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Suppression of invasion and metastasis in aggressive salivary cancer cells through targeted inhibition of *ID1* gene expression

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Abstract

Salivary gland cancer (SGC) represents the most common malignancy in the head and neck region, and often metastasizes to the lungs. The helix–loop–helix ID1 protein has been shown to control metastatic progression in many types of cancers. Using two different approaches to target the expression of *ID1* (genetic knockdown and progesterone receptor introduction combined with progesterone treatment), we previously determined that the aggressiveness of salivary gland tumor ACCM cells in culture was suppressed. Here, using the same approaches to target *ID1* expression, we investigated the ability of ACCM cells to generate lung metastatic foci in nude mice. Moreover, since both approaches would be challenging for applications in humans, we added a third approach, i.e., treatment of mice with a non-toxic cannabinoid compound known to down-regulate *ID1* gene expression. All approaches aimed at targeting the pro-metastatic *ID1* gene led to a significant reduction in the formation of lung metastatic foci. Therefore, targeting a key transcriptional regulator using different means results in the same reduction of the metastatic spread of SGC cells in animal models, suggesting a novel approach for the treatment of patients with aggressive SGC.

Keywords

Progesterone; Proliferation; Migration; Helix; loop; helix; *In vivo* experiments

Introduction

The spread of aggressive cancer is a multistep process that involves many genetic and epigenetic alterations, and is a devastating and ultimately fatal condition. A majority of available therapeutic strategies for cancer patients are nonspecific, have significant associated toxicities, and do not produce durable responses [1]. A critical barrier in the

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treatment of aggressive cancer is that few drugs have been developed to specifically target invasion and metastasis. The detailed molecular mechanisms controlling tumor progression and metastasis are still not well understood. Identifying these alterations is essential to develop more effective methods of diagnosis and treatment. Salivary gland cancers (SGCs) represent the most common malignancy in the head and neck region, and often metastasize to the lungs at an early stage of the disease [2,3].

It has previously been determined that ID1, a helix–loop–helix protein that acts as an inhibitor of basic helix–loop–helix transcription factors [4,5], was expressed at a high level in aggressive cancer cells (including SGCs) and human biopsies [6–8]. Importantly, *ID1* was identified as a key gene in a non-biased *in vivo* selection, transcriptomic analysis and functional verification validation of a set of human genes that mark and mediate cancer metastasis to the lungs [9]. Moreover, functional studies have demonstrated that ID1 correlated with abnormalities and proliferation/survival in cancer cells, as well as was required for tumor initiating functions during metastatic colonization of the lung microenvironment [10–12].

We previously transfected progesterone receptor (PR) isoforms into the ACCM metastatic SGC cell line, then observed how progesterone (Pg) treatment impacted their migration and invasion through the down-regulation of *ID1* gene expression [13]. Metastatic SGC cells transfected with PR exhibited lower levels of migration and invasion after Pg treatment than those treated only with an ethanol control. Our findings indicated that Pg could interact with PR to suppress invasion in SGC and suggested that Pg may be a viable treatment for human patients with salivary gland carcinomas. We also used a second approach in cells in culture where we utilized genetic knockdown to inhibit *ID1* gene expression [14]. We determined that proliferation, migration as well as invasion were significantly reduced upon *ID1* knockdown, indicating that ID1 was indeed a key regulator on SGC cell aggressiveness in culture.

In order to determine whether targeting the pro-metastatic gene, *ID1*, would represent a promising option for patients with SGC, it was important to investigate the consequences of such a targeting using relevant preclinical animal models. We therefore used the ACCM cells obtained from the two approaches described above, and we added a third approach using a non-toxic small molecule inhibitor of *ID1* expression to determine the effects of the modulation of *ID1* gene expression on the generation of lung metastatic foci in nude mice. Indeed, we recently described in breast cancer and in glioblastoma [15–17] that tumor aggressiveness was inhibited upon treatment with the non-psychoactive cannabinoid compound, cannabidiol (CBD).

We therefore investigated the effectiveness of CBD on SGC cell aggressiveness *in vivo* and compared it with the other approaches. All three different approaches aimed at targeting the pro-metastatic *ID1* gene led to a significant reduction in the number of metastatic foci in the lungs suggesting a novel approach for the treatment of patients with aggressive SGC that expresses high levels of ID1.

