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Silibinin inhibits ultraviolet B radiation-induced mast cells recruitment and bone morphogenetic protein 2 expression in skin at early stages in Ptch(+/-) mouse model of basal cell carcinoma

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Abstract

Around 80% of non-melanoma skin cancers (NMSCs) are basal cell carcinoma (BCC), still studies evaluating the efficacy of chemopreventive agents during early stage/s of BCC development are lacking. Accordingly, utilizing the well-established patched (Ptch)+/- mouse model of ultraviolet B (UVB) radiation-induced BCC formation, we excised skin samples from UVB exposed Ptch+/- and Ptch+/+ mice prior to tumor formation to study the promotion/progression of BCC and to determine the efficacy and target/s of silibinin, a well-known skin cancer chemopreventive agent. UVB exposure for 1 month increased the number of mast cells in Ptch+/- mice by ~48% ($P<0.05$), which was completely inhibited by silibinin. PCR profiler array analysis of skin samples showed strong molecular differences between Ptch+/+ and Ptch+/- mice which were either unexposed or UVB irradiated +/-silibinin treatment. Most notably, silibinin treatment significant decreased the expression of BMP-2, Bbc3, PUMA and Ccnd1 in Ptch+/- mice irradiated with silibinin + UVB. Additional studies showed that silibinin targets UVB-induced expression of BMP-2 in Ptch+/- mouse skin. Lastly, our studies found that silibinin strongly attenuates UVB-induced BMP-2 expression and DNA damage in Ptch+/- mouse skin ex vivo only after single UVB exposure. Together, our results suggest a possible role of mast cell recruitment and BMP-2 activation in the early stages of BCC development; these are strongly inhibited by silibinin suggesting its possible chemopreventive efficacy against BCC formation in long-term UVB exposure regimen.

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Keywords

Basal cell carcinoma; Silibinin; Chemoprevention; Mast cell; Bone morphogenetic protein 2

Introduction

There are about 3.5 million new cases of non-melanoma skin cancers (NMSCs) diagnosed in the United States each year corresponding to an overall lifetime risk of 1 in 5 people [1]. NMSCs are composed mainly of basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs); BCCs account for about 80% of skin cancers while SCCs make up about 16%. Whereas the cure rates for NMSCs are up to 99%, treatments are associated with significant morbidity, recurrence, and high cost to the health care system. The most important risk factor for BCCs and SCCs is solar ultraviolet (UV) light exposure, which can initiate and promote skin carcinogenesis. The enormity of the disease as well as main role of UVB in NMSCs initiation, promotion and progression demand the development of effective chemopreventive measures which refer to the use of non-toxic natural, synthetic or biological agents to prevent, suppress, and reverse the carcinogenic progression [1]. Candidates for skin cancer chemoprevention include patients with a history of NMSCs and who are at high risk of developing more NMSCs, or are at risk for invasive or metastatic NMSCs. Additionally, patients with numerous Actinic Keratosis (AK), NMSCs in high-risk locations, metastatic NMSCs, and solid organ transplant recipients are potential candidates for skin cancer chemoprevention. There are four FDA approved drugs for treating precancerous lesions or reducing cancer risk of AKs which are the pre-cursor lesions to SCCs [2]; on the contrary there is no chemopreventive agent approved for use against BCCs. This may be due, in part, to the failure of mutagenic agents or chemicals to induce BCCs in a murine model as it does for SCCs, and due to lack of clear understanding of early stages of BCC.

Of the current available pre-clinical BCC models, the *Ptch1*^{+/-} mice present an excellent model mimicking the human disease condition. These mice develop similar tumors, have similar abnormalities in Hh activation, and the disease condition is developed in the same tissue (skin), and is induced through the same environmental insults (UV or IR) as in humans [3]. When *Ptch1*^{+/-} mice are irradiated with IR they develop BCCs and trichoepitheliomas, and when irradiated with UVB they develop macroscopic tumors including BCCs, SCCs, and fibrosarcomas [3]. In earlier studies we have reported the strong chemopreventive efficacy of silibinin, a naturally occurring flavonolignan obtained from Milk thistle (*Silybum marianum* L., Family Asteraceae), against chemical- and UVB-induced photocarcinogenesis [4–8]. Recently, we reported silibinin's efficacy against BCC cells in cell culture as well as in an allograft mouse model [9]. Importantly, Silibinin treatment also inhibited the growth of hedgehog inhibitor resistant BCC cells via targeting the EGFR-MAPK-Akt pathway and hedgehog signaling [10]. However, chemopreventive efficacy of silibinin against UVB-induced basal cell carcinogenesis has not been studied yet. Here, we accessed the chemopreventive effects of silibinin against UVB-induced early molecular alterations in skin in a transgenic mouse model (*Ptch*^{+/-}) of BCCs that closely represents the human condition [11]. We collected early time point skin samples to both

determine the major molecular players involved in BCCs promotion and to understand the mechanism of silibinin's chemopreventive effects against BCC. Our results suggest strong chemopreventive efficacy of silibinin against UVB-induced recruitment of mast cells as well as inhibiting the BMP2 expression, which might be responsible for BCC formation over the period of months of UVB exposure.

