Antitumoral effects of calcitriol in basal cell carcinomas involve inhibition of Hedgehog-signaling and induction of vitamin D receptor-signaling and differentiation

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Abstract

Activation of the Hedgehog (Hh)-signaling pathway due to deficiency in the Hh receptor Patched1 (Ptch) is the pivotal defect leading to formation of basal cell carcinoma (BCC). Recent reports provided evidence of Ptch-dependent secretion of vitamin D3-related compound, which functions as an endogenous inhibitor of Hh-signaling by repressing the activity of the signal transduction partner of Ptch, Smoothened (Smo). This suggests that Ptch deficient tumor cells are devoid of this substance, which in turn results in activation of Hh-signaling. Here we show that the application of the physiologically active form of vitamin D3, calcitriol, inhibits proliferation and growth of BCC of Ptch mutant mice in vitro and in vivo. This is accompanied by the activation of the vitamin D receptor (Vdr) and induction of BCC differentiation. In addition, calcitriol inhibits Hh-signaling at the level of Smo in a Vdr-independent manner. The concomitant antiproliferative effects on BCC growth are stronger than those of the Hh-specific inhibitor cyclopamine, even though the latter more efficiently inhibits Hh-signaling. Taken together, we show that exogenous supply of calcitriol controls the activity of two independent pathways, Hh- and Vdr-signaling, which are relevant to tumorigenesis and tumor treatment. These data suggest that calcitriol could be a therapeutic option in the treatment of BCC, the most common tumor in humans.
Introduction

The Hh-signaling pathway regulates cell differentiation, organ patterning, and cell proliferation (1). In the absence of a ligand the activity of the Hh-pathway is inhibited due to an interaction between the Hh receptor Ptch and its partner Smo. Binding of Hh to Ptch, inactivating Ptch mutations, or activating Smo mutations may suspend this inhibition, resulting in the transcription of target genes including Gli1 (1). Thus aberrant (e.g. mutation-driven) Hh-signaling results in tumor formation (2). One prominent example is BCC, the most frequent tumor entity in humans, with active Hh-signaling due to mutations in Ptch.

Today the inhibition of the Hh-pathway is considered as a promising strategy in the treatment of these tumors. Thus specific Smo inhibitors such as cyclopamine and GDC0449 have been tested in several small, non-randomized clinical trials (2). GDC0449 was recently shown to elicit antitumoral effects in 18 out of 33 patients with locally-advanced or metastatic BCC (3). It remains to be elucidated if these promising results will be confirmed in a prospective, randomized and controlled study and if Hh targeting alone will be sufficient. In this context, a recent report describes resistance to GDC0449 due to a therapy-associated Smo mutation (4). As with other tumors, therapy of those associated with abnormal Hh-signaling may require targeting of additional signaling pathways.

Vitamin D₃ and its derivatives (e.g. EB1089; Paricalcitol) are known to have antitumoral effects on different cancer types (5) including squamous cell carcinoma (6, 7) or on hyperproliferative skin diseases such as psoriasis (8, 9). These effects comprise G0/G1 arrest, cellular differentiation, induction of apoptosis and modulation of inflammation or of different signaling pathways in tumor cells, as well as inhibiting tumor angiogenesis (5). Until now, the antitumoral effects of vitamin D₃ are explained by binding of the biologically active form of
vitamin D$_3$, calcitriol (1α,25-dihydroxy vitamin D$_3$), to the Vdr and the subsequent regulation of Vdr-bound genes.

Calcitriol is produced from vitamin D$_3$ by two hydroxylation steps in the liver and kidney, respectively, and to a lesser extent in other organs and in tumor cells (5). Through binding to Vdr, calcitriol regulates the transcription of Vdr target genes (5). This so-called “genomic” calcitriol/Vdr-signaling regulates a variety of physiological processes including cellular differentiation, especially in the skin (10, 11), proliferation, and apoptosis, and can be monitored by measuring transcription of the calcitriol metabolizing enzyme 24-hydroxylase (5). In addition, calcitriol elicits rapid, so-called “non-genomic” (i.e. transcription-independent) effects such as calcium influx (5).

