Coupling of COX-1 to mPGES1 for prostaglandin E_2 biosynthesis in the murine mammary gland

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Abstract **The mammary gland, like most tissues, produces measurable amounts of prostaglandin E_2 (PGE_2), a metabolite of arachidonic acid produced by sequential actions of two cyclooxygenases (COX-1 and COX-2) and three terminal PGE synthases: microsomal prostaglandin E_2 synthase-1 (mPGES1), mPGES2, and cytosolic prostaglandin E_2 synthase (cPGES). High PGE_2 levels and COX-2 overexpression are frequently detected in mammary tumors and cell lines. However, less is known about PGE_2 metabolic enzymes in the context of normal mammary development. Additionally, the primary COX partnerships of terminal PGE synthases and their contribution to normal mammary PGE_2 biosynthesis are poorly understood. We demonstrate that expression of COX-1, generally considered constitutive, increases dramatically with lactogenic differentiation of the murine mammary gland. Concordantly, total PGE_2 levels increase throughout mammary development, with highest levels measured in lactating tissue and breast milk. In contrast, COX-2 expression is extremely low, with only a modest increase detected during mammary involution. Expression of the G_s-coupled PGE_2 receptors, EP2 and EP4, is also temporally regulated, with highest levels detected at stages of maximal proliferation. PGE_2 production is dependent on COX-1, as PGE_2 levels are nearly undetectable in *COX-1*-deficient mammary glands. Interestingly, PGE_2 levels are similarly reduced in lactating glands of *mPGES1*-deficient mice, indicating that PGE_2 biosynthesis results from the coordinated activity of COX-1 and mPGES1. We thus provide evidence for the first time of functional coupling between COX-1 and mPGES1 in the murine mammary gland in vivo.**—Chandrasekharan, S., N. A. Foley, L. Jania, P. Clark, L. P. Audoly, and B. H. Koller. **Coupling of COX-1 to mPGES1 for prostaglandin E_2 biosynthesis in the murine mammary gland. *J. Lipid Res.* 2005.** 46: **2636–2648.**

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Prostaglandin E_2 (PGE_2), a lipid mediator produced by most mammalian tissues, regulates multiple biological processes under both normal and pathological conditions. In addition to being a key mediator of inflammation, PGE_2 was recently demonstrated to play an important role in epithelial cell physiology, particularly in gastrointestinal tissues. The biosynthesis of PGE_2 is achieved by sequential actions of three groups of enzymes. First, membrane-bound and secretory phospholipase A_2 isoforms convert phospholipids to arachidonic acid (AA). Next, the cyclooxygenases (COXs) convert AA into the unstable intermediate, prostaglandin endoperoxide (PGH_2). Finally, terminal PGE_2 synthase (PGES) enzymes isomerize PGH_2 into PGE_2. Two COX enzymes, COX-1 and COX-2, catalyze the rate-limiting step in PGE_2 biosynthesis (i.e., conversion of AA to PGH_2). COX-1 and COX-2, in addition to having different subcellular localizations, also have different tissue expression profiles. COX-1 is primarily associated with constitutive or "housekeeping" functions in normal tissues. In contrast, with the exception of some organs such as the kidney, testis, and the central nervous system, COX-2 expression is extremely low in most normal tissues and is induced by growth factors, cytokines, and proinflammatory stimuli. High COX-2 expression is also associated with pathological conditions, such as tissue damage and malignant transformation of gastrointestinal and mammary epithelium. The distinct functional roles of COX-1 and COX-2 in vivo are further supported by different physiological defects observed in *COX-1*-deficient (*COX-1*$^{-/-}$) and *COX-2*$^{-/-}$ mice (1, 2).

To date, three different genes with PGES activity have been cloned (3). The first PGES, microsomal prostaglan-

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E_2 synthase; LC-MS, liquid chromatography-mass spectrometry; mPGES, microsomal prostaglandin E_2 synthase; PGDH, NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase; PGE_2, prostaglandin E_2; PGH_2, prostaglandin endoperoxide; PR, progesterone receptor; TXB_2, thromboxane B_2; VEGF, vascular endothelial growth factor.

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din E_2 synthase-1 (mPGES1), was isolated as a microsomal protein and is a member of the MAPEG (for membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily (4, 5). mPGES1 expression is low in most normal tissues, although abundant and constitutive expression is detected in a limited number of organs, such as the lung, kidney, and reproductive organs. Interestingly, mPGES1 expression is increased in a number of cancers, including lung, gastric, and colorectal tumors, similar to that observed for COX-2 (6, 7). Additionally, the coordinated inducibility of COX-2 and mPGES1 by proinflammatory factors and their efficient cooperation in converting AA to PGE_2 in vitro (8) has led to the generally accepted model that these two enzymes are predominantly coupled for PGE_2 biosynthesis. This is further supported by recent studies demonstrating that peritoneal macrophages derived from *mPGES1*$^{-/-}$ mice are unable to synthesize PGE_2 in response to lipopolysaccharide treatment, similar to the defect observed in *COX-2*$^{-/-}$ mice (9, 10). Two additional proteins with PGES activity have been identified recently. The cytosolic isoform, cPGES, was first isolated as a molecular chaperone in complex with HSP90 and the progesterone receptor (PR). cPGES is expressed ubiquitously and is thought to mediate constitutive PGE_2 biosynthesis. In vitro studies demonstrate that when overexpressed, cPGES couples preferentially with COX-1 (11). However, cPGES is also reported to partner with COX-2 at much lower efficiency for basal PGE_2 synthesis in specific cell types, which express COX-2 constitutively (12). The exact physiological functions of cPGES and its relative contribution to COX-1- or COX-2-directed biosynthesis in vivo remain unclear at this time. A second membrane-bound PGES, mPGES2, was originally isolated from microsomal fractions of the bovine heart and the corresponding human and murine genes were identified. mPGES2 is more widely expressed in mammalian tissues, has broader substrate specificity, and bears similarity to glutaredoxin and thioredoxin. mPGES2 is expressed constitutively in various tissues, and unlike mPGES1 it is not induced by proinflammatory signals. Coexpression of COX-1, COX-2, and mPGES2 in human cell lines revealed that mPGES2 can partner with either COX isoform in vitro for PGE_2 biosynthesis, with a slightly increased preference for COX-2 (13). However, the participation of mPGES2 in COX-1- and COX-2-directed biosynthesis in vivo is unknown at this time.

The functions of PGE_2 and the COX enzymes in inflammation and many associated pathological conditions have been extensively characterized. With PGE_2 biosynthetic enzymes emerging as important players in epithelial cancer biology, particularly colorectal and mammary cancers, a number of studies have focused on defining the role of COX-2-derived PGE_2 in tumor formation and progression. In contrast, the biological roles of PGE_2 and PGE synthase enzymes in normal epithelial tissues are far less studied. Recent reports demonstrate cPGES and mPGES1 are differentially coexpressed with COX-1 and COX-2 in luminal and glandular epithelial cells of the endometrium, suggesting that they may form unique partnerships for PGE_2 synthesis during the menstrual cycle and the establishment of pregnancy (14–16). Additionally, mPGES1 expression is regulated by gonadotropin in granulosa cells of primate ovarian follicles, suggesting that increases in PGE_2 levels during ovulation may be primarily regulated by mPGES1 (17). However, little is known about the contribution of mPGES1 or its COX partnerships for PGE_2 biosynthesis during normal mammary homeostasis in vivo. Mammary gland development is characterized by the coordination of multiple biological processes that results in regulated phases of cellular proliferation, differentiation, and apoptosis. We have examined the expression patterns of all terminal PGE synthases and COXs during different stages of murine mammary gland development. We have also determined the expression levels of all PGE_2 receptor isoforms, EP1–EP4, at these stages. In this study, we report that high PGE_2 levels detected during lactation result from a dramatic induction of COX-1 RNA and protein. We also demonstrate that the G_s-coupled receptors EP2 and EP4 have similar expression profiles, with maximal levels detected during the proliferative phase of pregnancy. PGE_2 biosynthesis in the mammary gland is dependent on COX-1, as *COX-1*$^{-/-}$ mice, although able to lactate efficiently, have no detectable PGE_2 in mammary tissue or breast milk. PGE_2 biosynthesis also appears to be primarily dependent on mPGES1, because PGE_2 levels are reduced significantly in lactating glands of *mPGES1*$^{-/-}$ mice. In contrast, PGE_2 levels measured in lactating mammary glands of *mPGES2*$^{-/-}$ mice were not significantly different from those of wild-type controls. These findings provide evidence that although all three terminal PGE synthases are expressed in the murine mammary gland, during normal mammary development COX-1 and mPGES1 are the predominant functional partners in vivo.