Materials and methods

Cell cultures

Human ACCM (high metastatic potential) and ACC2 (low metastatic potential) cells derived from an adenoid cystic carcinoma of the salivary gland [18] were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China). They were cultured in phenol red-containing RPMI-1640 (from the University of California, San Francisco, CA) supplemented with 10% charcoal-stripped fetal bovine serum (FBS) and insulin (5 μg/ml; Sigma Chemical Co., St. Louis, MO) at 37 °C in the presence of 5% CO_2. ACCM cells were infected with control shRNA (Ctl shRNA) or with shRNA against *ID1* (*ID1* shRNA) purchased as lentiviral particles (sc-29356-V; Santa Cruz Biotechnology), selected with puromycin and pooled.

Chemicals

Progesterone (1000-fold stock, Sigma Chemical Co, St. Louis, MO) was added to ethanol to yield a final ethanol concentration of 0.1%. For each of the following assays, treatment cells were dosed once or twice daily with the progesterone–ethanol mixture; control cells received 0.1% ethanol only. CBD was obtained from National Institutes of Health through the National Institute of Drug Abuse. For the *in vivo* experiments, ethanol stocks of CBD were dissolved in a solution containing 2% ethanol, 2% Tween 80, and 96% saline.

Introduction of PR into the ACCM cells

Pooled cell populations of PR-expressing cells were prepared as previously described [13]. Briefly, a pSG5 plasmid was used to introduce vectors hPR1 and hPR2, which contained human PR cDNA coding for isoforms PR-B and PR-A, respectively. We selected three populations of ACCM cells (ACCM-PR1, -PR2, and -PR3) that expressed both PR-A and PR-B. The first of these cell populations was used for further experiments. As controls, we used T47D cells, which express the human PR, and MDA-MB231 cells, which express none.

Western blotting analysis

Cells were lysed in 2× Laemmli buffer and stored at −70 °C. A DC protein assay kit (Bio-Rad, Hercules, CA) was used to determine protein concentrations. Proteins (30 μg each) were separated by 10% SDS–PAGE and transferred onto membranes. Membranes were blocked for 1 hr at room temperature with TBST containing 10% nonfat milk, then incubated with the primary antibodies for 1 hr (PR-A or PR-B (C-20; Santa Cruz Biotechnology); actin (C4; Chemicon International); ID1 (BCH-1/ 195-14; Biocheck); vimentin (Ab-2; Thermo Scientific); VEGF (A-20; Santa Cruz Biotechnology)). Membranes were washed and incubated with secondary antibody (goat anti-rabbit or anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology)), then washed again and developed for enhanced chemiluminescence using the Amersham ECL or ECL-plus kit.

MTT assay

To quantify cell proliferation, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT) assay was used (Chemicon, Temecula, CA). Cells were seeded in 96-well

plates at 3×10^3 cells/well for the 7-day experiments and 9×10^3 cells per cm^2 for the 3-day experiments to obtain optimal cell density. Upon completion of the drug treatments, cells were incubated at 37 °C with MTT for 4 hr, and then isopropanol with 0.04 N HCl was added and the absorbance was read after 1 hr in a plate reader with a test wavelength of 570 nm. The absorbance of the media alone at 570 nm was subtracted, and % control was calculated as the absorbance of the treated cells/control cells × 100.

Scratch assay

Cells were seeded in 6-well plates at 1×10^5 cells per well. On the next day, a pipette tip was used to generate a scratch wound. Cultures were rinsed with media to remove detached cells, after which they were treated for 48 hr with medium containing 10% serum with 10 nM Pg. Photographic images were taken from each well immediately after scratching and again after 12, 24, and 36 hr. NIH ImageJ software [19] (National Institutes of Health, Bethesda, MD) was used to measure the remaining area not covered by migrated cells at each of these 3 time points. The experiment was performed in triplicate.

Matrigel invasion assay

Invasion assays were performed in modified Boyden chambers with 8-μm-pore filter inserts for 24-well plates (Becton, Dickson and Company, Franklin Lakes, NJ). Filters were coated with 12 μl of ice-cold Matrigel. Cells (5×10^4 per well) were pre-treated with ethanol or CBD for 48 hr, then added to the upper chamber. The lower chamber was filled with 300 μl of NIH-3T3 cell-conditioned medium. After a 16-hr incubation, cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy. Assays were performed in triplicate, and the results were averaged.

