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Aldo-keto Reductase 1C3 (AKR1C3) is overexpressed in skin squamous cell carcinoma (SCC) and affects SCC growth via prostaglandin metabolism

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Abstract

Aldo-keto reductase 1C3 (AKR1C3) is an enzyme involved in metabolizing prostaglandins (PGs) and sex hormones. It metabolizes PGD₂ to 9α,11β-PGF₂, diverting the spontaneous conversion of PGD₂ to the PPARγ agonist, 15-Deoxy-Delta-12,14-prostaglandin J2 (15d-PGJ₂). AKR1C3 is overexpressed in various malignancies, suggesting a tumor promoting function. This work investigates AKR1C3 expression in human non-melanoma skin cancers, revealing overexpression in squamous cell carcinoma (SCC). Effects of AKR1C3 overexpression were then evaluated using 3 SCC cell lines. AKR1C3 was detected in all SCC cell lines and its expression was upregulated in response to its substrate, PGD₂. Although attenuating AKR1C3 expression in SCC cells by siRNA did not affect growth, treatment with PGD₂ and its dehydration metabolite, 15d-PGJ₂, decreased SCC proliferation in a PPARγ-dependent manner. In addition, treatment with the PPARγ agonist pioglitazone profoundly inhibited SCC proliferation. Finally, we generated an SCC cell line that stably overexpressed AKR1C3 (SCC-AKR1C3). SCC-AKR1C3 metabolized PGD₂ to 9α,11β-PGF₂ 12 fold faster than the parent cell line and was protected from the anti-proliferative effect mediated by PGD₂. This work suggests that PGD₂ and its metabolite 15d-PGJ₂ attenuate SCC proliferation in a PPARγ-dependent manner, therefore activation of PPARγ by agonists such as Pioglitazone may benefit those at high risk of SCC.

Keywords

Aldo-keto reductase; PPARγ; Prostaglandin; Squamous cell carcinoma; Skin cancer

Introduction

Aldo-Keto Reductase 1C3 (AKR1C3) belongs to a large superfamily of NAD(P)(H)-dependent AKR enzymes which metabolize various substrates including prostaglandins and sex hormones [1]. In humans, the AKR1C subfamily consists of 4 enzymes termed AKR1C1, AKR1C2, AKR1C3 and AKR1C4 which despite their extensive structural overlap, exhibit different substrate binding preferences [2].

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The role of AKR1C3 in steroid hormone metabolism includes the conversion of the relatively weak androgen $4\text{-androstene-3,17-dione}$ to its potent derivative, testosterone, as well as the reduction of the weak estrogen, estrone, to the more potent steroid, $17\beta\text{-estradiol}$ [2]. AKR1C3 can therefore indirectly regulate steroid hormone receptor activation under physiological and pathological conditions. With respect to this, previous work has shown that AKR1C3 is over-expressed in hormone-dependent cancers such as prostate, breast and endometrium suggesting that the capacity of AKR1C3 to convert weak sex hormones to their more potent derivatives may generate proliferative signaling to support tumor progression [3–8].

Unlike other AKR1C members, AKR1C3 can synthesize $\text{PGF}_{2\alpha}$ from PGH_2 , an arachidonic acid derivative synthesized by cyclooxygenase [9]. Its role in prostaglandin metabolism also includes the conversion of PGD_2 , a pro-inflammatory lipid mediator, to $9\alpha,11\beta\text{-PGF}_2$ [9–11]. Thus, via its involvement in PG metabolism, AKR1C3 may regulate biological and pathological responses in hormone-independent tissues. In fact, AKR1C3 is overexpressed in various cancers originated from non-steroidogenic tissues such as kidney [12], central nervous system [13], pancreas, gastrointestinal tract and lung [14, 15]. AKR1C3 can activate carcinogens [16], modulate oxidative stress [17], and its polymorphisms is associated with risk for childhood leukemia [18] and bladder cancer [19]. Collectively, these observations suggest a tumor promoting function for AKR1C3 which is beyond its function in sex hormone metabolism. Interestingly, other AKR1C enzymes including AKR1C1 and AKR1C2 have been shown to be inducible in skin following UV light exposure which is the main cause of non-melanoma skin cancers [20].

