Microsomal prostaglandin E synthase 1 determines tumor growth in vivo of prostate and lung cancer cells

Hiromi Hanaka1, Sven-Christian Pawelzikb,1, John Inge Johnsen1, Marija Rakonjac2, Kan Terawaki3, Agnes Rasmuson1, Baldur Sveinbjörnssoncd, Martin C. Schumacher3, Mats Hamberg5, Bengt Samuelsson2, Per-Johan Jakobssonb, Per Kogner4, and Olof Rädmarka,2

1Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, 2Rheumatology Unit, Department of Medicine, 3Childhood Cancer Research Unit, Department of Woman and Child Health, and 4Department of Urology, Karolinska Institutet, S-171 77 Stockholm, Sweden; and 5Department of Biology and Histological Institute of Medical Biology, University of Tromsø, N9037 Norway

Contributed by Bengt Samuelsson, September 10, 2009 (sent for review June 17, 2009)

There is strong evidence for a role of prostaglandin E2 (PGE2) in cancer cell proliferation and tumor development. In PGE2 biosynthesis, cyclooxygenases (COX-1/COX-2) convert arachidonic acid to PGH2, which can be isomerized to PGE2 by microsomal PGE-synthase-1 (MPGES-1). The human prostate cancer cell line DU145 expressed high amounts of MPGES-1 in a constitutive manner. MPGES-1 expression also was detectable in human prostate cancer tissues, where it appeared more abundant compared with benign hyperplasia. By using shRNA, we established stable and practically complete knockdown of MPGES-1, both in DU145 cells with high constitutive expression and in the non-small cell lung cancer cell line A549, where MPGES-1 is inducible. For microsomes prepared from knockdown clones, conversion of PGH2 to PGE2 was reduced by 85–90%. This resulted in clear phenotypic changes: MPGES-1 knockdown conferred decreased clonogenic capacity and slower growth of xenograft tumors (with disintegrated tissue structure) in nude mice. For DU145 cells, MPGES-1 knockdown gave increased apoptosis in response to genotoxic stress (adriamycin), which could be rescued by exogenous PGE2. The results suggest that MPGES-1 is an alternative therapeutic target in cancer cells expressing this enzyme.

Results

High Constitutive Expression of MPGES-1 in DU145 Cells. Three human prostate cancer cell lines (DU145, PC3, and LNCaP) were analyzed for MPGES-1 protein expression (Fig. L4). High expression was found in DU145 cells. Comparison to known amounts of purified MPGES-1 protein on Western blots gave an estimate of about 10 ng per 100 μg of total protein. Weaker expression of MPGES-1 was found in PC3 cells, and in LNCaP samples MPGES-1 protein was not detectable. For the human lung adenocarcinoma cell line A549, up-regulation of MPGES-1 by IL-1β was confirmed (Fig. 2A). However, IL-1β did not change expression of MPGES-1 in the prostate cancer cell lines (Fig. S1). Hence, the high expression in DU145 is constitutive. Samples from DU145 cells typically showed more intense bands at 16,000 than analogous samples from A549 cells that had been treated with IL-1β, as illustrated in Fig. 2A.

A similar pattern was obtained when MPGES-1 enzyme activity was determined by incubations of microsomes with PGH2 (10 μM arachidonic acid | cyclooxygenase | eicosanoid | prostaglandin E2

Prostaglandin E2 (PGE2) is an eicosanoid with many functions; its role as a mediator of pain and fever in inflammatory reactions is of major importance. In PGE2 biosynthesis, cyclooxygenases (COX-1, COX-2) transform arachidonic acid to the endoperoxide PGG2, which is reduced to PGH2. Subsequently, PGE synthase (PGES) converts PGH2 into PGE2. Three PGESs are present in human cells: two microsomal and one cytosolic (also referred to as p23, a cofactor for Hsp90). Microsomal PGE2 synthase-1 (MPGES-1), belonging to the MAPEG family, is inducible by proinflammatory stimuli, such as IL-1β and LPS. As for COX-2, this induction is reduced by anti-inflammatory glucocorticoids (1–4). See Samuelsson et al. (5) for a recent review on MPGES-1.

