Published in final edited form as:

Cancer Prev Res (Phila). 2012 January ; 5(1): 51–60. doi:10.1158/1940-6207.CAPR-11-0256.

Stem Cell Antigen-1 Deficiency Enhances the Chemopreventive Effect of Peroxisome Proliferator-Activated Receptor Activation

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Abstract

Stem cell antigen-1 (Sca-1, Ly6A) is a glycerophosphatidylinositol (GPI)-anchored protein that was identified as a murine marker of bone marrow stem cells. Although Sca-1 is widely used to enrich for stem and progenitor cells in various tissues, little is known about its function and associated signaling pathways in normal and malignant cells. Here, we report that the absence of Sca-1 in the mammary gland resulted in higher levels of PPAR γ and PTEN, and a reduction of pSer84PPAR γ , pERK1/2 and PPAR δ . This phenotype correlated with markedly increased sensitivity of Sca-1 null mice to PPAR γ agonist GW7845 and insensitivity to PPAR δ agonist GW501516. Reduction of Sca-1 expression in mammary tumor cells by RNA interference resulted in a phenotype similar to the Sca-1 deficient mammary gland, as evidenced by increased PPAR γ expression and transcriptional activity, resulting in part from a lesser susceptibility to proteasomal degradation. These data implicate Sca-1 as a negative regulator of the tumor suppressor effects of PPAR γ .

Introduction

Stem cell antigen-1 (Sca-1, Ly6A) is a glycerophosphatidylinositol (GPI)-anchored protein that was first identified as a murine marker of bone marrow stem cells (1, 2), and has been used subsequently to enrich for stem and progenitor cells from a variety of tissues (3–10). In the mammary gland, Sca-1-expressing epithelial cells have the capacity to reconstitute the mammary gland in the cleared fat pad (5, 11) and to generate alveolar and ductal structures (12). These findings are consistent with enhanced fluorescence in the alveolar end buds (13) in heterozygous Sca-1^{+EGFP} (14), where the stem cell niche is believed to reside (15, 16). Even though, these studies suggest that Sca-1-expressing cells behave like stem and progenitor cells, it is unclear whether Sca-1 is a *bona fide* stem cell marker or serves as a growth modifier through its ability to prime multipotent cell populations (17, 18). In support of the latter possibility, Sca-1 expression is increased in mammary tumors arising in MMTV-Wnt1 (19) and MMTV-erbB2 (20) transgenic mice, and serves as a tumor initiating factor in murine mammary tumor cells by suppressing TGF- β signaling (21).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that regulate a plethora of signaling pathways controlling fatty acid β -oxidation, glucose

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest

utilization, cholesterol transport, adipocyte differentiation, cell proliferation and survival (22–28). In the context of mammary tumorigenesis, PPAR γ agonists inhibit carcinogen-induced preneoplastic mammary lesions (29) and delay mammary tumor development (30–32), while the opposite phenotype results from PPAR γ haploinsufficiency (33), expression of dominant-negative Pax8PPAR γ (34) and by treatment with PPAR δ agonists (35, 36). PPAR δ is also an etiologic factor in gastric carcinogenesis (37) and colon tumorigenesis (38, 39), although interpretation of the role of PPAR δ in latter process is dependent on the derivation of the knockout model (40).

The objectives of the present study were to determine if Sca-1 deficiency affected mammary gland development and tumorigenesis in response to the potent and highly selective PPAR γ agonist GW7845 (35). Here, we report that Sca-1 deficiency reduced the proliferation of mammary epithelium and markedly enhanced the chemopreventive effects of GW7845. This outcome correlated with increased PPAR γ and PTEN expression, and a reduction in the ratio of pSer84PPAR γ /PPAR γ , as well as the levels of PPAR δ and pERK1/2 in the mammary gland and tumors of Sca-1 deficient animals. Reduction of Sca-1 in mammary tumor cells by RNA interference produced a similar PPAR γ phenotype, and further demonstrated reduced proteasomal turnover and increased the transcriptional activity of PPAR γ . These results suggest that Sca-1 attenuates the tumor suppressor activity of PPAR γ through inhibition of post-translational processes that modulate its activity.

