, we therefore explored the possibility that mammary gland vascularization was defective in *mPGES-1*-null animals.

Microvessel density was scored in MGs from MMTV/NDL, *mPGES-1* +/+ and MMTV/NDL, *mPGES-1* −/− mice by quantitating the number of CD31-positive blood vessels observed in association with ductal or lobular units in each microscopic field (Figure 2A). The mean CD31-positive blood vessel count was significantly reduced in *mPGES-1*-null mammary tissues (Figure 2B; P<0.001), implicating mPGES-1 in mammary vascularization. Consistent with this observation, expression of *VEGF-A*, a key driver of angiogenesis, was significantly reduced in MGs lacking mPGES-1 (Figure 2C; P=0.02). The median *VEGF-A* transcript level in MGs from MMTV/NDL, *mPGES-1* −/− mice was only 32% of that in glands with wildtype *mPGES-1*. Our data suggest that mPGES-1 may regulate tumor growth at least in part via controlling vascular development.

3.3. Mammary aromatase activity is substantially reduced in mPGES-1-deficient MGs

Extensive data support the estrogen synthase aromatase as a potentially key effector of PGE_2 signaling in mammary neoplasia [47]. Expression of the *CYP19* gene encoding aromatase is

increased by PGE_2 signaling via a cascade involving cAMP and cyclic AMP response element-binding protein (CREB), resulting in promoter switching and upregulated aromatase expression and activity [48–53]. Activation of this axis has been identified both in breast neoplasia and in normal mammary tissues in the context of obesity [41, 42, 54–56], and is presumed to be an important determinant of NSAID-mediated suppression of postmenopausal breast cancer. Thus it was logical to test the effect of *mPGES-1* deficiency on mammary aromatase activity in our study. We observed an approximately 45% reduction in aromatase activity in MGs from MMTV/NDL mice lacking mPGES-1 (Figure 3; P=0.006). These data support the notion that mPGES-1-derived PGE_2 drives aromatase expression in MMTV/NDL mammary tissues.

4. Discussion

In this study we tested the role of mPGES-1 in mammary tumorigenesis by crossing *mPGES-1*-deficient mice with the MMTV/NDL breast cancer model, and quantitating the multiplicity of MIN tumors. Based on our previous study in which we knocked out *COX-2* in the MMTV/NDL strain [4], we anticipated that we would see a global reduction in tumor number. Unexpectedly however, only tumors above the 1.0mm threshold were affected by loss of mPGES-1 (Table 1). Our data are strikingly similar to findings from the Rosenberg group, who have studied the role of mPGES-1 in intestinal tumorigenesis. They found that small intestinal polyp multiplicity in $Apc^{\Delta 14/+}$ mice was substantially reduced by *mPGES-1* deletion [35]. However, only tumors > 1.0mm diameter were decreased, whereas the multiplicity of polyps ≤ 1.0mm in diameter was significantly increased. Together the parallel findings in our study and in the $Apc^{\Delta 14/+}$ model suggest that mPGES-1, and by extension PGE_2, may contribute to tumor growth rather than to initial tumor formation in some experimental systems. This finding does not hold true for all models: *mPGES-1* deficiency impacts carcinogen-induced neoplasia at all stages including decreasing aberrant crypt focus formation [35, 37], and unexpectedly, Elander and colleagues observed increased tumor formation in $Apc^{Min/+}$ mice in the context of *mPGES-1* deficiency [57].

Interestingly, direct comparison of the consequences of knocking out *COX-2* and *mPGES-1* in MMTV/NDL mice suggests that other COX-derived eicosanoids could contribute to early tumor formation in this strain, since both knockouts result in comparable magnitudes of reduction of intramammary PGE_2 levels (*COX-2* ko, 50% reduction [4]; *mPGES-1* ko, 59% reduction, Figure 1), but only *COX-2* nulls exhibit a reduction in tumors <1mm in diameter. Knocking out *mPGES-1* was previously shown to cause a greater than 90% reduction in PGE_2 levels in lactating mammary gland [43], consistent with data from other systems where mPGES-1 has been identified as the predominant source of PGE_2 [20–24]. Residual PGE_2 levels in MGs from MMTV/NDL, *mPGES-1*-null mice in our experiment may reflect the activity of other PGE synthases or potentially non-enzymatic isomerization. It is conceivable that selective suppression of PGE_2 synthesis leads to a synthetic “shunt”, resulting in increased conversion of the PGH_2 precursor to other protumorigenic eicosanoids in the *mPGES-1* nulls relative to the *COX-2* ko mice. This could provide a rational basis for the decreased magnitude of tumor protection afforded by *mPGES-1* ablation.