For many cancer cells, production of COX and lipoxygenase-derived eicosanoids promotes growth and survival (6, 7). Increased amounts of PGE2 were first found in colorectal adenomas and cancers, and nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit COX reduce the risk of developing colorectal cancer. However, COX-2 inhibitors are associated with cardiovascular side effects (for review, see refs. 5 and 8). Not only COX-2 but also high expression of MPGES-1 has been found in various cancers, including non-small cell lung cancer, colorectal cancer, breast cancer, and hepatocellular carcinoma (5). However, for prostate cancer cell lines as well as tissue, different observations have been made regarding expression of COX-2 (up- or down-regulation in relation to normal prostate epithelial cells and tissue; see refs. 9 and 10 for reviews). Interestingly, during proliferative inflammatory atrophy, which may precede prostate cancer, COX-2 was up-regulated (11), and NSAIDs may prevent development of benign prostatic hyperplasia (12). Recently, inhibition of cytosolic phospholipase A2 (provides arachidonic acid for eicosanoid biosynthesis) in the prostate cancer cell line PC3 was found to reduce xenograft tumor growth (13).

PGE2 promotes cancer cell growth and survival by several mechanisms, including increased proliferation, counteracted apoptosis, increased migration and invasiveness, angiogenesis, recruitment of myeloid suppressor cells to evade T-cell attack, and chronic inflammation. These effects are mediated via multiple signaling pathways, including cross-talk with Wnt and EGFR pathways (6, 8–10, 14–16). Thus, PGE2 has been implicated in many different types of cancer, and COX inhibitors are tested in animal models for cancer (e.g., neuroblastoma; ref. 17) and in the clinic regarding prostate cancer (10). Here, we show that the human prostate cancer cell line DU145 expresses large amounts of MPGES-1. Knockdown of MPGES-1 by shRNA gave considerably reduced tumorigenicity of both DU145 and the human lung cancer cell line A549. MPGES-1 was also detected in prostate cancer tissue samples.


The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

5S-C.P. and J.I.J. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: bengt.samuelsson@ki.se or olof.radmark@ki.se.

This article contains supporting information online at www.pnas.org/cgi/content/full/0910218106/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.0910218106

PNAS | November 3, 2009 | vol. 106 | no. 44 | 18757–18762

MEDICAL SCIENCES
for 1 min on ice. The highest activity (914 pmol PGE2; formed per 25 μg of microsomal protein per minute) was found for DU145, followed by PC3 and LNCaP (Fig. 1B). Please note that in the incubations of DU145 microsomes, almost all substrate was converted, limiting PGE2 formation. Thus, the MPGES-1 activity of DU145 microsomes is probably underestimated. This may also explain the apparently high MPGES-1 activity in PC3 microsomes in relation to the Western blot bands.

Generation of Stable MPGES-1 Knockdown Cells. In view of the high expression of MPGES-1 in DU145, this prostate cancer cell line was used for RNAi. For comparison, we also performed RNAi for MPGES-1 in A549 cells. Cells were transfected with five different shRNA plasmids by using Lipofectamine 2000, and stable transfectants were selected by resistance to puromycin. For DU145, clone A was found to be a practically complete knockdown clone, and this clone was used in the experiments described below. For A549, the selected complete knockdown clone was denoted clone a. The absence of MPGES-1 protein in the knockdown clones is shown in Fig. 2A. For A549 knockdown clone a, MPGES-1 was absent also after stimulation with IL-1β. After transfections with the negative control nontargeting shRNA plasmid, MPGES-1 was still expressed. These knockdown clones (DU145 knockdown clone A, A549 knockdown clone a) are stable; no MPGES-1 protein expression has been detectable up to 1.5 years after initial isolation. For DU145, efficient knockdown was demonstrated also by quantitative PCR. For WT DU145, the MPGES-1 to cyclophilin A (PPIA) mRNA ratio was 7%. For DU145 knockdown clone A, this was reduced to 0.3%. Expression of PPIA, considered a stable reference gene, was the same in WT and knockdown cells.

Knockdown was demonstrated also by MPGES-1 activity assays (Fig. 2B). For DU145 knockdown clone A, formation of PGE2 from PGH2 was reduced by 90% compared with WT DU145. For A549 knockdown clone a, formation of PGE2 from PGH2 was reduced by 85% compared with WT A549 (cells treated with IL-1β). As shown, MPGES-1 activity remained inducible by IL-1β in A549 transfected with the negative control shRNA plasmid. In a separate experiment, MPGES-1 activity was demonstrated also for DU145 transfected with this negative control. The constitutive MPGES-1 activity was higher for WT DU145 compared with WT A549 treated with IL-1β (Fig. 2B).

Expression of COX-2 in A549 and DU145 Cells. As previously published (2–4), expression of COX-2 protein in A549 cells was strongly induced by IL-1β, whereas for noninduced cells, COX-2 expression was weak (or not detectable). As observed previously (18), COX-2 expression in DU145 cells was relatively weak, but with some variation, as shown in Fig. 3. Treatment with IL-1β (1 ng/mL; 24 h) resulted in a slightly increased COX-2 Western blot band for DU145 cells, but not to the same extent as for A549 (Fig. 3). After 24 h, TNF-α (10 ng/mL) had no effect on COX-2 expression in DU145, although COX-2 in A549 increased about 2- to 3-fold. COX-2 was found also in the MPGES-1 knockdown clones (both A549 and DU145); no correlation could be observed regarding expression of these two enzymes (Fig. S2).

