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Microsomal prostaglandin E synthase-1 in both cancer cells and hosts contributes to tumour growth, invasion and metastasis

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mPGES-1 (microsomal prostaglandin E synthase-1) is a stimulus-inducible enzyme that functions downstream of COX (cyclo-oxygenase)-2 in the PGE_2 (prostaglandin E_2)-biosynthesis pathway. Although COX-2-derived PGE_2 is known to play a role in the development of various tumours, the involvement of mPGES-1 in carcinogenesis has not yet been fully understood. In the present study, we used LLC (Lewis lung carcinoma) cells with mPGES-1 knockdown or overexpression, as well as mPGES-1-deficient mice to examine the roles of cancer cell- and host-associated mPGES-1 in the processes of tumorigenesis *in vitro* and *in vivo*. We found that siRNA (small interfering RNA) silencing of mPGES-1 in LLC cells decreased PGE_2 synthesis markedly, accompanied by reduced cell proliferation, attenuated Matrigel™ invasiveness and increased extracellular matrix adhesion. Conversely, mPGES-1-overexpressing LLC cells showed increased proliferating and invasive capacities. When implanted subcutaneously into wild-type mice, mPGES-1-silenced cells formed smaller xenograft tumours than did control cells. Furthermore, LLC tumours grafted subcutaneously into mPGES-1-knockout mice grew more slowly than did those grafted into littermate wild-type mice, with concomitant decreases in the density of microvascular networks, the expression of pro-angiogenic vascular endothelial growth factor, and the activity of matrix metalloproteinase-2. Lung metastasis of intravenously injected LLC cells was also significantly less obvious in mPGES-1-null mice than in wild-type mice. Thus our present approaches provide unequivocal evidence for critical roles of the mPGES-1-dependent PGE_2 biosynthetic pathway in both cancer cells and host microenvironments in tumour growth and metastasis.

Key words: knockout mouse, metastasis, microsomal prostaglandin E synthase-1, prostaglandin E_2, tumorigenesis.

INTRODUCTION

Numerous studies on rodent cancer models and human cancers have shown that NSAIDs (non-steroidal anti-inflammatory drugs) have antineoplastic properties [1]. A well-known effect of the NSAIDs is their ability to inhibit the enzyme COX (cyclo-oxygenase) and thereby to suppress PG (prostaglandin) synthesis. PGE_2, the most common PG, is involved in tumour progression by inducing angiogenesis, invasion and metastasis in several solid tumours [2]. Biosynthesis of PGE_2 from arachidonic acid, which is spatiotemporally supplied from membrane phospholipids by the action of phospholipase A_2, is catalysed sequentially by COX and PGES (PGE synthase) [3]. COX catalyses the insertion of molecular oxygen into arachidonic acid to form the unstable intermediate PGG_2, which is rapidly converted into PGH_2 by the peroxidase activity of the same enzyme. Of the two COX isoforms, COX-1 is expressed constitutively in most tissues and is generally responsible for the production of PGs that control normal physiological functions, whereas COX-2 is inducible in response to mitogens, cytokines and cellular transformation. High levels of constitutive expression of COX-2 and its product PGE_2 have been detected in various cancer cells and tissues. Moreover, pharmacological, cell biological and gene targeting studies investigating COX-2 and EPs (PGE receptors) have demonstrated that PGE_2 produced through the COX-2-dependent pathway contributes to the progression of several types of cancer [4,5].

PGES catalyses the conversion of PGH_2, produced by COX-1 or COX-2, into PGE_2. Thus far, three PGES enzymes, mPGES (microsomal PGES)-1, mPGES-2 and cPGES (cytosolic PGES), have been identified [6–9]. Among these PGES isozymes, mPGES-1 is induced by pro-inflammatory stimuli and down-regulated by anti-inflammatory glucocorticoids, as in the case of COX-2, and is functionally coupled with COX-2 in marked preference to COX-1 [7,10,11]. Induction of mPGES-1 expression and its function have been observed in various diseases and systems in which COX-2-driven PGE_2 has been implicated, such as rheumatoid arthritis, febrile response, reproduction, bone metabolism, cardiovascular function, stroke and Alzheimer's disease [12,13]. Furthermore, it has previously been reported

Abbreviations used: COX, cyclo-oxygenase; cPGES, cytosolic prostaglandin E synthase; DMEM, Dulbecco's modified Eagle's medium; $dmPGE_2$, 16,16-dimethyl prostaglandin E_2; ECM, extracellular matrix; EP, prostaglandin E receptor; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; KD, knockdown; KO, knockout; LLC, Lewis lung carcinoma; MMP, matrix metalloproteinase; mPGES, microsomal prostaglandin E synthase; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PGES, PGE synthase; RT, reverse transcriptase; siRNA, small interfering RNA; TBS, Tris-buffered saline; TBS-Tween, TBS containing 0.05% Tween 20; VEGF, vascular endothelial growth factor; WT, wild-type.

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[2] Professor Kudo died on April 27, 2008. We greatly miss him as a scientist and a friend. We offer sincere thanks to all the friends, colleagues and former collaborators of Professor Kudo who showed him kindness during his lifetime.

that mPGES-1 is constitutively expressed in several cancers, most of which also express COX-2 constitutively [14,15]. We have reported that the forcible transfection of mPGES-1 in combination with COX-2, but not with COX-1, into HEK (human embryonic kidney)-293 cells led to cellular transformation with a concomitant and robust increase in PGE_2 [14]. Transgenic mice overexpressing both COX-2 and mPGES-1 developed metaplasia, hyperplasia and tumorous growth in the glandular stomach with heavy macrophage infiltration [16,17]. It has also been suggested that the PGE_2 produced through the COX-2-dependent pathway may regulate cancer–host communications that influence tumour progression. Studies using mice null for COX-2 or EPs have revealed that stromal cells around cancer cells express COX-2 and synthesize PGE_2, which, in tumour niches, may act on stromal cells in an autocrine fashion to induce the production of pro-angiogenic factors and consequent angiogenesis, as well as on cancer cells in a paracrine fashion to promote their growth, survival, adhesion and motility [5,18–20]. Although several studies, including our own, have found, by immunohistochemistry, that mPGES-1 is expressed in both stromal cells and cancer cells in tumour tissues [14,21], the contribution of mPGES-1 expressed in either cell population to tumour progression has not yet been fully elucidated.

Although the inhibition of COX-2-mediated PGE_2 formation represents a promising chemopreventive strategy for reducing the risk of cancer, the cardiovascular side effects associated with COX-2 inhibitors, which most likely result from the blunting of anti-thrombotic prostacyclin (PGI_2), have previously been found to limit their use [22,23]. From this viewpoint, selective blockage of the biosynthesis of PGE_2 without affecting other prostanoids appears to be feasible for cancer chemoprevention with the potential for improved tolerability over NSAIDs. To better evaluate the efficacy of mPGES-1 inhibition in relieving symptoms of cancer, the present study used lung carcinoma cells with mPGES-1 KD (knockdown) or overexpression, as well as mice null for mPGES-1. Our results provide evidence that mPGES-1 in both cancer cells and hosts contributes to tumorigenesis *in vitro* and *in vivo*.

EXPERIMENTAL

Cells

LLC (Lewis lung carcinoma) cells, which were originally isolated from C57BL/6 mice, were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FCS (fetal calf serum) under a humidified atmosphere containing 5 % CO_2. To establish mPGES-1-KD and -overexpressing LLC cells, we transfected these cells with a pRNA-U6.1/Hygro siRNA (small interfering RNA) expression vector (GenScript) harboring an mPGES-1-directed siRNA target sequence [5′-GGCCTTTGCCAACCCCGAG-3′ (residues 126–144 in the open reading frame)] and a pcDNA3.1 expression vector (Invitrogen) containing mouse mPGES-1 cDNA respectively, using Lipofectamine™ 2000 (Invitrogen). After the transfection of these plasmids, LLC cells were cultured in medium containing 1 mg/ml G418 (Invitrogen) to establish stable clones. As a control, LLC cells transfected with an empty vector (pRNA-U6.1/Hygro or pcDNA3.1) were used (referred to as mock cells hereafter).

