Supplemental Information

Supplemental Data

1. IL-1β-positive LoVo SCC CM induces production of PGE_2 by MSCs

To identify the factors secreted by LoVo cells that were responsible for inducing PGE_2 production in MSCs, we isolated eight single-cell LoVo clones (SCCs) and compared the abilities of their respective conditioned media (CM) to induce expression in MSCs of the two rate-limiting enzymes responsible for PGE_2 synthesis – COX2 and mPGES1 – in addition to measuring the resulting secreted PGE_2. With the exception of SCC34, the CM of all remaining SSCs induced robust increases in MSCs of PGE_2, COX2 and mPGES1 (Supplementary Fig. S1Ea, CM of SCC23 as a representive sample, S1Eb). We then used cytokine arrays to screen SCC34 CM and SCC23 CM (as a positive, PGE_2-producing control) and found that SCC34 CM, which failed to induce PGE_2 production, contained elevated levels of IL-1ra, an IL-1 receptor antagonist, relative to SCC23 CM (data not shown). We also found that the seven SSCs that (including SCC23) elicited PGE_2 production produced significant amounts of IL-1β in contrast to SSC34, which did not produce this interleukin (Supplementary Fig. S1Ec). Hence, those LoVo SCCs whose CM were able to induce PGE_2 production expressed high levels of IL-1β and

low levels of IL-1ra in contrast to SSC34, which exhibited the opposite set of traits.

2. Migration of MSCs to tumors in vivo

As described in the text of this report, unlike LoVo or HCC1806 cells, MDA-MB-453 cells do not secrete IL-1 and do not induce PGE_2 or cytokines upon coculture with MSCs (Fig. 1C). To confirm and extend our cell culture observations on the interactions between carcinoma cella and MSCs, we implanted green fluorescent protein (GFP)-expressing populations of either LoVo or HCC1806 cells subcutaneously in the right flanks of mice and GFP-expressing MDA-MB-453 tumors in the left flanks. Red fluorescent protein tdTomato-expressing MSC cells (tdTomato-MSCs) (1) were then administered intravenously via the tail vein, after palpable tumors had formed. MSCs were indeed recruited to both LoVo and HCC1806 carcinomas, but not to the MDA-MB-453 tumors (Supplementary Fig. S3A, top); these results were confirmed by immunofluorescence analyses (Supplementary Fig. S3A, bottom).

In a model of experimental metastasis, we injected Lovo-GFP or HCC1806-GFP cells intravenously, to seed lung metastases. After an interval of 3 weeks, tdTomato-MSCs were injected intravenously. The tumors were then examined for the presence of MSCs two weeks later (Supplementary Fig. S3B). As observed with

subcutaneously implanted tumors, MSCs localized to disseminated LoVo and HCC1806 metastases. In contrast, for the MDA-MB-453 tumors, which do not secrete this IL-1, MSCs failed to localize to tumors.

Previous work had demonstrated that MSCs are recruited to various types of tumors, and that IL-1 is a factor responsible for the recruitment of bone marrow cells to tumor stroma (2, 3). Our observations, taken together with the previous observations of others, indicate that IL-1 secretion by carcinoma cells plays a dual role, first recruiting MSCs to the tumor-associated stroma and then eliciting production of PGE_2 and cytokines by these cells.

3. ALDH activity as a useful marker for identifying LoVo and HCC1806 cancer stem cells

Aldehyde dehydrogenase (ALDH) has been used as a marker both of normal tissue stem cells and of CSCs present in carcinomas arising in various tissues (4, 5). Among 94 single cell clones (SCCs) that we isolated from bulk LoVo cells, four LoVo SCC clones, termed SCC+ clones, expressed elevated ALDH1 levels, while the remaining 90 clones, termed SCC- clones, did not express detectable ALDH1 (Supplementary Fig. S4A, left). The LoVo SCC+ clones contained round, clumpy cells and tended to be readily detached

from the culture dish (Supplementary Fig. S4A, right), while LoVo SCC- clones were composed of flat cells that adhered strongly to the culture dish (Supplementary Fig. S4A, right). When bulk LoVo cells were sorted based on their ALDH activity (Supplementary Fig. S4B, panel a), $ALDH^{high}$ LoVo cells isolated by FACS were morphologically similar to LoVo SCC+ clones (Supplementary Fig. S4B, panel b). $ALDH^{low}$ LoVo cells isolated by FACS and LoVo SCC- clones also resembled one another morphologically. We chose 4 SCC+ and 5 SCC- clones and implanted cells from each of these clones subcutaneously in SCID mice. The SCC+ clones (5×10^5 cells/injection) grew far more vigorously than did LoVo SCC- clones. The masses of SCC+ primary tumors were ~60-fold higher than those of SSC- clones (Supplementary Fig. S4C). To further validate this notion, $ALDH^{high}$ and $ALDH^{low}$ LoVo cell populations were flow-sorted, based on their ALDH activity (Supplementary Fig. S4Ba), and implanted in SCID mice (Fig. 4Ca). The frequencies of TICs in $ALDH^{high}$ and $ALDH^{low}$ LoVo cells were 4×10^{-5} - 3×10^{-4} and 7×10^{-7} - 5×10^{-6} respectively (Fig. 4Cb). The $ALDH^{high}$ LoVo cell population contained approximately 60-fold more TICs than did comparable numbers of $ALDH^{low}$ LoVo cells. Similar results were observed for 4 HCC1806 SSCs; HCC1806 SCC+ clones, expressing elevated ALDH (Supplementary Fig. S5A), exhibited higher tumor-initiating frequencies and gave rise to larger primary tumors than did HCC1806 SCC- clones (Supplementary Fig. S5B).