Recent data suggest a crosstalk between vitamin D$_3$ and Hh-signaling. By medium transfer experiments Bijlsma et al. provided first evidence for a Ptch-dependent secretion of vitamin D$_3$ compounds. They also showed that vitamin D$_3$ inhibits the Hh-pathway at the level of Smo (12). This suggests that, in addition to or instead of a direct protein-protein interaction, Ptch may repress Smo via secretion of a vitamin D$_3$ derivative (12).

The possibility of a Ptch-dependent secretion of a vitamin D$_3$ derivative with Smo-inhibitory properties opens new perspectives for therapies of tumors that arise due to mutations in Ptch. One would expect that inactivation of Ptch results in a disrupted secretion of this derivative. This should result in a potential deprivation of the vitamin D$_3$ derivative, and in lack of Smo-inhibition. If this hypothesis is true, the concerted action of active Hh- and inactive Vdr-signaling may be the driving forces leading to enhanced cell proliferation, compromised differentiation and ultimately to tumor formation. It follows that it should be possible to revert or at least to impede these processes by administration of the respective vitamin D$_3$ derivative.
Here we investigated the effect of the biologically active vitamin D₃ derivative calcitriol on Vdr- and Hh-signaling, growth, apoptosis and differentiation of Ptch-deficient BCC cells in vitro and in vivo using the Ptch<sup>flax/flax</sup> Rosa26CreERT<sup>2+/−</sup> (Ptch<sup>flax/flax</sup> ERT2<sup>2+/−</sup>) mouse model for BCC (13). In addition, we compared its effects with those of cyclopamine and sought to unravel the molecular mechanisms underlying the calcitriol-mediated effects on Hh-signaling.
Material and methods

Compounds

Calcitriol (Sigma-Aldrich, Germany) and cyclopamine (Toronto Research Chemicals Inc., Canada) were dissolved in ethanol (EtOH). Final concentrations for in vitro experiments are indicated in the respective experiments and correspond to those normally used in cell culture (5, 14, 15). For in vivo use, calcitriol was diluted individually for each animal in 20 µl EtOH/1200 µl sterile sunflower oil (Sigma-Aldrich) to obtain a final concentration of 40 or 100 ng/kg in 50 µl.

Animals and treatment of tumor-bearing Ptchflx/flx ERT2+/− mice with calcitriol

Conditional Ptchflx/flx ERT2+/− mice were randomized into 2 groups and BCC were induced in all animals by intramuscular injection of 100 µg tamoxifen as described (13, 16). Tumors of this animal model lack the expression of wt Ptch alleles and are therefore deficient in Ptch (13, 17). Starting points of the daily intraperitoneal (i.p.) treatment with 100 ng/kg calcitriol or vehicle were day 0 or 60 after BCC induction. Treatment of each cohort was conducted until day 90 after BCC induction. For RNA isolation and histological examinations skin samples were collected from tails 24 h after the last calcitriol injection.

Mice were fed with calcium- and phosphate-reduced and vitamin D3-free food (ssniff Spezialdiäten, Soest, Germany; E15312-14) (18) one week before and during the injection period, and for one week thereafter.

All animals were treated and housed in accordance with the German animal protection law.

Measurement of tumor size

BCC size was measured on hematoxylin and eosin (H&E) stained sections using the area calculation tool of the software CellF (Olympus Soft Imaging Solutions GmbH; Germany).
Analysis of calcium blood serum values

100 µl of blood were collected from the retroorbital plexus. Serum calcium concentrations were measured using an O-cresolphthaleine-based assay (cobas, Roche Diagnostics GmbH, Germany).

Histopathology and Immunohistochemistry

BCC and normal skin (NS) from the tail were embedded in paraffin for histological analyses or were used for isolation of total RNA. The identity of BCC was confirmed by examination of H&E stained sections. Paraffin sections were stained using an anti-Ki67- and anti-active caspase 3-antibody as described (19).