MATERIALS AND METHODS

Animals

C57BL/6 and B6/D2 females were purchased from Jackson Laboratories and Taconic Laboratories, respectively. Eight to 10 week virgin females were mated for isolation of mammary glands at gestation, lactation, and involution. Pregnancy was confirmed by detection of vaginal plugs. The first day of plug visualization was counted as day 0.5. *COX-1*$^{-/-}$ females were generated by intercrosses of *COX-1*$^{+/-}$ mice on the 129/B6D2 mixed genetic background. C57BL/6 *EP2*$^{-/-}$ congenic pairs were generated by backcrossing *EP2*$^{-/-}$ mice to the C57BL/6 strain for 12 generations (18). *mPGES1*$^{-/-}$ mice were maintained on the DBA/1lacJ background. *mPGES2*$^{-/-}$ and *mPGES2*$^{+/+}$ mice were generated by heterozygous intercross and were on the 129/B6D2 mixed genetic background. All animal colonies were maintained in accordance with institutional animal guidelines.

Measurement of PGE_2

The inguinal mammary glands without the lymph nodes were quickly dissected from each animal at the chosen developmental stage and snap-frozen in liquid nitrogen. Approximately 150 mg of frozen tissue was pulverized and then homogenized in ice-cold PBS containing 1 mM EDTA and 10 μM indomethacin. Homogenates were mixed with chilled 75% ethanol and treated with

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acetic acid to precipitate proteins. Prostaglandins were purified from tissue supernatants using Amprep Octadecyl C18 mini columns (Amersham Biosciences) according to the manufacturer's instructions. Prostaglandins were eluted with ethyl acetate, evaporated over N_2 at 40°C, and resuspended in ELISA assay buffer. Total (intracellular and secreted) steady-state PGE_2 levels were measured using the PGE_2 Monoclonal ELISA Kit (Neogen Corp.), which detects PGE_2 levels in the range of 0.10–4.00 ng/ml. ELISA was performed in duplicate for each sample. The cross-reactivity of the PGE_2 monoclonal ELISA to other eicosanoids (prostaglandin A_1, A_2, F_{1a} F_{2a}, and D_2 and leukotriene B_4) was <1%.The reactivity of the PGE_2 monoclonal antibody to isomeric PGE_2 analogs such as 8-iso-PGE_2, however, has not been determined, and PGE_2 levels measured by ELISA could include, in some part, the levels of isomeric compounds detected by this antibody. For measurement of PGE_2 levels in breast milk, lactating females were mildly anesthetized and milk let down was stimulated by intraperitoneal administration of oxytocin. Aliquots (250 μl) of milk were collected and diluted in 1× PBS/EDTA containing 10 μM indomethacin. PGE_2 was extracted as described above. The lower limit of detection for PGE_2 is 0.2 ng/ml milk. Measurement of prostanoids, PGE_2, PGD_2, PGF_2, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 (TXB_2) by liquid chromatography-mass spectrometry (LC-MS) was performed as described previously. (19) The lower limit of detection for PGE_2 by LC-MS is 0.0902 ng/mg protein. A representative chromatogram for the PGE_2 standard with retention times for PGE_2 (6.49) and PGD_2 (6.75) is shown in Fig. 5A below. Isomeric analogs of PGE_2, such as 8-iso-PGE_2 and 11-β-PGE_2, however, were not separated. The amounts of PGE_2 measured by LC-MS, therefore, potentially include the levels of these isomeric compounds as well (see Fig. 5D below).

Isolation of total RNA and Northern blotting

Inguinal mammary glands were dissected from three C57BL/6 females for each developmental stage: virgin, gestation, lactation, and involution. Samples were pooled, snap frozen in liquid nitrogen, divided randomly into three aliquots, and stored at −80°C. Pulverized frozen tissue was homogenized in RNA-Bee reagent (Tel-Test, Inc.), and total RNA was isolated according to the manufacturer's instructions. Twenty micrograms of total RNA per sample was electrophoresed on 1.2% (w/v) formaldehyde-agarose gels. RNA was then transferred to nitrocellulose (Immobilon NC; Amersham Biosciences) or nylon (Hybond-XL; Amersham Biosciences) membranes by capillary transfer overnight. Northern blots were hybridized with [^{32}P]dCTP (Amersham Biosciences)-labeled murine cDNA probes for the different PGE_2 metabolism and receptor isoform genes. cDNA probes for *cPGES* and *mPGES2* were derived from commercially available EST clones (Invitrogen Laboratories).

Real-time quantitative RT-PCR analysis

Total RNA was extracted from frozen tissue aliquots for each stage of mammary gland development as described above. Thirty to 50 μg of total RNA was further purified using Qiaprep RNA-easy columns (Qiagen), and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer apparatus. For each sample, cDNA was generated from 5 μg of purified total RNA using Multiscribe Reverse Transcriptase and random primers provided in the High-Capacity cDNA Archive Kit, according to the manufacturer's instructions (Applied Biosystems). Five nanograms of cDNA was used in amplification reactions performed using the Taqman PCR Universal Master Mix kit (Applied Biosystems) according to the manufacturer's instructions. All amplifications were performed on a Stratagene Mx300P cycler. Expression of *mPGES1*, *mPGES2*, *cPGES*, and *COX-1* was detected using the following Taqman primer and probe sets (Applied Biosystems): *COX-1*, Mm00477214_m1; *COX-2*, Mm00478374_m1; *cPGES*, Mm00727367_s1; *mPGES1*, Mm00452105_m1; *mPGES2*, Mm00460181_m1; *PGDH*, Mm00515121_m1. Expression levels were normalized to murine β_2-microglobulin used as the internal reference: Mm00437762 (Applied Biosystems). Expression of *COX-2* at different stages of mammary gland development (**Fig. 1C**) was measured by real-time quantitative PCR and normalized to the mouse β-actin gene (internal reference) for each developmental stage. Primers and probes for β-actin have been described previously (20). The following primers were used for the amplification of *COX-2*: forward, 5′ GTG CCA ATT GCT GTA CAA GC 3′; reverse, 5′ ATG ATG TGT ACG GCT TCA GG 3′. All reverse transcription and amplification reactions for *COX-2* were performed as described previously (20). Quantification of samples was performed using the comparative Ct (ΔΔCt) method for all of the above genes, as described in the Assays-on-Demand Users Manual (Applied Biosystems). Changes in expression levels during stages of pregnancy, lactation, and involution were calculated by the formula $x = 2^{(-\Delta\Delta Ct)}$ and expressed as fold changes compared to the resting virgin (10 week) mammary gland.

Isolation of protein and Western blotting

Total protein was isolated from inguinal mammary glands at different developmental stages by homogenizing frozen tissue aliquots in lysis buffer (50 mM HEPES, 0.15 M NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol containing freshly added protease inhibitors: 500 μM Na_3VO_4, 50 μM Na_2MoO_4, 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM PMSF). Homogenates were centrifuged at 14,000 rpm for 5 min at 4°C, and protein content of the supernatant was measured using BCA reagent (Pierce, Ltd.). Two hundred microliter aliquots of total protein were denatured by boiling at 95°C for 5 min and electrophoresed on 8% SDS-PAGE gels. Proteins were transferred using the Transblot™ apparatus (Bio-Rad) to Immobilon™ polyvinylidene difluoride membranes (Millipore) by electrolytic transfer. Membranes were blocked in 5% nonfat milk in 1× PBS containing 0.05% Tween 20 for 1 h at room temperature. Goat anti-mouse COX-1 antibody was used at a 1:200 dilution (Santa Cruz Biotechnology). Blots were incubated with primary antibodies diluted in 5% nonfat milk in 1× PBS containing 0.05% Tween 20 for 1–2 h at room temperature. Donkey anti-goat secondary antibodies (Santa Cruz Biotechnology) were diluted 1:10,000 in 5% nonfat milk in 1× PBS containing 0.05% Tween 20 and applied to membranes for 30 min at room temperature. Membranes were incubated with ECL reagent, and signal was detected by autoradiography after exposure to Hyperfilm™ (Amersham Biosciences). Mouse anti-β-actin (Sigma) was used at a dilution of 1:5,000, and goat anti-mouse secondary antibody (Pierce, Ltd.) was used at a 1:10,000 dilution.