Zymography analysis

Proliferating ACCM cells (1×10^6 in 100-mm-diameter dishes) were shifted to serum-free medium for 2 days, after which they were given 8 ml of fresh serum-free medium. Forty-eight hours later, after twice-daily ethanol or progesterone treatment, the conditioned medium was collected and concentrated 10- to 15fold. The concentrated medium was analyzed on gelatin substrate gels, as we previously described [20]. Gelatinase activities were visible as clear bands, indicative of proteolysis of the substrate protein. Polyacrylamide gel electrophoresis was performed on an identical quantity of the conditioned medium; this served as a control for the protein loading.

In vivo experiments

For all the experiments, 6–8 week old female athymic *nu/nu* mice were used. Ten mice per group were used. Mice were cared for as previously described [15] and studies were carried out in accordance with the National Institutes of Health guidelines involving experimental neoplasia and our approved IACUC protocol.

For the experiments investigating the effects of *ID1* knockdown, lung metastases were generated in mice after i.v. injection of 5×10^5 human ACCM cells infected with control shRNA (Ctl shRNA) or with shRNA against *ID1* (*ID1* shRNA). The number of total lung

metastatic foci was compared between groups. All animals were sacrificed 8 weeks after inoculation and lungs harvested.

For the experiments investigating the effects of Pg treatment, transfected ACCM cells (at 50,000 cells in 200 μl of culture medium) were injected into the lateral flanks of ovariectomized mice. Treatments were performed on several groups of mice: two injected with ACCM-CTL cells and two given ACCM-PRCL1 cells. On day 3, mice were injected with a pellet containing either slow-release Pg compound or a placebo. All animals were euthanized 12 weeks after inoculation and lungs harvested.

For the experiments investigating the effects of CBD on ACCM cells, mice were injected i.v. through tail vein with 200 μl of RPMI containing 0.25×10^5 cells. Two days after the injection, the tumor bearing mice were injected i.p. once a day, for 5 days, with vehicle (control) or CBD for approximately 8 weeks, at which time the mice were euthanized and the lungs were harvested.

Statistical analyses

Differences between treatment and control groups were investigated using Student's *t*-tests or Mann–Whitney U-tests. Significance was defined as $p < 0.05$. All statistical tests were performed using Statcel2 software (Statcel2, OMS, Tokyo).

Results

Effect of *ID1* downregulation using genetic knockdown on SGCs in culture and *in vivo*

As presented in Fig. 1A, we confirmed the decrease in *ID1* gene expression in ACCM cells infected with lentiviral particles containing *ID1* shRNAs that we previously used to knock down *ID1* in breast cancer cells [16]. The levels of proliferation and invasion in these cells (shRNA against *ID1* (*ID1* shRNA)) were significantly reduced upon *ID1* knockdown compared to control cells (Ctl shRNA) (Fig. 1B), as well as the levels of MMP-9 expression determined using zymography analysis (Fig. 1C). Lung metastases were generated in mice after injection of these human ACCM cells infected with control Ctl shRNA or with *ID1* shRNA. We found a significant decrease in the number of total lung metastatic foci in the *ID1* shRNA group compared to the Ctl shRNA group (Fig. 1D and E), suggesting that ID1 not only controls the aggressive phenotype of SGCs in culture but also in a mouse model of metastasis. Besides the decrease in MMP-9 expression associated with *ID1* knockdown, we also determined that vimentin and VEGF expressions were down-regulated in these cells, which may further explain the reduction in their aggressive phe-notype (Fig. 1A).

Effect of the re-establishment of PR on the phenotype of SGCs

As shown in Fig. 2A (upper panel), ACCM cells (ACCM-Ctl) do not express form A and/or form B of the progesterone receptor (PR). This is also true for the negative control, the MDA-MB231 breast cancer cells, whereas the positive controls, T47D breast cancer cells, express both subtypes of PR. However, upon transfection with the two forms of the receptor, we isolated three populations of ACCM cells (ACCM-PR1, -PR2, and -PR3) that expressed

both PR-A and PR-B. The first of these cell populations that showed a pattern of isoform expression similar to the positive control (T47D cells) was used for further experiments.