Subsequent analyses in our study focused on identifying potential mechanistic explanations for the observed reduction in tumor growth in *mPGES-1*-deficient animals. Based on the known link between PGE_2 and angiogenesis [1, 44, 45], we first explored the impact of *mPGES-1* ablation on mammary vascularization. Consistent with the observed reduction in PGE_2 levels, both microvessel density and *VEGF-A* expression were significantly decreased in *mPGES-1* null MGs (Figure 2). These data are consistent with our previous reports of decreased vasculature in *COX-2* null MGs, and increased vascular development in *COX-2* transgenic glands [4, 46]. Previous reports similarly implicate mPGES-1 in angiogenesis

[38, 58, 59], with a clear role for stromal mPGES-1 identified by transplant studies. Consistent with a role for mPGES-1 in angiogenesis, correlations have been observed between levels of mPGES-1 and proangiogenic factors in some human cancers [60]. Given the well-established requirement for neovascularization for tumor growth, reduced angiogenesis provides a plausible explanation for the observed reduction in mammary tumor growth in *mPGES-1* knockout mice.

Also of interest was to determine the impact of *mPGES-1* ablation on the activity of the estrogen synthetase aromatase. PGE_2 is an established regulator of the *CYP19* gene encoding aromatase, acting via a clearly defined pathway involving cAMP and CREB, and ultimately resulting in increased transcription from cAMP-sensitive promoters [48–53]. MMTV/NDL MGs lacking mPGES-1 exhibited similar magnitudes of reduction in aromatase activity and PGE_2 levels (Figures 1 & 3), consistent with our previous data from *COX-2* knockout mice [52]. These data suggest that local estrogen production in mammary tissues is impaired in the absence of mPGES-1.

The role of estrogen receptor (ER) signaling in HER2/neu-driven breast cancer is complex. *HER2*-overexpressing human breast carcinomas tend to lack ER expression, as do invasive cancers in *HER2/neu* transgenic strains. Nevertheless, treatment of post-pubertal MMTV/*neu* mice with a selective estrogen receptor modulator (SERM) delays mammary tumor formation, implicating estrogen signaling in HER2/neu-induced tumor development [61]. In this context, estrogen may regulate mammary tumor formation through direct effects on epithelial cells prior to loss of ER expression during development of invasive lesions. Alternatively, the role of estrogen in *HER2/neu*-driven tumor formation may be primarily regulation of angiogenesis through interaction with ER-expressing stromal cells [62–64]. The ability of estrogen to regulate angiogenesis is well established [65], but the capacity of estrogen to promote ER-negative breast tumor growth via modulation of stromal cells in the tumor microenvironment is a comparatively recent discovery [62–64]. These findings suggest the possibility that the decreased vascularization observed in *mPGES-1*-null mammary glands could be a consequence not only of attenuation of PGE_2-driven synthesis of proangiogenic factors, but also of decreased PGE_2-dependent estrogen synthesis impacting stromal angiogenic responses.

In summary, using a genetic approach we have established a role for mPGES-1 in mammary tumor growth and angiogenesis. Multiplicity of HER2/neu-induced MIN lesions ≥1mm in diameter is reduced by *mPGES-1* nullizygosity, with corresponding reductions in mammary PGE_2, aromatase activity and angiogenesis. These data suggest mPGES-1 as a potential anti-breast cancer target, based on the reported upregulation of mPGES-1 in tumor epithelium in almost four-fifths of human breast cancers, as well as in DCIS [31]. Relative safety of this approach compared with COX-2 inhibition is suggested by the lack of prothrombotic phenotype associated with knocking out *mPGES-1* [14]. Importantly, we have recently identified that breast adipose inflammation with consequent upregulation of the PG-aromatase-estrogen signaling axis is associated with overweight and obesity [41, 42, 56], which may provide at least a partial explanation for the increased risk of breast cancer associated with obesity in post-menopausal women. Findings of the present study suggest mPGES-1 as a potential target for intervention to reduce the increased breast cancer risk associated with obesity in the post-menopausal setting.