Densitometric analysis

Autoradiographs were scanned, and density of signal for bands of interest was measured using ImageQuant software. To generate normalized fold changes in gene expression during murine mammary gland development, the signal density of GAPDH was measured for each sample in the blot. Because the representation of housekeeping gene RNAs decreases compared to milk protein RNAs in the late-pregnant and lactating mammary gland, we determined the "mean" GAPDH expression level per blot. A correction factor based on this mean was then applied to each sample to get normalized "corrected" expression levels for every gene at each developmental stage. The expression levels at pregnancy, lactation, and involution stages were then expressed

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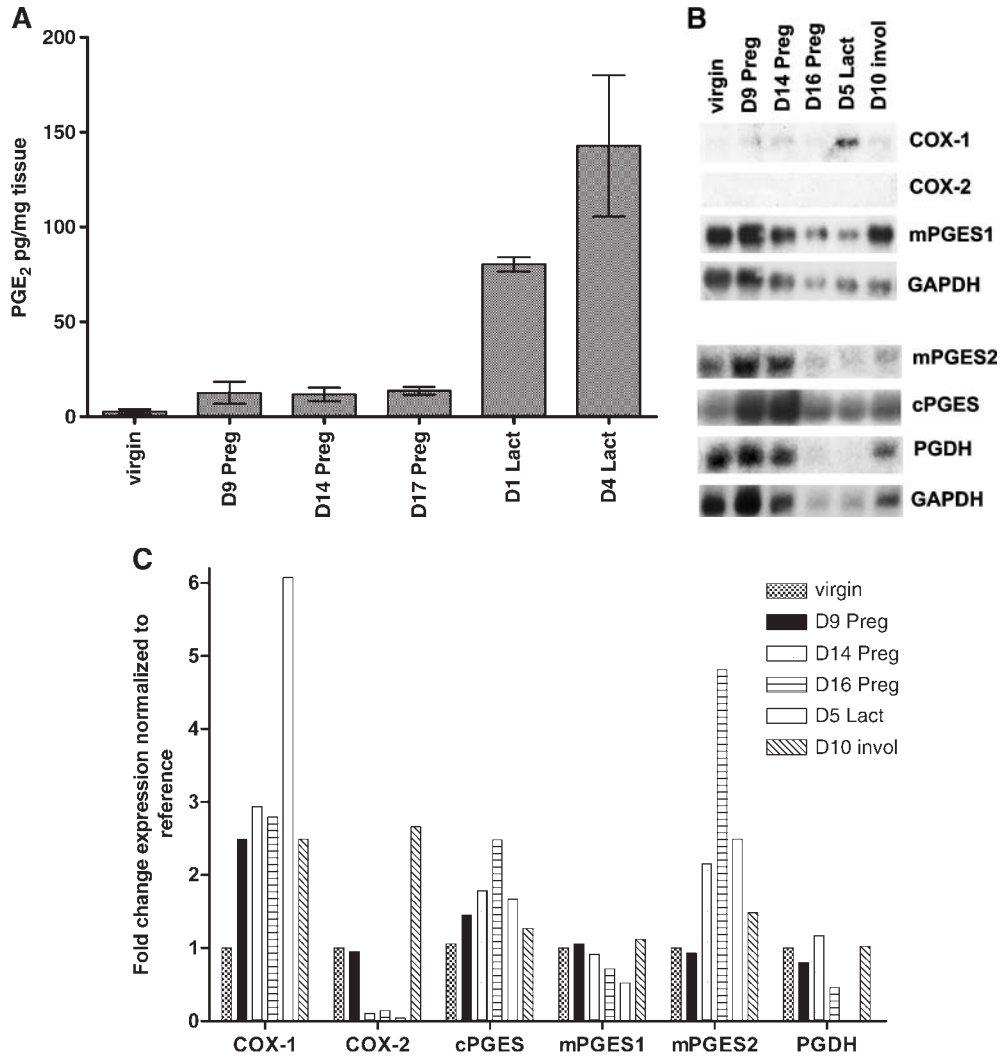

Fig. 1. Differential expression of prostaglandin E_2 (PGE_2) biosynthesis enzymes during murine mammary gland development. A: Total cellular PGE_2 levels were measured by ELISA in inguinal mammary glands derived from mice at the following stages: 10 week virgin, day 9 of pregnancy, day 14 of pregnancy, day 17 of pregnancy, day 1 of lactation, and day 4 of lactation. PGE_2 values represent means ± SEM; n = 3 animals per stage. B: Northern blot analysis of steady-state RNA levels of genes regulating PGE_2 biosynthesis and catabolism at different points of mammary gland development. Total RNA was isolated from inguinal mammary glands pooled from three separate mice at the following stages: 10 week virgin, day 9 of pregnancy, day 14 of pregnancy, day 16 of pregnancy, day 5 of lactation, and day 10 of involution. Twenty micrograms of total RNA was electrophoresed on 1.2% agarose gels and transferred to nylon membranes by capillary transfer. Membranes were probed with [^{32}P]dCTP-labeled murine cDNA probes corresponding to the genes of interest and murine GAPDH cDNA for normalization of samples. COX, cyclooxygenase; cPGES, cytoplasm prostaglandin E_2 synthase; mPGES, microsomal prostaglandin E_2 synthase; PGDH, NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase. C: Expression of genes was quantified by densitometric analysis of scanned autoradiographs. Gene expression levels at every developmental stage were normalized to the mean GAPDH expression per blot. Normalized levels at each stage were then expressed as fold changes relative to the 10 week virgin gland. Expression of *COX-2* was detected by real-time quantitative PCR, normalized to β-actin, and then expressed as fold changes relative to the 10 week virgin gland.

as fold changes compared with the levels detected in the resting virgin (10 week) mammary tissue.

Statistical analysis

All statistical analyses were performed applying the appropriate statistical tests mentioned in Results using the JMPIN (version 5.1) statistical software package (SAS).

RESULTS

PGE_2 biosynthetic enzymes are differentially expressed during normal mammary gland development

Total steady-state PGE_2 concentrations in the mammary gland were first measured at various developmental stages, including the mature virgin gland, early pregnancy, mid

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pregnancy, lactation, and involution, by ELISA. PGE_2 levels increased modestly during pregnancy compared to the virgin gland. However, levels increased dramatically during lactation (~19-fold higher at day 4 lactation compared with day 17 pregnancy) compared with even late gestational time points (Fig. 1A). Comparison of total PGE_2 levels at different stages of mammary gland development using the Tukey-Kramer HSD multiple comparison test revealed that increases in PGE_2 levels during lactation were significantly different ($P < 0.05$) from those detected during all pregnancy stages. Consistent with this, high levels of PGE_2 were also measured in breast milk expressed from lactating mammary glands (data not shown). Because the biosynthesis of PGE_2 is regulated by a number of enzymes, we next examined the steady-state RNA levels of *COX-1*, *COX-2*, and all three terminal PGE synthases at different developmental stages (Fig. 1B). Expression levels at each developmental stage were normalized to *GAPDH* (internal reference) and compared with those of the resting virgin gland (Fig. 1C). While expression of *COX-2* could not be detected at any of the stages by Northern blotting (Fig. 1B, top panel), *COX-1* expression increased dramatically during lactation compared with the virgin gland (Fig. 1B, C, day 5.0 lactation). We further examined the levels of *COX-2* and *COX-1* mRNA using real-time PCR. Low expression of *COX-2* was detected at each stage, with the highest levels measured on day 10 of involution (Fig. 1C). The highest levels of *COX-1* were detected during lactation, consistent with results obtained from Northern blot analysis (data not shown). The expression of *cPGES* increased during pregnancy, reaching maximal levels by late pregnancy (Fig. 1C, day 16 pregnancy), and returned to levels comparable to those of the resting virgin gland by day 10 of involution. Similarly, expression levels of the recently isolated *mPGES2* also increased during mid pregnancy and remained high during lactation. *mPGES1* expression was easily detectable by Northern blotting at all stages of mammary gland development but did not vary much during pregnancy or lactation compared with the virgin gland. The expression of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH), the enzyme primarily responsible for PGE_2 degradation, was measured in each developmental stage. *PGDH* is expressed moderately throughout mammary gland development, and total RNA levels do not change measurably during early pregnancy compared with those in the virgin gland. In contrast, *PGDH* expression was reduced to nearly half of that detected in the virgin gland on day 16 of pregnancy and was undetectable in day 5 lactating tissue (Fig. 1B, C).

Expression of the PGE_2 receptors EP2 and EP4 is maximally induced during pregnancy in the normal mammary gland

The intracellular effects of PGE_2 are mediated by four receptors, EP1–EP4, which activate diverse signaling pathways, potentially regulating multiple biological functions. Additionally, these receptors have unique tissue expression profiles. To facilitate the understanding of PGE_2 functions in mammary tissue homeostasis, we also examined the expression of all receptor isoforms during normal mammary gland development. The steady-state levels of *EP1*, *EP2*, *EP3*, and *EP4* RNA were analyzed by Northern blotting (**Fig. 2A**). Expression levels were normalized to *GAPDH* and compared with those of the resting virgin gland (Fig. 2B). *EP3* expression was high in the virgin gland and through the early and mid pregnancy stages. *EP3* RNA levels were reduced dramatically by day 16 of pregnancy and in day 5 lactating tissue. *EP1* expression, in contrast, was not detectable by Northern blotting at any developmental stage. The expression of *EP2*, although barely detectable in the virgin gland, increased nearly 200-fold by late gestation (Fig. 2B, day 16 pregnancy), remained high during lactation, and returned to levels comparable to those of the virgin gland by day 10 of involution. Expression levels of *EP4*, although much lower than those of *EP2*, also increased dramatically by day 16 of gestation. Similar to *EP2*, *EP4* RNA levels remained higher in the lactating gland and returned to levels comparable to those of the resting virgin gland by the completion of involution (Fig. 2B). Expression of *EP1* was further analyzed by real-time quantitative PCR. In accordance with the Northern blot experiments, EP1 mRNA levels, although readily detected in the kidney, were extremely low and barely detectable at all stages of mammary development (data not shown). Because the maximal expression of *EP2* precedes peak PGE_2 levels detected in the mammary gland, we investigated the possibility that EP2 may regulate PGE_2 synthesis in an autocrine manner. PGE_2 levels, therefore, were measured in mammary glands of $EP2^{-/-}$ mice at the developmental stages described above. PGE_2 levels were found to be comparable to those of wild-type mice in virgin $EP2^{-/-}$ mammary glands. Furthermore, no significant changes in PGE_2 levels were detected in mammary glands of $EP2^{-/-}$ mice compared with their wild-type counterparts at day 17 of pregnancy, when *EP2* expression would be maximal, or on day 4 of lactation (Fig. 2C).