Materials and Methods

Mice

Sca-1 null (KO) mice were bred from heterozygous Sca-1 mice kindly provided by Dr. William L. Stanford, University of Toronto (41). Mice were screened by PCR, and Sca-1 deficiency in the mammary gland was confirmed by FACS and western blotting.

Mammary carcinogenesis

Five week-old wild-type (WT) C57BL/6 mice and Sca-1 KO mice of the same background were treated with medroxyprogesterone (MP) and DMBA as previously described (30, 35). Briefly, mice were injected s.c. with 15 mg MP suspension (Depo-ProveraTM), followed by four weekly doses of 1 mg DMBA/0.1 ml cottonseed oil administered by gavage (8, 21). All animals were fed diets of Purina rodent chow 5001 supplemented with either 0.005% (w/w) GW7845 (provided by Dr. Brad Henke, GlaxoSmithKline) or 0.005% GW501516 (provided by the Chemoprevention Branch, NCI) beginning one day after the last dose of DMBA.

Antibodies

The source, use and dilutions of antibodies were the following: FITC-mouse anti-Sca-1 (553335, BD Biosciences, FACS, 1:200), rat anti-Sca-1 (553333, BD Biosciences, western, 1:1,000), mouse anti-PPAR γ (sc-7273, Santa Cruz Biotechnology, western, 1:300), mouse anti-pSer84PPAR γ (sc-28001-R, Santa Cruz Biotechnology, western, 1:1,000), rabbit anti-PPAR δ (48–6600, Zymed Laboratories, western, 1:1,000), mouse anti-PTEN (7974, Santa Cruz Biotechnology, western, 1:1,000), pERK1/2 (4376S, Cell Signaling, western, 1:1,000), ERK1/2 (9102, Cell Signaling, western, 1:1,000), anti-pRB (9308, Cell Signaling, IHC, 1:100), anti-Ki-67 (M7249, Dako, IHC, 1:50) and mouse anti- β -actin (A-5441, Sigma-Aldrich, western, 1:5,000).

Cell culture

Cell line 34T was generated from a mammary adenocarcinoma induced by MP/DMBA in heterozygous Sca-1^{+/EGFP} mice (34). Briefly, tissue was excised and minced into 1 mm pieces under sterile conditions, and digested in DMEM/F12 medium supplemented with 5%

FBS, 100 U/ml collagenase I, 100 U/ml hyaluronidase, 100 U/ml penicillin, 100 U/ml streptomycin, 10 µg/ml gentamicin and 2.5 µg/ml amphotericin B. Cell lines were maintained in DMEM medium supplemented with 5% FBS (34). 34T cells and 34T cells in which Sca-1 was reduced by transduction with a lentivirus-expressed a Sca-1 shRNA (D8 cells) were generated as previously described (21).

Western blotting

Western blotting was carried out as previously described (34). Briefly, either the cell pellet at 4°C or tissue was frozen in liquid nitrogen and pulverized in a mortar and pestle, were mixed with lysis buffer containing: 0.1% SDS, 0.5% NP-40, phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, and protease inhibitor cocktail (Roche Diagnostics). Following incubation on ice for 30 min, lysates were cleared by centrifugation for 15 min at 13,000 × g at 4°C. Protein concentrations were determined by the Coomassie Plus Protein Assay (Pierce), and 50 µg of lysate was separated in a 4–12% NuPAGE Bis-Tris gel (Invitrogen). After wet transfer, membranes were blocked for 1 hr at room temperature in 5% non-fat dry milk in TBS (pH 7.4) containing 0.1% Tween 20. Primary antibody was incubated overnight at 4°C. Secondary antibody was incubated for 1 hr at room temperature, and proteins were visualized with either SuperSignal West Pico or SuperSignal West Dura (Pierce).

Immunohistochemistry

IHC analysis was carried out as previously described (30, 34, 35).