Animals

C57BL/6 and BALB/c mice were obtained from the Saitama Animal Center. mPGES-1-KO (knockout) mice were established as described previously [12,13], and backcrossed at least three generations with C57BL/6 mice or ten generations with BALB/c mice. Female mPGES-1-KO mice and littermate WT (wild-type) mice (7-weeks-old) were used in each experiment. The mice were housed in microisolator cages in a pathogen-free barrier facility, and all procedures involving animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Showa University, in accordance with the Standards Relating to the Care and Management of Experimental Animals in Japan.

Cell growth assay

Cells were seeded at 6×10^4 cells/well in six-well plates or 1.5×10^5 cells/flask in T-25 culture flasks in culture medium in the presence or absence of 10 nM NS-398 (Cayman Chemicals), a COX-2 selective inhibitor, or 1 mM $dmPGE_2$ (16,16-dimethyl PGE_2) (Cayman Chemicals), a metabolically stable analogue of PGE_2. After culture for 72 h, the cells were collected by trypsinization and counted in a Bright-line haemocytometer in the presence of Trypan Blue.

Western blot analysis

Aliquots of samples (20 μg of protein equivalents) were subjected to SDS/PAGE using 10 % (w/v) gels under reducing conditions. The separated proteins were electroblotted on to nitrocellulose membranes (Schleicher & Schuell) with a semi-dry blotter (Bio-Rad). After blocking with 3 % (w/v) dried non-fat skimmed milk in TBS (Tris-buffered saline) (pH 7.4) containing 0.05 % Tween 20 (TBS-Tween), the membranes were probed with the respective antibodies for 2 h {1:2000 dilution for antibodies against mPGES-1 [14], mPGES-2 (Cayman Chemicals), cPGES [11], COX-1 and -2 (Santa Cruz Biotechnology), EP1, 2, 3 and 4 (Cayman Chemicals), and VEGF (vascular endothelial growth factor; Sigma); and 1:2500 dilution for anti-mouse α-tubulin antibody (Zymed Laboratories) in TBS-Tween}. After washing with TBS-Tween, the membranes were incubated with horseradish peroxidase-conjugated anti-goat (for COX-1, COX-2 and VEGF), anti-rabbit (for mPGES-1, mPGES-2, cPGES and EP1–4) and anti-mouse (for α-tubulin) IgG antibodies (1:5000 dilution in TBS-Tween) for 1 h, and visualized with the ECL (enhanced chemiluminescence) Western blot system (PerkinElmer Life Sciences), as described previously [7,13,14]. Amounts of individual proteins relative to that of α-tubulin were estimated from their signal intensities on the Western blots using Lane and Spot Analyzer (ATTO).

Determination of PG levels

For the measurement of PGs in tissues, mouse tissues were washed twice with Hank's balanced salt solution containing 10 μM indomethacin (Sigma) before homogenization. The supernatants obtained from the tissue homogenates were adjusted to pH 3.0 with 1 M HCl and passed through a Sep-Pak C_{18} cartridge (Waters), and the retained PGs were eluted with 8 ml of methanol, as described previously [13]. A trace amount of [^{3}H]PGE_2 (Cayman Chemicals) was added to the samples before passage through the cartridges to calibrate the recovery of the PGs. The sample solvents were evaporated, and then the PGs were dissolved in an aliquot of buffer and assayed with commercial enzyme immunoassay kits for individual PGs (Cayman Chemicals). Likewise, aliquots of the supernatants of cultured cells were subjected to the enzyme immunoassay for PGs.

Adhesion assay

Cells were plated at 2×10^5 cells in 35-mm dishes, which were pre-treated with collagen, fibronectin or laminin (BD Biosciences). After incubation for 60 min at 37 °C, the cells were fixed with Carnoy solution and then stained with Giemsa solution for 60 min. Adherent cells were counted in three fields at ×40 magnification using a microscope and J image software.

Invasion assay

Cell invasiveness was evaluated using a BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences) according to the manufacturer's instructions. In brief, cells (1.2×10^6 cells in 0.5 ml) suspended in DMEM containing 3 % (v/v) FCS and 1 % (v/v) sodium pyruvate (Invitrogen) were seeded on the top of the gel in each chamber. DMEM containing 10 % (v/v) FCS (0.75 ml) was added as a source of chemoattractants into the bottom wells of the plate. After 16 h of incubation, cells that had invaded on to the lower surface of the chamber were fixed with methanol for 5 min, and stained with Crystal Violet. Non-invasive cells on the upper surface were removed with a cotton bud, and the membrane was cut. The number of invading cells was quantified by counting them under a light microscope. Statistical significance was determined using the Student's *t* test.

Tumour implantation model

LLC cells (1×10^6) in 100 μl of PBS were injected subcutaneously into 8-week-old female mice. Tumour growth was assessed by the measurement of two bisecting diameters in each tumour using calipers. The size of the tumour was determined by direct measurement of the tumour dimensions. The volume was calculated according to the equation: $V = (L \times W^2) \times 0.5$, where V = volume, L = length and W = width [35]. On day 14 after tumour implantation, the mice were anaesthetized and killed by dislocation of the cervical spine, and the tumour tissues were dissected, weighed and then fixed in 10 % (v/v) formalin for histochemical analyses. The intratumoral blood vessels in the most intensive neovascularization areas were quantified by staining of the sections with haematoxylin and eosin followed by silver. For each tumour, five random images were captured at ×400 magnification. Only areas of viable tumour tissue were imaged; necrotic regions were excluded. The individual microvessels were counted. The structure of individual microvessels was clearly differentiated from tumour cells on silver staining. The final vascular density score for the tumour represents an average of all scored fields.

Lung metastasis model

LLC cells (1×10^6) in 100 μl of PBS were intravenously injected into the lateral tail veins of 8-week-old female mice. Then, 14 days later, the mice were anaesthetized and killed, and the lungs were removed and weighed. Finally, the lungs were placed in Bouin's solution for 24 h and then photographed.

Determination of haemoglobin levels in tumour tissues

The dissected tumour tissues were washed, cut into small pieces with scissors, and homogenized with a Polytron homogenizer in a SET buffer [250 mM sucrose, 0.5 mM EDTA and 20 mM Tris/HCl (pH 7.4)] containing 10 μM indomethacin, 1 mM PMSF and 0.5 % Triton X-100. The tissue homogenates obtained were centrifuged at 600 ***g*** for 5 min, and an aliquot (200 μl) of the supernatant was centrifuged again at 14 000 ***g*** for 30 min at 4 °C. Concentrations of haemoglobin in the supernatant were then determined spectrophotometrically by measuring the absorbance at 540 nm using a haemoglobin assay kit (Wako).

RT (reverse transcriptase)–PCR

Total RNA was isolated from cells and tissues using TRIzol® (Invitrogen). Synthesis of cDNA was performed with 2 μg of the total RNA and avian myeloblastosis virus RT according to the manufacturer's instructions supplied with the RNA PCR kit version 2.1 (Takara Biomedicals). Subsequent amplifications of the partial cDNA fragments were performed using 0.5 μl of the reverse-transcribed mixture as a template with a set of specific oligonucleotide primers (Sigma) as follows: (i) mouse VEGF, sense 5′-GATGAAGCCCTGGAGTGC-3′ and antisense 5′-TCCCAGAAACAACCCTAA-3′; and (ii) mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase), sense 5′-TCGTGGATCTGACGTGCCGCCTG-3′ and antisense 5′-CACCACCCTBTTGCTGTAGCCGTAT-3′. The PCR mixtures were subjected to 30 cycles of amplification by denaturation (30 s at 94 °C), annealing (30 s at 57 °C) and elongation (30 s at 72 °C). The PCR products were analysed by 1 % (w/v) agarose gel electrophoresis with ethidium bromide.