COX-1 is necessary for PGE_2 biosynthesis in the normal mammary gland

Because the increase in *COX-1* RNA by day 5 of lactation paralleled the increase in PGE_2 levels during lactation, we examined COX-1 protein expression levels in greater detail at all developmental time points. COX-1 protein levels increased dramatically by day 1 of lactation (**Fig. 3A**), remained high on day 4 of lactation, similar to the steady-state RNA levels, and returned to levels comparable to those of the virgin gland by day 10 of involution. On longer exposure of the Western blot, low levels of COX-1 expression were also detected on days 9 and 14 of pregnancy and on day 10 of involution (data not shown). Therefore, we measured PGE_2 levels in lactating mammary glands derived from $COX\text{-}1^{+/+}$, $COX\text{-}1^{+/-}$, and $COX\text{-}1^{-/-}$ mice. PGE_2 was almost undetectable in mammary glands of $COX\text{-}1^{-/-}$ lactating glands ($P = 0.007$, $COX\text{-}1^{+/+}$ vs. $COX\text{-}1^{-/-}$, by Student's *t*-test) and in breast milk ex-

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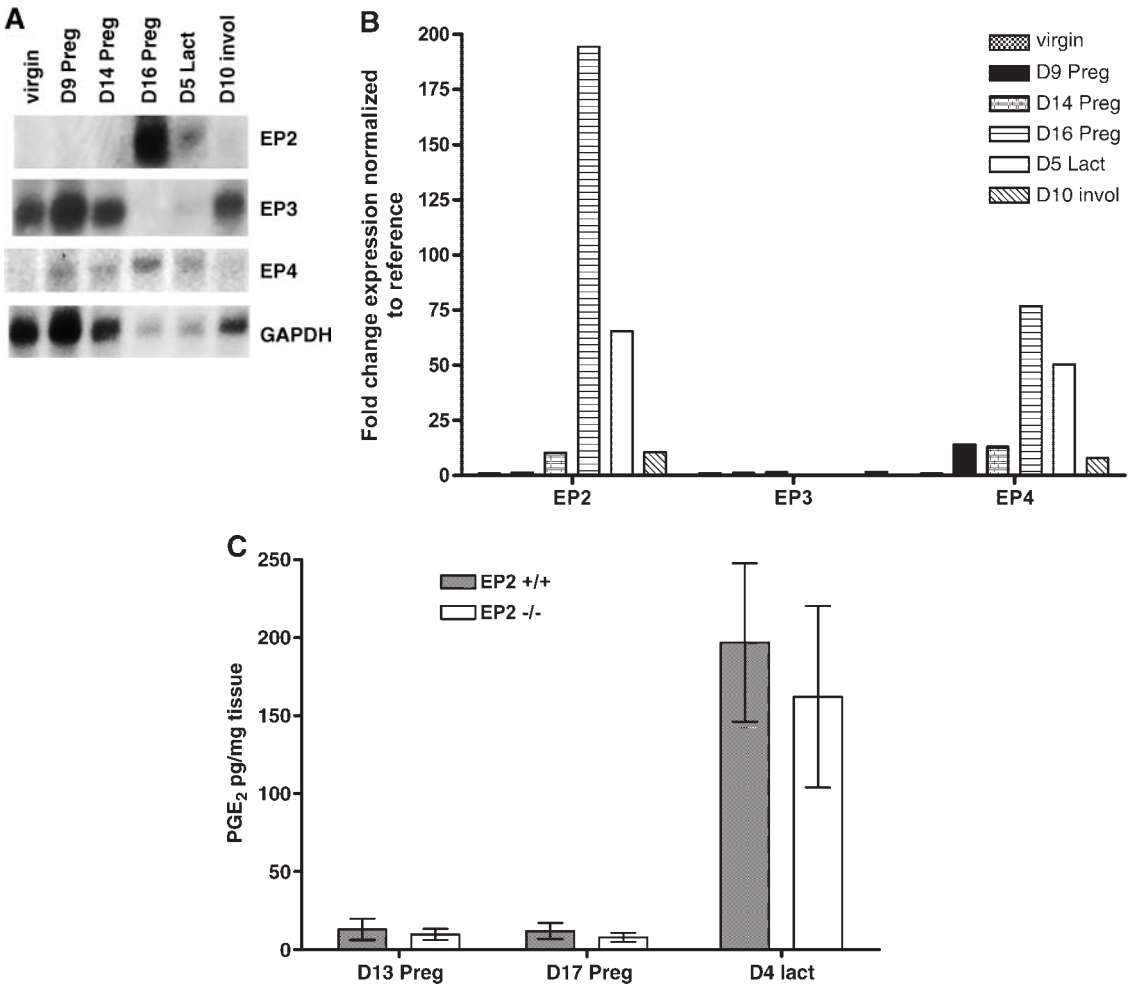

Fig. 2. Differential expression of PGE_2 receptor isoforms during murine mammary gland development. A: Northern blot analysis of steady-state expression levels of PGE_2 receptor (subtypes EP2, EP3, and EP4) RNAs at different stages of mammary gland development. Total RNA was isolated from inguinal mammary glands pooled from three separate mice at the following developmental stages: 10 week virgin, day 9 of pregnancy, day 14 of pregnancy, day 16 of pregnancy, day 5 of lactation, and day 10 of involution. Twenty micrograms of total RNA was electrophoresed on 1.2% agarose gels and transferred to nylon membranes by capillary transfer. Membranes were probed with [^{32}P]dCTP-labeled cDNA probes corresponding to each EP receptor. Membranes were probed with murine GAPDH cDNA for normalization of expression. B: Expression of genes was quantified by densitometric analysis of scanned autoradiographs. Gene expression levels were normalized to GAPDH (internal reference) and then expressed as a fold change at each stage compared with the 10 week virgin gland. C: Total PGE_2 levels in inguinal mammary glands of $EP2^{+/+}$ and $EP2^{-/-}$ mice at the following developmental stages: day 13 of pregnancy, day 17 of pregnancy, and day 4 of lactation. PGE_2 was extracted, and total cellular levels were measured by ELISA. Values are presented as means ± SEM for three separate animals per stage.

pressed from *COX-1*$^{-/-}$ lactating females compared with their wild-type littermates (Fig. 3C). Interestingly, PGE_2 levels were also reduced significantly in *COX-1*$^{+/-}$ glands compared with wild-type controls ($P = 0.014$, *COX-1*$^{+/+}$ vs. *COX-1*$^{+/-}$, by Student's *t*-test) (Fig. 3B). Concordantly, intermediate COX-1 protein levels were detected in the mammary glands of lactating *COX-1*$^{+/-}$ females compared with wild-type females (Fig. 3B). Whole-mount and histological analyses of *COX-1*$^{-/-}$ mammary glands did not reveal any abnormalities compared with wild-type controls (data not shown). To verify that loss of COX-1 did not alter the expression profiles of other PGE synthases, we analyzed the expression of *mPGES1* and *mPGES2* in *COX-1*$^{-/-}$ glands on day 4 of lactation by real-time quantitative PCR. *mPGES1* levels were increased slightly in *COX-1*$^{-/-}$ lactating glands compared with *COX-1*$^{+/+}$ mice, but this difference was not statistically significant (Fig. 3D). Similarly, *mPGES2* and *cPGES* expression levels (data not shown) were not significantly different between *COX-1*$^{-/-}$ and *COX-1*$^{+/+}$ lactating mammary tissues.