We determined that, upon treatment with Pg, *ID1* gene expression (measured by Western blotting) (Fig. 2A (lower panel)) and MMP-9 protein expression (measured by zymography) (Fig. 2B) were down-regulated in ACCM-PR cells. Pipette scratching produced similar-sized wounds in both ACCM-PR and control cell cultures (Fig. 2C) at 0 hr. At 12, 24, and 36 hr after wound infliction, a greater percentage of the scratched area was filled in cultures of control cells (ACCM-Ctl) than in those transfected with human PR (ACCM-PR) (all $p < 0.001$). This suggests that PR expression significantly reduces migration activity upon treatment with Pg by reducing the expression of ID1 and MMP-9. Further, lung tumor metastasis (as measured by the total number of tumor nodules in the lung) was significantly lower in mice receiving the ACCM-PR + Pg treatment than in any of the other treatment groups ($p < 0.01$) (Fig. 2D). Together, these results indicate that circulating Pg prevents the spread of PR-positive tumor cells and that this effect occurs in conjunction with the down-regulation of the pro-metastatic gene, *ID1*.

Effect of *ID1* inhibitor on the aggressiveness of SGCs in culture and *in vivo*

We found that non-toxic cannabinoid compound, cannabidiol (CBD), was able to significantly down-regulate *ID1* gene expression in SGC cells (Fig. 3A). This is in agreement with our previous studies in breast cancer and glioblastoma [15,17,21]. ACCM cells that have high metastatic potential were more sensitive to inhibition of *ID1* by CBD in comparison to the ACC2, a line with low metastatic potential. We therefore used ACCM cells to determine the effect of CBD on cell aggressiveness using proliferation as well as invasion assays. As shown in Fig. 3B (upper panel: proliferation; lower panel: invasion), CBD was able to decrease both proliferation and invasion of ACCM cells as expected for cells that underwent a major decrease in *ID1* expression. We next determined whether CBD would inhibit metastatic progression of ACCM cells *in vivo*. Mice were injected i.v. with ACCM cells and, two days later, they were then injected i.p. once a day, for 5 days, with vehicle (control) or two doses of CBD until the completion of the study, at which time the mice were euthanized and the lungs were harvested (Fig. 3C). As shown in Fig. 3D, CBD produced a significant ($p < 0.05$) dose-dependent reduction in the number of metastatic foci in the lungs.

Discussion

In a series of experiments in culture and *in vivo*, we determined that a significant decrease in the expression of the pro-metastatic gene, *ID1*, was associated with a down-regulation of proliferation, migration and/or invasion of aggressive salivary cancer cells, as well as with their metastatic spread. Cumulatively, these results highlight the importance of a transcriptional regulator in the aggressiveness and spread of SGC tumors, and suggest that *ID1* expression could be a valuable prognostic tool and therapeutic target for patients with salivary gland cancers.

The path of cancer progression is determined by alterations in the regulatory mechanisms of proliferation and migration/invasion. The expression of ID1 protein (an inhibitor of basic

helix–loop–helix transcription factors) has been reported to be dysregulated in over twenty types of cancer, and suggested as a key determinant of tumorigenesis and/or metastasis in a wide range of tissues [21–23]. In ACCM cells, *ID1* knockdown led to a strong reduction of cell invasion and to a significant decrease in cell proliferation. We also observed a significant reduction in expression of markers associated with EMT (vimentin), angiogenesis (VEGF) and invasion (MMP-9). Importantly, we determined that the individual targeting of *ID1* gene expression in SGC cells also led to a down-regulation of lung metastatic foci after injection in nude mice, suggesting that *ID1* expression represents a key event during tumor progression in SGC.