Real-time RT–PCR

Single-stranded cDNA was generated using 1 μg of total RNA as a template and avian myeloblastosis virus RT, using a high capacity reverse transcriptase kit (Applied Biosystems). Real-time PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems) and StepOne (Applied Biosystems) according to the manufacturer's instructions. The PCR primer sets used were: (i) mouse EP2, sense 5′-GCTGTGCTCGCCTGCAA-3′ and antisense 5′-CGACGGTGCATGCGAAT-3′; (ii) mouse EP4, sense 5′-CATCATCTGTGCCATGAGCAT-3′ and antisense 5′-GCTGTAGAAGTAGGCGTGGTTGA-3′; (iii) mouse VEGF, sense 5′-TACCTCCACCATGCCAAGTG-3′ and antisense 5′-TGGGACTTCTGCTCTCCTTCTG-3′; (iv) mouse MMP (matrix metalloproteinase)-2, sense 5′-GGACCCCGGTTTCCCTAA-3′ and antisense 5′-CAGGTTATCAGGGATGGCATTC-3′; (v) mouse MMP-9, sense 5′-AGTGGGACCATCATAACATCACAT-3′ and antisense 5′-TCTCGCGGCAAGTCTTCAG-3′; (vi) mouse α5 integrin, sense 5′-ATGGCTCAGACATCCACTCC-3′ and antisense 5′-GGTCATCTAGCCCATCTCCA-3′; and (vii) mouse β1 integrin, sense 5′-GGTGTCGTGTTTGTGAATGC-3′ and antisense 5′-TGACGCTAGACATGGACCAG-3′. The expression levels of EP2, EP4, MMP-2, MMP-9 and integrins were normalized with those of mouse GAPDH with a primer set of 5′-ATGTGTCCGTCGTGGATCTGA-3′ and 5′-ATGCCTGCTTCACCACCTTCT-3′. Results represent an average of several independent experiments.

Gelatin zymography

Lysates of cells and tissues (27 μg of protein equivalents) were subjected to SDS/PAGE (10 % gels), with 1 mg/ml gelatin substrate being incorporated into the gels. Following electrophoresis, the gels were soaked in 2.5 % Triton X-100 to remove SDS, rinsed with 10 mM Tris/HCl (pH 8.0), and transferred to a bath containing 50 mM Tris/HCl(pH 8.0), 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ at 37 °C for 18 h. The gels were then stained with 0.1 % Coomassie Blue in 45 % methanol and 10 % acetic acid.

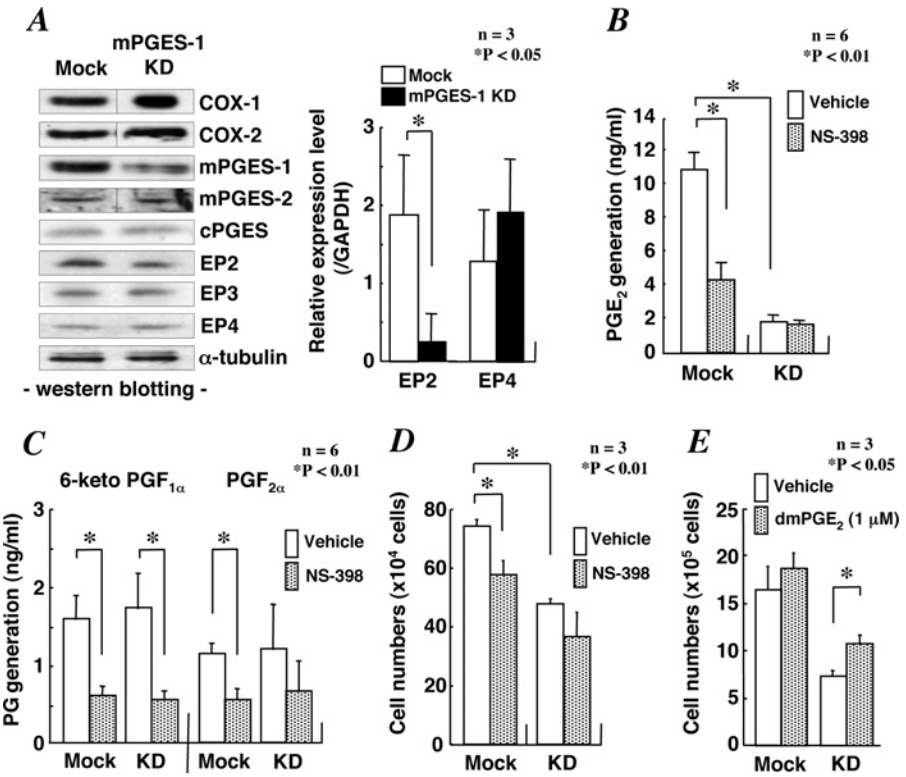

Figure 1 PGE_2 production and cell proliferation in mPGES-1-silenced LLC cells *in vitro*

(**A**) Expression of PGE_2 biosynthetic enzymes and PGE receptors in mPGES-1-KD and control (mock) cells was assessed by immunoblotting (left-hand panel). Equal amounts of cell lysates (20 μg of protein equivalents) were separated by SDS/PAGE and analysed by Western blotting at the same time with the corresponding antibodies to allow for a direct comparison. Representative results of at least three experiments are shown. Expression levels of EP2 and EP4 mRNAs in KD and mock cells were evaluated by quantitative RT–PCR, with GAPDH mRNA used for normalization (right-hand panel). (**B**–**D**) Effects of mPGES-1 silencing and 10 nM NS-398, a COX-2 selective inhibitor, on production of PGE_2 (**B**), 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (**C**), and on cell proliferation (**D**). mPGES-1-KD and mock cells were seeded at 6×10^4 cells/well in six-well culture dishes in the presence or absence of 10 nM NS-398. After culture for 3 days, the cells were collected and counted in a Bright-line haemocytometer in the presence of Trypan Blue, and the supernatants were taken for enzyme immunoassay of several PGs. (**E**) Effect of $dmPGE_2$ on cell proliferation. mPGES-1-KD and mock cells were seeded in T-25 culture flasks at 1.5×10^5 cells/flask in culture medium in the presence or absence of 1 μM $dmPGE_2$. After culture for 3 days, the cells were collected and counted. Values are the means $\pm$ S.E.M. Similar results were obtained in three independent cell lines of mPGES-1-KD and control cells.

RESULTS

Reduced tumorigenic potential of mPGES-1-silenced LLC cells *in vitro*

We established mPGES-1-KD LLC cells by means of an siRNA-silencing strategy, as described in the Experimental section. The expression levels of mPGES-1 and other enzymes involved in PGE_2 synthesis in mPGES-1-KD and mock cells were examined by Western blot analysis (Figure 1A). The expression level of mPGES-1 was reduced by $\sim$80 % in mPGES-1-KD cells relative to mock cells, whereas those of COX-1 and -2 and other PGE synthases (cPGES and mPGES-2) in both cell lines were approximately the same (Figure 1A, left-hand panel). Furthermore, we found that, among the four EP receptor subtypes that can bind to PGE_2 [24], LLC cells expressed detectable levels of EP2, EP3 and EP4 proteins, among which the level of EP2 protein was reduced in mPGES-1-KD cells relative to mock cells, whereas the levels of EP3 and EP4 were unaffected by mPGES-1 silencing (Figure 1A, left-hand panel). The reduction in EP2, but not EP4, expression in the KD cells was verified by quantitative RT–PCR (Figure 1A, right-hand panel) and quantifying individual immunoblots by a densitometer (Supplementary Figure S1 at http://www.BiochemJ.org/bj/425/bj4250361add.htm). The amounts of PGE_2 released into the medium during culture were decreased in mPGES-1-KD cells relative to mock cells (Figure 1B), whereas those of 6-keto $PGF_{1\alpha}$ (a non-enzymatic hydrolytic product of PGI_2) and $PGF_{2\alpha}$ were comparable between mPGES-1-KD cells and mock cells (Figure 1C).