These observations suggested that ALDH1 might serve as a useful marker for identifying LoVo and HCC1806 cell subpopulations that are enriched for CSCs.

ALDH activity can be used to identify a CSC-enriched population in LoVo cells. To find markers to further enrich CSCs in $ALDH^{high}$ LoVo cells, we analyzed expression of the known CSC markers CD133, LGR5 and CD44 in $ALDH^{high}$ and $ALDH^{low}$ LoVo cells. The mRNA levels of the CSC markers in freshly isolated $ALDH^{high}$ and $ALDH^{low}$ LoVo cells were compared by qPCR. The ALDH1 mRNA level of $ALDH^{high}$ LoVo cells was 65-fold higher than that of and $ALDH^{low}$ LoVo cells (Supplementary Fig. S6A), confirming the successful separation of $ALDH^{high}$ and $ALDH^{low}$ LoVo cells. Compared to $ALDH^{low}$ LoVo cells, $ALDH^{high}$ LoVo cells expressed a 5-fold and 19-fold excesses of LGR5 and CD133 mRNA. In contrtast, the CD44 mRNA levels are similar in $ALDH^{high}$ and$ALDH^{low}$ LoVo cells (Supplementary Fig. S6A). Flow cytometry analysis confirmed that the number of $CD133^{+}$ cells is enriched in $ALDH^{high}$ LoVo cells (Supplementary Fig. S6B).

CD133 has previously been used to enrich CSCs in LoVo cells (6). In addition, $ALDH^{high}$/$CD133^{+}$ markers have been used to define CSCs in a variety of cancer cells, including colon, ovarian and liver cancer cells (7-10). To determine whether MSCs can increase the population of cells that are double-positive for two presumptive CSC markers, we co-cultured tdTomato-MSCs and LoVo cells for five days, isolated the LoVo

cells and determined the percentage of $ALDH^{high}CD133^{+}$ cells by flow cytometry (Supplementary Fig. S6C). While the percentage of $ALDH^{high}$ cells following this coculture increased from 20% to 38% (Fig 4Ca), the percentage of $ALDH^{high}CD133^{+}$ cells increased from 1.4% to 16%. (Supplementary Fig. S6C). Co-culture with MSCs caused a 10-fold increase of $ALDH^{high}CD133^{+}$ CSCs. To characterize the biochemical and biological properties of the CSC population induced in LoVo cells by MSC coculture, it will be necessary to identify an appropriate subset of CSC markers and characterize more fully their tumor initiating properties.

4. LoVoMSC cocultures secrete PGE_2-dependent angiogenic factors that stimulate new blood vessel formation

To verify the possibility that the interaction between cancer cells and MSCs increases tumor angiogenesis, we performed a Matrigel plug assay to measure the increase of blood vessel formation caused by the interaction of LoVo cells and MSCs. Concentrated CM from LoVo, MSC, and LoVoMSC cultures were mixed with Matrigel and implanted subcutaneously into nude mice. Some LoVoMSC media were conditioned in the presence of indomethacin or indomethacin + PGE_2. Plugs were isolated after seven days (Supplementary Fig. S9) and hemoglobin levels were measured to provide a

quantitative measure of angiogenesis. LoVoMSC CM induced significantly more blood vessel formation in the plugs than did LoVoCM or MSCCM. Inclusion of indomethacin in LoVoMSC cocultures blocked production of angiogenic factors; the indomethacin inhibition of angiogenic factor induction was rescued by PGE_2. In LoVoMSC coculture, MSCs secrete some angiogenic factors in a PGE_2-dependent manner.

5. Blocking PGE_2 signaling decreases MSC-induced $ALDH^{high}$ cancer stem cells

To test whether MSCs increased the numbers of $ALDH^{high}$ LoVo CSCs through secreted PGE_2, we inhibited PGE_2 synthesis in LoVoMSC cocultures with a COX2 inhibitor, and inhibited PGE_2 signaling with an EP4 receptor antagonist in similar cocultures. Coculture with tdTomato-MSCs for 5 days increased the percentage of $ALDH^{high}$ LoVo cells from 10% to 30.8% (Supplementary Fig. S10). About half of this MSC-elicited increase of $ALDH^{high}$ LoVo cells was blocked by the NS398 COX2 inhibitor, and 75% of the NS398-imposed reduction was reversed by adding exogenous PGE_2. In addition, the GW627368X EP4 receptor antagonist blocked 88% of the MSC-induced increase of $ALDH^{high}$ LoVo cells. These data indicated that MSCs generated increased numbers of $ALDH^{high}$ CSCs in a PGE_2-dependent manner.