This work investigates the expression and function of AKR1C3 in non-melanoma skin cancers. Results suggest the expression of AKR1C3 may facilitate squamous cell carcinoma growth in the setting of inflammation, and that the $\text{PPAR}\gamma$ agonist pioglitazone may be a useful treatment to decrease SCC growth.

Materials & Methods

SCC cell lines

Freshly isolated SCC tumors from sun-exposed areas were excised from patients by Moh's surgery. In order to eliminate any possible surface contaminants, tumors were submerged in 70% ethanol for 10 minutes followed by 3 washes in sterile PBS. Using a scalpel, the outer surface of the tumor, including all necrotic areas was discarded and the viable tumor core was isolated and transferred to a sterile 30 mm tissue culture dish. The tumor core was very finely minced and suspended in a solution of 2000 units of Collagenase type 1 (MP Biomedicals, Cat # 195109) reconstituted in 1 ml KGM (Invitrogen Cat#17005042) and 1ml sterile DPBS and allowed to dissociate for 2hrs at 37°C . Processed tissue transferred to a 15ml conical tube and spun at 1000 rpm for 4 minutes. The supernatant was aspirated, and the tumor was resuspended in 2 ml of 0.25% trypsin-EDTA solution and trypsinized at 37°C for 30 more minutes. The trypsin reaction was quenched with 2ml FBS, the suspension filtered through a $100\mu\text{m}$ filter and then spun at 1000 rpm for 8 minutes to form a pellet. The supernatant was aspirated and the pellet was resuspended in KGM and transferred to collagen (Pure Col, Advanced BioMatrix) pre-coated T-25 flasks and allowed to adhere.

Fibroblasts were removed from the initial culture by brief trypsinization that allowed fibroblasts, but not SCC cells to detach. Cells were routinely fed every 3–5 days with serum-free KGM containing 5ng/ml EGF, 20µg/ml BPE, 100 U/ml penicillin and 100 µg/ml streptomycin. SCC-13 cell line was purchased from Harvard Skin Disease Research Center (<http://rheinwaldlab.bwh.harvard.edu/cell.html>) and maintained under the same cultivating conditions described above.

Immunohistochemistry

Samples were prepared and stained for AKR1C3 using the ImmunoCruz Staining System (Santa Cruz Biotechnology, Santa Cruz, CA) and ABC kit (Vector Laboratories, Burlingame CA) according to the manufacturer's protocol using 1:50 dilution of the monoclonal anti-human AKR1C3 antibody. Sections were washed in TBST 3 times followed by 10 minutes incubation in AEC reagent (Vector Laboratories, Burlingame CA). Positive immunoreactive cells, identified by red stain, were visualized using a light microscope.

Cellular AKR1C3 activity

Cellular enzymatic activity of AKR1C3 was assessed by measuring the conversion of PGD₂ to 9α11β-PGF₂ using ELISA (catalog: 516521, Cayman Chemical, Ann Arbor, MI) as previously described [21]. Cells were seeded in 6 well plates and allowed to reach 90% confluence. Cells were treated with 1µM PGD₂ (0.5ml/well) in growth medium without supplements for the indicated times. 9α11βPGF₂ in each condition was determined in supernatants by ELISA following manufacturer directions and results were normalized to total protein. Each condition was evaluated for 9α11βPGF₂ by averaging a minimum of 2 OD measurements.

Cell proliferation assessment by Click-iT assay

Cell proliferation was determined using Click-iT assay according to the protocol provided with Click-iT EdU Imaging Kit (Invitrogen #C10337). Cells were plated in chamber slides in growth media (KGM + additives) until 60% confluent. Cells were then treated as indicated. Proliferating cells were visualized using a fluorescence microscope (Nikon Eclipse E800 equipped with a Spot RT3 camera, Nikon, Melville, NY). Pictures were obtained using Spot advanced software (Diagnostic Instruments, Sterling Heights, MI). Five random fields from each condition were captured at X10 magnification and the number of proliferating cells normalized to total cell count in each field was determined. Results were averaged and presented as percent proliferation compared with DMSO treated controls.