PGE_2 synthesis in vivo is mediated by mPGES1 and COX-1 coupling

As described above, the PGE_2 synthases mPGES1, mPGES2, and cPGES are expressed at varying levels in the mammary gland, with *mPGES2* and *cPGES* RNA levels increasing by mid pregnancy. However, the expression profiles of these synthases do not exactly coincide with that of COX-1 or with the total PGE_2 levels measured, particularly during lactation. Although in vitro studies have demonstrated that cPGES couples preferentially with COX-1, mPGES1 is thought to be the primary partner for COX-2-

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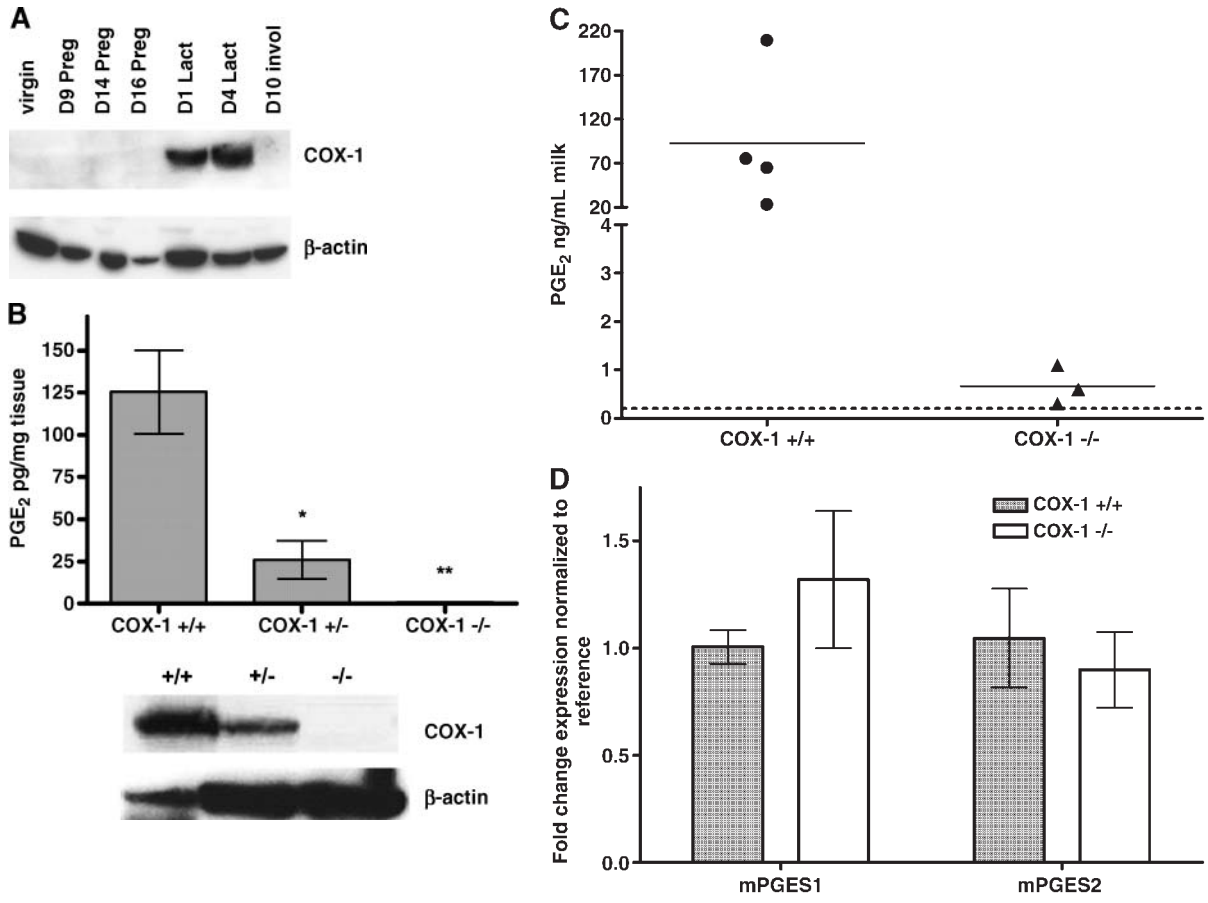

Fig. 3. COX-1 mediates the increased production of PGE_2 during lactation in vivo. A: COX-1 protein levels increase during lactation in the mammary gland. Total cellular proteins were isolated from inguinal mammary glands at the following stages of development: 10 week virgin, day 9 of pregnancy, day 14 of pregnancy, day 16 of pregnancy, day 1 of lactation, day 4 of lactation, and day 10 of involution. Two hundred mircrogram total protein aliquots were electrophoresed on 8% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membrane, and the blot was incubated with anti-mouse COX-1 antibody. The blot was also probed with murine β-actin antibodies to control for loading. B: Top panel, total PGE_2 levels were measured in inguinal mammary glands of *COX-1*$^{+/+}$, *COX-1*$^{+/-}$, and *COX-1*$^{-/-}$ mice on day 4 of lactation by ELISA. Values represent means ± SEM for six *COX-1*$^{+/+}$, three *COX-1*$^{+/-}$, and three *COX-1*$^{-/-}$ mice. * $P < 0.05$, *COX-1*$^{+/+}$ versus *COX-1*$^{+/-}$; ** $P < 0.01$, *COX-1*$^{+/+}$ versus *COX-1*$^{-/-}$, by Student's *t*-test. Bottom panel, representative Western blot of COX-1 expression levels in *COX-1*$^{+/+}$, *COX-1*$^{+/-}$, and *COX-1*$^{-/-}$ mammary glands on day 4 of lactation. Total protein was isolated from inguinal mammary glands, and 200 μg protein aliquots were electrophoresed on 8% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membrane, and the blot was incubated with anti-mouse COX-1 and anti-mouse β-actin antibodies. C: PGE_2 levels were measured in breast milk expressed from *COX-1*$^{+/+}$ (n = 4) and *COX-1*$^{-/-}$ (n = 3) mice at days 12–18 postpartum by ELISA as described previously. The lower limit of detection for PGE_2 is 0.2 ng/ml breast milk and is represented as a dotted line. D: Microsomal *mPGES1* and *mPGES2* expression levels are not altered in *COX-1*$^{-/-}$ mammary glands. mRNA levels of *mPGES1* and *mPGES2* were measured in total RNA (means ± SEM) isolated from *COX-1*$^{+/+}$ (n = 3) and *COX-1*$^{-/-}$ (n = 3) day 4 lactating mammary glands by real-time quantitative PCR. Expression levels of genes were normalized to β_2-microglobulin (internal reference) and are presented as fold change (arbitrary units) in *COX-1*$^{-/-}$ mice relative to wild-type animals.

directed PGE_2 biosynthesis. In contrast, mPGES2 was demonstrated to cooperate with either COX-1 or COX-2 in vitro. To clearly define the COX-1 coupling selectivity of PGE_2 synthases in mammary tissue in vivo, we measured PGE_2 levels in lactating mammary glands of *mPGES1*$^{-/-}$ mice. As shown in **Fig. 4A**, PGE_2 levels are reduced significantly in *mPGES-1*$^{-/-}$ mice compared with their wild-type counterparts ($P = 0.0053$, *mPGES1*$^{+/+}$ vs. *mPGES1*$^{-/-}$, by Student's *t*-test). To confirm that the decrease in PGE_2 levels measured in *mPGES1*$^{-/-}$ mammary glands was not attributable to alterations in the expression levels of either the COX genes or other terminal PGE synthases, we next measured the expression levels of *COX-1*, *COX-2*, and all PGE_2 synthases in lactating mammary glands of *mPGES1*$^{-/-}$ mice by real-time quantitative PCR (Fig. 4B). As described previously, extremely low *COX-2* expression levels were detected in both *mPGES1*$^{+/+}$ and *mPGES1*$^{-/-}$ lactating glands (data not shown). *COX-1* levels were identical in both *mPGES1* wild-type and *mPGES1*$^{-/-}$ lactating mammary glands, as expected. *mPGES2* expression levels were not significantly different in *mPGES1*$^{-/-}$ glands compared with those in wild-type controls. Although *cPGES* levels were slightly decreased in *mPGES1*$^{-/-}$ lactating mammary glands, this change was not statistically significant compared with *mPGES1*$^{+/+}$ mice. To further investigate the contribution of mPGES2 to PGE_2 production in vivo, we

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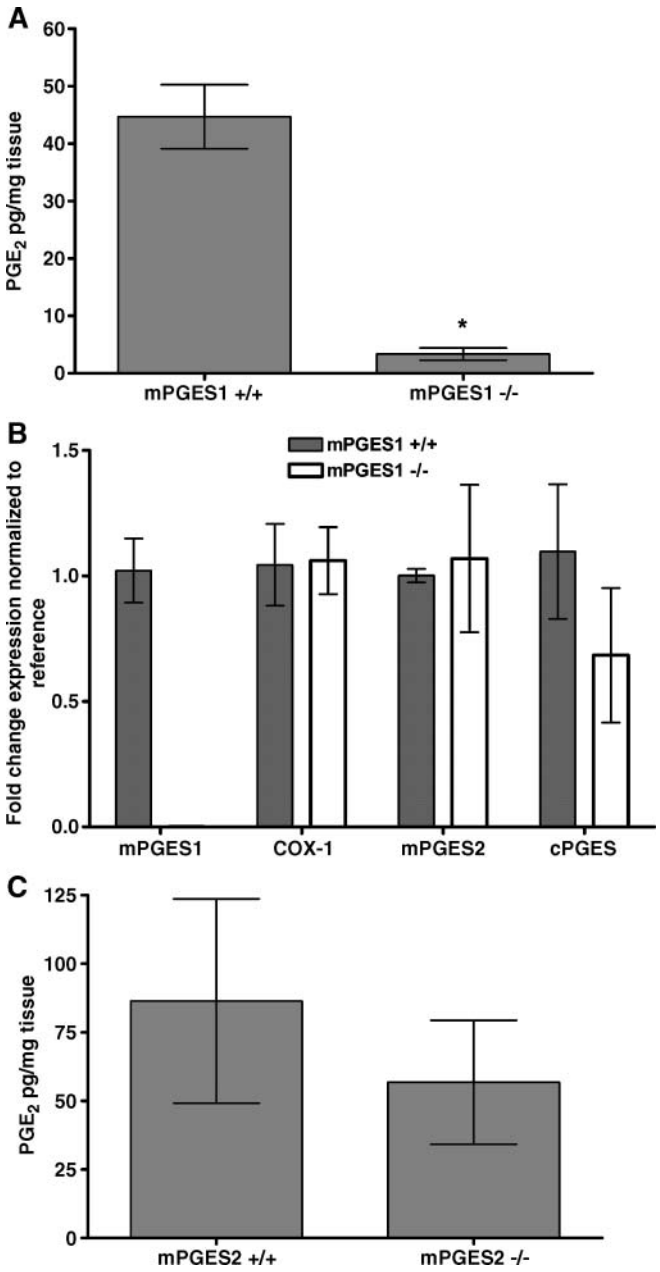