Because there are few available human salivary gland tumor cell lines, and therefore no appropriate model in which to study the Pg-PR system, we used PR-transfected metastatic ACCM cells. We previously reported that aggressiveness was reduced in PR-deficient human aggressive breast cancer cells after PR was reintroduced and treated with Pg [20]. Further, we found that Pg could act as an inhibitor of SGC cell proliferation [13]. In these two studies, we reported that progesterone-induced growth inhibition was accompanied by both up-regulation of p21, which generally plays a critical role in regulating cell growth inhibition [13]. Here, we report that Pg also inhibited PR-transfected ACCM cell migration and this effect was accompanied by a downregulation of ID1 and MMP-9, a protein active in the degradation of the basement membrane and extracellular matrix during cancer progression. Importantly, the Pg-PR system had a significant influence on SGC cells *in vivo*, as demonstrated by the inhibition of tumor metastasis.

Our previous data showed that cannabidiol (CBD) represented a non-toxic plant derived compound that, at therapeutic doses, was able to reduce *ID1* expression and corresponding breast cancer metastasis and glioblastoma invasion, while not affecting the viability of normal cells [15,17,24]. Here, we establish ID1 as a key regulator of both invasion and proliferation in SGC cells, and demonstrate that the non-toxic cannabinoid, CBD, down-regulates *ID1* expression and tumor aggressiveness in culture and *in vivo*. A greater understanding of this phenomenon may lead to more effective therapies for cancer patients including the additional refinement of cannabinoid analogs targeting *ID1*. We expect our efforts to ultimately translate to the development of future clinical trials with non-toxic compounds that target the expression of *ID1*, a master regulator of SGC aggressiveness. With its lack of systemic toxicity and psychoactivity [24], CBD is an ideal candidate agent in this regard and may prove useful in combination with front-line agents for the treatment of patients with aggressive SGC tumors. While we demonstrate CBD is able to directly target SGC cell aggressiveness, we cannot rule out the possibility that CBD may in part produce antimetastatic activity through modulation of the tumor microenvironment.

Besides surgical resection and radiation of SGCs, there are no other effective therapies. Since adverse effects caused by chemotherapy often threaten the life of a patient, the adoption of adjuvant therapy should be carefully considered. Reducing *ID1* expression (a gene whose expression is absent in healthy adult tissues) could therefore provide a rational therapeutic strategy for the treatment of aggressive SGCs.

Ethics

The protocol of the animal studies has been approved by the Committee on the Ethics of Animal Experiments of Ehime University and by the IACUC at California Pacific Medical Center Research Institute, and all efforts were made to minimize suffering.

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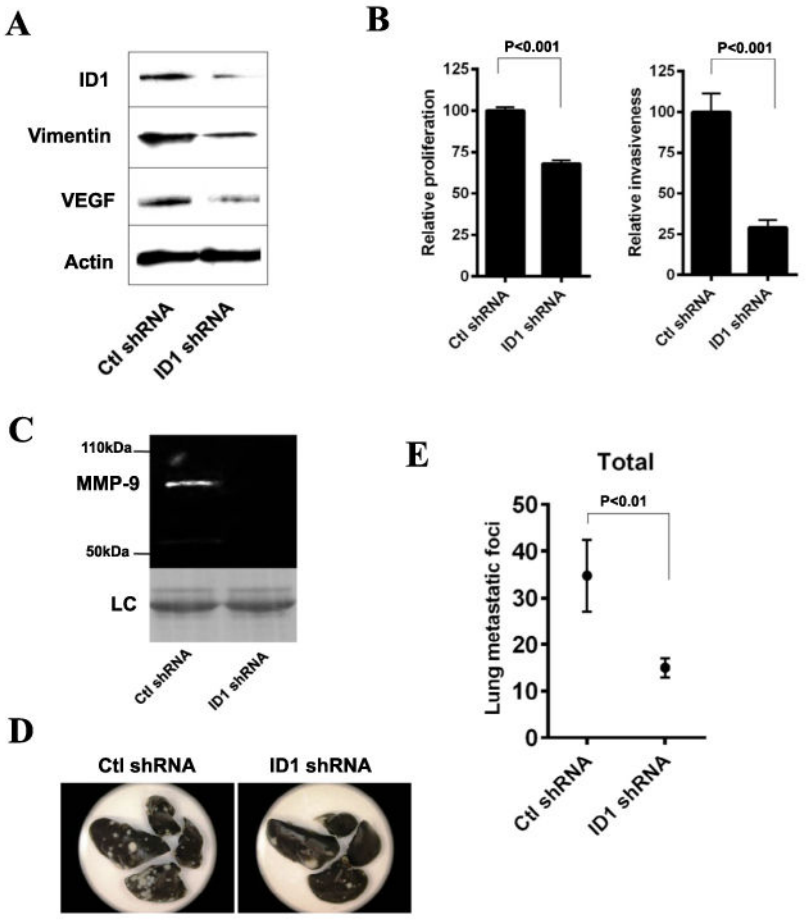

Fig. 1.