Materials and methods

Antibodies and reagents

The antibodies for BMP-2 and PUMA were from Abcam (Cambridge, MA). The toluidine blue, Harris hematoxylin, and Eosin were obtained from Sigma Aldrich (St. Louis, MO) as well as the GAPDH forward and reverse primers. The β -galactosidase staining set was purchased from Roche (Indianapolis, IN). The BMP-2 primers for qPCR were from Qiagen (Valencia, CA). The anti-thymine dimer antibody (CPD) was from Kamiya (Seattle, WA).

Ptch heterozygous mice

Ptch^{tm1Mps}/J mice were purchased from Jackson laboratories, herein referred to as Ptch^{+/-} mice. Breeding pairs were set up with Ptch^{+/+} females and Ptch^{+/-} males to generate both genotypes for the studies as previously published [12]. The male Ptch^{+/+} and Ptch^{+/-} mice were used for the studies reported here. Ear clippings were excised from each mouse and sent to Transnetyx, Inc (Cordova, TN) for genotyping of ptch heterozygosity as previously published [12].

Short term skin analysis

The UVB light source was four FS-40-T-12-UVB sunlamps with UVB Spectra 305 Dosimeter (Daavlin Co, Bryan, OH) emitting 80% radiation within 280 to 340 nm with a peak at 314 nm monitored with a SEL 240 photodetector, 103 filter, and 1008 diffuser attached to IL1400A Research Radiometer. Ptch^{+/+} and Ptch^{+/-} 6 to 8 week old mice were divided into the following groups: control (untreated/unexposed), UVB irradiated with 240 mJ/cm² 3 times per week on M, W, F; topically applied silibinin (9 mg/200 μ l) 5 times per week on M-F, topically applied silibinin for 30 minutes followed by UVB exposure M, W, F or no UVB on T and Th. Skin punch biopsies were collected once per month for 3 months. Mice were anesthetized prior and during the biopsy procedure with isoflurane, and had the hair shaven off the biopsy locale. Additionally, mice were administered carprofen subcutaneously prior to the procedure (5 mg/kg) to relieve any pain. Next, the biopsy site was cleaned with chlorohexidine followed by alcohol and a 6 mm punch biopsy was removed. A drop of bupivacaine was applied to the open site after making the skin punch. The wound was closed with a wound clip. A second biopsy was performed immediately after the first, on a separate part of the skin following a similar procedure as above. After 7 to 10 days the wound clip was removed. Following the surgical procedure, the mice were administered carprofen for 2 to 3 days. The UVB irradiation and silibinin treatments were continued following the surgical procedure. The skin punch biopsies were divided up to be formalin fixed, snap frozen or embedded in OCT for later processing.

Ear study

Ptch+/+ and Ptch+/- mice were sacrificed and ears were immediately excised from the mice. The dorsal and ventral portions of the ears were pulled apart. The ears were placed dermis side down onto media (DMEM with 10% FCS). The ears were separated into groups: Control (untreated, unexposed), UVB irradiated, pre-treatment with silibinin (2.25mg/50µl) topically on the ear for 1 h (prior to sacrificing the mouse) followed by UVB radiation herein referred to as Sb+UV in vivo, and silibinin pre-treatment with 100 µM in media for 1 h followed by UVB radiation herein referred to as Sb+UV ex vivo. The mouse ears were irradiated with UVB (100 mJ/cm²) and incubated overnight at 37°C. Twenty-four hours later the samples were fixed in formalin and slides were then processed for IHC and CPD staining as detailed below.

IHC

Paraffin-embedded sections (5 µm) of the skin and ear tissue were deparaffinized, rehydrated, and antigen retrieval was performed using sodium citrate buffer (0.01M; pH 6.0) for 20 min in a pressure cooker. Sections were quenched of endogenous peroxidase activity and incubated with primary antibodies: BMP-2 (1:500) or PUMA (1:100) or PBS (negative control) overnight. The next day, sections were incubated with appropriate secondary biotinylated antibody followed by streptavidin. Color development was achieved by incubation with DAB followed by counter staining with Harris hematoxylin. Microscopic analyses were conducted using a Zeiss Axioscope 2 microscope. Photomicrographs were captured using a Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. Other details pertaining to the IHC staining are described in recent studies [13,14]. Briefly, quantification of nuclear staining was done by counting brown-positively stained cells and total number of cells at 10 to 12 randomly selected fields at 400× magnification. Immunoreactivity (represented by intensity of brown staining) was scored as 0 (no staining), +1 (very weak but uniform cytoplasmic staining), +2 (weak but uniform cytoplasmic staining), +3 (moderate with peripheral tumor areas with strong patchy cytoplasmic staining) and +4 (strong with both nuclear and cytoplasmic staining). Quantification of IHC data is represented as mean ± SEM of 3–5 samples in each group.