Subcellular Localization of MPGES-1 and COX-2 in A549 and DU145 Cells. By using confocal microscopy and double stain, subcellular localization of MPGES-1 and COX-2 was determined before and after cell stimulation with IL-1β. For A549, the intensity of both
MPGES-1 and COX-2 was higher for stimulated cells, in accordance with Western blotting. Staining was most intense around the nucleus, fading off toward the cell periphery. Colocalization (yellow) of MPGES-1 and COX-2 can be observed, particularly around nuclei in cells stimulated with IL-1β (Fig. S3A). Thus, the general view that these two enzymes function together in biosynthesis of PGE₂ is supported by the immunocytochemistry pattern in A549 cells.

For DU-145 cells, strong staining for MPGES-1 was seen in nonstimulated cells and in cells stimulated with IL-1β (Fig. S3B). However, the localization appears more distinctly perinuclear in IL-1β-stimulated cells. A weak but similarly perinuclear ring can also be observed for COX-2 after IL-1β. The two enzymes thus colocalize also in DU145 cells, although the much stronger signal for MPGES-1 (green) compared with COX-2 (red) yields a less clear composite yellow, compared with A549 cells (Fig. S3A).

Inhibition of MPGES-1 Expression Impairs DU145 and A549 Clonogenicity. Clonogenic assay was performed to determine whether MPGES-1 expression determines the tumorigenic potential of DU145 and A549 cells. In both cell lines, the clonogenic capacity was significantly reduced (P < 0.001) in cells stably transfected with shRNA against MPGES-1 (Fig. 4A). The reduction was more pronounced for A549 knockdown cells (23% of WT) compared with DU145 knockdown cells (47% of WT). After transfection with the negative control nontargeting shRNA plasmid, there was a slight reduction in DU145 clonogenicity, but not so for A549 cells. These results indicate a less malignant phenotype after MPGES-1 knockdown.

Knockdown of MPGES-1 Reduces the Tumorigenic Potential of DU145 and A549 Cells in Vivo. Next, we investigated the effect of MPGES-1 knockdown on tumor development in vivo. DU145 and A549 WT tumor cells, nontargeting shRNA control cells, and MPGES-1 knockdown cells were injected into hind flanks of nude mice, and tumor formation was monitored. The time to tumor take, defined as the number of days for a tumor in the animal to reach a volume of 0.2 mL, was prolonged after knockdown of MPGES-1 for both DU145 and A549 knockdown cells. For DU145 knockdown clone A, the median time to tumor volume 0.2 mL was more than doubled compared with WT cells (53 vs. 18 days; Fig. 5A). In addition, DU145 knockdown clone A cells did not develop tumors in 48% of the injection sites (Fig. 4B). For A549 cells, similar results were obtained. Only 5 of 20 injections of A549 knockdown clone resulted in tumors (0.2 mL) at the end of the experiment, 72 days after tumor cell injection (Fig. 4C). When mice were injected with cells (DU145, A549) transfected with negative control nontargeting shRNA plasmid, tumor growth was not significantly delayed. These results show that MPGES-1 is important for the tumorigenic potential of these cells.

The histology of DU145 xenografts was examined. Tumors growing from DU145 WT cells consisted of homogeneous viable tumor tissue, whereas MPGES-1 knockdown DU145 cells displayed large areas with massive necrosis and nonviable tumor parenchyma (hematoxylin/eosin stain; Fig. S4A). DU145 WT tumors contained Ki-67-positive cells throughout, whereas there was only a small rim of viable cells (Ki-67⁺) along the periphery of the MPGES-1 knockdown tumor (Fig. S4B).