Effect of *ID1* downregulation using genetic knockdown on SGCs in culture and *in vivo*. (A) Comparison of ID1, vimentin and VEGF expression in ACCM cells infected with Ctl shRNA or *ID1* shRNA, and using Western analysis. Actin was used as a control for protein integrity and quantification. (B) Cell proliferation (left panel) and cell invasion (right panel) were investigated in ACCM cells infected with Ctl shRNA or *ID1* shRNA. (C) Gelatin zymogram analysis showing MMP-9 activity in ACCM cells infected with Ctl shRNA or *ID1* shRNA. (D) The pictures are representative of lung tumor formation after injection of ACCM cells infected with Ctl shRNA or *ID1* shRNA. (E) The number of lung metastatic foci was compared between ACCM cells infected with Ctl shRNA or *ID1* shRNA.

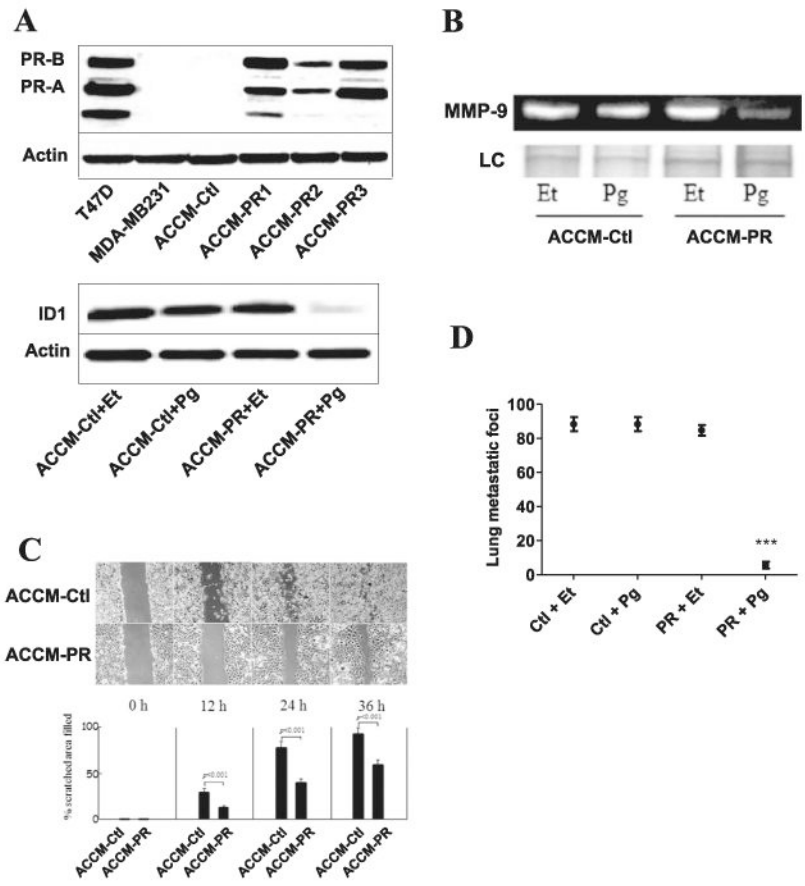

Fig. 2.

Effect of the re-establishment of PR on the phenotype of SGCs. (A) Expression of PR isoforms (upper panel) and ID1 protein (lower panel) in T47D, control MDA-MB231, and PR-transfected ACCM cell populations using Western blotting. Actin was used as a control for protein integrity and quantification. (B) Gelatin zymogram analysis showing MMP-9 activity 48 hr after treatment with Pg. (C) Results of the scratch assays on cultures of progesterone-treated ACCM-Ctl and ACCM-PR cells. NIH ImageJ was used to measure (at 0, 12, 24 or 36 hr) the area of the scratch remaining after cell migration. (D) Number of lung metastatic foci in mice injected with ACCM-Ctl or ACCM-PR cells, and treated or not with Pg.