CPD staining

Paraffin embedded sections (5 µm) of the skin tissue were deparaffinized, rehydrated and incubated in 1N HCl for 30 minutes at room temperature. Sections were washed in PBS and then incubated in proteinase K (20 µg/ml) for 30 min at 37°C. Afterwards sections were quenched of endogenous peroxidase activity then incubated in blocking solution Cas block (1:10) for 15 min. The sections were incubated in anti-thymine dimer antibody (Kamiya) at a dilution of 1:100 for 2 hours at room temperature. The color development was achieved by incubation in DAB followed by counterstaining with Harris hematoxylin. The number of positively stained cells was counted and total number of cells at 10 to 12 randomly selected fields at 400x magnification.

PCR profiler array

Total RNA was isolated from skin according to manufacturer's protocol for RNeasy fibrous tissue kit (Qiagen, Valencia, CA). Once RNA was isolated, a total of 250 ng of RNA per sample was used for reverse transcription using RT² First Strand kit (Qiagen). PCR array was done using Qiagen PCR array system (Valencia, CA). Real-time PCR was done with mouse signal transduction pathway finder PCR array (Qiagen) on the Applied Biosystems 7500 Real-time PCR system. The program was as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. In each group, three skin samples were used for PCR array analysis. Relative fold changes of gene expression were calculated according to the manufacturer's (Qiagen) instructions and the manufacturer's software was available online.

Statistical analysis

Statistical analysis was performed using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA) and Qiagen data analysis software for real time (RT)-PCR profiler array. Data was analyzed using analysis of variance and a statistically significant difference was considered at $P < 0.05$.

Results

Silibinin attenuates UVB-induced mast cells in *Ptch*^{+/-} mice

In order to study the promotional aspect of BCC induction we irradiated both *Ptch*^{+/-} and *Ptch*^{+/+} mice with UVB as detailed above and acquired three skin punch biopsies at 1, 2 and 3 month after first UVB exposure. Skin punch biopsies were analyzed for histological changes through H&E staining. In both groups, the UVB irradiated skin had a thickened epidermis compared to controls (Figure 1A). Additionally we found that the nuclei were more euchromatic and bigger in size than in control cells, they more closely resembled the basal layer nuclei. When mice were treated with silibinin (with or without UVB), the skin histology more closely resembled that of the unexposed control groups wherein the nuclei did become pyknotic and appeared smaller. The most profound histological effect we observed was upregulation of mast cell numbers with UVB irradiation; therefore, we next quantified this increase. Slides were stained with toluidine blue to facilitate mast cell identification. We found UVB treatment significantly increased the number of mast cells in *Ptch*^{+/-} mice by about 48% compared to control mice ($p < 0.05$) and silibinin treatment inhibited that effect significantly (Figure 1B and 1C). In *Ptch*^{+/+} mice we did not observe an increase in mast cells following UVB exposure (Figure 1B and 1C), suggesting this could be a specific pathway in the development of BCCs.

Differential expression profile of signal transduction pathways in *Ptch*^{+/+} and *Ptch*^{+/-} mice

To unravel the mechanism associated with the ability of UVB irradiated *Ptch*^{+/-}, but not *Ptch*^{+/+}, mice to develop BCCs, we conducted a PCR profiler array analysis of several genes involved in the signal transduction pathways in 1 month skin punch biopsies. We chose the 1-month time point because these mice are in the early tumor promotion stages of BCC development. A clustering analysis of the expression of 84 genes involved in signal

transduction of a variety of pathways showed strong differences between Ptch^{+/+} and Ptch^{+/-} mice that were unexposed, UVB irradiated and silibinin treated (Figure 2). The genes with higher correlation coefficients across different samples are clustered together by rows.