Concurrently, mPGES-1-KD cells grew more slowly than did mock cells (Figure 1D). NS-398, a COX-2-selective inhibitor, suppressed PGE_2 generation and cell proliferation in mock cells by $\sim$60 % and $\sim$25 % respectively, whereas no further decreases in PGE_2 production and cell growth were found in mPGES-1-KD cells (Figures 1B and 1D). Production of 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ was equally sensitive to NS-398 in both cell lines (Figure 1C). Furthermore, cell growth of mPGES-1-KD cells was partially (even if not completely, probably because EP2 expression was reduced in the KD cells; see above) restored by addition of an optimal concentration (1 μM) of $dmPGE_2$, a synthetic analogue of PGE_2 (Figure 1E). These results suggest that the PGE_2 produced through the COX-2/mPGES-1 pathway is partially required for the proliferation of LLC cells.

In the process of tumour metastasis, cancer cells from the primary tumour must invade the ECM (extracellular matrix). There are many reports demonstrating that malignant tumour cells often possess high invasive activity [25]. Thus we next assessed the effect of mPGES-1 silencing on the invasive activity of LLC cells using Matrigel™ invasion chambers. As shown in Figure 2(A), the number of mPGES-1-KD cells migrating across the Matrigel™-coated filter was markedly fewer than that of mock cells. Since it is known that the PGE_2 signalling activates the invasive potential of cancer cells by increasing the expressions

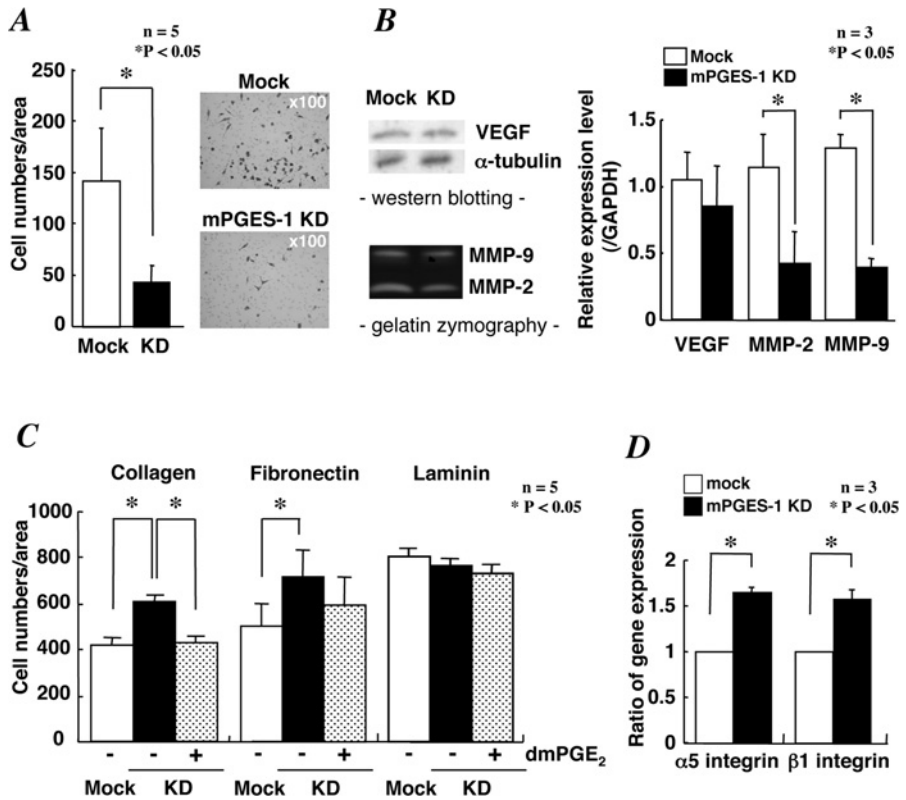

Figure 2 siRNA silencing of mPGES-1 in LLC cells reduces their malignant potential *in vitro*

(**A**) mPGES-1-KD and mock cells [1.2×10^6 cells in DMEM containing 3% (v/v) FCS] were seeded on to the upper wells of BD BioCoat™ Matrigel™ Invasion Chambers. DMEM containing 10% (v/v) FCS was added as a source of chemoattractants into the bottom wells of the plates. After 16 h of incubation, cells that had invaded on to the lower surface of the chambers were fixed, stained with Crystal Violet and counted (left-hand panel). Values are the means ± S.E.M. of five independent experiments. Representative photographs of mPGES-1-KD and mock cells that invaded across the Matrigel™-coated inserts are shown (right-hand panel). (**B**) The expression of VEGF protein in mPGES-1-KD and mock cells was assessed by immunoblotting (upper panel). The cell lysates (20 μg of protein equivalents) were separated by SDS/PAGE and then subjected to Western blot analysis using anti-VEGF and anti-α-tubulin antibodies. The activities of MMP-2 and -9 in mPGES-1-KD and mock cells were assessed by gelatin zymography (lower panel). Lysates of cells (27 μg of protein equivalents) were subjected to SDS/PAGE containing 1 mg/ml gelatin. Following electrophoresis, the gels were incubated in a bath containing 1 μM $ZnCl_2$ at 37 °C for 18 h. The gels were then stained with 0.1% Coomassie Blue. The expression levels of mRNAs for VEGF, MMP-2 and MMP-9 were evaluated by quantitative RT–PCR, with GAPDH mRNA used for normalization (means ± S.D.; $n = 3$) (right-hand panel). (**C**) Adhesion of mPGES-1-KD and mock cells to ECM proteins. mPGES-1-KD or mock cells (2×10^5 cells) were seeded on to 35-mm dishes coated with collagen, fibronectin or laminin in culture medium in the presence or absence of 1 μM $dmPGE_2$. After allowing cells to attach for 60 min at 37 °C, non-adherent cells were removed by washing. Adherent cells were fixed and stained with Giemsa solution and counted in three fields at ×40 magnification using a microscope and J image software. Values are the means ± S.E.M. of five independent experiments. (**D**) Expression of α5 and β1 integrins in mPGES-1-KD and mock cells. Total RNA was isolated from mPGES-1-KD and mock cells and subjected to quantitative RT–PCR using specific primers of α5 (left-hand panel) and β1 (right-hand panel) integrins and those of GAPDH as a reference. The results of quantitative RT–PCR were normalized with their expression in mock cells being regarded as 1 (means ± S.D., $n = 3$). Similar results were obtained in two independent cell lines of mPGES-1-KD and control cells.

of VEGF, a pro-angiogenic factor, and MMPs, which hydrolyse type IV collagen localized in the basement membrane [25], we next examined the effects of mPGES-1 silencing on the expression of VEGF and the activities of MMP-2 and -9 by Western blot analysis, gelatin zymography and quantitative RT–PCR. As shown in Figure 2(B), the activities of MMP-2 and -9 in the conditioned medium from mPGES-1-KD cells were lower than those in the medium from mock cells. Consistently, expression levels of both MMP-2 and -9 mRNAs were significantly reduced in the KD cells relative to control cells. In contrast, the levels of VEGF protein and mRNA were unaffected by mPGES-1 silencing (Figure 2B). These results suggest that the silencing of mPGES-1 diminishes *in vitro* migration of LLC cells by reducing the PGE_2-induced expression of MMP-2 and -9.

The ability of cancer cells to adhere to the ECM influences their motility and invasion in tumour tissues *in vivo*, and metastatic tumour cells show decreased ECM-adherent activity *in vitro* [25,26]. Therefore we next studied the attachment of mPGES-1-KD or mock cells to culture dishes pre-coated with distinct ECM components. The results showed that the numbers of mPGES-1-KD cells adhering to collagen and fibronectin, but not to laminin, were significantly increased as compared with those of mock cells (Figure 2C). Moreover, the increased adhesion of mPGES-1-KD cells to collagen was reversed by $dmPGE_2$ to the level of mock cells, and adhesion of the cells to fibronection also showed a similar trend (Figure 2C). Consistent with the increased binding of mPGES-1-KD cells to collagen and fibronectin, the expressions of their receptor components, α5 and β1 integrins, were elevated in the KD cells relative to control cells (Figure 2D). Thus we conclude that mPGES-1 supplies a majority of the PGE_2 that enhances the malignant potential (in terms of ECM invasion and adherence) of cancer cells.