Expression of MPGES-1 and COX-2 in Xenograft Tumors. Expression of MPGES-1 and COX-2 in excised xenograft tumor tissues was analyzed by Western blot analyses. Reduced expression of MPGES-1 was detected in tumors derived from DU145 knockdown clone A, compared with tumors derived from WT cells and from DU145 transfected with nontarget shRNA (Fig. S4A). For A549 cells, expression of MPGES-1 was similar in all xenograft tumors (Fig. S5B). These observations suggest that the xenograft tumor tissues (DU145, A549) contain different amounts of stromal and endothelial cells that express MPGES-1. Also, COX-2 was present in all tumors and most abundant in xenografts from A549 cells (in which COX-2 is highly inducible).
Expression of MPGES-1, COX-2, and Androgen Receptor in Prostate Tissue Samples. Cancer and benign hyperplasia tissues were obtained from prostate surgery. From pieces of frozen tissue, Western blot samples were prepared (Fig. 6, five cancer with Gleason grade 6 or 7, and five benign; see Table 1). Expression of MPGES-1 was clearly detectable in two of the prostate cancer samples (nos. 6 and 9), whereas weaker bands were observed for the remaining three cancer samples. In four of the five benign hyperplasia samples, MPGES-1 was undetectable; only in sample 4 could a very faint band be observed. Thus, from these 10 samples, MPGES-1 appears to be more abundant in prostate cancer tissue compared with benign hyperplasia. Expression of COX-2 was seen in four of the benign samples and in three of the cancer samples. Thus, COX-2 varied in prostate tissue samples, as published previously (9, 10). Androgen receptor was clearly detected in four of the benign samples and in four of the cancer samples.

MPGES-1 Knockdown Sensitizes DU145 Cells to Adriamycin. Caspase activation after treatment with adriamycin reflects apoptosis by the intrinsic (mitochondria-dependent) pathway (19). As indicated by the 6-fold increase in accumulated cytokeratin 18 fragments, genotoxic stress-induced apoptosis was considerably increased after knockdown of MPGES-1 in DU145 cells (Fig. 7A). Interestingly,

Table 1. Donors of prostate tissues analyzed in Fig. 6

<table>
<thead>
<tr>
<th>Sample, no.*</th>
<th>Age, y</th>
<th>Preoperative PSA in plasma, ng/mL</th>
<th>Gleason score of the prostatectomy specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 b</td>
<td>59.2</td>
<td>7.2</td>
<td>–</td>
</tr>
<tr>
<td>2 b</td>
<td>56.6</td>
<td>6.8</td>
<td>–</td>
</tr>
<tr>
<td>3 b</td>
<td>50.2</td>
<td>3.9</td>
<td>–</td>
</tr>
<tr>
<td>4 b</td>
<td>61.9</td>
<td>2.7</td>
<td>–</td>
</tr>
<tr>
<td>5 b</td>
<td>73.5</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>6 c</td>
<td>44.7</td>
<td>19</td>
<td>4 + 3 = 7</td>
</tr>
<tr>
<td>7 c</td>
<td>70.5</td>
<td>5.4</td>
<td>3 + 4 = 7</td>
</tr>
<tr>
<td>8 c</td>
<td>58.6</td>
<td>17</td>
<td>3 + 4 = 7</td>
</tr>
<tr>
<td>9 c</td>
<td>70.9</td>
<td>4.3</td>
<td>3 + 3 = 6</td>
</tr>
<tr>
<td>10 c</td>
<td>56.7</td>
<td>0.8</td>
<td>3 + 3 = 6</td>
</tr>
</tbody>
</table>

PSA indicates for prostate-specific antigen.

*In this column, b indicates benign, and c indicates cancer.

Fig. 5. Western blot analyses of MPGES-1 in xenograft tumor tissues. Tumors were excised from the injection sites (hind flanks) of NMRI nu/nu mice and snap-frozen in liquid nitrogen. Pieces of frozen tissue were solubilized (M-PER plus sonication), and aliquots (37.5 μg) of total protein were applied to SDS/PAGE gels. After electroblotting, membranes were incubated with antibodies to MPGES-1, COX-2, and β-actin (see Materials and Methods). (A) Tumors derived from DU145 cells. (B) Tumors derived from A549 cells.

Fig. 6. Western blot analyses of prostate tissues (five cancer and five benign hyperplasia). Samples were prepared from frozen tissues as described in Materials and Methods. For analysis of MPGES-1 in lanes 1–8, 200 μg of protein was applied to SDS/PAGE gels, and Cayman antibody no. 160140 was used. For lanes 9–10, 80 μg of protein was applied, and the in-house MPGES-1 antibody was used. For analysis of COX-2 and androgen receptor, 37.5 μg was analyzed. For antibodies, see Materials and Methods.