In order to understand baseline changes in Ptch^{+/+} and Ptch^{+/-} mice, the fold changes in Ptch^{+/-} mice were compared to wild-type mice (Table 1, top panel). There was a significant decrease in baseline expression levels of Cyclin-dependent kinase inhibitor 1A (P21, Cdkn1a), GADD45 β , Glutamate-cysteine ligase, catalytic subunit (Gclc), Heme oxygenase (decycling) 1 (Hmox1), Lactate dehydrogenase A (Ldha), Retinoblastoma 1 (Rb1), and Serine (or cysteine) peptidase inhibitor, clade E, member 1 (Serpine 1) in Ptch^{+/-} mice compared to wild-type mice. We next sought to compare expression levels of UVB irradiated Ptch^{+/+} and Ptch^{+/-} mice (Table 1, bottom panel). In Ptch^{+/-} mice the expression of BMP-2, Hairy/enhancer-of-split related with YRPW motif 1 (Hey1), and Inhibitor of DNA binding 1 (Id1) were significantly upregulated compared to Ptch^{+/+} mice. Additionally the expression of CCAAT/enhancer binding protein (C/EBP), delta (Cebpd), Hmox1, Leucine-rich alpha-2-glycoprotein 1 (Lrg1), Sorbin and SH3 domain containing 1 (Sorbs1), TNF, and WNT1 inducible signaling pathway protein 1 (Wisp1) were significantly downregulated in Ptch^{+/-} mice.

Silibinin targets UVB-induced expression of Lrg1 and several other genes in Ptch^{+/+} mice

The fold change of UVB irradiated wild-type mouse gene expression is shown in Table 2. UVB irradiation only induced the expression of 2 genes in wild-type mice in our gene set, those 2 genes were Lrg1 and Bcl2-associated X protein (Bax). Pathways involved with these target genes include STAT3 and p53; Lrg1 is induced by STAT3 while Bax is a pro-apoptotic protein induced by p53. In contrast, in wild type mice, UVB irradiation downregulated the genes: Acyl-CoA synthetase long-chain family member 3 (Acsl3), BMP-4, Ccnd2, Ferritin heavy chain 1 (Fth1), GADD45 β , Id1, Ldha, Rb1, Solute carrier family 2 (facilitated glucose transporter), member 1 (Slc2a1), Thioredoxin reductase 1 (Txnrd1), and WNT2b. Pathways involved with these target genes include PPAR, Hedgehog, WNT, oxidative stress, TGF β , Notch, Hypoxia, and p53. Ptch^{+/+} mice only develop SCCs when UVB irradiated, so the pathway changes observed could be attributed to promotion of SCCs carcinogenesis. Silibinin significantly attenuated the expression of Lrg1 in UVB treated wild-type mice. Other genes affected by silibinin with greater than 2 fold induction include increased expression of Carbonic anhydrase 9 (Car9) and Fos-like antigen 1 (Fosl1) and decreased expression of Hmox1, WNT3a, and WNT 6 (data not shown). The effects of these changes may be more important in SCC pathogenesis than BCC pathogenesis since BCCs do not form in the Ptch wild-type mice.

Silibinin targets UVB-induced expression of PUMA in Ptch^{+/-} mice

UVB radiation significantly induced expression of Ccnd1 and Glutamate-cysteine ligase, catalytic subunit (Gclc) in Ptch^{+/-} mice which could be related to BCCs pathogenesis (Table 3). Interestingly Ccnd1 is a downstream target gene of Hh signaling and WNT signaling pathways while Gclc is integral to oxidative stress signaling. Additionally, UVB irradiation decreased expression of Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (Herpud1) and TNF which are target genes for

the TGF β and TNF signaling pathways. Importantly, silibinin significantly inhibited expression of BMP-2, BCL2 binding component 3 (Bbc3, PUMA), and Ccnd1 in Ptch+/- mice irradiated with UVB (Table 3). Silibinin also attenuated gene expression for Car9, Erythropoietin (Epo), Fatty acid binding protein 1, (Fabp1), Hairy and enhancer of split 5 (Drosophila) (Hes5), Hey1, Hey2, Heyl, Hmox1, Interferon gamma (Ifng), MMP7, Thioredoxin 1 (Txn1), WNT1, WNT3a, and WNT6 (data not shown).

We next investigated the protein levels of PUMA (Bbc3) to see if protein expression changed similarly to that of the mRNA levels. PUMA was increased following UVB radiation in Ptch+/- mice, though not statistically significantly (Figure 3). Silibinin decreased the level of PUMA following UVB exposure, though not in a statistically significant manner.