Generation of AKR1C3-overexpressing cells (SCC-AKR1C3)

The AKR1C3-pLNCX2 retroviral construct was kindly provided by Dr. Trevor Penning (University of Pennsylvania, Philadelphia, PA). HEK293T packaging cells and additional reagents were kindly provided by Dr. Glynis Scott and Dr. Lei Xu (University of Rochester, Rochester, NY). AKR1C3-pLNCX2 or empty vector was added to HEK293T packaging cells and media from these cells was collected and added to SCCAM1 cells. Stable transfectants were selected by the inclusion of 0.6mg/ml neomycin to the growth medium.

Western blot analysis

Protein extraction and western blot procedures were carried out as previously described [21]. For all experiments 50µg protein was used and the primary antibodies were diluted as follows: Anti-AKR1C3 1:1000; anti-human β-actin 1:2000.

Reagents

Monoclonal mouse anti-human AKR1C3 (ab49680) and polyclonal rabbit anti-human β-actin (ab8227) were obtained from Abcam (Abcam, Cambridge, MA). Common Antibody Diluent purchased from BioGenex (BioGenex, San Ramon, CA). PGD₂, 9α11β-PGF₂, 15d-PGJ₂ and GW9662 purchased from Cayman Chemical (Cayman Chemical, Ann Arbor, MI) and dissolved in dimethyl sulfoxide (DMSO) to give a stock concentration of 10mM.

Statistical analysis

The results are presented as means ± SEM. The appropriate t-test was performed using Microsoft Excel or GraphPad Prism. A P-value < 0.05 was considered statistically significant.

Results

AKR1C3 is overexpressed in SCC while not detected in the tumor mass of BCC

To investigate the expression of AKR1C3 in non-melanoma skin cancers, 6 human SCCs and 7 BCCs were analyzed by immunohistochemistry. Strong immunoreactivity with AKR1C3 antibody (red) was observed in the tumor mass of all SCCs while its expression in tumor nodules of all the tested BCCs was undetectable (figure 1a). In normal skin samples, modest immunoreactivity was most evident in the upper epidermal layers. Staining of the tumor stroma and vasculature of both types of cancer was also moderately positive.

AKR1C3 is expressed in SCC cell lines and is up-regulated by its substrate, PGD₂

To further investigate the possible role of AKR1C3 in SCC, AKR1C3 expression was investigated in 3 SCC cell lines derived from sun-exposed skin compared with normal keratinocytes. Expression of AKR1C3 protein was detected in all SCC cell lines (figure 1b). It was also observed that AKR1C3 expression was dependent on SCC cell confluence with stronger expression noted in highly confluent cells (not shown). To evaluate the effects of AKR1C3 on SCC proliferation, its expression was knocked down using siRNA and SCC cell proliferation was assessed by ClickiT assay. Despite profound knockdown (varied between 39.5–84%) of AKR1C3 expression in the SCC cultures, proliferation was not altered compared with mock-transfected cells (Figure 2S). In addition, we tested the effects of steroid hormones and PGs which are metabolized by AKR1C3 on its expression. We found that PGD₂ but not 9α11β-PGF₂ induced AKR1C3 expression in each cell line (representative data shown in figure 1c), while testosterone and 17β-estradiol had no effect (data not shown).