Fig. 7. Adriamycin-induced apoptosis in wild type cells and MPGES-1 knockdown clones. (A) Apoptosis assay. Cells (7 × 10⁴) were seeded on 96-well plates and allowed to attach for 24 h. New medium containing 300 nM adriamycin was added, and after 24 h cells were lysed, and cytokeratin 18 fragments were analyzed by ELISA. For each condition, two incubations were analyzed (n = 2), and results are given ± SD. (B) Western blot analysis of caspase-3 fragment in DU145 cells—WT and MPGES-1 knockdown clone A—after treatment with adriamycin (300 nM) for 24 h. Cells were lysed with M-PER buffer, and 50-μg aliquots of total protein were applied to the SDS/PAGE gel. β-Actin analysis shows equal loading. After electroblotting, the membrane was incubated with an antibody for caspase-3 cleaved at Asp-175.
PGE2 could rescue DU145 MPGES-1 knockdown cells against the effect of adriamycin, whereas WT cells were resistant (Fig. 8A). DU145 MPGES-1 knockdown cells were sensitive to adriamycin, and similar results were obtained regarding DU145 cell viability. This was also the case for A549 cells. Because Western blot analysis showed similarly high expression of MPGES-1 in DU145 and PC3, the delayed growth of xenograft tumors from injected MPGES-1 knockdown clones suggests a reduced capacity of the cancer cells to establish and proliferate at the injection site. This is in line with COX inhibitors causing reduced invasion of DU145 and PC3 through Matrigel and reduced release of matrix metalloproteases (21). The low viability of tumors growing from knockdown cells may also depend on defective angiogenesis; PGE2 leads to VEGF release in prostate cancer cell lines (22).

Previously, COX-2 expression was reduced with shRNA in the breast cancer cell line LM2-4175, and tumor growth in immunocompromised mice was monitored (23). For these cells, knockdown only of COX-2 gave a limited effect; however, combined knockdown of four genes (COX-2, epiregulin, MMP1/2) almost completely abrogated tumor growth. In comparison, it thus appears that MPGES-1 knockdown gives a more pronounced effect than separate COX-2 knockdown. In addition to cell type-specific PGE2 requirements, a possible reason may be that COX-2 knockdown cells can be supplied with PGH2 from stroma cells by transcellular metabolism (24). A549 cells were recently found to metabolize PGH2 derived from HUVECs (25). Furthermore, although COX-2 is generally assumed to be the major PGH2 producer in tumors (8, 10), there is also evidence of a role for COX-1. Thus, knockdown of COX-1 in the cervix carcinoma cell line Hep2 reduced PGE2 formation (26), and COX-1 is weakly expressed in, for example, DU145 cells (18). PGE2 may thus be formed in cancer cells lacking COX-2.

An interesting question is whether PGE2 formed by other cells in the tumor tissue may promote tumor growth, as does PGE2 produced by the cancer cells (6). Xenograft tumors deriving from the MPGES-1 knockdown clones (both DU145 and A549) expressed PGE2, probably reflecting the presence of other cell types (stroma, vessels, leukocytes) in the tumor tissue. In addition, COX-2 was present in all tumors. Furthermore, COX-1 and the other two PGE synthases may be expressed in the cancer cells as well as in stromal cells of the xenografts. Thus, PGE2 could potentially be formed also in the MPGES-1 knockdown xenografts. Nevertheless, growth of xenograft tumors from both A549 and DU145 knockdown clones in nude mice was compromised. These observations suggest that expression of MPGES-1 in the cancer cells of tumor tissue is important and cannot be entirely substituted by PGE synthases in the stromal compartment of the tumor. This conclusion is supported by Kamei et al. (27), who found that HEK293 cells transfected with COX-2 and MPGES-1 became tumorigenic, whereas this was not the case for HEK293 cells treated with PGE2.

COX-2 can be up-regulated by p53, and this was shown to reduce apoptosis induced by genotoxic stress (28). Also, celecoxib potentiated the effect of adriamycin on neuroblastoma tumors (29). We found that knockdown of MPGES-1 led to increased adriamycin-induced apoptosis of DU145 cells in culture, and that exogenous PGE2 could rescue against this adriamycin-induced cell death. DU145 cells express two of the four PGE2 receptors (EP2 and EP4) (30). These receptors are usually associated with the plasma membrane, but EP4 and EP3a have also been localized in the nuclear membrane, but EP4 and EP3a have also been localized in the nuclear membrane.
and survival, and that inhibition of M-PGES-1 is a therapeutic option for cancers that express this enzyme.

Materials and Methods

Generation of Stable M-PGES-1 Knockdown Cells. Prostate cancer cell lines DU145 and non-small cell lung carcinoma cell line A549 were transfected with shRNA plasmids by using Lipofectamine 2000 (Invitrogen). Stably transfected clones were isolated with puromycin (8 μg/mL). The sequence of the shRNA insert is: 5'-CCGGAACUCATGGGAACATCTACTGTCGTTGCTACCATGTCGTTGTCTTGTGTT-3', and unedited residues correspond to nucleotides 236–258 in M-PGES-1 mRNA.

Immunoblot Analysis. Cell and tissue samples were dissolved in mammalian protein extraction reagent (M-PER) with Complete protease inhibitor mixture (Roche).