Fig. 4. mPGES1 is necessary for increased PGE_2 synthesis in the lactating mammary gland. A: Total PGE_2 levels were measured by ELISA in inguinal mammary glands of *mPGES1*$^{+/+}$ and *mPGES1*$^{-/-}$ mice on day 4 of lactation. Values represent means ± SEM for *mPGES1*$^{+/+}$ (n = 4) and *mPGES1*$^{-/-}$ (n = 5) mice. * $P < 0.01$, *mPGES1*$^{+/+}$ versus *mPGES1*$^{-/-}$, by Student's *t*-test. B: Real-time quantitative PCR analysis of expression levels of *mPGES1*, *COX-1*, *mPGES2*, and *cPGES* in total RNA isolated from day 4 lactating mammary glands of *mPGES1*$^{+/+}$ (n = 4) and *mPGES1*$^{-/-}$ (n = 4) mice. Expression levels (means ± SEM) were normalized to β_2-microglobulin (internal reference) and are presented as fold change (arbitrary units) in *mPGES1*$^{-/-}$ glands compared with mean wild-type expression levels. C: PGE_2 levels measured by ELISA in mammary glands of *mPGES2*$^{-/-}$ mice and wild-type littermates on day 4 of lactation. PGE_2 levels are expressed as means ± SEM for *mPGES2*$^{+/+}$ (n = 4) and *mPGES2*$^{-/-}$ (n = 4) animals.

measured PGE_2 levels in day 4 lactating mammary glands of *mPGES2*$^{-/-}$ mice, recently generated in our laboratory (L. Jania, L. P. Audoly, and B. H. Koller, unpublished data). PGE_2 levels were decreased slightly in *mPGES2*$^{-/-}$ mice but not significantly different from those detected in wild-type controls ($P = 0.5$, *mPGES2*$^{+/+}$ vs. *mPGES2*$^{-/-}$, by Student's *t*-test). More interestingly, PGE_2 production was not reduced to levels comparable to those measured in *mPGES1*$^{-/-}$ or *COX-1*$^{-/-}$ mice (Figs. 3A, 4A). To further verify that PGE_2 production during lactation was mediated primarily by mPGES1, we measured levels of PGE_2 and several other prostanoids by LC-MS in lactating mammary glands of *mPGES1*$^{+/+}$, *mPGES1*$^{+/-}$, and *mPGES1*$^{-/-}$ mice. Representative chromatograms for the PGE_2 standard (**Fig. 5A**), a high PGE_2 sample from a *mPGES1*$^{+/+}$ mammary gland (Fig. 5B), and a low PGE_2 sample from a *mPGES1*$^{-/-}$ gland are shown in Fig. 5C. PGE_2 levels were not significantly different between *mPGES1*$^{+/+}$ and *mPGES1*$^{+/-}$ mice, although a nearly 50% reduction in PGE_2 was measured in the *mPGES1*$^{+/-}$ mice. PGE_2 levels were decreased significantly in mammary glands of *mPGES1*$^{-/-}$ mice compared with *mPGES1*$^{+/-}$ mice ($P = 0.036$) (Fig. 5D). Although mean PGE_2 levels were decreased substantially (nearly 10-fold) in *mPGES1*$^{-/-}$ glands compared with wild-type controls, this difference did not reach statistical significance because of the high variability in the levels of PGE_2 measured in *mPGES1*$^{+/+}$ mice. No significant differences in the levels of 6-keto-$PGF_{1\alpha}$ and PGF_2 were measured in *mPGES1*$^{-/-}$ mammary glands compared with either *mPGES1*$^{+/+}$ or *mPGES1*$^{+/-}$ mice (data not shown). Interestingly, PGD_2 levels, which were extremely low and largely below the threshold of detection in *mPGES1*$^{+/+}$ and *mPGES1*$^{+/-}$ mice, increased nearly 3- to 4-fold in *mPGES1*$^{-/-}$ samples. A similar trend was also observed for the levels of TXB_2 measured in *mPGES1*$^{-/-}$ lactating mammary glands (data not shown).

DISCUSSION

The mammary gland is a complex and dynamic organ composed of multiple tissue types. The physiology of the mammary gland is regulated by systemic factors like the ovarian-pituitary axis hormones, local growth factors, and products of various biochemical and metabolic pathways. Although the role of PGE_2 and enzymes of this biosynthetic pathway, particularly the COXs, is being extensively studied in the context of breast cancer, little is known about their functions in normal mammary gland tissue homeostasis. The postnatal development of the adult murine mammary gland is well defined and regulated. Specific cellular processes, changes in tissue architecture, differential gene expression, and activation of different biosynthetic pathways characterize each developmental stage. To examine the overall profile of PGE_2 biosynthesis through this developmental cycle, we first measured total PGE_2 levels in the mammary gland at different stages of adult development. PGE_2 levels are barely detectable in the pubescent gland (10 weeks), by which time the ductal architecture of the gland has been completely established. PGE_2 levels in-

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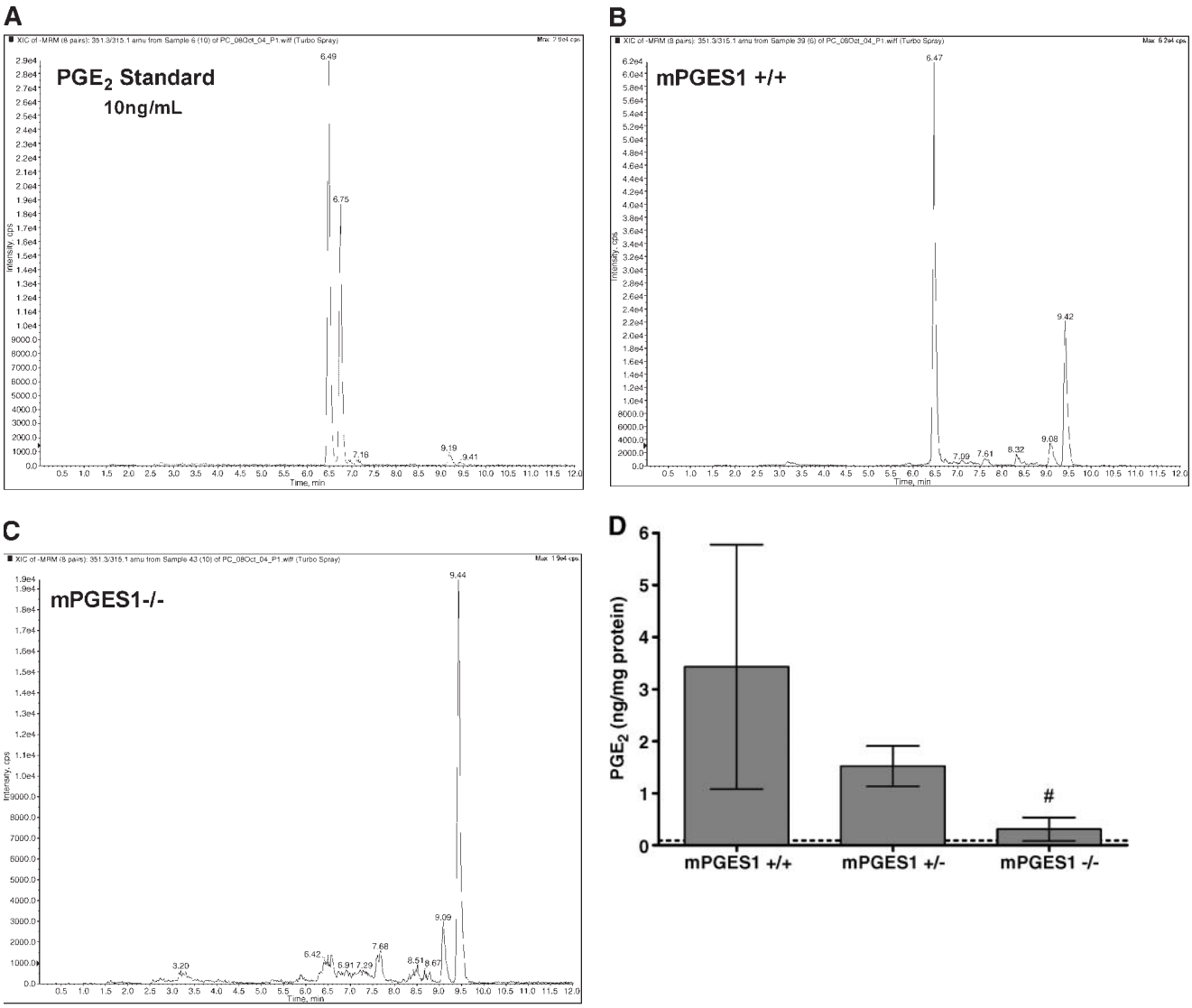