Increased tumorigenic potential of mPGES-1-overexpressing LLC cells *in vitro*

We next established LLC cells that stably overexpressed mPGES-1 and examined whether these cells (as opposed to

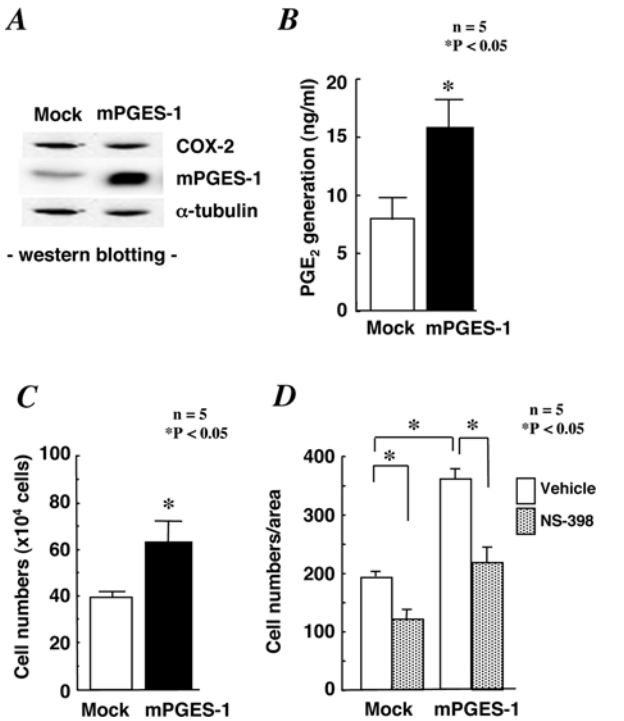

Figure 3 Increased PGE_2 generation, proliferation and invasion of mPGES-1-overexpressing LLC cells

(**A**) Expression of COX-2 and mPGES-1 in mPGES-1-overexpressing and mock cells was assessed by Western blot analysis. Representative results of at least three experiments are shown. (**B** and **C**) Effects of mPGES-1 overexpression on PGE_2 generation (**B**) and cell growth (**C**). mPGES-1-overexpressing and mock cells were seeded at 6×10^4 cells/well in six-well culture dishes. After culture for 2 days, the cells were collected and counted, and the supernatants were taken for a PGE_2 enzyme immunoassay. Values are the means $\pm$ S.E.M. of five independent experiments. (**D**) mPGES-1-overexpressing and mock cells (1.2×10^6 cells in the presence or absence of 10 nM NS-398) that had invaded through BD BioCoat™ Matrigel™ Invasion Chamber inserts over 16 h were counted. Values are the means $\pm$ S.E.M. of five independent experiments. Similar results were obtained in two independent cell lines of mPGES-1-overexpressing and mock cells.

mPGES-1-KD cells) would show increased proliferation and invasion. mPGES-1-transfected LLC cells expressed more mPGES-1 protein than did mock cells, whereas the expression of COX-2 protein was unaltered (Figure 3A). PGE_2 release into the medium during culture was increased by ~2-fold in mPGES-1-overexpressing cells relative to mock cells (Figure 3B). As shown in Figure 3(C), the growth rate of mPGES-1-overexpressing cells was significantly faster than that of mock cells. Moreover, the Matrigel™ invasion chamber assay revealed that the invasive activity of mPGES-1-overexpressing cells was notably higher than that of mock cells (Figure 3D). Additionally, treatment of both mock and mPGES-1-overexpressing LLC cells with NS-398 reduced the invasion by ~40 % (Figure 3D). These results confirm that the increased production of PGE_2 through the COX-2/mPGES-1 pathway in cancer cells facilitates their proliferation and invasion.

Silencing of mPGES-1 in cancer cells reduces tumorigenesis *in vivo*

To assess the contribution of mPGES-1 in cancer cells to tumour development *in vivo*, we grafted mPGES-1-KD and mock LLC cells subcutaneously into BALB/c mice, and the development of the solid tumour around the injection sites was evaluated on day 14 after implantation. Remarkably, both the volume and mass of xenografts derived from mPGES-1-KD cells were significantly smaller than those derived from mock cells (Figures 4A and 4B). The levels of prostanoids (PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$) in the homogenates of xenografts did not differ significantly between the genotypes, even though PGE_2 tended to decrease in the xenografts from mPGES-1-KD cells compared with those from mock cells (Figure 4C). The expression of VEGF, as assessed by RT–PCR and quantitative RT–PCR, was reduced in mPGES-1-KD xenografts as compared with control xenografts (Figure 4D). Considering that VEGF expression was unaltered by silencing of mPGES-1 in LLC cells *in vitro* (Figure 2B), the reduction of VEGF expression in mPGES-KD xenografts *in vivo* may reflect the action of mPGES-1-derived PGE_2 from cancer cells on proximal stromal cells in tumour microenvironments.

Reduced tumour growth and angiogenesis in mPGES-1-KO mice

The observations described above indicate that cancer cell-associated COX-2 and mPGES-1 co-operatively produce PGE_2, which accelerate multiple steps of malignant progression, including cell proliferation, invasion and ECM adhesion. In terms of the pathological circumstances, it has been reported that COX-2 and mPGES-1 are expressed not only in tumour cells, but also in the stromal cells (mainly in infiltrating macrophages) surrounding them [14,21]. To investigate the contribution of host-associated mPGES-1 to tumour growth, we next grafted parental LLC cells subcutaneously into either mPGES-1-KO or WT mice, and evaluated the development of solid tumour around the injection sites. Although the absence of mPGES-1 in the host mice influenced neither the engraftment rate nor the growth of tumours during the first 5 days after implantation, tumours grafted into the mPGES-1-KO mice were significantly smaller in size than those grafted into the WT mice after day 6 and beyond (Figure 5A). On day 14 after implantation, the tumour mass in mPGES-1-KO mice was reduced to half of that in WT mice (Figure 5B, left-hand panel). The levels of PGE_2 in homogenates of the dissected tumour tissues were nearly 50 % less in mPGES-1-KO mice than in WT mice, whereas those of $PGF_{2\alpha}$ were unaffected by the host mPGES-1 deficiency (Figure 5B, right-hand panel).

Histological examination of the xenografts revealed that the vascularization of tumour tissues was markedly reduced by the lack of host mPGES-1 expression (Figure 5C, indicated by arrowheads). The vascular density was approx. 60 % lower in tumours grafted in mPGES-1-KO mice than those grafted in WT mice (Figure 5D, left-hand panel). The haemoglobin contents in the tumour tissues, which appeared to be well correlated with tumour neovascularization upon histological examination [30], were also reduced in mPGES-1-KO mice in comparison with that in WT mice (Figure 5D, right-hand panel). The reduction of tumour angiogenesis in mPGES-1-KO mice caused a large area of central necrosis in the xenografts of mPGES-1-KO, but not WT, mice (Figure 5C, indicated by an asterisk). We further found that the expression levels of the VEGF protein and mRNA in tumour tissues were also markedly lower in mPGES-1-KO mice than in WT mice (Figure 5E, left-hand panel). The decrease in VEGF protein in the xenografts of mPGES-1-KO mice was confirmed by quantification of the relative abundance of VEGF to α-tubulin using a densitometer (Figure 5E, right-hand panel). These results suggest that host mPGES-1-derived PGE_2 plays a pivotal role in tumour-associated VEGF production and accompanying angiogenesis. Again, considering that VEGF expression was