Silibinin targets UVB-induced expression of BMP-2 in Ptch+/- mice

BMP-2 has shown both tumor promotion and tumor suppression characteristics; however the role BMP-2 plays in UVB-induced BCCs has not been investigated yet. We found strong upregulation of BMP-2 in Ptch+/- mice when compared to wild-type mice (Fig 2). Additionally UVB radiation further upregulated BMP-2 expression in Ptch+/- mice (1.4 fold) and silibinin was shown to attenuate that in our PCR profiler array (Fig 2). We sought to further confirm our PCR array results through RT-PCR analysis and found that BMP-2 mRNA expression was increased 3.6 fold in Ptch+/- mice compared to Ptch+/+ mice (Figure 4A). Additionally the UVB irradiated Ptch+/- mice expressed an even higher level of BMP-2 (5.05 fold change) when compared to UVB irradiated Ptch+/+ mice (2.2 fold change). Treatment with silibinin could bring those increased levels back down to 1.3 fold of the control level (similar to wild-type mice controls). Next we IHC stained the skin tissue to measure BMP-2 protein levels and found that BMP-2 expression was increased by 85% in Ptch+/- mice in comparison to wild-type mice (Figure 4B–C). Additionally we observed a similar trend in upregulation of BMP-2 by 45% ($p < 0.05$) in UVB irradiated Ptch+/- mice compared to Ptch+/+ mice. Lastly, we observed that UVB induced BMP-2 protein expression (by IHC) by 30% in Ptch+/- mice compared to control, and silibinin treatment decreased that expression by 14% (Figure 4B–C).

Silibinin strongly attenuates UVB-induced BMP-2 expression and DNA damage in Ptch+/- mouse skin ex vivo.

In order to understand how early BMP-2 is induced following UVB exposure, we irradiated both Ptch+/+ and Ptch+/- ex vivo mouse ears with a single dose of UVB and analyzed BMP-2 expression 24 hours later. UVB radiation induced BMP-2 expression, and silibinin treatment significantly reduced that response by 73% ($p < 0.05$, Sb+UV in vivo) and 39% (Sb +UV ex vivo) in Ptch+/- mice. Ptch+/+ mice had 87% ($p < 0.05$) less UVB-induced expression of BMP-2 than Ptch +/- mice (Figure 5A–B). Additionally, the level of BMP-2 with silibinin treatment was slightly increased in Ptch+/+ mice though not statistically significant, further suggesting BMP-2 to be important for BCC induction.

Lastly, we determined the amount of DNA damage present after a single UVB exposure by assessment of thymine dimer formation (CPDs). We found significantly less DNA damage

evidenced by CPD levels in UVB irradiated Ptch+/- mice compared to UVB irradiated wild-type mice (6.8 versus 22.9, $p < 0.001$) (Figure 5C–D). This may suggest that the repair capacity of Ptch+/- mice is faster than Ptch+/+ mice or the Ptch+/- mice had less DNA damage to begin with. A time-course study would need to be employed to investigate this further. Silibinin treatment decreased the percent of CPDs by 31% (Sb+UV in vivo) and 98% (Sb+UV ex vivo) ($p < 0.001$) in Ptch+/+ mice and 86% (Sb+UV in vivo) and 80% (Sb+UV ex vivo) in Ptch+/- mice ($p < 0.05$), showing strong efficacy in both groups.

Discussion

BCCs are the most common malignancy and chemopreventive efforts against these carcinomas have been unsuccessful so far. Therefore, we investigated the use of silibinin as a potential chemopreventive agent against the early stages of BCC formation. Indeed, we found a clear and strong efficacy of silibinin. This is an important finding since the pathogenesis of SCCs and BCCs differ greatly and these results provide evidence that silibinin can inhibit the formation of both types of NMSCs.

To elucidate the mechanism of silibinin's actions, we acquired skin punch biopsies from both the Ptch+/- and Ptch+/+ mice at 1–3 months following UVB exposure and respective silibinin treatments. First, we analyzed histological changes between groups and found an increase in epidermal thickness following UVB exposure as expected [15]. There was an increase in mast cell number following UVB exposure in Ptch+/- mice but not in Ptch+/+ mice. This is an interesting observation since mast cells and stem cell factor (SCF) are increased in BCCs but not SCCs, when compared to normal skin [16–18]; SCF promotes proliferation and differentiation of both immature and mature mast cells. This finding may suggest the importance of mast cells in BCCs pathogenesis. The effect of mast cells on the development of cutaneous malignancies (BCCs and melanoma) are most likely mediated via immunosuppression, enhancement of angiogenesis, disruption of ECM, and promotion of tumor mitosis [19]. Silibinin treatment significantly decreased the UVB-induced mast cell increase in Ptch+/- mice. Skin mast cells reside in the dermis and are not activated by UVB directly since UVB cannot penetrate into the dermis. UVB however could indirectly activate mast cells through two mechanisms that involves neuropeptides: (1) isomerization of the photoreceptor trans-urocanic acid (UCA) to cis UCA in the epidermis that promotes neuropeptide secretion which stimulates mediators from mast cells and (2) irradiated keratinocytes secrete nerve growth factor (NGF) which sustains release of neuropeptides from sensory nerves [20,21]. Further studies are warranted to examine if silibinin can also inhibit the activation of mast cells in addition to decreasing their numbers. Previous reports have shown silibinin to inhibit mast cell degranulation through NF- κ B [22], suggesting a potential role for silibinin to inhibit mast cell recruitment as well as mast cell activation.