6. MSCs were induced to differentiate into FSP- and EDA-FN-positive fibroblasts by LoVoCM

MSCs recruited to tumors differentiate into a variety of mesenchymal cell types soon after their arrival in the tumor-associated stroma (11). Consequently, we evaluated several markers that could be used to identify MSCs and MSC-derived cells. Fibroblast Surface Protein (FSP) is a marker of fibroblasts (12), while EDA-containing fibronectin (EDA-FN), a myofibroblast-associated fibronectin splice variant, is required for myofibroblast differentiation (13). α-smooth muscle actin (α-SMA) is commonly regarded as a myofibroblast marker (14). Using these markers, we found that, when MSCs were cultured in LoVoCM, they expressed FSP and EDA-FN. However, they expressed little, if any, α-SMA (Supplementary Fig. S11).

7. ALDH^{high} CSCs are located near COX2-expressing regions in human colorectal cancer tumors

To ascertain whether COX2-PGE$_2$ signaling is relevant to the behavior of primary human tumors, we determined, in excised tumor samples, whether ALDH^{high} tumor cells are located near the regions of stroma expressing COX2, the likely source of PGE$_2$ production. In human colorectal adenocarcinomas with abundant stroma (Supplementary

Fig. S12A). Indeed, we detected strong COX2 expression in small populations of mesenchymal cells (e.g., the cells indicated by arrowheads in Supplementary Fig. S12B). In these samples, most carcinoma cells near the COX-2-expressing cells also exhibited high ALDH1 levels (e.g., the cells indicated by the arrows in Supplementary Fig. S12B). [In these sections, carcinoma cells were identified by their enlarged cell nuclei (15)]. Conversely, ALDH-positive cells were sparse in regions located at some distance from the COX2-expressing cells (Supplementary Fig. S12A, Merge 2).

Supplemental Materials and Methods

Cell Culture

SW1116, MDA-MB-231 and MDA-MB-453 cells were grown in DMEM containing 10% fetal bovine serum with penicillin-streptomycin. LoVo, HCC1806 and BT549 cells were grown in RPMI1640 containing 10% fetal bovine serum with penicillin-streptomycin. SUM149 and SUM159 cells, provided by S. Ethier (Wayne State University), were grown in DMEM/F12 (1:1) containing 5% FBS, 5 μg/ml insulin and 2 μg/ml hydrocortisone. Human bone-marrow derived MSC (Sciencell) were grown in Mesenchymal Stem Cell Medium (MSCM, Sciencell). MSCs used in the all experiments were not grown beyond the eighth passage.

Fluorescence activated cell sorting (FACS) and flow cytometry

Cells were prepared according to standard protocols and suspended in 2%FBS/PBS prior to FACS. 7-AAD (BD Biosciences) was used to exclude dead cells. Cells were sorted on a BD FACSAria SORP and analyzed on a BD LSRII, using BD FACSDiva Software (BD Biosciences).

Creation of stable cell lines

Green fluorescent protein-labeled LoVo, HCC1806 and MDA-MB-453 cells and tdTomato fluorescent protein-labeled MSCs were made as described before (16). Short hairpin RNAs (shRNA) targeting the mRNAs encoding IL-1α, IL-β or cox-2 were expressed from pLKO.1-puro (Open Biosystems); the target sequences of these shRNA hairpins are as follows: shIL1α-1: CCAATGACTCAGAGGAAGAAA, shIL1α-2: GCTTACCTTCAAGGAGAGCAT, shIL1β-1: CACATGGGATAACGAGGCTTA, shIL1β-2: CCTGCGTGTTGAAAGATGATA, shcox2-1: GCAGATGAAATACCAGTCTTT, shcox2-2: CCATTCTCCTTGAAAGGACTT, shsc: CAACAAGATGAAGAGCACCAA. All stable cell lines were generated via retroviral infection using HEK293T cells, as previously described (17), followed by selection with puromycin or by cell sorting.