FACS

Single cell suspensions of primary mammary epithelial cell cultures were prepared from WT and Sca-1 KO mice as previously described (34). Briefly, cells were detached from the flask with 0.05% trypsin, filtered through a 45 µm cell strainer, washed twice with 1X PBS and blocked for 15 min on ice with 1X PBS supplemented with 3% FBS. Cells were washed twice with 1X PBS, fixed with 1% paraformaldehyde in PBS and stored in the dark at 4°C until sorting. Cells (5×10⁶/ml) were incubated for 1 hr at room temperature with a 1:400 dilution of either FITC-Sca-1 (clone E13-161.7, BD Biosciences) or a conjugated IgG of the same isotype as a negative control. Sorting was performed on a FACS Aria flow cytometer (Becton Dickinson) and analyzed by FACS Express Denovo software by the Flow Cytometry/Cell Sorting Shared Resource, LCCC.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. One µg of RNA was reverse transcribed in a total volume of 20 µl using the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). PCR was performed in triplicate in an ABI 7900 instrument (Applied Biosystems, Foster City, CA) using SYBRGreen detection (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. qRT-PCR primers were designed using the Integrated DNA Technologies primer design tool (42). Efficiencies of all primer sets were validated using a standard curve of five serial cDNA dilutions in water in duplicate. Primers were acceptable if the deviation from the slope of the standard curve was <0.3, and if the melting curve showed only one product. The expression of each target gene was normalized to the expression of GAPDH, and the relative quantification method was applied using SDS2.3 software (Applied Biosystems, Foster City, CA). Primers were the following:

PTEN: forward, TGTAAGCTGGAAAGGGACG, reverse,
CCTCTGACTGGGAATTGTGAC

FABP3: forward, TCATCCATGTGCAGAAGTGG, reverse,
CTTCTCATAAGTCCGAGTGCTC

Gene microarray analysis

Total RNA was extracted from tumors of wild-type and Sca-1 KO mice using an RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. cRNA synthesis was carried out using the Affymetrix (Santa Clara, CA) protocol with minor modifications as described (30). Biotin-labeled cRNA was fragmented for 35 min at 94°C and hybridized overnight to an Affymetrix mouse 430A 2.0 GeneChip® representing approximately 14,000 annotated mouse genes by the Macromolecular Analysis Shared Resource, Lombardi Comprehensive Cancer Center, Georgetown University. Hybridization signals were detected with an Agilent Gene Array scanner, and grid alignment and raw data generation performed with Affymetrix GeneChip® Operating software 1.1. Gene expression data with a signal ≥ 300 and with ≥ 2.5 -fold change are listed in Table S1. Gene ontology analysis categorizing sets of four or more genes at $P < 0.05$ was performed with Pathway Studio 7.1 (Aridane Genomics, Inc., Rockville, MD) (Table S2). Array data have been deposited in the GEO database under accession no. GSE31954.

Statistical analysis

The significance ($P < 0.05$) of data presented as the mean $\pm$ SEM was determined using a two-tailed Student's *t*-test. Differences between groups of two or more variables were determined by the two-tailed Fisher's Exact test, and survival curves were analyzed by Pearson's log rank test.

Results

The Sca-1 deficient mammary gland

Sca-1 KO mice were bred from heterozygous mice and confirmed to have loss of Sca-1 by FACS analysis (Fig. 1A). The effect of Sca-1 deficiency on mammary gland development was initially measured in whole mounts. Sca-1 KO mice exhibited a reduction in ductal invasion of the fat pad at seven weeks of age (*Ctl*, Fig. 1B), and responded less vigorously to MP treatment in comparison to WT mice (*MP*, Fig. 1B). The phenotype of the Sca-1 deficient gland was further assessed by determining expression of the anti-proliferative marker p21^{Cip1} and proliferative marker phosphoRB (pRB). Sca-1 KO mice expressed increased p21^{Cip1} (Fig. 1C) and reduced pRB (Fig. 1C), suggesting that the ductal epithelium was less poised to respond to a stimulus. This was confirmed, in part, by the reduced expression of the proliferative marker Ki-67 in Sca-1 KO mice in response to MP treatment (Fig. 1D), although there was no significant difference in Ki-67 between untreated WT and Sca-KO mice. This suggests that p21^{Cip1} and pRB are sensitive predictors of the proliferative potential of the tissue.