A low level (~3%) of Ptch+/- mice develop microscopic BCCs sporadically starting at 2 months of age and chronic UVB radiation exponentially enhances the quantity of BCCs that occur, wherein 100% of mice develop microscopic BCC lesions after 6 months of chronic UVB irradiation [11]. Ptch gene normally functions to repress transcription of multiple target genes including Ptch, TGF β , and WNT. Therefore, when Ptch is mutated it can be deduced that Hh becomes activated and transcription of multiple target genes occurs. In our

studies we found that baseline expression of *Ptch*^{+/-} mice showed decreased mRNA of genes related to TGF β , oxidative stress, hypoxia and p53 signaling. Sonic Hh regulates hair follicle growth and morphology in the skin and its expression is localized to the hair follicles, which could explain why we didn't see relatively high Hh expression in controls since we were measuring the levels in the whole skin. Though, upon chronic UVB irradiation we observed significant increase in target genes associated with Hh, TGF β and Notch signaling (BMP-2, Hey1, Id1) in *Ptch*^{+/-} mice compared to *Ptch*^{+/+} mice. These changes may shed light into what contributes to BCCs tumor development.

In order to understand the changes that are important for SCC pathogenesis versus BCC pathogenesis, we analyzed the *Ptch*^{+/+} mice as a model for SCCs and *Ptch*^{+/-} mice as a model for BCC. The *Ptch*^{+/+} mice do not develop BCC lesions when irradiated chronically with UVB but they do develop SCCs lesions. The *Ptch*^{+/-} mice develop both BCCs and SCCs. Interestingly, a new target for SCCs development was found in these studies, wherein we found a significant increase in *Lrg1* levels in UVB irradiated *Ptch*^{+/+} mice when compared to un-irradiated mice. *Lrg1* has been found to be involved in cell proliferation, immune response, migration, apoptosis, and angiogenesis [23–25]. *Lrg1* is overexpressed in the serum or tumor tissues of a variety of cancers such as bladder, ovarian, biliary tract, non-small cell lung, and colon cancer [23,26–30]. Additionally, *Lrg1* has been shown to be induced by STAT3 and several studies have shown the importance of STAT3 in skin carcinogenesis [31]. STAT3 has been shown to be required for the development of DMBA-TPA tumors, since STAT3 deficient mice do not develop tumors following DMBA-TPA treatments [32]. Similarly, in UVB induced skin carcinogenesis, STAT3 overexpression increased tumor susceptibility and STAT3 deficiency lowered tumors when compared to wild-type mice [33]. The induction of *Lrg1* in the *Ptch*^{+/+} mice was significantly downregulated by silibinin. This suggests a new target of silibinin in UVB-induced (SCCs) skin carcinogenesis. Perhaps *Lrg1* is also a new target in SCCs development. Analyzing the *Lrg1* expression levels in SCCs of human origin would be of interest. It is important to note is that these changes we have cataloged in our studies were found in C57Bl/6 mice and may not be the same for other mouse models. The hairless mice, for example are more susceptible to tumorigenesis than haired mice, and elevated inflammation has been shown to play a role in that increased susceptibility [34].

The promotion of BCCs are not as well studied, as is the promotion of SCCs. Herein, we found significant changes in *Ccnd1* which was upregulated by UVB radiation in *Ptch*^{+/-} mice. *Ccnd1* is a direct target gene for Gli1, implying Hh signaling; *Ccnd1* is also a target of other signaling pathways. It is primarily involved in cell proliferation and plays a key role in regulating the G1/S phase transition of cell cycle progression. Importantly, silibinin was found to significantly downregulate its expression. Another interesting new finding was the upregulation of BMP-2 following UVB radiation. Bone morphogenetic proteins (BMPs) belong to the TGF β family which binds to cell surface receptors and can initiate cell signaling that control cell growth, differentiation, and migration [35]. BMPs elicit their cellular effects through specific type I and type II serine/threonine receptors. The type II receptors phosphorylate the type I receptors in a ligand dependent fashion. Once activated the type I receptors phosphorylate receptor specific SMADs such as R-SMAD 1/5/8 in the cytoplasm [36]. The signal is then transduced via the complex formed by R-Smad 1/5/8 and

Co-Smad4 and subsequently translocated to the nucleus to regulate the expression of specific BMPs target genes. Additionally BMPs can act via MAPK signaling pathways to exert their effects [36].