Invasion assay

tdTomato-labeled carcinoma cells (2×10^4 cells/well), MSCs (2×10^4 cells/well), or carcinoma cells-MSCs were added to the upper chambers of BD Matrigel™ Invasion Chambers, 8.0 μm (BD biosciences), containing DMEM with 2.5% FBS. Neutralizing antibodies (anti-IL-6 [AF-206-NA; 100 ng/ml], anti-IL-8 [AF-208-NA; 1 μg/ml], anti-GRO-α [AF-275; 100 ng/ml], and anti-RANTES antibodies [AF-278-NA; 1 μg/ml], goat IgG [1 μg/ml] R&D systems), NS398 (50 μM, Cayman Chemical) and PGE_2 (100 nM, Cayman

Chemical) were added into the both upper and bottom chambers, as indicated in the figure legends. After 72 h, residual cells were removed from the top of the membrane and the cells on underside of the membrane were washed in PBS, and fixed in 4% paraformaldehyde. The tdTomato-cells that migrated through the membrane during the incubation period were counted in five randomly selected regions. All experiments were done in the presence of a serum gradient, 2.5% in the upper chamber; 12.5% in the bottom chamber.

Western blotting

Total protein was extracted with RIPA lysis buffer. Nuclear and cytoplasmic extracts were prepared by using NE-PER kit (Pierce). Concentrations were determined using the Bradford protein assay (Bio-Rad). Protein lysates were resolved on a 4%–12% Bis-Tris Gel, transferred to PVDF membranes, probed with primary antibodies overnight at 4 °C and then with HRP-linked secondary antibodies (GE Healthcare) and visualized with ECL reagent (Thermo Scientific). The following antibodies were used: anti-COX2 (Thermo Fisher Scientific), anti-mPGES1 (Cayman Chemical), anti-GAPDH (Santa Cruz Biotechnology), anti-β-actin (Abcam), anti-ALDH1 (BD biosciences), anti-IL-1-β (R&D Systems), anti-FSP (Abcam) and anti-EDA-FN1 (Millipore), anti-E-Cadherin (BD biosciences), anti-vimentin (Thermo Fisher Scientific), anti-fibronectin (BD biosciences),

anti-Snail, anti-p473-Akt, anti-pan-Akt, anti-p21-GSK3α, anti-p9-GSK3β, anti-GSK3β, anti-p33/37/41-β-catenin, anti-p552-β-catenin, anti-β-catenin (Cell Signaling Technology), Lamin B1 (Abcam).

ALDH activity assay

ALDH activities of the carcinoma cells were measured with the *ALDEFLUOR*® fluorescent reagent system (STEMCELL Technologies), according to the manufacturer's protocol.

Immunofluorescence

The tissue paraffin sections were deparaffinized, hydrated and blocked with normal serum. The sections were incubated with primary antibodies overnight at 4 °C and then with secondary antibodies (Invitrogen) for 1 h at room temperature. Cell nuclei were visualized with DAPI (Sigma). Slides were mounted with SlowFade Gold antifade reagent (Invitrogen). The sections of human breast primary tumors were obtained from US Biomax.

PGE_2 and cytokine assays

Carcinoma cells (1.25 x 10^5 cells/well), MSCs (2.5 x 10^5 cells/well), or carcinoma cell-MSC cocultures were grown in 6-well tissue culture plates or 0.4 μm transwell plates. After the times of incubation indicated in the figure legends, the supernatants were

collected and the concentrations of PGE_2, IL-6, IL-8, GRO-α, RANTES, IL-1α, IL-1β and IL-1ra were determined by ELISA as described in the manufacturers' protocols. PGE_2 levels were measured using a PGE_2 EIA assay kit (GE healthcare). Human IL-6, IL-8, GRO-α, RANTES, IL-1α, IL-1β and IL-1ra levels were measured using Quantikine kits (R&D systems). Three replicate samples were included in each experiment.

Conditioned media

LoVo (7.5 x 10^5 cells/well), SW1116 (7.5 x 10^5 cells/well), MSCs (1.5 x 10^6 cells/well) or LoVo(7.5 x 10^5 cells/well)MSCs(1.5 x 10^6 cells/well) were grown in 10-cm tissue culture plates with 15 ml DMEM-10%FBS medium for 120 h. The supernatants of the cultures were collected and centrifuged at 2000 rpm for 10 min to remove floating cells and cell debris. The supernatants were the conditioned media used in the experiments.

In vivo Matrigel plug assay

LoVo, SW1116, MSC, LoVoMSC cocultures and SW1116MSC cocultures were grown in 10-cm culture plates in 15 ml DMEM-10%FBS, containing vehicle, 100 μM indomethacin, and/or 500 nM PGE_2 for 120 h. Five-fold concentrated CM used in the Matrigel plug assay was derived from by centrifugation at 12,500g (Microcon YM-3 centrifugal filter devices; Millipore, Bedford, Massachusetts) for 30 min. The YM-3 filter cuts off molecules smaller than 300, eliminating indomethacin and PGE_2.

Nude mice (four/group) were injected subcutaneously with 100 µl of concentrated conditioned medium mixed with 450 µl growth factor reduced Matrigel (BD Scientific). Plugs were removed from anaesthetized mice after 8 days, washed with PBS and examined with a dissection microscope. Plugs were then suspended in 0.5 ml PBS and centrifuged (5 min, 1000 g). Supernatant optical densities were determined at 415 nm.