PGE2 Synthase Activity Assay. Cells were detached from culture dishes and sonicated on ice. After centrifugation, micromolar pellet fractions were incubated with PGH2 (10 μM) for 1 min on ice. After solid-phase extraction, samples were analyzed by reverse-phase HPLC.

Fluorescence Microscopy. DU145 and A549 cells growing on chamber slides were fixed with methanol at −20 °C for 5 min. Samples were incubated with anti-M-PGES-1 and anti-COX-2 antisera, and fluorescent secondary antibodies and were analyzed with an LSM 510 Laser Scanning Microscope (Carl Zeiss).

Clonogenic Assay. To determine colony formation, DU145 and A549 cells were seeded (150 cells per well) in six-well plates. After 12 days, colonies (>75 cells) with 50% plate efficiency were counted.

Animals and Xenografting. Female NMRI nu/nu mice (4–8 weeks old) were s.c. injected in both hind flanks with the following tumor cell variants: 2 × 106 DU145 cells or 5 × 105 DU145 M-PGES-1 knockdown clone A2. For the same variants of A549, 5 × 106 cells were injected. A tumor was considered to be established once it had reached a volume of 0.2 mL. For immunohistochemical analysis, sections from paraffin-embedded tissue were incubated with primary antibody Ki-67.

Human Prostate Samples. Prostate specimens were obtained (fresh frozen) from prostate cancer patients undergoing radical prostatectomy. Histopathological staining confirmed the presence of tumor or benign tissue. Cancer samples were graded by using the Gleason score (Table 1).

Apoptosis and Cell Viability Assays. Cytokeratin 18 fragments in cell lysates were determined by ELISA. The appearance of activated caspase-3 was confirmed by Western blotting. Cell viability was determined by mitochondrial dehydrogenase assay (cleavage of the tetrazolium salt WST-1).

Additional Information. For more detailed information on all Materials and Methods, see SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Johan Dixelius and Christer Betsholtz for kind help and access to LSM 510. This work was supported by the Swedish Research Council, European Union Grants LSHM-CT-2004-00553 and FP7-Health-201668, the Swedish Children's Cancer Foundation, the Stockholm Cancer Society, and the Swedish Cancer Foundation. H.H. was supported by the Nakatomi Foundation, the Naito Foundation, the Scandinavia-Japan Saskatchewan Foundation, and the Nakayama Foundation for Human Science.
### Supporting Information

#### Hanaka et al. 10.1073/pnas.0910218106

### SI Materials and Methods

#### Cell Culture.** Human prostate cancer cell lines DU145 (ATCC HTB-81), PC3 (ATCC CRL-1435), and LNCaP (ATCC CRL-1740) were grown in RPMI-1640 medium (Sigma) supplemented with 10% FBS (Sigma). Human non-small cell lung carcinoma cell line A549 (ATCC CRL-2271) was cultured in Ham’s F-12 medium with 10% FBS.

#### Generation of Stable MPGES-1 Knockdown Cells.** Cells growing in six-well tissue culture dishes (BD Falcon) were transfected with plasmid DNA (MISSION shRNA pLKO.1-puro plasmids; Sigma) by using Lipofectamine 2000. Stably transfected clones growing in the presence of puromycin (8 μg/mL) were isolated and assayed for expression of MPGES-1. For both DU145 and A549 cells, transfection with plasmid containing insert TRCN-0000045936 yielded MPGES-1 knockdown clones. The sequence of the shRNA insert is: 5’-CCGGGAACAGCATGAGACCATCTACATCGAGTAGATGGTCTCCATGTCGTTC TTTTT-T-3’, and underlined residues correspond to nucleotides 236–258 in MPGES-1 mRNA. For negative control experiments, cells were transfected with the nontarget shRNA control vector (MISSION SHC002; Sigma).

#### Immunoblot Analysis.** Cell samples were dissolved in M-PER (Themo Fisher Scientific) with addition of Complete protease inhibitor mixture (Roche). Also, xenograft tumor samples and prostate tissues were dissolved in M-PER and Complete, and these samples also were sonicated. Protein concentrations were determined with Protein Assay Kit II (Bio-Rad). Samples typically containing 37.5 μg of protein were separated by SDS/PAGE under reducing conditions and transferred to nitrocellulose membranes (Amersham). Membranes were probed with an in-house anti-MPGES-1 antibody (1:5,000 dilution) (1), followed by incubation with a peroxidase-conjugated goat anti-rabbit antibody (1:5,000; Sigma) and visualized with an ECL plus chemiluminescence detection system (GE Healthcare). For prostate tissues, an anti-MPGES-1 antibody from Cayman (no. 160140) also was used (1:1,000 dilution). Autoradiography exposure time (Hyperlifm ECL, Amersham, GE Healthcare) was typically 1 min. For COX-2 analysis, mouse monoclonal antibodies were from Cayman (no. 160112) or from Alex (ALX-804-112). For androgen receptor, mouse monoclonal antibodies (sc-7305; Santa Cruz Biotechnology) were used. β-Actin was probed with a goat polyclonal antibody from Santa Cruz Biotechnology (sc-1616), followed by incubation with an alkaline phosphatase-conjugated rabbit anti-goat antibody, and was visualized with nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