The BMPs roles in cancer are sometimes contradictory since they have been shown to be linked with carcinogenesis and tumor progression though sometimes also appear to act as tumor suppressors [37]. Mostly, BMPs appear to be involved in metastasis, EMT, altered cell behavior and angiogenesis in human cancer [38]. In the skin, BMPs appears to play an anti-tumor role, though the studies so far have been limited to chemical induced skin carcinogenesis models or transgenic mouse models that do not lead to BCCs [39]. In our studies, we found silibinin to significantly downregulate the UVB-induced expression of BMP-2. BMP-2 is expressed in basal keratinocytes in the embryonic epidermis and insufficient BMP signaling leads to hair follicle loss. A potential target of BMP-2 is SOX-9 as evidenced in fibroblast and osteosarcoma cells [40], and SOX-9 has been shown to be a marker for BCCs in humans [41]. SOX-9 is found to be expressed in sebaceous and sweat glands as well as in the outer root sheath of the hair follicles and hair stem cells. Another potential nuclear effect of the BMP pathway is through RUNX3, which has also been shown to be overexpressed in human BCCs. The question remains in our studies whether SOX-9 or RUNX3 are downstream effectors of BMP-2 and may additionally contribute to BCCs tumorigenesis.

These studies presented here confirm that the pathogenesis of BCCs and SCCs are indeed quite different. We did not observe overlap between pathways that were activated following UVB exposure in *Ptch*^{+/+} and *Ptch*^{+/-} mice. Pathways activated in *Ptch*^{+/-} mice following UVB exposure involved Hh and WNT signaling while in *Ptch*^{+/+} mice involved STAT3 and p53. A novel finding in this study is the role BMP-2 and *Ccnd1* may have in BCCs pathogenesis and that silibinin can regulate both of them. Additionally we found BMP-2 to be upregulated after a single UVB exposure in *Ptch*^{+/-} mice but not in *Ptch*^{+/+} mice which may suggest an early role of BMP-2 in the promotion of BCCs. BMPs may play a role as cytokines however it is yet to be investigated whether BMP-2 and the infiltration of mast cells are connected. Overall, these studies have shown that silibinin targets UVB-induced BMP-2 expression which might be a key event in its efficacy against BCC formation in future long-term studies. Additionally, we have shown the varying pathogenesis of SCCs and BCCs in our model system, and that silibinin has proven to be an efficacious multi-target agent, capable of chemoprevention for both types of NMSCs.

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Abbreviations:

AK	Actinic keratosis
BCC	Basal cell carcinoma
BMP2	Bone morphogenetic protein 2

C/EBP	CCAAT/enhancer binding protein
CPD	Cyclobutane pyrimidine dimers
Lrg1	Leucine-rich alpha-2-glycoprotein 1
NMSCs	Non-melanoma skin cancers
Ptch	Patched
SCCs	Squamous cell carcinomas
SCF	Stem cell factor
Sorbs1	Sorbin and SH3 domain containing 1
TNF	Tumor necrosis factor
UVB	Ultraviolet B
Wisp1	WNT1 inducible signaling pathway protein 1

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Figure 1. Effect of silibinin on the level of mast cells induced by UVB radiation. Skin punch biopsies were excised from Ptch^{+/+} and Ptch^{+/-} mice after 1 month of UVB exposure, fixed in formalin, and sections were cut as detailed in ‘Materials and Methods.’ Sections were stained for H&E and toluidine blue to quantify mast cells. Representative photographs are shown for (A) H&E and (B) mast cells. (C) Quantitative data for mast cells are shown with the mean $\pm$ SEM from 5 individual skin samples for each group. *P<0.05; NS: Not Statistically Significant

Figure 2.

The effect of silibinin on PCR array analysis of various signal transduction pathways. Skin punch biopsies were excised from *Ptch*^{+/+} and *Ptch*^{+/-} mice after 1 month of UVB exposure; RNA was isolated from skin and converted to cDNA as described in 'Materials and Methods.' RT-PCR CT values were normalized to housekeeping gene (B2M). Clustering analysis of the expression of 84 genes related to signal transduction of a variety of pathways. Genes with higher correlation coefficients across different samples are clustered together by rows. Each row represents a single gene labeled with the gene name whereas each column represents a group (3 samples per group). The color scale at the bottom represents the magnitude of gene expression. Expression levels greater than the mean are shaded in red and those lower than the mean are shaded in green.

Figure 3.

The effect of silibinin on UVB-induced PUMA expression. Ptch^{+/-} skin punch biopsies after 1 month of UVB exposure from control and treated groups were subjected to IHC analysis. **(A)** Representative photographs are shown for PUMA. **(B)** Quantitative data for PUMA showing the mean ± SEM from 3 individual skin samples for each group. NS: Not Statistically Significant

Figure 4.