Sca-1 deficiency enhances PPAR γ expression in the mammary gland

To further characterize the mammary gland of Sca-1KO mice, tissue lysates were probed by western blotting for PPAR γ , pSer84PPAR γ , PPAR δ , PTEN, pERK1/2 and ERK1/2 (Fig. 2A). Sca-1 deficient tissue expressed a three-fold higher level of PPAR γ than WT tissue (Fig. 2B), as well as a reduction in the ratio of pSer84PPAR γ /PPAR γ (Fig. 2C). These changes were accompanied by a marked reduction in PPAR δ (Fig. 2D), a 50% reduction in the percentage of pERK1/2/ERK (Fig. 2E), and an increase in PTEN expression (Fig. 2A). These results are consistent with reduced proteasome-dependent turnover of hypophosphorylated Ser84PPAR γ (43).

Sca-1 KO mice are sensitized to the chemopreventive effects of PPAR γ agonist GW7845

Since Sca-1 KO mice expressed a significantly higher ratio of PPAR γ /PPAR δ , mammary carcinogenesis (30) was evaluated in animals administered diets supplemented with either PPAR γ agonist GW7845 or PPAR δ agonist GW501516 beginning one day after administration of the last dose of DMBA (35) (Fig. 3). Tumorigenesis developed more slowly in Sca-1 KO mice than in WT mice, and KO mice treated with GW7845 exhibited increased survival and an 80% reduction in tumor formation in comparison to WT mice (Fig. 3A, B). There was no significant delay in tumor formation in WT mice treated with GW7845 (Fig. 3A, B). In contrast, GW501516 produced an increased rate of tumorigenesis in WT mice, whereas, Sca-1 KO mice were unaffected by this treatment (Fig. 3C, D). Sca-1 KO mice tended to present with a higher percentage of adenocarcinomas and a lower percentage of squamous cell carcinomas than WT mice (30), although it did not reach statistical significance (Table 1). Analysis of the adenocarcinomas from WT and KO mice (Fig. 4A) generally phenocopied the changes found in the mammary gland (Fig. 2A), as shown by the increase in PPAR γ levels (Fig. 4B) and reduction in the ratio of pSer84PPAR γ /PPAR γ (Fig. 4C), as well as decreased expression of PPAR δ (Fig. 4D). Although, the percentage of pERK1/2/ERK was reduced in tumors from Sca-1 KO mice (Fig. 4E), it did not approach the changes observed in the mammary gland.

Adenocarcinomas from WT and KO mice were also assessed for changes in gene expression. Tumors from KO mice expressed ≥ 2.5 -fold changes in 182 genes (Table S1), of which 108 genes exhibited increased expression and 74 genes reduced expression. Reduced gene expression in tumors from KO mice was associated predominantly with proteolytic and developmental processes, whereas increased gene expression was associated with the cell cycle, mitosis, and cell division (Table S2).

Sca-1 negatively regulates PPAR γ expression by reducing proteasomal turnover

The mechanism by which Sca-1 deficiency increased PPAR γ expression was further examined in mammary tumor cell line 34T using RNA interference. Cells were transduced with a lentivirus expressing either an EGFP shRNA as a control or a Sca-1 shRNA (D8 cells) to reduce Sca-1 expression (21) (Fig. 5A). Reduction of Sca-1 increased PPAR γ two-fold and reduced the ratio of pSer84PPAR γ /PPAR γ by 70% (Fig. 5B). These changes were accompanied by a 50% reduction in the ratio of pERK1/2/ERK (Fig. 5B), as well as an increase in PTEN expression (Fig. 5A). Treatment of cells with GW7845 further enhanced PPAR γ levels in both cell lines (5C), which was consistent with ligand-induced stability and/or autoactivation. To determine if increased PPAR γ expression was due to reduced turnover, cells were treated with the proteasome inhibitor lactacystin (Fig. 5D). Lactacystin increased PPAR γ levels to a greater extent in 34T cells than in D8 cells (Fig. 5D), and correlated with the reduced ratio of pSer84PPAR γ /PPAR γ in these cells. Higher expression of PPAR γ in D8 cells correlated with the increased expression of two PPAR γ -dependent genes, PTEN and FABP3, which was consistent with the higher ratio of the activated hypophosphorylated species of Ser84PPAR γ in these cells (44–46) (Fig. 5E). Overall, these results suggest that Sca-1 deficiency in the mammary gland increases PPAR γ expression and activity through a combination of effects resulting from ligand stabilization, decreased ERK1/2-dependent phosphorylation and reduced susceptibility to proteasomal degradation.