Pharmacological mPGES-1 inhibitors for antiinflammatory and antineoplastic applications are currently under development. Evaluation of numerous compounds has identified several promising candidates [66–71], including some with *in vivo* analgesic/antiinflammatory properties, although no clinical studies have thus far been reported. Preclinical evaluation of these molecules in animal cancer models has been hampered by structural dissimilarities

between human and rodent mPGES-1 enzymes at the active site, which render mouse and rat orthologs insensitive to multiple compounds that have activity towards human mPGES-1. Nevertheless, mPGES-1 inhibitor-mediated suppression of tumor xenograft growth has recently been reported [59], providing important proof-of-principle for the validity of mPGES-1 inhibition as an anticancer approach.

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Highlights

- Knocking out mPGES-1 suppresses mouse mammary tumor growth
- Angiogenesis is reduced by genetic ablation of mPGES-1
- Aromatase activity is substantially reduced in mPGES-1-deficient mammary glands

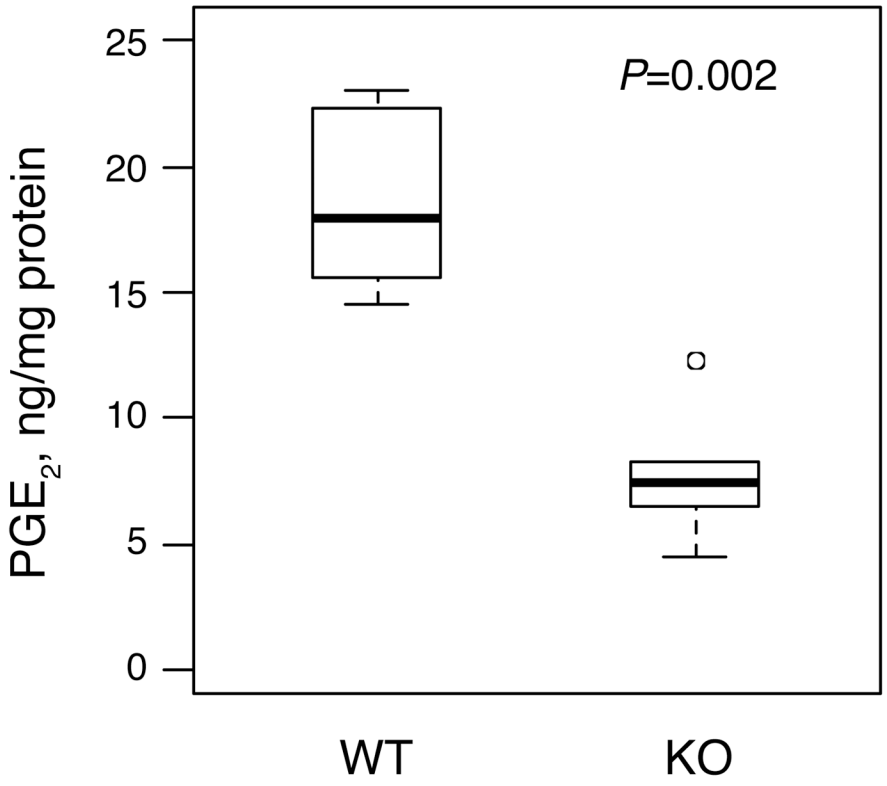

Fig. 1.

Mammary PGE_2 levels are markedly decreased in *mPGES-1* knockout MMTV/NDL mice. MGs were harvested from 20 week old virgin female mice that were MMTV/NDL, *mPGES-1* +/+ (WT) or MMTV/NDL, *mPGES-1* −/− (KO), and PGE_2 levels were assayed by ELISA. Mammary PGE_2 levels were reduced from 18.0 [14.5, 23.0] ng/mg protein (median [range], n=6) in MMTV/NDL, *mPGES-1* +/+ samples to 7.4 [4.5, 12.3] ng/mg protein in MMTV/NDL, *mPGES-1* −/− samples (*P*=0.002; Wilcoxon rank-sum test).