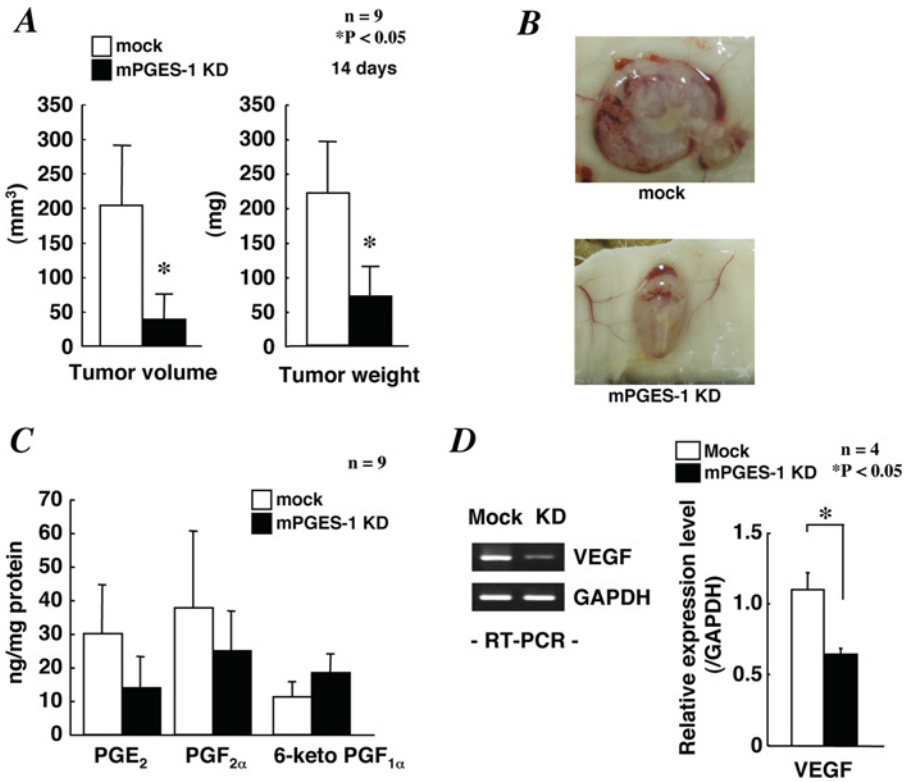

Figure 4 siRNA silencing of mPGES-1 in cancer cells reduces tumorigenesis *in vivo*

(**A**–**C**) mPGES-1-KD or mock cells (10^6 cells) were injected into the subcutaneous tissue of female BALB/c mice. On day 14 after implantation, tumour volume was scored according to the formula $V = (L \times W^2) \times 0.5$ (**A**, left-hand panel). The tumour tissues were photographed at $\times$ 100 magnification (**B**), dissected and weighed (**A**, right-hand panel). Values are means $\pm$ S.E.M. ($n = 9$). (**C**) Amounts of PGE_2, $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ in homogenates of the tumour tissues were quantified by enzyme immunoassay (means $\pm$ S.E.M., $n = 9$). (**D**) Expression of VEGF in tumour xenografts of mPGES-1-KD and mock cells was assessed by RT–PCR followed by electrophoresis (left-hand panel) and quantitative RT–PCR (right-hand panel), with the expression of GAPDH as a reference (means $\pm$ S.D., $n = 4$).

unaltered in mPGES-1-KD cells *in vitro* (Figure 2B), the reduced expression of this angiogenic factor in tumour tissue in mPGES-1-KO mice may reflect the impact of mPGES-1-derived PGE_2 on tumour microenvironments rather than on tumour cells *in vivo*.

We further examined the role of host-associated mPGES-1 in tumour metastasis by intravenous injection of parental LLC cells into mPGES-1-KO and WT mice. After 14 days, macroscopic metastases, as assessed by Bouin's staining, were found in the lungs of both genotypes, but the number and size of metastatic foci (Figure 6A) and the mass (Figure 6B, left-hand panel) of the lungs were significantly reduced in mPGES-1-KO mice compared with WT mice. Levels of PGE_2 were reduced by nearly 50 % in homogenates of the lungs from mPGES-1-KO mice relative to WT mice (Figure 6B, right-hand panel). Moreover, as assessed by RT–PCR and quantitative RT–PCR, VEGF expression in the metastasized lungs was significantly lower in mPGES-1-KO mice than in WT mice (Figures 6C and 6E). Gelatin zymography revealed that the activity of MMP-2 in the metastasized lung tissues was mitigated in mPGES-1-KO mice in comparison with that in WT mice (Figure 6D), and the levels of both MMP-2 and -9 mRNAs were significantly lower in the lung of mPGES-1-KD mice than in that of WT mice (Figure 6E). Collectively, these results indicate that host mPGES-1-driven PGE_2 plays a role in promoting tumour development and metastasis, which was associated with increased VEGF expression and MMP-2 and -9 activation.

DISCUSSION

Although the concept that the PGE_2 produced through the COX-2-dependent pathway participates in the pathogenesis of several types of cancer has been well established in the past decade based on a series of genetic studies employing mice ablated for the biosynthetic enzymes (COX-2) and receptors (EPs) or pharmacological studies employing inhibitors or agonists/antagonists fairly specific for them, the contribution of a step between COX-2 and EPs, namely PGES enzymes that convert COX-2-produced PGH_2 into PGE_2, to cancer development has remained incompletely understood. In this context, we have previously shown that co-transfection of mPGES-1 in combination with COX-2 into HEK-293 cells leads to a malignant phenotype [14]. In our continuing efforts to gain further insights into the role of mPGES-1 in tumorigenesis, we used two approaches in the present study. First, we performed siRNA-mediated silencing and overexpression of mPGES-1, which enabled us to address the complementary effects of endogenous compared with overexpressed mPGES-1 in cancer cells on tumorigenic potentials (growth, invasion and ECM binding *in vitro* and tumour xenograft propagation *in vivo*). Secondly, implantation of carcinoma cells into mPGES-1-KO mice and WT mice allowed us to evaluate the contribution of mPGES-1 expressed in tissue microenvironments to the development and metastasis of tumours in proximal and distant tissues. Our results clearly indicate that mPGES-1 expressed in both cancer cells and

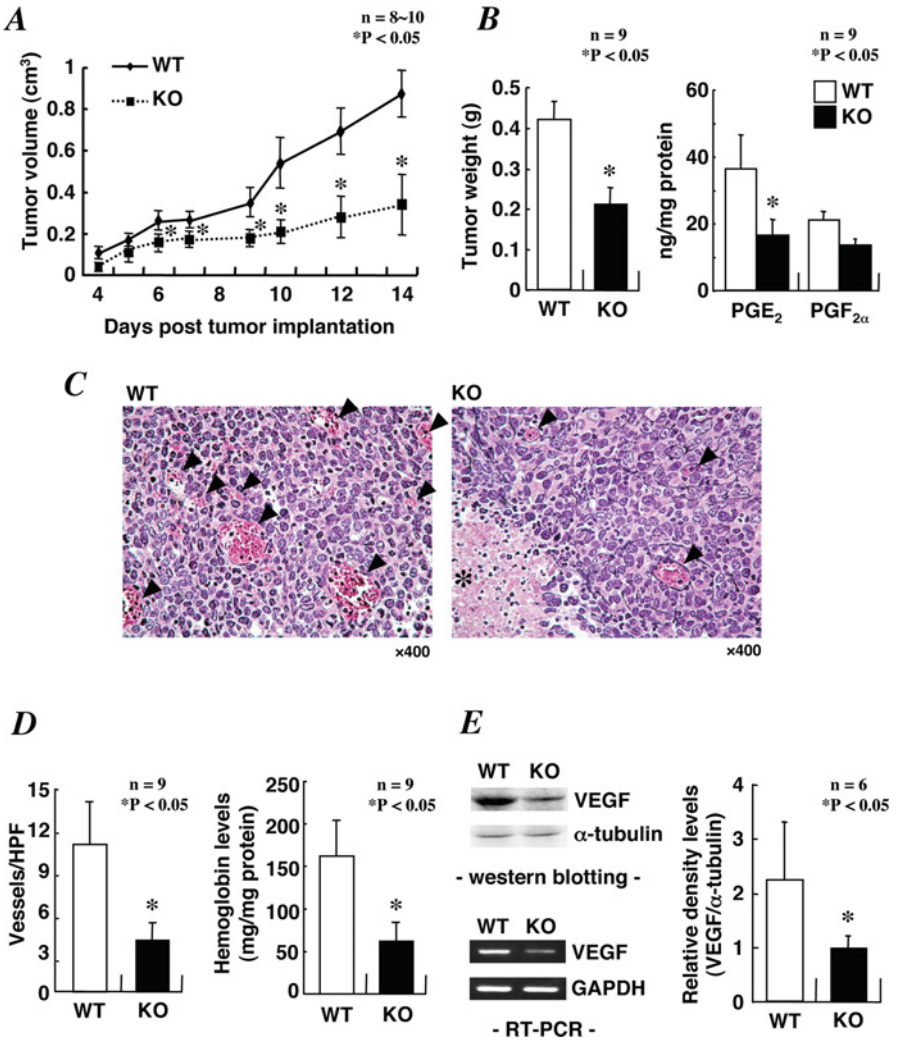

Figure 5 Growth of LLC cells subcutaneously implanted into mPGES-1-KO and WT mice