Discussion

Although Sca-1 is commonly used as a marker of stem and early progenitor cells in the mammary gland and other organs, its function in normal and malignant tissues has remained largely undefined. In the present study, Sca-1 deficiency enhanced the chemopreventive action of PPAR γ agonist GW7845, and negated the tumor promoting action of PPAR δ

agonist GW501516. These opposing actions have been previously described in WT FVB mice (35), but the almost complete protection against tumorigenesis by GW7845 in Sca-1 KO mice is most remarkable. The enhanced chemopreventive effect of GW7845 was associated with increased PPAR γ and a reduced ratio of pSer84PPAR γ /PPAR γ , and correlated with increased expression of the PPAR γ -driven gene, PTEN (47). However, the latter finding could also be explained by alterations in post-translational processes. We have found no evidence by gene profiling of either the KO mammary gland (unpublished results) or tumors from KO mice (Tables S1 and S2) for enhanced PTEN mRNA expression. The Sca-1 'knockdown' phenotype in mammary tumor cell line D8 correlated with increased tumor suppressor signaling, in part through TGF β ligand GDF10 and possibly increased PTEN levels (21). In the present study, D8 cells also expressed moderately higher PPAR γ levels and a lower relative percentage of pSer84PPAR γ and pERK1/2, which are consistent with the phenotype of the KO mammary gland. Since ERK-dependent phosphorylation at Ser84PPAR γ has been reported to inhibit transcriptional activity (44–46) and promote proteasomal degradation (43), our data suggest that PPAR γ is regulated through a post-translational rather than by a transcriptional mechanism (47). One explanation for this effect is that ERK1/2 is activated downstream of Ras signaling by dominant-negative regulation of PPAR γ (34), an alteration that may set into motion repression of PPAR γ signaling and negate some of the effects of PPAR γ agonists, whereas, the opposite occurs in KO mice. A second level of regulation may also result from changes in the ratio of PPAR γ to PPAR δ as was evident in both the KO mammary gland and tumors. Increased expression of PPAR δ leads to inhibition of PPAR γ target genes, and has been postulated to serve as a "gateway receptor" that can modulate PPAR γ -mediated processes, such as adipogenesis (48). These opposing actions of PPAR γ and PPAR δ agree with their opposite functions in mammary tumorigenesis (25, 34, 35). Since there are more than 1,000 genes in the human genome containing PPAR response elements, which encompass 25 biological processes, including mitogen-activated protein kinase signaling (49), the mechanism by which PPAR signaling ultimately affects tumorigenesis is likely to occur directly, as well as through its modulation of ancillary processes, such as angiogenesis, inflammation and immune function (25, 50).

In addition to the negative regulation of the tumor suppressors PPAR γ and PTEN in Sca-1 KO mice, Sca-1 was recently shown to block TGF- β signaling through a unique GDF10/Smad3 signaling pathway (21). It is interesting that PPAR γ also regulates the tumor suppressor gene RASSF5 (51), a suppressor of the ERK pathway (52), which could additionally contribute to the antiproliferative effects of Sca-1 deficiency. Overall, these actions may account for the association of Sca-1 with metastasis (53–55), mammary tumor initiating cell capacity in MMTV-Wnt1 and MMTV-ErbB2 mice (19, 20) and BCR-ABL-induced chronic myeloid leukemia (56), as well as radiation resistance (57, 58). Although, the Sca-1/Ly6A gene is missing in the human genome (59), there are Ly6 family members that may serve a similar purpose in breast cancer. In this context, the 8q24.3 amplicon identified in breast cancer includes the Ly6 locus (60), Ly6D is a marker of basal-type breast cancer (61) and micrometastases in head and neck cancer (62), and Ly6K is a marker of breast (63) and head and neck (64) cancer. However, a comprehensive analysis of Ly6 gene expression and their function in breast and other cancers has not been reported.