PGD₂ and 15d-PGJ₂ reduce SCC cell proliferation in a PPAR γ -dependent manner

Following the observation that AKR1C3 expression is upregulated in SCC cells specifically in response to PGD₂, we evaluated the effects of PGD₂ and its metabolites on SCC proliferation. Each SCC cell line was treated for 24 hours with PGD₂, its AKR1C3-mediated metabolite 9 α 11 β -PGF₂, or with the PGD₂ spontaneous dehydration product, 15d-PGJ₂. Proliferation was then assessed using a Click-iT assay. Representative detailed dose response data obtained in our SCCAM1 cell line are shown (figure 2a). 15d-PGJ₂ and PGD₂ but not 9 α 11 β -PGF₂ inhibited cell proliferation dose-dependently. Treatment with 15d-PGJ₂ reduced proliferation more effectively than PGD₂ at all tested concentrations where 5 μ M of 15d-PGJ₂ completely eliminated SCC proliferation. In contrast, 9 α 11 β -PGF₂ had no significant effect on SCC proliferation at any tested dose.

15d-PGJ₂ is a spontaneous dehydration product of PGD₂ and a potent activator of PPAR γ . To evaluate whether the anti-proliferative effects induced by PGD₂ and 15d-PGJ₂ were mediated by PPAR γ activation, cells were treated with 1.5 μ M 15d-PGJ₂ or 7 μ M of PGD₂ in the presence of the PPAR γ inhibitor, GW9662, and cell proliferation was assessed (Figure 2b and 2c). The presence of PGD₂ and 15d-PGJ₂ reduced SCC proliferation to 51% and 27% of DMSO-treated control cells, respectively. Although treatment with 1 μ M GW9662 itself modestly reduced SCC proliferation compared to DMSO treated controls, its combination with PGD₂ and 15d-PGJ₂ dose-dependently reversed the anti-proliferative effects mediated by both prostaglandins. The presence of 1 μ M GW9662 partially restored the proliferation rate of SCC cells treated with 15d-PGJ₂ and completely reversed the anti-proliferative effects mediated by PGD₂.

Since the proliferation of all SCC lines was profoundly inhibited by treatment with 15d-PGJ₂, we next tested the effects of the PPAR γ agonist, Pioglitazone, on SCC proliferation. Each SCC line was treated with Pioglitazone as indicated for 24 hours and cell proliferation was evaluated as before (fig 2d). Little effect was evident at the lowest dose tested, but exposure to 50 or 100 μ M Pioglitazone effectively inhibited cell growth. Taken together, this data implies that the anti-proliferative effect mediated by PGD₂ is due to its spontaneous conversion to 15d-PGJ₂ followed by activation of PPAR γ .

AKR1C3 over-expressing SCC cells are protected from the anti-proliferative effects of PGD₂

To evaluate whether AKR1C3 overexpression protects SCC cells from PGD₂ *in vitro*, we used SCCAM1 cells to generate an SCC cell line that stably over-expresses AKR1C3 by means of retroviral infection. The SCC cell line was infected with either AKR1C3 over-expressing retroviral construct (SCC-AKR1C3) or with an empty vector (SCC-control). AKR1C3 over-expression in SCC-AKR1C3 cells was confirmed by western blot analysis (Figure 3a). To verify that the over-expressed AKR1C3 is enzymatically active, we compared the capacity of SCC-AKR1C3 and SCC-control cells to mediate the AKR1C3 specific conversion of exogenous PGD₂ to 9 α 11 β -PGF₂ by ELISA (Figure 3b). Concentrations of 9 α 11 β -PGF₂ were markedly higher in supernatants of SCC-AKR1C3 cells compared with SCC-controls at all tested time points.

Finally, the effect of AKR1C3 overexpression on proliferation of SCC cells treated with PGD₂ was evaluated. SCC-AKR1C3 and SCC-control cells were treated with the indicated doses of PGD₂ for 24 hours, and proliferation was assessed by Click-IT assay. While treatment with 10 μ M PGD₂ reduced SCC-control proliferation to 44% of control, SCC-AKR1C3 cells retained approximately 70% of their proliferative capacity (Figure 3c). This resistance to PGD₂ suggests that AKR1C3 overexpression in SCCs may protect tumors from the anti-proliferative effects mediated by this PG.

Discussion

AKR1C3 has been mostly studied in malignancies of classical steroidogenic tissues such as breast and prostate [3, 7, 8, 22]. This is due to its involvement in generating potent sex hormones such as testosterone and 17 β -estradiol. However, recent work has shown altered AKR1C3 expression in tumors of various tissues such as lung, brain and kidney suggesting a tumorigenic function beyond its role in sex hormone metabolism [12, 13].