Fig. 5. PGE_2 levels were measured in inguinal mammary glands of *mPGES1*$^{+/+}$ (n = 5), *mPGES1*$^{+/-}$ (n = 4), and *mPGES1*$^{-/-}$ (n = 4) animals on day 4 of lactation by liquid chromatography-mass spectrometry. A: Representative chromatogram for the PGE_2 standard (10 ng/ml) in which PGE_2 has the peak with 6.49 retention time. (PGD_2 has a retention time of 6.75 in this chromatogram.) B: Representative chromatogram for a high-PGE_2 sample (mPGES1$^{+/+}$) based on the 6.4 retention time peak. C: Representative chromatogram for a low-PGE_2 (*mPGES1*$^{-/-}$) sample. D: PGE_2 levels are presented as means ± SEM. # Statistically significant ($P < 0.05$) difference in PGE_2 levels between *mPGES1*$^{+/-}$ and *mPGES1*$^{-/-}$ mice. The lower limit of quantitation for PGE_2 is 0.0902 ng/mg protein and is indicated by the dotted line.

crease modestly during pregnancy, which is marked by high epithelial proliferation, but increase dramatically during lactation (Fig. 1A). Thus, high PGE_2 levels appear to be associated with terminal differentiation, rather than proliferation of mammary epithelium during normal gland development. These findings are also consistent with those previously reported for the rodent mammary gland (21), in which PGE_2 levels were found to be lower in the resting virgin gland and highest in lactating mammary glands.

PGE_2 metabolic enzymes are reported to have tissue-specific expression patterns and are putatively regulated by specific stimuli and cellular stresses. To identify the primary enzymes modulating PGE_2 concentrations in the normal mammary gland, we examined the steady-state RNA levels of all the genes implicated in PGE_2 biosynthesis and catabolism at different developmental stages. *COX-1* RNA was extremely low in the virgin gland, but levels increased nearly 6-fold during lactation. In contrast, *COX-2* expression levels in normal mammary tissue were not detectable by Northern blotting in our experiments. However, *COX-2* expression was detected at very low levels at all developmental time points by real-time quantitative RT-PCR. A 3-fold increase in *COX-2* mRNA levels measured in the involuting gland (Fig. 1C) possibly reflects the contribution of infiltrating macrophages and neutrophils activated by massive tissue remodeling and inflammation during mammary gland regression (22). Similar to *COX-1*, *mPGES1* expression was easily detected by Northern blotting at all mammary developmental time points. However, unlike *COX-1*, *mPGES1* levels varied little during preg-

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nancy or lactation. The two putative synthases, *mPGES2* and *cPGES*, are also highly expressed in the mammary gland. In contrast to *mPGES1*, *cPGES* and *mPGES2* levels are increased at mid pregnancy (days 14–16 of pregnancy) and lactation compared with the resting virgin gland. PGE_2 is rapidly catabolized to a less active 15-keto metabolite by PGDH. We show for the first time that *PGDH* is expressed in the mammary gland at most developmental stages, suggesting that PGE_2 concentrations can be locally modulated in mammary tissue. Interestingly, we were unable to detect any *PGDH* RNA during lactation. The downregulation of *PGDH* may thus contribute to the maintenance of high PGE_2 levels in the lactating mammary gland.

The intracellular effects of PGE_2 are mediated by four receptors, EP1–EP4, which have widely varying tissue distributions. They act through different G-proteins and signaling intermediates, thus modulating multiple and sometimes opposing cellular functions in a tissue. EP2 and EP4 are coupled to G_s and when activated cause increases in intracellular cAMP levels. EP3 is coupled to both G_i and G_q and upon activation increases intracellular Ca^{2+} levels. It is still unclear which specific G-protein is coupled with the EP1 receptor, although activation of EP1 also results in increased intracellular Ca^{2+} levels (23). The diversity of receptor-mediated signaling further increases the complexity of defining the functions of PGE_2 in normal mammary gland homeostasis. Therefore, we analyzed the steady-state RNA levels of all EP genes during mammary development. We were unable to detect *EP1* expression by Northern blotting, and *EP1* mRNA levels were only barely above the threshold of detection, even by real-time quantitative PCR analysis, at all stages of development. *EP3*, however, was expressed highly at all mammary developmental stages except late pregnancy (day 16), when expression levels were significantly reduced. EP3 RNA levels were reduced to nearly half of those detected in the virgin gland at day 5 of lactation and returned to levels comparable to those of the virgin gland by day 10 of weaning. Interestingly, *EP2* expression, which was undetectable in the virgin gland, was dramatically induced at late pregnancy (day 16). On day 5 of lactation, *EP2* RNA levels, although diminished relative to late gestation, remained high compared with the virgin gland. *EP4* has an expression profile similar to *EP2*, although the RNA is expressed at much lower levels. This is in contrast to most tissues that coexpress these receptors, in which *EP4* is usually detected at much higher levels (24, 25). It has been suggested that higher EP4 levels are maintained across most tissues in part because of the faster desensitization of EP4 compared with EP2 (26). Although the specific receptor activities of EP2 and EP4 still remain to be determined, in the mammary gland, EP2 would potentially predominate in mediating PGE_2 effects because of its higher expression levels and slower desensitization rates. Because maximal EP2 levels are detected before lactation and high expression levels are maintained during lactation, we explored the possibility that EP2 may regulate PGE_2 synthesis in an autocrine manner. Evidence for this feedback mechanism has been documented in $EP2^{-/-}$ mice: *COX-2* RNA levels were decreased significantly in the colonic epithelium of $EP2^{-/-}$ mice compared with wild-type mice (27). Additionally, PGE_2 has been demonstrated to increase COX-2 expression levels in vitro, further supporting the possibility of such a feedback loop (28). PGE_2 levels in mammary glands derived from virgin or mid-pregnant $EP2^{-/-}$ mice were not significantly different from those of wild-type controls. Although a small decrease in PGE_2 levels was detected in the lactating glands of $EP2^{-/-}$ mice, this difference was not statistically significant (Fig. 2C), suggesting that EP2 is not necessary for PGE_2 synthesis.

The coordinated temporal regulation of EP2 and EP4, coupled with the increase in total PGE_2 levels by late pregnancy, before peak lactogenic differentiation, suggests that PGE_2 may regulate different cellular functions during this phase. These potentially include angiogenesis and/or cytoprotection of the mammary epithelium from the increased stresses of hyperproliferation during pregnancy. During normal mammary development, there is a significant increase in angiogenesis at mid pregnancy to meet the nutrient requirements of the expanded epithelium and concordantly an increase in vascular endothelial growth factor (VEGF) expression (29, 30). PGE_2 has been demonstrated to promote angiogenesis by increasing the expression of proangiogenic factors such as VEGF (31, 32). Additionally, colorectal tumors in $EP2^{-/-}$ $Apc^{\Delta 716}$ mice have a marked reduction in angiogenesis and expression levels of proangiogenic factors, including VEGF (33). Alternatively, PGE_2 via EP2 (and/or EP4) may promote epithelial cell survival by negatively regulating apoptosis in mammary epithelial cells during pregnancy. COX-1-derived PGE_2 was recently demonstrated to promote crypt cell survival and protect the gastrointestinal epithelium from radiation-induced apoptosis via EP2. Crypt cell apoptosis was increased dramatically in the jejunum of $EP2^{-/-}$ mice after irradiation compared with wild-type mice (34, 35). PGE_2 has also been demonstrated to increase the expression of the antiapoptotic protein Bcl-2 in vitro (36). During the rapid cell division characteristic of pregnancy, mammary epithelial cells may accumulate mutations as a result of insufficient time for repair. By allowing such cells to escape programmed cell death, EP2 (and EP4) may facilitate the accelerated and timely expansion of the mammary epithelium, which eventually undergoes lactogenic differentiation and, finally, apoptosis during involution. Whole-mount analysis of $EP2^{-/-}$ mid-pregnant mammary glands did not reveal any significant changes in ductal branching or lobuloalveolar development (data not shown). Although $EP4^{-/-}$ mice seem to have less profuse lobuloalveolar growth compared with wild-type mice on day 16 of pregnancy, it is difficult to ascertain whether this defect is mammary-specific or secondary to the reduced fertility observed in these mice (data not shown). Further detailed analysis of receptor-deficient mammary glands and localization of the receptors to specific mammary cell types will be necessary to determine which cellular processes are regulated by EP2 and EP4 during pregnancy.