Another intriguing finding was that Sca-1 deficient mammary gland exhibited a reduced basal proliferative state as determined by p21^{Cip1}, pRB and Ki-67 expression, as well as a reduced response to progestin stimulation (Fig. 1). This phenotype likely contributed to the delay in tumor formation following carcinogenesis, and to the enhanced response to GW7845. However, Sca-1 deficiency did not alter lactation and involution in multiparous animals (results not shown), suggesting that Sca-1 likely functions in a developmentally and physiologically stochastic manner. This agrees with its role in modulating the proliferation of multipotent hematopoietic cells (17), and the tumorigenicity of mammary tumor cells

(21). Thus, Sca-1 may act as a rheostat of proliferation under specific conditions, as well as cooperate with other signaling pathways. In this regard, Sca-1 KO mice were deficient in the stem and progenitor cell marker, Musashi1 (65), suggesting a link to the Notch and Wnt pathways under its control in mammary epithelial (66) and breast cancer cells (67). Thus, Sca-1 appears to be a master modulator of tumor suppressor function that ultimately impinges on tumorigenesis and the action of chemopreventive agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support

NIH grant 1NO1 CN43302-WA19 (R.I. Glazer). This investigation was conducted using the Animal Research, Flow Cytometry, Genomics and Epigenomics, and Microscopy and Imaging Shared Resources. This study was supported by Award P30CA051008 from the National Cancer Institute, NIH, and by Grant 1G20RR025828-01 from the NIH for Rodent Barrier Facility Equipment. The content does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

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Fig. 1. (A) Phenotype of Sca-1 KO mice. FACS analysis indicates the absence of Sca-1. (B) Ductulogenesis is diminished in Sca-1 KO mice. Whole mounts of the mammary gland of seven-week old WT (a) and KO (b) indicates a lesser degree of duct elongation in KO mice (0.6 cm) vs. WT mice (1.0 cm). Treatment of six-week old WT (c, e) and KO (d, f) mice with 15 mg medroxyprogesterone (MP) resulted in less proliferation in KO mice after 7 days. The line indicates the extent of ductal invasion. Inset indicates 5× magnification. The difference in ductal invasion between WT and KO mice differed significantly ($P < 0.01$, $N = 5$) by the two-sided Student's *t* test.

(C) The Sca-1 KO mammary gland exhibits a lower proliferative potential. KO mice exhibit increased expression of the antiproliferative marker, p21^{Cip1}, and less expression of the proliferative marker, pRB, than WT mice. Magnification 400×. (D) The Sca-1 KO mammary gland exhibits an impaired proliferative response to MP. Animals were treated as in (B) and proliferation was monitored after 7 days by Ki-67 labeling. Sca-1 KO mice showed less Ki-67 labeling after MP treatment. Magnification 400×. Bar graph indicates the number of Ki-67-positive cells per duct based on a total of 40–50 ducts. Although, there was no significant difference in Ki-67 expression between control WT and KO mice ($P>0.05$), their response to MP differed significantly ($P<0.001$, $N=16$) by the two-sided Student's *t* test.

Fig. 2. Sca-1 deficiency increases PPAR γ , and reduces pSer84PPAR γ , PPAR δ and pERK. (A) Mammary gland lysates from two wild-type (WT) and two KO (KO) mice were probed by western blotting for pSer84PPAR γ , PPAR γ , pERK1/2, ERK1/2, PPAR δ , PTEN and β -actin. (B) Bar graph of PPAR γ (PPARg) levels shown in (A). (C) Bar graph of the ratio of pSer84PPAR γ /PPAR γ (pPPARg/PPARg) shown in (A). (D) Bar graph of PPAR δ (PPARd) levels shown in (A). (E) Bar graph of the ratio of pERK1/2/ERK1/2 shown in (A). All values were normalized to actin.

Fig. 3.