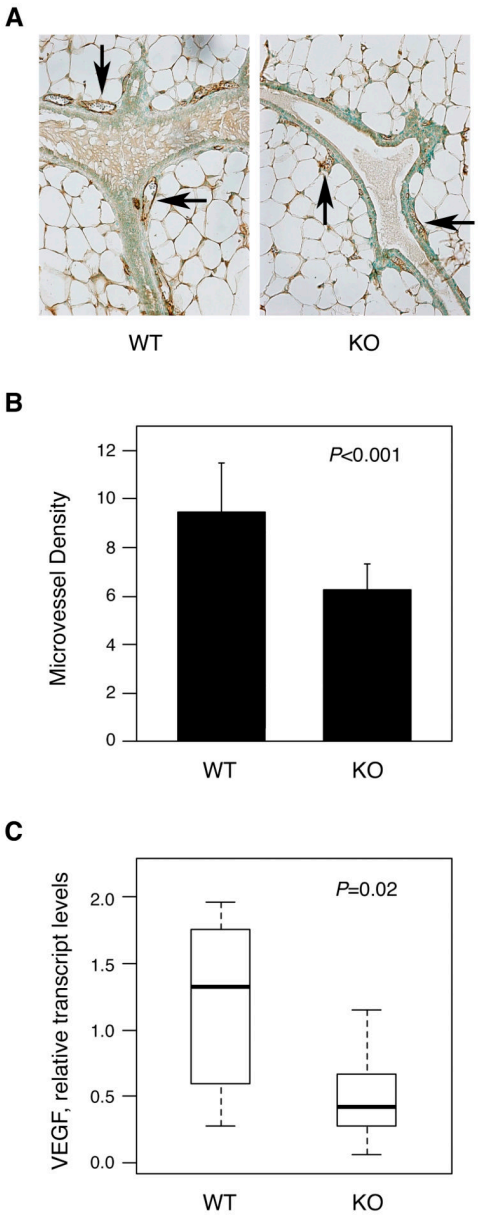

Fig. 2.

Mammary gland vascularization is reduced in *mPGES-1*-null MMTV/NDL mice. (**A, B**) Microvessel density is reduced in *mPGES-1*-null tissue. MG tissue sections from 20 week old virgin MMTV/NDL females that were *mPGES-1* wildtype (WT) or *mPGES-1* null (KO) were immunohistochemically stained with anti-CD31 antibody. Several microscopic fields were evaluated for each animal. The number of CD31-positive blood vessels associated with a ductal or lobular unit was scored in each microscopic field, and a mean value was calculated for each mouse. The mean CD31-positive blood vessel count in WT mice was significantly greater than that in KO mice: 9.45+/−2.05 (mean+/−SD, n=10) *vs* 6.26+/−1.07 (mean+/−SD, n=8); $P<0.001$ (Student t-test). Panel A shows representative images for *mPGES-1* wildtype (WT) and *mPGES-1* null (KO) mammary glands. Examples of CD31-positive blood vessels are indicated by arrows. Panel B shows the data obtained from numerical evaluation of anti-CD31-stained tissue sections. (**C**) VEGF levels are strikingly

reduced in *mPGES-1*-null tissue. Transcript levels of *VEGF-A* were assayed in MGs harvested from 20 week old virgin female mice that were MMTV/NDL, *mPGES-1* +/+ (WT) or MMTV/NDL, *mPGES-1* −/− (KO). Relative *VEGF-A* transcript levels (normalized to GAPDH) were reduced from 1.33 [0.28, 1.96] (median [range], n=10) in MMTV/NDL, *mPGES-1* +/+ samples to 0.42 [0.06, 1.15] in MMTV/NDL, *mPGES-1* −/− samples (P=0.02; Wilcoxon rank-sum test).