In this work we characterized the expression levels of AKR1C3 in non-melanoma skin cancers, demonstrating that AKR1C3 was markedly expressed by malignant SCC cells while its expression was undetected in tumor nodules of BCC. This finding aligns with previous observations showing that AKR1C3 overexpression was detected in malignancies with a more differentiated phenotype. For example, high AKR1C3 expression was detected in renal cell carcinoma, papillary urothelial carcinoma, glial neoplasms, meningiomas, lung SCC and adenocarcinoma compared with reduced or undetectable AKR1C3 expression in tumors with more embryonic phenotypes of the same tissue [12, 13, 15].

The current work provides further support for AKR1C3 being a marker of tumors with more differentiated characteristics, and suggests it may be worthwhile to assess AKR1C3 as a prognostic marker for cutaneous SCC.

The tumor mass of SCC is comprised of a heterogeneous population of malignant keratinocytes at various stages of differentiation. Different levels of AKR1C3 expression were detected within the epithelial SCC mass, with stronger immunoreactivity found in areas of moderately differentiated keratinocytes. Interestingly, this distribution pattern aligns with its relatively higher expression in the spinous and granular layers of normal epidermis and in differentiated cultured keratinocytes [21].

To study the role of AKR1C3 in cutaneous SCC, we established two new skin SCC cell lines from tumors arising on sun-exposed skin (SCCAM1 & SCCJV) and used one existing SCC line (SCC13). We further confirmed the epithelial origin as well as malignant potential of the newly established lines (Figure S1). Our SCC cells expressed keratin 5 (figure S1a), maintained a proliferative phenotype for as many as 70 passages and were able retain their proliferative capacity following an exposure for several days to concentrations of calcium sufficient to induce terminal differentiation of normal keratinocytes (figure S1b). Furthermore, subcutaneous injections of the SCC cell lines into immunocompromised mice resulted in the formation of SCC-like tumors *in vivo* (figure S1c). Taken together, these cell

lines meet typical criteria for malignancy validating them as a model for studying molecular mechanisms in skin SCC [23–25].

Our work demonstrates that AKR1C3 is expressed in each cell line studied; however, attenuation of its expression did not modulate SCC cell growth (figure S2). Our examination therefore looked at the way in which SCC cells might respond to substrates and products formed by the action of AKR1C3. Under our experimental conditions, estrogen and testosterone had no effect on SCC proliferation or on the enzyme expression (data not shown). Since AKR1C3 is also involved in PG metabolism, we examined the effects of relevant prostaglandins on SCC proliferation and this enzyme expression. Interestingly, among the tested PGs, only PGD₂, a substrate of AKR1C3, induced its upregulation in SCC cells suggesting the existence of a positive feedback loop wherein in the presence of high PGD₂ concentrations, SCC cells up-regulate its metabolizing enzyme. It would be interesting to evaluate whether PGD₂ induces AKR1C3 in other cancer cell lines especially of hormone-dependent malignancies such as breast and prostate. Upregulation of AKR1C3 due to high PGD₂ levels in the microenvironment of hormone-dependent cancers may indirectly result in increased potent sex hormone synthesis leading to excess activation of proliferative signaling pathways.

Large numbers of inflammatory cells with the capacity to synthesize and secrete the pro-inflammatory mediator PGD₂ are often observed in SCC tumors. Increased numbers of mast cells, the major PGD₂ secreting cell type, have been detected in SCC of the lip [26], oral cavity [27], cervix [28] and esophagus [29]. Parizi *et al* assessed the number of mast cells in SCC of the mouth compared with SCC of the skin. They found the total number of mast cells were elevated in the tumor site compared with a marginal area, and that SCC of the skin demonstrated higher mast cell concentrations, in some cases more than 200 cells/mm² [30]. Therefore, it is reasonable to assume the presence of high levels of PGD₂ within the tumor microenvironment.