#### Quantitative Real-Time PCR.** Total RNA was extracted from cells with TRizol (Invitrogen), and cDNA synthesis was performed by using oligo(dT) primers (Applied Biosystems) and SuperScript II reverse transcriptase (Invitrogen). Relative quantification analysis was performed with a 7300 Real-Time PCR System and sequence detection software (Applied Biosystems). Samples were denatured (95 °C for 10 min), followed by 40 cycles (95 °C for 15 sec, annealing and elongation at 60 °C for 1 min). PPIA was analyzed as endogenous control to normalize variations in cDNA quantities from different samples.

#### PGE2 Synthase Activity Assay.** Cells were detached from culture dishes by addition of trypsin/EDTA for 10 min at 37 °C and collected by centrifugation. After resuspension in 0.1 M KP buffer, pH 7.5; 0.5 M sucrose; 1 mM reduced glutathione (GSH); and Complete protease inhibitor mixture, cells were sonicated 3 × 20 sec on ice. The cell homogenate was centrifuged at 10,000 × g, 4 °C for 15 min, and the supernatant was further fractionated at 100,000 × g, 4 °C for 2 h. Pellets (microsomal fraction) were resuspended in 0.1 M KP, pH 7.5; 2.5 mM GSH; and 10% (vol/vol) glycerol. After determination of protein concentration, 25-μg aliquots in 100 μL of buffer were incubated with PGH2 (10 μM) for 1 min on ice. Reactions were stopped by addition of 400 μL of 25 mM FeCl3, 50 mM citric acid, and 2.5 mM 11-β-PGE2 (internal standard). Solid-phase extraction was performed with Oasis HLB SPE columns (30 mg; 1 mL) from Waters. Samples were analyzed on a Waters 2790 HPLC system equipped with a NovaPak C18 column (3.9 × 150 mm; 4-μm particle size) eluted with water/acetonitrile/formic acid (62:38:0.0025, vol/vol/vol) at 0.3 mL/min. The absorbance of the eluate was monitored with a Waters 996 diode array detector set at 195 nm.

For preparation of PGH2, arachidonic acid (1 mM) was stirred for 2 min with a suspension of the microsomal fraction of sheep vesicular gland homogenate in the presence of 5 mM L-1-tropanthol as the reducing agent. Extraction with diethyl ether followed by purification by normal-phase HPLC using a solvent system of 2-propanol-hexane-acetic acid (2.5:97.5:0.002; vol/vol/ vol) afforded >95% pure material, which was dissolved in dry aceton and stored at −80 °C until use.

#### Fluorescence Microscopy.** DU145 and A549 cells growing on chamber slides (Lab-Tek II; Nunc) were fixed with methanol at −20 °C for 5 min. Samples were incubated with anti-MPGES-1 antisera (1:300 dilution) and anti-COX-2 antisera (1:50 dilution; Cayman 160112), followed by FITC-conjugated anti-rabbit IgG (1:200; Invitrogen) and Alexa Fluor 633 goat anti-mouse IgG (1:200; Invitrogen) for 1 h at room temperature. After three washes with PBS, samples were treated with Slow-Fade Antifade Kit (Invitrogen). The fluorescent signal was observed with an LSM 510 Laser Scanning Microscope System.

#### Clonogenic Assay.** To determine colony formation, DU145 and A549 cells were seeded (150 cells per well) in six-well Cell plates (Sarstedt). After 12 days of incubation, cell cultures were rinsed three times with PBS, samples were treated with Slow-Fade Antifade Kit (Invitrogen). The fluorescent signal was observed with an LSM 510 Laser Scanning Microscope System.

#### Animals and Xenografting.** Female NMRI nu/nu mice (4–8 weeks old; Taconic Laboratories) were maintained at five animals per cage and were given sterile water and food ad libitum. Mice were s.c. injected in both hind flanks with the following tumor cell variants: 2 × 106 DU145 WT, DU145 nontarget shRNA control, or DU145 MPGES-1 knockdown clone A. For the same variants of A549, 5 × 106 cells were injected. Tumor development was monitored daily up to 72 days after cell injection; a tumor was considered to be established once it had reached a volume of 0.2 mL. Statistical significance of differences between populations was analyzed by the Mann–Whitney U test and the Kruskal–Wallis test (nonparametric ANOVA) followed by Dunn’s multiple-comparison test, respectively. All statistical tests were two-sided. Animal experiments were approved by the regional

For immunohistochemical analysis, sections from paraformaldehyde-fixed, paraffin-embedded tissue were incubated with primary antibody Ki-67 (NeoMarkers) overnight at 4 °C. A secondary HRP-SuperPicture Polymer detection kit with appropriate secondary antibodies (Invitrogen) was used together with diaminobenzidine tetrahydrochloride substrate chromogen to visualize nuclear immunopositivity.