A total of 10^5 cells were injected into the subcutaneous tissue of female mPGES-1-KO and WT mice. (**A**) Tumour volumes were scored on the indicated days as described in the Experimental section. (**B**) On day 14 after implantation, the tumour tissues were dissected and weighed (left-hand panel). Amounts of PGE_2 and $PGF_{2\alpha}$ in homogenates of the tumour tissues were quantified by enzyme immunoassay (right-hand panel). Results are presented as the means ± S.E.M. ($n = 9$). (**C**) Tumour tissues were cut out, fixed in formalin, and stained with silver, haematoxylin and eosin for histochemical analysis. Microvessels and necrotic regions are shown by arrowheads and an asterisk respectively. (**D**) Vascular density (left-hand panel) and haemoglobin content (right-hand panel) were determined as described in the Experimental section. Results are presented as the means ± S.E.M. ($n = 9$). HPF, high-power field. (**E**) Expression levels of VEGF protein (upper panel) and mRNA (lower panel) in the tumour tissues were determined by Western blot and RT–PCR analysis respectively. Representative results of six independent experiments are shown. On Western blot analysis, the expression level of VEGF protein was quantified by a densitometer, with the expression level of α-tubulin used for normalization (means ± S.D., $n = 6$) (right-hand panel).

hosts is capable of promoting proliferation and invasion of cancer cells *in vitro* and *in vivo*.

Role of mPGES-1 in cancer cells

We found that PGE_2 generation and cell proliferation were reduced by mPGES-1 knockdown (Figures 1A, 1B and 1D) and conversely enhanced by mPGES-1 overexpression (Figures 3A–3C) in LLC cells (a PGE_2-dependent mouse lung carcinoma cell line; [27]), indicating the role of mPGES-1, acting downstream of COX-2, in providing the mitogenic PGE_2. In accordance with the impact on cell proliferation, the gene silencing of mPGES-1 decreased, whereas overexpression of mPGES-1 increased, the invasive activity of LLC cells (Figures 2A and 3D). The process by which tumour cells break out from their site of origin and metastasize to distant sites requires an ability to invade through the ECM and underlying mesenchymal cells. PGE_2 stimulates the invasion of cancer cells through up-regulating MMP-2 and MMP-9 (type IV collagenases), as shown by the observations that MMP-2 is increased in COX-2-overexpressing or PGE_2-treated tumour cells [25,26,28,29], and that MMP-9 is induced by COX-2/EP4 signalling in macrophages [30]. We found that the expression of MMP-2 and -9 in mPGES-1-KD cells were lower than that in mock cells (Figure 2B), suggesting the contribution of these matrix-degrading proteases to the mPGES-1-dependent invasiveness of LLC cells. In addition to adhesion to and degradation of the ECM, detachment from the ECM is also an important step in the metastasis of tumour cells [25,26]. As shown in Figure 2(C), both collagen- and fibronectin-adherent activities were significantly increased in mPGES-1-KD cells, and treatment with $dmPGE_2$ reversed these activities consistently. Accordingly, the expression of integrin $\alpha5\beta1$ (a major adhesion molecule of fibronectin) in mPGES-1-KD cells was higher than that in

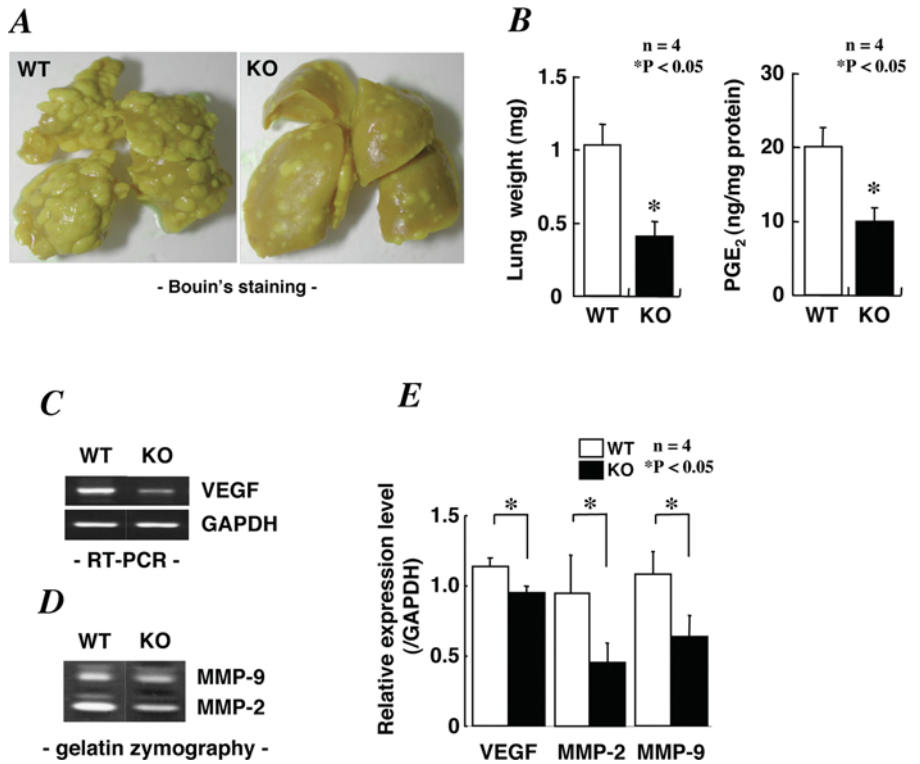

Figure 6 Reduced lung metastasis in mPGES-1-KO mice

A total of 10^5 cells were injected into the lateral tail veins of female mPGES-1-KO and WT mice on the BALB/c background. On day 14 after implantation, the mice were killed, and the removed lungs were weighed (**B**, left-hand panel) and then stained with Bouin's solution (**A**). Amounts of PGE_2 in homogenates of the metastasized lung tissues were measured by enzyme immunoassay (**B**, right-hand panel). (**C**) Expression of VEGF and GAPDH mRNAs were assessed by RT–PCR. (**D**) Activities of MMP-2 and -9 were evaluated by gelatin zymography. Results are presented as the means $\pm$ S.E.M. ($n = 4$) in (**B**) and representative results of four independent experiments are shown in (**A**, **C** and **D**). (**E**) The expression levels of mRNAs for VEGF, MMP-2 and MMP-9 were evaluated by quantitative RT–PCR, with GAPDH mRNA used for normalization (means $\pm$ S.D., $n = 4$).

mock cells (Figure 2D), an observation that is reminiscent of our previous report that co-overexpression of COX-2 and mPGES-1 in HEK-293 cells results in a marked reduction in a subset of integrins [14]. Thus mPGES-1-driven PGE_2 may regulate the ECM-adherent activity of cancer cells by altering the expression of integrins.