PGD₂ is a relatively unstable mediator, with an apparent half-life of 30 minutes in plasma [31], after which it is spontaneously dehydrates to form 15d-PGJ₂, a potent physiological PPAR γ agonist [32]. The current work shows that PGD₂ and its spontaneous dehydration product 15d-PGJ₂, but not 9 α 11 β -PGF₂, an AKR1C3-mediated metabolite of PGD₂, significantly attenuate SCC cell proliferation. Treatment with the selective PPAR γ inhibitor GW9662 reversed the anti-proliferative effects of both mediators, supporting the role of PPAR γ activation in this effect.

To model the observed overexpression of AKR1C3 in SCC and to test its possible function in modulating prostaglandin-induced changes in SCC proliferation, we generated AKR1C3 overexpressing SCC cells (SCC-AKR1C3) which metabolized PGD₂ to 9 α 11 β -PGF₂ faster than parental cells (12.3 fold on average). Since 9 α 11 β -PGF₂ and 15d-PGJ₂ share the same precursor, the observed elevation of 9 α 11 β -PGF₂ in SCC-AKR1C3 cells treated with PGD₂ must also be accompanied by a reduction of 15d-PGJ₂ levels. This relationship has been documented in similar experiments done in AKR1C3 overexpressing MCF-7 breast cancer cells. Using LC/MS analysis, the authors showed a decrease in the amount of spontaneous dehydration of PGD₂ to 15d-PGJ₂ by instead producing an accumulation of 9 α 11 β -PGF₂

[11]. Because of their increased capacity to convert exogenous PGD₂ to 9α11β-PGF₂, SCC-AKR1C3 cells can better maintain their proliferative capacity following PGD₂ challenge, compared with parental SCC-control cells. Statistically significant differences between the proliferation of SCC-AKR1C3 and SCC-control cells were only observed at relatively high concentrations of PGD₂. This could be explained by the robust capacity of PGD₂ to induce endogenous expression of AKR1C3 in SCC-control cells which likely partially protected them from PGD₂ effects during treatment.

Since 15d-PGJ₂ was able to inhibit SCC growth, we wanted further evidence that this action was mediated by PPARγ activation. Recent work has demonstrated the potential contribution of PPARγ to skin cancer in a mouse photocarcinogenesis model [33]. Pioglitazone is an oral medication utilized in clinical practice for treatment of diabetes. We therefore tested the PPARγ activator Pioglitazone on SCC proliferation in our cell lines. These experiments showed Pioglitazone profoundly inhibited SCC cell line proliferation (Figure 2d). Daily dosing with Pioglitazone produces serum levels over time which are similar to those used in the experiments reported here (50 μM). In addition, pioglitazone has two active metabolites [34, 35]. When tested for selectivity of activation for all PPARs, Pioglitazone was found to be a modest activator of PPARα, a robust activator of PPARγ with little effect on other PPARs [36]. The efficacy of Pioglitazone in suppressing proliferation in the 3 cell lines tested here suggests that it may be effective clinically for individuals with a high risk of developing skin cancer, such as individuals with transplants on immunosuppressive therapy. More in vitro work testing SCC cell lines is needed.

We propose a model (figure 4) in which over-expression of AKR1C3 in SCC can benefit malignant cells due to their enhanced capacity to metabolize PGD₂ to 9α11β-PGF₂, resulting in decreased levels of the anti-proliferative and pro-apoptotic metabolite 15d-PGJ₂ within the tumor microenvironment. The profound effects observed here on SCC proliferation by agents that influence PPARγ activation support the idea that manipulation of PPARγ activity in individuals prone to skin cancer may have therapeutic benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Alon Mantel and Amanda Carpenter-Mendini performed and analyzed all experiments besides the ones showed in figures 1b, 2d and S2 that were done by JoAnne VanBuskirk. SCCAM1, SCC-AKR1C3 and SCC-control established by Alon Mantel. SCCJV cell line was generated by JoAnne VanBuskirk. Manuscript was written and edited by Alon Mantel and Alice Pentland.