As shown in Figs. 1A and 3A, increased COX-1 protein expression paralleled the significant increase in tissue PGE_2

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levels during lactation. Furthermore, PGE_2 was almost undetectable in *COX-1*$^{-/-}$ lactating mammary glands and in breast milk expressed from *COX-1*$^{-/-}$ lactating females compared with wild-type littermates. These data demonstrate that COX-1 is necessary for PGE_2 biosynthesis in the normal mammary gland. Although the expression of the recently identified COX-1b protein (also referred to as COX-3) in the murine mammary gland has not been determined, it remains possible that COX-1b, if expressed, could also contribute to PGE_2 biosynthesis during lactation. Because *cPGES* and *mPGES2* expression levels were increased during mid pregnancy (Fig. 1C), we hypothesized that these synthases could partner with COX-1 for PGE_2 synthesis during lactation in vivo. cPGES has been demonstrated to work in concert with COX-1 for PGE_2 synthesis in vitro by coexpression of these proteins. It is unclear at present which COX isoform mPGES2 preferentially partners with in vivo, although in vitro studies suggest a modest preference for COX-2. mPGES1, on the other hand, is thought to partner specifically with COX-2 for delayed PGE_2 biosynthesis during inflammatory responses. To clarify these mechanisms, we first assessed PGE_2 levels in lactating mammary glands derived from *mPGES1*$^{-/-}$ and *mPGES1*$^{+/+}$ females. Surprisingly, in *mPGES1*$^{-/-}$ mice, PGE_2 levels were decreased significantly compared with both wild-type mice (Fig. 4A) and heterozygous mice (Fig. 5B). This decrease did not result from changes in expression levels of C*OX-1* (Fig. 4B) or from increases in the expression of the catabolic enzyme *PGDH* (data not shown). Together with the observation that *COX-2* expression is barely detectable in the normal gland, these data strongly suggest that mPGES1 and COX-1 are primary functional partners in vivo for mammary PGE_2 biosynthesis, particularly during lactation. Evidence for such a partnership in vivo is growing. Studies have demonstrated that in the acetic acid-induced pain hypersensitivity model, *mPGES1*$^{-/-}$ mice have reduced writhing responses similar to those observed in *COX-1*$^{-/-}$ but not in *COX-2*$^{-/-}$ mice (10, 37). Additionally, *mPGES1*$^{-/-}$ macrophages are deficient in both acute and delayed PGE_2 biosynthesis, similar to defects observed in macrophages isolated from *COX-1*$^{-/-}$ and *COX-2*$^{-/-}$ mice, respectively. From these data, it was inferred that mPGES1 could pair with either COX isoform (10), instead of being coupled exclusively with COX-2. A recent study demonstrating that mPGES1 and COX-1 cooperate for basal PGE_2 biosynthesis in the murine stomach (38) further supports our findings. These data suggest that a functional COX-1/mPGES1 pairing may be common in certain tissue types under normal physiological conditions.

Because PGE_2 levels in *mPGES1*$^{-/-}$ lactating glands are extremely reduced despite high expression levels of *mPGES2* and *cPGES*, and because PGE_2 levels are not significantly different in *mPGES2*$^{-/-}$ lactating mammary glands (Fig. 4C) compared with wild-type mice, it appears that cPGES and mPGES2 do not contribute significantly to PGE_2 biosynthesis in mammary tissue. However, mean PGE_2 levels measured in *mPGES1*$^{-/-}$ mice (3.33 pg/mg tissue; Fig. 4A) remained higher than those detected in *COX-1*$^{-/-}$ mice (0.96 pg/mg tissue; Fig. 3B). It is difficult to ascertain the significance of this difference, because *mPGES1*$^{-/-}$ and *COX-1*$^{-/-}$ mice are maintained on different genetic backgrounds. The residual PGE_2 levels detected in *mPGES1*$^{-/-}$ mammary glands may result from experimental variability or reflect nonenzymatic formation of PGE_2 (39–41). Alternatively, it remains possible that cPGES may contribute to basal PGE_2 production in the mammary gland, potentially via a COX-1 partnership, especially because *cPGES* is expressed abundantly at all stages of mammary gland development. Analysis of *cPGES*$^{-/-}$ mice, when available, will help define the primary COX partnership and determine the contribution of this enzyme to PGE_2 biosynthesis in the mammary gland. Although *mPGES1*$^{-/-}$ mice produced barely detectable PGE_2 levels during lactation, no increases in *mPGES1* RNA levels were observed in wild-type mice at this stage (Fig. 1B, C). It is possible that changes in mPGES1 protein levels, enzymatic activity, or cellular compartmentalization may be responsible for increasing the efficiency of PGH_2 catalysis and PGE_2 production. Alternatively, the availability of substrate (i.e., the conversion of AA to PGH_2 by COX-1) may be the principal regulatory step. This is partly supported by the significant reduction of PGE_2 levels detected in lactating glands of *COX-1*$^{+/-}$ mice compared with *COX-1*$^{+/+}$ controls (Fig. 3B). LC-MS measurements of multiple eicosanoids in *mPGES1*$^{-/-}$ mammary glands revealed that PGD_2 levels were significantly increased in *mPGES1*$^{-/-}$ lactating mice compared with both wild-type and *mPGES1*$^{+/-}$ mice. Similarly, increased levels of TXB_2 were measured in *mPGES1*$^{-/-}$ mammary samples. Although it is difficult to interpret these data because PGD_2 and TXB_2 levels measured by LC-MS in *mPGES1*$^{+/+}$ and *mPGES1*$^{+/-}$ mammary glands are largely below the limit of quantitation, these observations are qualitatively similar to those reported recently by Boulet et al. (38). They propose that increased basal PGD_2 and TXB_2 levels in murine *mPGES1*$^{-/-}$ stomach potentially result from an increased shunting of excess unused PGH_2 substrate to alternative prostanoid pathways. A recent report also demonstrates that prostaglandin production is redirected in *mPGES1*$^{-/-}$ macrophages, resulting in the increased synthesis of TXB_2 and 6-keto-$PGF_{1\alpha}$ (42). However, it remains possible that the increased PGD_2 levels observed in *mPGES1*$^{-/-}$ glands simply result from the lack of specificity in detecting PGD_2.

Our study also suggests that transcription of PGE_2 metabolism genes and PGE_2 effectors is regulated tightly during normal mammary gland development, although, at present, very little is known about these mechanisms. Although COX-1 expression is constitutive in most tissues, it appears to be specifically regulated during murine mammary epithelial differentiation. It is unclear at this time whether the increases in *COX-1* RNA and protein levels during lactation represent increases in transcription, changes in RNA stability, or increased translation. The murine *COX-1* promoter is relatively uncharacterized, and one study suggests that the promoter has functional glucocorticoid response elements with weak responsiveness to progesterone (43–45). Therefore, we can speculate that

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COX-1 transcription may be positively modulated by the activity of glucocorticoid receptor hormones in combination with progesterone during lactation. Information about the regulation of the murine *PGDH* promoter is currently limited (46). However, a recent study demonstrates that the human *PGDH* promoter is activated by PRs, PR-A and PR-B (47). Interestingly, PR RNA levels are higher during pregnancy and lowest during lactation, similar to the *PGDH* expression profile in the murine mammary gland (48). It is possible, therefore, that *PGDH* transcription is positively regulated by progesterone. Consequently, *PGDH* expression would be low when PR levels are decreased, thus allowing high amounts of PGE_2 to be maintained during lactation. The temporal expression profiles of *EP2* and *EP4* suggest that ovarian hormones, specifically estrogen and progesterone, may directly or indirectly regulate these genes. Expression of *EP2* and *EP4* is regulated by ovarian estrogen and progesterone during implantation in the luminal epithelium of the uterus (49, 50). Additionally, novel PR binding sites have been identified recently in the murine *EP2* promoter (51), suggesting that EP2 may be differentially regulated in the mammary gland by progesterone.

In conclusion, our study demonstrates that PGE_2 metabolism is temporally regulated during normal murine mammary gland development. PGE_2 production in the murine mammary gland appears to be dependent on the functional pairing of COX-1 and mPGES1, and this report demonstrates for the first time that mPGES1 is the primary synthase mediating PGE_2 biosynthesis during normal mammary homeostasis. PGE_2 effectors, particularly the G_s-coupled receptors EP2 and EP4, are also regulated in a temporal manner. Furthermore, the disconnection between points of maximal PGE_2 synthesis and receptor expression levels suggests that PGE_2 may perform distinct functions at different stages of mammary development. PGE_2 via the actions of EP2/EP4 may regulate mammary-specific cellular functions during pregnancy, whereas high amounts of PGE_2 secreted into breast milk may modulate infant physiology or immune responses (52, 53). Specifically, PGE_2 may promote intestinal maturation or motility in the infant (54, 55) or be cytoprotective during postnatal development of the intestinal mucosa. Identifying specific mammary cell populations that express the different PGE synthases and receptors, coupled with the analysis of mammary epithelial proliferation and apoptosis in receptor and *mPGES1*$^{-/-}$ mice, will help define the normal physiological functions of PGE_2 in the murine mammary gland.

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