Importantly, *in vivo* tumour growth after subcutaneous engraftment of mPGES-1-KD cells was less obvious than that after engraftment of replicate mock cells (Figure 4), implying that mPGES-1 contributes to the supply of the major pool of the PGE_2 mediating tumour proliferation both *in vitro* and *in vivo*. Gene disruption of EP2 resulted in a reduced number and size of intestinal polyps in *Apc*-mutant mice, a model for human familial adenomatous polyposis [31], and the *Apc* mutation is accompanied by aberrant activation of β-catenin signalling, which is accelerated by PGE_2 through the EP2–Gsα axis [32,33]. In another model, disruption of the gene for EP1, EP2 or EP4 suppressed the development of colorectal cancer induced by carcinogens [34–36]. Transactivation of EGF (epidermal growth factor) receptors by the PGE_2/EP1, 2, or 4 signalling via protein kinase A and c-Src led to increased cell growth and invasion of carcinoma cells [37–39]. In a positive-feedback loop, COX-2-derived PGE_2 acts on EP2, leading to the elevation of intracellular cAMP, which in turn up-regulates the expressions of both COX-2 and EP2 [40]. Although the identification of EPs participating in the propagation of LLC xenografts is beyond the scope of the present study, the co-ordinated reduction of mPGES-1 and EP2 in mPGES-1-KD cells (Figure 1A) suggests that COX-2/mPGES-1-derived PGE_2 may stimulate EP2 signalling in an autocrine/paracrine fashion to facilitate LLC cell proliferation and, accordingly, defects in mPGES-1 may lead to down-regulation of EP2 and eventually reduce the cellular sensitivity to PGE_2.

While this paper was in preparation, two groups reported opposite effects of mPGES-1 deficiency in intestinal tumorigenesis. Nakanishi et al. [41] showed that the genetic deletion of mPGES-1 ameliorates the development of intestinal tumours in both $Apc^{\Delta 14}$-dependent and carcinogen-induced models, whereas Elander et al. [42] demonstrated that intestinal polyposis is exacerbated in mPGES-1-null Apc^{min} mutant mice, probably because of the metabolic shift from PGE_2 towards other pro-tumorigenic lipid mediators. Although the reason for the discrepancy between these two studies is unclear, our present results appear to be in line with the former study and thus support the feasibility of targeting mPGES-1 for cancer chemoprevention. Critically, although the experimental design of the previous studies did not allow a precise distinction between the contribution of cancer cell- and host-associated mPGES-1 to tumour development, our present approach clearly underscores the importance of mPGES-1 pools both in cancer cells (as discussed above) and in microenvironments (see below).

Role of mPGES-1 in host microenvironments

As is COX-2, mPGES-1 is expressed in stromal cells as well as in cancerous cells in several types of tumour [14,15]. Given that stromal COX-2 and PGE_2 of the host can influence the

development of grafted tumours [20,43], we used mPGES-1-KO mice to evaluate the potential roles of the host stromal mPGES-1 in tumour growth, and found that tumour growth at a proximal site and metastasis into a distant tissue were significantly reduced in mPGES-1-KO mice relative to WT mice (Figures 5 and 6). This result provides an additional line of genetic evidence that the COX-2/mPGES-1-derived PGE_2 from host stromal cells, in addition to that from cancer cells (see above), is important for tumorigenesis *in vivo*. The induction of mPGES-1 in tumour stromal tissues may be ascribed to the migration and expansion of host inflammatory cells and neovascular endothelial cells, since previous immunohistochemical analyses have demonstrated a preferential localization of mPGES-1 in macrophage-like cells infiltrating into the stromal tissues in proximity to cancer cells [14], and since massive macrophage infiltration and microvessel formation have been observed in the stroma of gastric hyperplasia in COX-2/mPGES-1-double transgenic mice [16,17].

As shown in Figure 5, vascular density and VEGF expression in tumour xenografts were decreased in mPGES-1-KO mice. It has been reported that angiogenesis induced by either endogenous COX-2 or exogenous PGE_2 was accompanied by increased VEGF expression and was abolished by a VEGF-directed antisense oligonucleotide [19]. Moreover, the growth, VEGF expression and angiogenesis of LLC-implanted tumours were markedly suppressed in EP3-KO mice [43], a phenotype that is reminiscent of mPGES-1-KO (the present study) and COX-2-KO mice [20]. These studies indicate that, in the tumour milieu, both cancer cells and adjacent stromal cells synthesize (via COX-2/mPGES-1) PGE_2, which in turn acts on the particular population of EP3-expressing stromal cells to induce the production of VEGF and consequent angiogenesis [43]. Consistently, Nakanishi et al. [41] have reported very recently that genetic deletion of mPGES-1 in *Apc*-mutant mice caused marked and persistent suppression of intestinal cancer growth in association with a disorganized vascular pattern. In addition to tumour growth and associated angiogenesis, lung metastasis of LLC cells across the blood circulation was also decreased in mPGES-1-KO mice as compared with replicate WT mice (Figure 6). The metastatic phenotype observed in mPGES-1-KO mice was similar to that observed in MMP-2-KO mice, in which focal xenograft propagation and lung metastasis of LLC cells were reduced [44]. In agreement, MMP-2, as well as MMP-9, expression in the metastasized lung tissues was markedly lower in mPGES-1-KO mice than in WT mice (Figures 6D and 6E). Thus, in the metastatic foci, the PGE_2 derived from the host mPGES-1 may lead to the increase in the activities of MMP-2 and -9, which, in co-operation with VEGF, may promote the invasion of cancerous cells into the adjacent and distant tissues, thereby allowing subsequent expansion and metastasis of the tumour.

In conclusion, the results of the present study suggest that both cancer cell-associated and host-derived mPGES-1 is critical for tumour growth and metastasis. The mPGES-1-driven PGE_2 signalling on stromal cells may be functionally linked to the induction of potent pro-angiogenic and matrix-degrading factors, which in turn would facilitate tumour development. Previous studies have shown that, unlike the specific inhibition of COX-2, which predisposes to cardiovascular risk, gene ablation of mPGES-1 in mice shows minimal unfavourable effects on the circulation system [12]. Therefore an mPGES-1 inhibitor would exhibit a chemopreventive action on various tumours by attenuating both cancer cell- and stromal cell-derived PGE_2, thereby serving as a novel therapeutic tool for cancer. Future clinical studies will address the important question of the efficacy and safety of mPGES-1 inhibition in human diseases.

AUTHOR CONTRIBUTION

Daisuke Kamei designed and performed the experiments, analysed the data and contributed to writing the manuscript; Yuka Sasaki also performed the experiments; Yoshihito Nakatani, Masataka Majima, Yukio Ishikawa and Toshiharu Ishii helped in manuscript and scientific discussions; Satoshi Uematsu and Shizuo Akira provided the resources for the work; Ichiro Kudo designed the experiments; and Makoto Murakami and Shuntaro Hara designed the experiments and also wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

Microsomal prostaglandin E synthase-1 in both cancer cells and hosts contributes to tumour growth, invasion and metastasis

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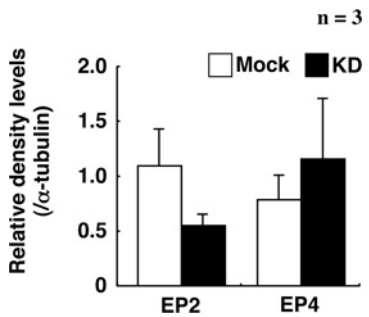

Figure S1 Reduced expression level of EP2 protein in mPGES-1-silenced LLC cells *in vitro*

Expression of EP2 and EP4 in mPGES-1-KD and control (mock) cells was assessed by immunoblotting. Representative results of at least three experiments are shown. On Western blot analysis the expression levels of EP2 and EP4 protein were quantified by a densitometer, with the expression level of α-tubulin used for normalization (means $\pm$ S.D., $n = 3$).

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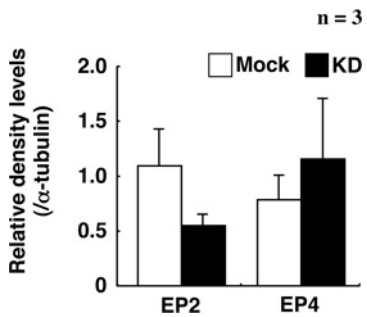

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