Cytokine array assay

Cytokine levels in the conditioned medium were screened with RayBio® Human Cytokine Antibody Array G Series 1000 kit (RayBiotech), according to the manufacturer's protocols.

RNA preparation and qPCR analysis

RNA preparation and cDNA synthesis were performed using RNeasy Plus Mini Kit (Qiagen) and High Capacity RNA-to-cDNA Kit (Applied Biosystem), respectively, both according to the manufacturer's protocol. A cDNA sample prepared from 500 ng total RNA was used for each PCR. The PCR reactions using SYBR Green Mix I (Roche Diagnostics), data collection, and data analysis were performed on the LightCycler® 480 System (Roche Diagnostics). The thermal cycling parameters for the PCR were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 10 sec, 49°C for 7 sec, and 72°C for 25 sec. The relative mRNA quantity was normalized against the relative quantity

of GAPDH mRNA in the same sample.

IL-1α: TGTATGTGACTGCCCAAGATGAAG/AGAGGAGGTTGGTCTCACTACC, IL-1β: CCACAGACCTTCCAGGAGAATG/ GTGCAGTTCAGTGATCGTACAGG, IL-1ra: ATGGAGGGAAGATGTGCCTGTC/ GTCCTGCTTTCTGTTCTCGCTC, COX-2: CGGTGAAACTCTGGCTAGACAG/ GCAAACCGTAGATGCTCAGGGA, mPGES-1: GAGGATGCCCTGAGACACGGA/ CCAGAAAGGAGTAGACGAAGCC, 15-PGDH: TGGAGGTGAAGGCGGCATCATT/ GAGCGTGTGAATCCAACTATGCC, IL-6: AGACAGCCACTCACCTCTTCAG/ TTCTGCCAGTGCCTCTTTGCTG, IL-8: TCCTTTTCCGCCAGGCTTACCA/ GGCACGATGAAGCCAAAGGTGT, GRO-α: AGCTTGCCTCAATCCTGCATCC/ TCCTTCAGGAACAGCCACCAGT, EP1: TCATGGTGGTGTCGTGCATCTG/ GGATCTGGTTCCAGGAGGCAAG, EP2: GACCACCTCATTCTCCTGGCTA/ AACCTAAGAGCTTGGAGGTCCC, EP3: CCTTCAAGGTTCTGTGCTCAGC/ CATCAGCTTAGCTGGACACTGC, EP4: TACTCATTGCCACCTCCCTGGT/ GACTTCTCGCTCCAAACTTGGC, GAPDH: GTCTCCTCTGACTTCAACAGCG/ ACCACCCTGTTGCTGTAGCCAA, NANOG: CTCCAACATCCTGAACCTCAGC/ CGTCACACCATTGCTATTCTTCG, Oct4: CCTGAAGCAGAAGAGGATCACC/ AAAGCGGCAGATGGTCGTTTGG, SOX2: GCTACAGCATGATGCAGGACCA/ TCTGCGAGCTGGTCATGGAGTT, SOX9:

AGTACCCGCACTTGCACAAC/ CGTTCTTCACCGACTTCCTC, Angptl4:

GATGGCTCAGTGGACTTCAACC/ TGCTATGCACCTTCTCCAGACC, MMP2:

AGCGAGTGGATGCCGCCTTTAA/ CATTCCAGGCATCTGCGATGAG, MMP7:

TCGGAGGAGATGCTCACTTCGA/ GGATCAGAGGAATGTCCCATACC, MMP9:

GCCACTACTGTGCCTTTGAGTC/ CCCTCAGAGAATCGCCAGTACT, Twist1:

GCCAGGTACATCGACTTCCTCT/ TCCATCCTCCAGACCGAGAAGG, TGFβ3:

CTAAGCGGAATGAGCAGAGGATC/ TCTCAACAGCCACTCACGCACA, Jag1:

TGCTACAACCGTGCCAGTGACT/ TCAGGTGTGTCGTTGGAAGCCA, Wnt3a:

ATGAACCGCCACAACAACGAGG/ GTCCTTGAGGAAGTCACCGATG, FGF4:

CGTGGTGAGCATCTTCGGCGT/ GTAGGACTCGTAGGCGTTGTAG, PDGFRA:

GACTTTCGCCAAAGTGGAGGAG/ AGCCACCGTGAGTTCAGAACGC.

Animal experiments

All research involving animals complied with protocols approved by the MIT Committee on Animal Care. The number of cells, the route of injection and the time for tumor growth are indicated in the figure legends. For the experiments concerned with tracking of MSCs to subcutaneous tumors, the locations of tdTomato-labled MSCs and GFP-labeled tumors in vivo were observed using an IVIS Spectrum-bioluminescent and fluorescent imaging system (*Caliper* Life Sciences). For the experiments concerned with tracking of

MSCs to lung metastases, the locations of tdTomato-labled MSCs and GFP-labeled tumors in isolated lung tissue were observed by dissection microscopy. In experiments evaluating tumor initiation and growth, the tumors were isolated and weighed at the end of each experiment. To measure tumor-initiating cell (TIC) frequency, serial dilutions of cancer cell suspensions were injected subcutaneously into nude mice. TIC frequencies of the samples were determined using the ELDA webtool (18).