Sca-1 deficiency enhances the chemopreventive effect of a PPAR γ agonist, and attenuates the tumor promoting action of a PPAR δ agonist. (A) Survival curve following treatment with MP/DMBA and maintenance on a diet supplemented with 0.005% GW7845. Only GW7845-treated KO mice exhibited a significant reduction in tumorigenesis ($P=0.001$ by the log rank test), which developed more slowly in KO mice ($P=0.033$). The number (N) of mice per group was: WT (N=10), WT+GW7845 (N=8), KO (N=19), KO+GW7845 (N=9). (B) Cumulative tumor incidence in GW7845-treated mice. Animals were treated as in (A). KO mice treated with GW7845 exhibited a significant reduction in tumorigenesis vs. control KO mice ($P=0.001$ by the log rank test). (C) Survival curve following treatment with MP/DMBA and maintenance on a diet supplemented with GW501516. Carcinogenesis was induced as in A, except that animals were administered a diet supplemented with 0.005% GW501516. Only WT mice treated with GW501516 exhibited a significant increase in tumorigenesis ($P=0.03$ by the log rank test), which developed more slowly in KO mice vs. WT mice ($P=0.009$). The number (N) of mice per group was: WT (N=10), WT+GW501516

(N=5), KO (N=10), KO+GW501516 (N=6). (D) Cumulative tumor incidence in GW501516-treated mice. Animals were treated as in (C). Only KO mice treated with GW501516 exhibited a significant increase in tumorigenesis ($P=0.03$) by the log rank test).

Fig. 4. Sca-1 deficient adenocarcinomas express increased PPAR γ , and reduced pSer84PPAR γ , PPAR δ and pERK. (A) Lysates from adenocarcinomas induced in two wild-type (WT) and two KO (KO) mice by MP/DMBA were probed by western blotting for pSer84PPAR γ , PPAR γ , pERK1/2, ERK1/2, PPAR δ , PTEN and β -actin. (B) Bar graph of PPAR γ levels shown in (A). (C) Bar graph of the ratio of pSer84PPAR γ /PPAR γ shown in (A). (D) Bar graph of PPAR δ levels shown in (A). (E) Bar graph of the ratio of pERK1/2/ERK1/2 tumors shown in (A). All values were normalized to actin.

Fig. 5.

Reduction of Sca-1 increases PPAR γ expression. (A) 34T mammary tumor cells were transduced with either a GFP shRNA (34T) or a Sca-1 shRNA (D8) and probed by western blotting for expression of Sca-1, pSer84PPAR γ , PPAR γ , pERK1/2, ERK1/2, PTEN and β -actin. Reduction of Sca-1 increased PPAR γ and reduced the percentage of pSer84PPAR γ and pERK. (B) Bar graphs of the western blots shown in (A). (C) GW7845 increases PPAR γ expression. 34T and D8 cells in (A) were treated overnight with 0.1 μ M GW7845. Bar graph indicates that GW7845 enhanced PPAR γ levels to a greater extent in D8 cells than in 34T cells. (D) Effect of proteasome inhibition on pSer84PPAR γ and PPAR γ expression. 34T and D8 cells were treated overnight with 1 μ M lactacystin. Bar graph indicates that PPAR γ

expression is increased to a greater extent in 34T cells than in D8 cells. (E) Reduction of Sca-1 increases PTEN and FABP3 mRNA expression. 34T and D8 cells were analyzed for PTEN and FABP3 mRNA levels by qRT-PCR and all values were normalized to actin. There was a significant difference between 34T and D8 cells in PTEN mRNA levels ($P<0.01$, $N=3$) and FABP3 mRNA levels ($P<0.001$, $N=3$) by the two-sided Student's *t* test. (F) Schematic of the regulation of PPAR γ by Sca-1. Sca-1 is envisioned to suppress PTEN in part through ERK-mediated inhibition of PPAR γ . Phosphatidylinositol 3-kinase (PI3K)-dependent Ras/ERK signaling results in increased inhibition of PPAR γ by ERK-mediated phosphorylation on Ser84, which in turn reduces PTEN transcription. The opposite effect occurs during Sca-1 deficiency, which results in reduced progenitor cell expansion, delayed tumorigenesis and increased sensitivity to PPAR γ agonists.

Table 1

Tumor histopathology. Wild-type C57BL/6 and Sca-1 KO mice were the control groups fed standard rodent chow in Fig. 3. The number of tumors and the percentage of each type is indicated. There was no significant difference in the percentage of each histotype between wild-type and Sca-1 KO mice by Fisher's Exact test.

Tumor Histology	Wild-type	Sca-1 KO
Adenocarcinoma	6 (43%)	14 (56%)
Adenosquamous/Squamous	4 (28.5%)	2 (8%)
Myoepithelial	4 (28.5%)	9 (36%)