The effect of silibinin on UVB-induced BMP-2 expression in Ptch^{+/+} and Ptch^{+/-} mice. **(A)** Ptch^{+/+} and Ptch^{+/-} mice skin punch biopsies after 1 month of UVB exposure from control and treated groups were analyzed for mRNA level of BMP-2 through RT-PCR analysis. The relative fold change are shown in comparison to Ptch^{+/+} control. The skin punch biopsies were also analyzed for BMP-2 protein expression via IHC analyses. **(B)** Representative photographs are shown for BMP-2. **(C)** Quantitative data for BMP-2 showing the mean $\pm$ SEM from 3 individual skin samples for each group. * $P < 0.05$, ** $P < 0.001$ versus as labeled in the figure.

Figure 5.

The effect of silibinin on UVB-induced BMP-2 expression and DNA damage in Ptch^{+/+} and Ptch^{+/-} mice. Ears were excised from mice and separated into dorsal and ventral parts. The ears were placed dermis side down and exposed to 100 mJ/cm² UVB, cultured for 24 hours, and then fixed in formalin. Mice were treated with silibinin either before sacrificed topically (2.25 mg/50 μ l) on the ear (Sb+UV_A) or in media (100 μ M) after the ears were excised (Sb+UV_B) and then exposed to UVB 1 hour later. The ears were analyzed for BMP-2 protein expression and thymine dimer formation (CPDs) via IHC analyses. Representative photographs are shown for (A) BMP-2 and (C) CPDs. Quantitative data for (B) BMP-2 and (D) CPDs showing the mean $\pm$ SEM from 4 ears per group. *P< 0.05, **P<0.001 versus as labeled in the figure.

Table 1:

Baseline and UVB induced fold changes in Ptch+/- mice

Baseline fold change in control Ptch+/- compared to control Ptch+/+ mice			
Gene	Fold Change	p value	Pathway
Cdkn1a	0.499	0.013	TGFβ signaling
Gadd45b	0.423	0.016	TGFβ signaling
Gclc	0.579	0.032	Oxidative stress
Hmox1	0.505	0.028	Oxidative stress
Ldha	0.485	0.040	Hypoxia
Rb1	0.649	0.005	p53 signaling
Serpine1	0.367	0.034	Hypoxia signaling
Fold change in UVB exposed (+/-) mice in comparison to UVB exposed (+/+) mice.			
Gene	Fold Change	p value	Pathway
Bmp2	2.147	0.015	Hedgehog, TGFβ
Cebpd	0.524	0.033	Stat3 induced
Hey1	2.855	0.046	Notch
Hmox1	0.224	0.036	Oxidative stress
Id1	1.768	0.003	Notch
Lrg1	0.169	0.000	Stat3-induced
Sorbs1	0.501	0.027	PPAR signaling
Tnf	0.447	0.034	NF-κB signaling
Wisp1	0.663	0.029	Wnt signaling

Table 2:

Relative fold changes in UVB irradiated and silibinin treated Ptch+/+ mice

Fold change in UVB exposed (+/+) mice in comparison to control (+/+) mice.			
Gene	Fold change	p value	Pathway
Acsl3	0.706	0.031	PPAR signaling
Bax	1.898	0.026	p53
Bmp4	0.522	0.012	Hedgehog
Ccnd2	0.587	0.032	Wnt
Fth1	0.714	0.040	Oxidative stress
Gadd45b	0.509	0.030	TGFβ
Id1	0.637	0.019	Notch signaling
Ldha	0.637	0.046	Hypoxia signaling
Lrg1	2.259	0.027	Stat3 induced
Rb1	0.514	0.035	p53
Slc2a1	0.500	0.007	Hypoxia
Txnrd1	0.658	0.019	Oxidative stress
Wnt2b	0.472	0.005	Hedgehog
Fold change in silibinin treated (+/+) mice compared to UVB exposed (+/+) mice.			
Lrg1	0.425	0.045	Stat3-induced

Table 3:

Relative fold changes in UVB irradiated and silibinin treated Ptch+/- mice

Fold change in UVB exposed (+/-) mice in comparison to control (+/-) mice.			
Gene	Fold change	p value	Pathway
Ccnd1	2.957	0.017	Hedgehog, Wnt
Gclc	1.331	0.020	Oxidative stress
Herpud1	0.547	0.035	TGFβ
Tnf	0.398	0.030	NF-κB
Fold change in silibinin treated (+/-) mice in comparison to UVB exposed (+/-) mice.			
Gene	Fold change	p value	Pathway
Bbc3 (PUMA)	0.238	0.016	p53
Bmp2	0.147	0.003	Hedgehog, TGFβ
Ccnd1	0.475	0.008	Hedgehog, Wnt