Statistical analysis

Chi-squared test was used to compared the two groups of the data in Fig. 3G, 4A and Fig. S8. Student's t test (two-tailed) was used to compare the two groups of the data in the rest of the figures. $p<0.05$ was considered significant.

Supplemental Figure Legends

Fig. S1. IL-1 secreting carcinoma cells are able to induce PGE_2 from MSCs. (A) PGE_2 levels, COX2 mRNA levels and mPGES1 mRNA levels from carcinoma cell lines. Data are means ± SD, n = 3. mRNA levels were analyzed by qPCR. (B) PGE_2 is induced by soluble factors secreted from LoVo cells. [LoVo][MSC] cells were cultured in transwells. PGE_2 levels in CM of LoVo cells, MSCs, LoVoMSC coculture and [LoVo][MSC] cultures are shown. Data are means ± SE, n = 3. ***$p < 0.005$, compared to that in LoVo medium. (C) PGE_2, GRO-α, IL-6 and IL-8, but not RANTES, are induced in MSC by soluble factors released from LoVo cells. Levels of these factors were measured in the media of MSC cultures after treatment with LoVoCM, SWCM or DME for 48 h. Data are means ± SE, n = 3. ***$p < 0.005$, compared to that in DME-treated MSC. (D) COX2 and mPGES1 expression is stimulated in MSCs by soluble factors released from LoVo cells. LoVo (L), SW1116 (S) and MSC (M) cultures were treated for 48 h with LoVoCM, SWCM, MSCCM or control DME. COX2, mPGES1 and GAPDH proteins in cell lysates were analyzed by Western blot. (E) IL-1β-positive LoVo SCC CM induces PGE_2 accumulation and the expression of enzymes for PGE_2 production in MSCs. MSCs were treated with DME, IL-1-negative SCC34 CM or IL-1-positive SCC23

CM, After the 48 h incubation, media were collected for PGE_2 assay. Data are means ± SE, n = 3. ***$p < 0.005$, compared to that in MSC+DME (a). COX2, mPGES1 and β-actin in MSC cell lysates were analyzed by Western blot (b). IL-1β and β-actin levels in SCC cell lysates were analyzed by Western blot (c). (F) IL-1α, IL-1β, and IL-1ra mRNA expression levels in carcinoma cells were measured by qPCR, and normalized to GAPDH levels. Data are means ± SE, n = 3. (G) Cocultures of IL-1-secreting carcinoma cells (CC) and MSCs have increased COX2. COX2 and GAPDH levels in CC cultures, MSC cultures, and CC/MSC cocultures were examined by western blotting. CC + MSC lanes are western blots of carcinoma cell and MSC lysates mixed in equal amounts; CCMSC, lysate of carcinoma cell and MSC coculture. (H) IL-1 signaling is required for PGE_2 production in MSCs induced by cancer cell CM. MSCs were treated with cancer cell CM, cancer cell CM with IL-1α+β neutralizing antibodies (1 μg/ml or 5 μg/ml), or with IL-1ra (1000 ng/ml). After 36 h incubation, media were collected and assayed for PGE_2. To determine the 100% (crl) value for CM induced PGE_2 production in MSCs, the basal PGE_2 levels in cancer cell CM were subtracted from the PGE_2 levels in MSCs treated with cancer cell CM. The additional bars show the PGE_2 induction by carcinoma cell CM in the precence of vehicle, IL-1α+β neutralizing antibodies, or IL-1ra. Data are means ± SE, n = 3.

Fig. S2. IL-1 and LoVoCM induce PGE_2, IL-6, IL-8 and GRO-α expression in various mesenchymal cells. (A) PGE_2, GRO-α, IL-6, IL-8 and RANTES are induced in LoVoMSC coculture in different patterns. Levels of these factors in CM of LoVoMSC cocultures were measured at the time points indicated. Levels of the factors are expressed relative to their individual maximal values. Data are means ± SE, n = 3. *$p < 0.05$, ***$p < 0.001$, comparing RANTES to any of the cytokines. (B) COX2, mPGES1, IL-6, IL-8 and GRO-α mRNA levels were measured by qPCR in MSCs treated with IL-1 for 0, 30, 60, 120, and 720 minutes. Maximal mRNA levels are set as 100 %. Data are means ± SE, n = 3. (C) IL-6, IL-8 and GRO-α mRNA levels were measured by qPCR in MSCs treated for 36 h with conditioned media from LoVo cells expressing shRNAs against IL1α+IL1β (LoVoshIL1αβ-1, LoVoshIL1αβ-2), IL1β (LoVoshIL1β-1, LoVoshIL1β-2), or a scrambled sequence (LoVoshsc). The mRNA levels in LoVoshsc CM-treated MSCs are set as 100 %. Data are means ± SE, n = 3 (D) Mesenchymal cells, including human mesenchymal stem cells (MSC), human fibroblast cells (F1 and F2), human intestinal myofibroblast (CCD-18CO) and patient-derived breast MSCs (breast MSC), were treated for 48 hours with DME or LoVoCM. Media were assayed for PGE_2. Data are means ± SE, n = 3. (E) Mesenchymal cells, including human