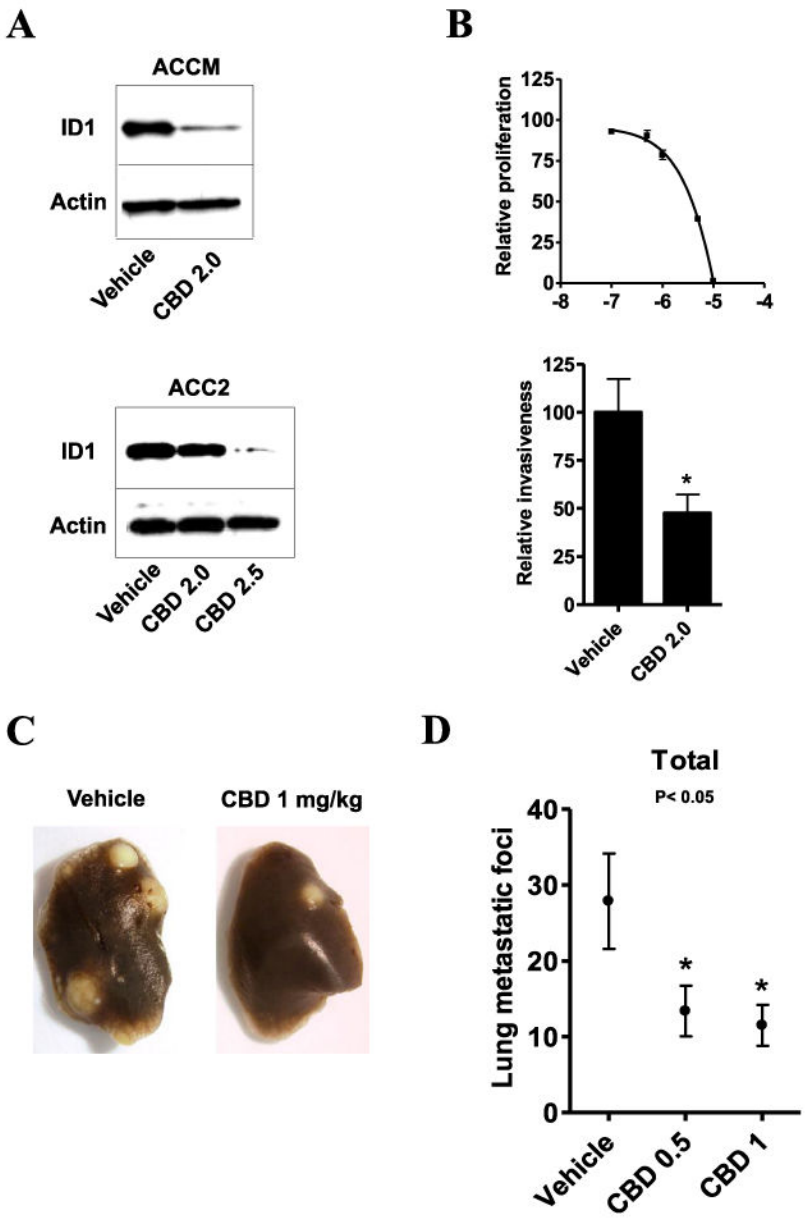

Fig. 3.

Effect of an *ID1* inhibitor on the aggressiveness of SGCs in culture and *in vivo*. (A) Expression of ID1 protein in ACCM cells (upper panel) or ACC2 cells (lower panel) treated with vehicle (control) or CBD. Actin was used as a control for protein integrity and quantification. (B) Cell proliferation (upper panel) and cell invasion (lower panel) were investigated in ACCM cells treated with various concentrations of CBD (upper panel) or with vehicle (control) or 2 μM CBD (lower panel). (C) The pictures are representative of lung tumor formation after injection of ACCM cells and treated with vehicle (control) or 1 mg/kg CBD. (D) The number of lung metastatic foci was compared after injection of ACCM cells and treatment with vehicle (control), 0.5 mg/kg CBD or 1 mg/kg CBD.