**Human Prostate Samples.** All prostate specimens were obtained from prostate cancer patients undergoing radical prostatectomy. Specimens with clinically unilateral palpable tumors (pT2) were fresh frozen just after surgical removal of the prostate. In all cases, histopathological staining confirmed the presence of tumor or benign tissue. Cancer samples were graded by using the Gleason score (Table 1). Typically, Western blot samples were prepared from pieces of frozen tissue (approximately 2 × 2 × 2 mm; see immunoblot descriptions above. This study was approved by the Stockholm ethics committee (Dnr. 02-472; extended 26/11-03). In addition, approval for tissue retrieval from the tumor biobank for urological malignancies was guaranteed (Dnr. 01-353).

**Apoptosis Assay.** The intermediate filament cytokeratin 18 is cleaved at Asp-396 by caspase-9 during early apoptosis and by caspase-3 and caspase-7 during the execution phase. The concentration of cytokeratin 18 fragments in cell lysates was determined by the Apoptosense ELISA kit (Peviva). The appearance of activated caspase-3 was confirmed by Western blot (antibody no. 9661 from Cell Signaling Technology).

**Cell Viability Assay.** Cell viability was determined by mitochondrial dehydrogenase assay (cleavage of the tetrazolium salt WST-1). Cells were plated in 96-well plates (5,000 or 10,000 cells per well) in 200 μL of medium. After culture overnight, reagents were added as indicated. After the indicated times, medium was removed, and adherent cells were washed once with PBS. WST-1 cell proliferation reagent (20 μL; Roche) was added, samples were incubated at 37 °C for 2 h, and absorbance was measured at 450–620 nm. Each experiment was performed in duplicate.

Fig. S1. Western blot analyses of MPGES-1 in three prostate cancer cell lines stimulated with IL-1β. Cells were cultured in presence of IL-1β (1 ng/mL) for 72 or 24 h. Cells growing on dishes were lysed with M-PER buffer, and 37.5-μg aliquots of total cellular protein were applied to SDS/PAGE gels. After electroblotting, membranes were incubated with antibody to MPGES-1 (see Materials and Methods).

<table>
<thead>
<tr>
<th></th>
<th>DU145</th>
<th></th>
<th>PC3</th>
<th></th>
<th>LNCaP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>72h</td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
</tr>
<tr>
<td>24h</td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
<td><img src="https://example.com" alt="Image" /></td>
</tr>
</tbody>
</table>
Western blot analyses of MPGES-1 and COX-2 in WT and MPGES-1 knockdown (KD) cell lines. For DU145, samples were prepared for WT and knockdown clone A. For A549, samples were from WT and knockdown clone a. Cells growing on dishes were lysed with M-PER buffer, and 37.5-μg aliquots of total cellular protein were applied to SDS/PAGE gels. After electroblotting, membranes were incubated with antibodies to MPGES-1 and COX-2 (see Materials and Methods).

<table>
<thead>
<tr>
<th></th>
<th>DU145</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KD</td>
</tr>
<tr>
<td>mPGES-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. S2. Western blot analyses of MPGES-1 and COX-2 in WT and MPGES-1 knockdown (KD) cell lines. For DU145, samples were prepared for WT and knockdown clone A. For A549, samples were from WT and knockdown clone a. Cells growing on dishes were lysed with M-PER buffer, and 37.5-μg aliquots of total cellular protein were applied to SDS/PAGE gels. After electroblotting, membranes were incubated with antibodies to MPGES-1 and COX-2 (see Materials and Methods).
Immunocytochemistry of MPGES-1 and COX-2 in (A) A-549 and (B) DU145 cells. Cells were grown on chamber slides with or without IL-1β (1 ng/mL) for 24 h and were double-stained as described in Materials and Methods. Green is MPGES-1, and red is COX-2. In the merged images, yellow indicates colocalization. The width of each panel corresponds to approximately 150 μm.
Fig. S4. Histology of xenograft tumors growing from DU145 cells: WT and MPGES-1 knockdown clone A. (A) Hematoxylin/eosin staining. (B) Immunohistochemistry with Ki-67 antibody.