mesenchymal stem cells (MSC), human fibroblast cells (F1 and F2), human intestinal myofibroblast (CCD-18CO) and patient-derived breast MSCs (breast MSC), were treated for 48 hours with DME, LoVoCM or IL-1α+IL-1β (1 ng/ml). mRNA levels of COX2, mPGES1, PDGH, IL-6, IL-8 and GRO-α were measured by qPCR. Data are means $\pm$ SE, n = 3. (F) PGE_2 potentiates IL-1-induced COX2, IL-6, IL-8 and GRO-α in MSCs. mRNA levels of these factors were measured by qPCR in MSCs treated with vehicle (crl), IL-1α (0.1 ng/ml), IL-1β (0.1 ng/ml), PGE_2 (0.1 μM), IL-1α+PGE_2, IL-1β+PGE_2 for 3 h. Data are means ± SE, n = 3. (G) EP receptor mRNA levels in the cells indicated were measured by qPCR and normalized to the level of GAPDH mRNA. Data are means $\pm$ SE, n = 3.

Fig. S3. MSCs are recruited to tumors in vivo. (A) MSCs migrate to subcutaneous LoVo and HCCC1806 cell tumors, but not to subcutaneous MDA-MB-453 tumors. LoVoGFP or HCC1806GFP cells and MDA-MB-453GFP were implanted in the left and right flanks of SCID mice. After tumors formed, tdtomato-MSCs were injected into the mice via the tail-vein. Top panel: the distributions of carcinoma cells and MSCs were monitored with non-invasive fluorescent imaging in the shaved middle segments of the mouse bodies. Arrows indicate the location of tumors. The strong tdTomato signals on

the unshaved parts of the bodies are autofluorescence from mouse hair. Bottom panel: the distribution of MSCs was monitored by immunofluorescence in tumor sections, using an antibody against tdTomato RFP (in red) and DAPI for cell nuclei (in blue). (B) MSCs associate with lung metastases of LoVo-GFP and HCC1806-GFP carcinoma cells. GFP-carcinoma cells were administered to SCID mice by tail-vein injection. Three weeks later tdTomato-labeled MSCs were administrated via tail-vein injection. Lung tissues were isolated 2 weeks after MSC injection and imaged with a dissection microscope. The tdTomato (red) signal shows the distribution of MSCs; the GFP (green) signal shows the location of tumors.

Fig. S4. LoVo cancer stem cells are ALDH-positive. (A) Expression of ALDH1 in LoVo cell clones. LoVo single cell clones (SCC) were isolated from LoVo cells. ALDH1 and b-actin in cell lysates were analyzed by Western blot. (Left panel). SCC+ and SCC- LoVo cells have distinct morphologies (Right panel). (B) $ALDH^{high}$ LoVo and $ALDH^{low}$ LoVo cells isolated from a LoVo cell culture population by an ALDH activity flow cytometry procedure (panel a) have morphologies similar to LoVo SCC+ and SCC- cells. Pictures were taken 36 h after sorting and initiation of cell culture (panel b). (C) ALDH-positive SCC+ cells form vigorously growing tumors. SSCs were injected into SCID mice (1×10^6

cells/injection with Matrigel). After four weeks, mice were sacrificed and the tumors were isolated and weighed. Data are means ± S.E.

Fig. S5. The frequency of tumor initiating cells is increased in ALDH1-postive HCC1806 SCCs. (A) SCCs were isolated from HCC1806 cells. Both ALDH positive and ALDH negative HCC1806 cells were isolated (left panel). The two SCC types exhibit different morphologies (right panel). (B) ALDH-positive HCC1806 SCCs form vigorously growing tumors. HCC1806 SSCs were injected into SCID mice. After four weeks mice were sacrificed and the tumors were isolated and weighed. Filled circles indicate individual tumor weights; Open circles indicate no tumor grew at the site of injection. Bars indicate means ± SE, $^{**}p < 0.001$, $^{***}p < 0.005$. (C) Co-culture with MSCs increases ALDH1 expression in HCC1806 cells. HCC1806GFP cells were cultured alone or with MSCs. After five days, the GFP-positive carcinoma cells were isolated by sorting, and ALDH1 and β-actin in carcinoma cell lysates were analyzed by Western blot.