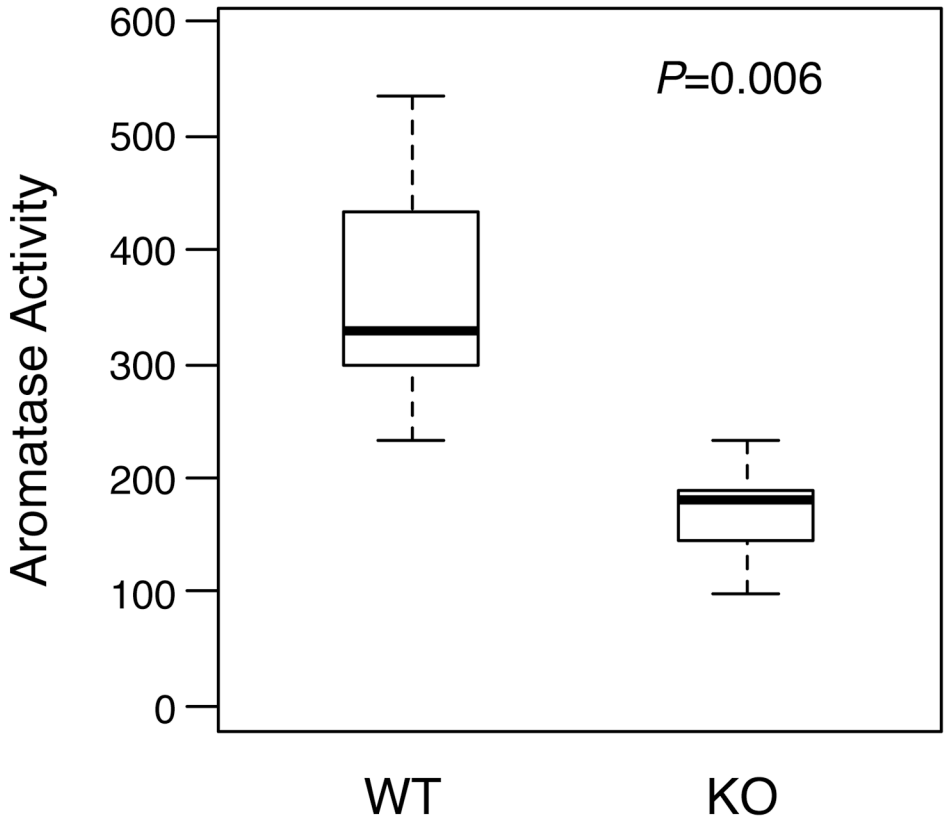

Fig. 3.

Aromatase activity is substantially reduced in MGs from *mPGES-1*-deficient MMTV/NDL mice. Aromatase activity was assayed in microsomes prepared from MGs harvested from 20 week old virgin female mice that were MMTV/NDL, *mPGES-1* +/+ (WT) or MMTV/NDL, *mPGES-1* −/− (KO). Aromatase activity was assayed by measuring tritiated water release from 1β-[^{3}H]-androstenedione. Mammary aromatase activity was reduced from 328 [234, 534] fmoles/μg protein/hr (median [range], n=6) in MMTV/NDL, *mPGES-1* +/+ samples to 182 [98, 234] fmoles/μg protein/hr in MMTV/NDL, *mPGES-1* −/− samples (*P*=0.006; Wilcoxon rank-sum test).

Table 1

Effect of *mPGES-1* deficiency on mammary tumor multiplicity

Genotype (n)	Tumors ≥0.25mm (Mean +/− sd)	*P* vs control ‡	Tumors ≥1.0mm (Mean +/− sd)	*P* vs control
MMTV/NDL, *mPGES-1* +/+ (22)	14.34 +/− 9.29	-	1.05 +/− 1.13	-
MMTV/NDL, *mPGES-1* +/− (20)	11.2 +/− 4.58	0.336	0.25 +/− 0.5	0.008
MMTV/NDL, *mPGES-1* −/− (30)	12.8 +/− 6.76	0.724	0.42 +/− 0.76	0.025

‡Comparisons effected with MMTV/NDL, *mPGES-1* +/+