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Figure 1. AKR1C3 overexpression in tumor mass of SCC and its induction by PGD₂
(a) AKR1C3 expression was evaluated in six cases of SCC, seven cases of BCC and six cases of normal buttock skin by immunohistochemical staining. Representative figures demonstrating AKR1C3 expression in normal skin and within the tumor mass (T) and tumor stroma (S) of SCC and BCC. Strong immunoreactivity was observed in SCC while undetected in BCC. Normal skin showed moderate expression mainly in suprabasal layers (arrows). Bar=30 μ M. **(b)** expression of AKR1C3 in cell lines SCCAM1, SCCJV and SCC13. To evaluate the expression of AKR1C3 in the different SCC cell lines, cells cultured at confluence for 24 hours following by protein extraction and western blot analysis (n=3; representative blot is shown). **(c)** PGD₂-induced AKR1C3 expression in SCC cells. SCC cells were treated for 24 hours with various doses of PGD₂ or its AKR1C3-mediated metabolite 9 α ,11 β PGF₂ as indicated and AKR1C3 expression was assessed by western blot. A representative experiment in which SCCAM1 was used is shown.

Figure 2. PGD₂ and 15d-PGJ₂ attenuated SCC cell proliferation in a PPAR γ -dependent manner (a) SCCAM1 cells were treated with the indicated concentration of PGD₂, 15d-PGJ₂, 9 α ,11 β -PGF₂ or DMSO for 24 hours followed by cell proliferation assessment by Click-iT assay. Data presented is the average of 3 experiments ($\pm$ SEM), *P<0.005, **P=0.012. Similar trends were observed in SCCJV and SCC13 cell lines (not shown). To study the involvement of PPAR γ activation in the anti-proliferative effects mediated by 15d-PGJ₂ and by its precursor PGD₂, SCCAM1 cells were treated with 1.5 μ M 15d-PGJ₂ (b) or 7 μ M PGD₂ (c) in the presence of the indicated concentration of the PPAR γ specific inhibitor GW9662 (GW). Cell proliferation was assessed by Click-iT assay. n=3, *P<0.05. Similar trends were

obtained with SCCJV and SCC13 cells (not shown). **(d)** The effects of Piogliazone on SCC proliferation. Each SCC cell line was treated for 24h as indicated and cell proliferation was assessed by Click-iT assay. (n=3)

Figure 3. Over-expression of AKR1C3 in SCCAM1 cells increases their capacity to metabolize PGD₂ to 9α,11β-PGF₂ and protects against PGD₂ anti-proliferative effects

(a) SCCAM1 cells were stably infected with either an AKR1C3 over-expressing retroviral construct (SCC-AKR1C3) or with empty vector (SCC-control). Over-expression of AKR1C3 was confirmed by western blot, n=2. (b) Over-expression of AKR1C3 in SCC cells increases their capacity to metabolize PGD₂ to 9α,11β-PGF₂. Cells were treated with PGD₂-containing media for the indicated times and 9α,11β-PGF₂, was measured by ELISA (n=2). (c) SCC-AKR1C3 or SCC-control cells were incubated for 24 hours with various doses of PGD₂ as indicated. Cell proliferation was then assessed by Click-iT assay. n=3, *P=0.0026.

Figure 4. AKR1C3 overexpression in SCC protects against the anti-proliferative effects mediated by PGD₂- a proposed model

PGD₂ is produced by either activated mast cells or other cell types within the tumor microenvironment of SCCs. PGD₂ is non-enzymatically converted to 15d-PGJ₂. This spontaneous metabolite of PGD₂ inhibits SCC proliferation in a PPAR_γ-dependent manner. The model proposes that when SCC cells overexpress the PGD₂ metabolizing enzyme AKR1C3, they are protected from 15dPGJ₂-mediated inhibition of proliferation, instead producing 9α11βPGF₂. In addition, our work suggests that direct activation of PPAR_γ via Pioglitazone inhibits SCC proliferation and therefore this drug may be further evaluated for treating cutaneous SCC patients.