Fig. S6. MSCs increase $ALDH^{high}/CD133^{+}$ CSC-enriched LoVo cells. (A) mRNA levels of the CD133 and LGR5 cancer stem cell markers are increased in $ALDH^{high}$ LoVo cells. Levels of ALDH1, LGR5, CD133 and CD44 mRNA were compared by qPCR in $ALDH^{high}$

and ALDH^{low} LoVo cells. Data are means ± SE, n = 3. (B) CD133^{+} cells are enriched in the ALDH^{high} LoVo cell population. ALDH activity and CD133 expression in LoVo cells were analyzed by flow cytometry. The percentages of CD133^{+} cells in ALDH^{high} and ALDH^{low} LoVo cells are plotted. Data are means ± SE, n = 3. (C) LoVo cells were cultured either alone (upper panel) or with dTtomato-MSCs (lower panel). After 5 days, ALDH activity and CD133 expression of LoVo cells was analyzed by flow cytometry. The percentages indicate the percentage of ALDH^{high}/CD133^{+} LoVo cells.

Fig. S7. LoVo cells cultured with tdTomato-MSCs have increased numbers of tumor initiating cells. LoVo cells were cultured either alone, or with tdTomato-MSCs. After 5 days, LoVo cells were isolated by FACS, then injected (5×10^4 or $5x10^3$ cells/injection) injected into mice. After 6 weeks, the tumor numbers were determined (A). The estimated frequency of TICs for each group and the lower and upper values of the range of frequency (B), as estimated by Extreme Limiting Dilution Analysis (ELDA). (C) HCC1806 cells cultured with tdTomato-MSCs have increased numbers of tumor initiating cells. Cells were cultured as in panel C. After isolating HCC1806 cells by sorting, cells (5×10^4 cells/injection) were injected into mice. After 6 weeks, the mice were sacrificed and the tumors were isolated and weighted. Filled circles indicate

individual tumor weights; Open circles indicate no tumor grew at the site of injection. Bars are means ± SE, **$p < 0.01$

Fig. S8. PGE_2-induced tumor initiation in vivo was not further potentiated by IL-6, IL-8, Gro-α and RANTES. LoVo cells treated with vehicle, PGE_2, or PGE_2 plus the cytokines (IL-6, IL-8, Gro-α and RANTES) for 5 days were injected into SCID mice. After 6 weeks, mice were sacrificed and tumors were isolated. The table shows the tumor formation for each group of mice. The tumor-initiating frequency of the LoVo cells with each treatment was calculated with ELDA. The fraction of nonresponding injections vs. the numbers of injected cells is plotted in the graph. The dashed lines indicate the upper and lower ranges of the predicted fraction of nonresponding injections and the solid lines show the averages. The lines of the injection of the PGE_2 plus the cytokines-treated LoVo cells (green lines) mostly overlap with those of PGE_2-treated cells (red lines), which suggests that the cytokines did not significantly further increase the PGE_2-induced tumor initiation.

Fig. S9. LoVoMSC cocultures secrete PGE_2-dependent angiogenic factors. Concentrated CM from LoVo, MSC, and LoVoMSC cultures were mixed with Matrigel

and implanted subcutaneously into nude mice. Some LoVoMSC media were conditioned in the presence of indomethacin or indomethacin + PGE_2. Isolated Matrigel plugs were photographed seven days after implantation (left panel). Hemoglobin in the plugs was isolated and concentrations were measured (right panel). Data, presented as $O.D._{415}$ nm, are means ± SD, n = 3.

Fig. S10. COX2-PGE_2-EP4 signaling is required for the increase of $ALDH^{high}$ LoVo cells induced by MSCs. LoVo cells were cultured with MSCs, PGE_2, NS398 or GW627368X, as indicated in the panel. After 5 days, ALDH activity levels in LoVo cells were analyzed by flow cytometry. The percentages indicate the percentage of $ALDH^{high}$ LoVo cells; i.e., the LoVo cells with ALDH activity beyond the indicated thresholds.

Fig. S11. LoVoCM induces fibroblast and myofibroblast markers in MSCs. MSCs were treated with LoVo conditioned medium or with DME for 2 days and 5 days. Fibroblast marker protein (FSP), the myofibroblast markers (EDA-FN1 and α-SMA), and β-actin were analyzed in cell lysates by Western blot.

Fig. S12. ALDH^{high} CSCs are located near COX2-expressing regions in human colorectal cancer tumors. (A) Immunofluorescence analyses were performed on human colorectal adenocarcinoma sections using antibodies against ALDH1 (in green) and COX2 (in red). Merge1 and Merge2 are from the same sections, shown in different magnification. cell nuclei were stained with DAPI (in blue). Scale bar = 100 μm. (B) images shown in panel B were taken at higher magnification. Scale bar = 16 μm. The arrows indicate carcinoma cells, which have enlarged nuclei. The arrowheads indicate stromal cells.

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