Cell lines and primary cell culture of BCC

The fibroblast cell line PtcHFlox/floxERT2+/− was established from dermis of a PtcHFlox/floxERT2+/− mouse. PtcH−/− cells are stable PtcH-deficient cells derived from tamoxifen-treated PtcHFlox/floxERT2+/− fibroblasts. Smo−/− and Vdr−/− fibroblasts have been described in (20) and (21), respectively. All fibroblast cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal calf serum (FCS) and 1% penicillin/streptomycine (PS).

The murine BCC cell line ASZ001 was established from UV-induced BCC of PtcHneo12/+ mice and maintained as described (22). All cell lines have been tested and authenticated before using by genotyping PCRs on genomic DNA as described (13, 20, 21).

Skin punches were isolated from BCC-bearing PtcHFlox/floxERT2+/− mice 30, 40 and 60 days after tumor initiation by tamoxifen. Punches were maintained for 10 days in culture as reported (16).

Cell culture experiments
For gene expression analysis or 5-bromo-2'-deoxyuridine (BrdU) incorporation and caspase assays, 100,000 or 4,000 cells/well were seeded in 6-well- and 96-well-plates, respectively. For siRNA-mediated knockdown of Vdr expression, PtcH^{flor/flor} ERT2^{+/−} cells were transfected with Vdr-specific or control siRNA as described below. After 24 h, the cells were washed and incubated for additional 48 h with calcitriol, cyclopamine or ethanol as indicated in the respective experiments.

Cell proliferation was measured after BrdU-pulsing for the last 22 h using a Cell Proliferation BrdU ELISA (Roche Diagnostics GmbH).

Activity of Caspase 3 and 7 was measured using the Caspase-Glo® 3/7 Assay (Promega) and a microplate reader (Biotek instruments inc., Bad Friedrichshall, Germany) according the manufacturer’s instructions.

Shh-N-conditioned medium (Shh-N-CM) or respective control medium (CoM) were obtained from HEK293-Shh (HEK293-Shh express one of the 3 mammalian Hh proteins Sonic hedgehog (Shh)) or HEK293 cells, respectively, as described (14).

Knockdown of Vdr expression in PtcH^{−/−} and PtcH^{flor/flor} ERT2^{+/−} cells was achieved by using a Vdr-specific short interfering RNA (siRNA) (5'-CAGGCGGAGCATGAAGCGCAA-3'). Scrambled siRNA (AllStars negative, Qiagen, Germany) was used as control siRNA. 75 ng siRNA and 4.5 µl HiPerFect (Qiagen, Germany) were mixed with 100 µl DMEM and incubated for 10 minutes. Simultaneously, 15,000 cells/well were seeded in DMEM/10% FCS/2% PS in 24-well-plates. Subsequently, the siRNA/HiPerFect-mix was added to the cells. After 24 h the cells were incubated with the respective media (as indicated in the experiments) supplemented with vehicle or 10 nM calcitriol. After additional 48 h the cells were collected and used for subsequent experiments.

For activation of Hh-signaling pathway in Smo^{−/−} fibroblasts the cells were transfected with a plasmid expressing hSMO (15, 23) using RotiFect following the manufacturer’s instructions.
After 6 h the cells were incubated with media containing 10 nM calcitriol, 5 µM cyclopamine or ethanol for 48 h.

Data shown are representative for at least three independent experiments each performed in triplicate.

Reverse transcription, RT-PCR and quantitative real time-PCR-analyses

Total RNA was extracted using TriReagent (Sigma-Aldrich). For skin samples the RNeasy fibrous tissue mini kit (Qiagen, Germany) was used. Synthesis of cDNA and primer combinations for amplification of 18S rRNA, Gli1 and Keratin10 (K10) transcripts used for quantitative real-time PCR (qRT-PCR) were described previously (16, 19). Primer pairs used for quantitative real time-PCR-(qRT-PCR) analysis of Gli2, Vdr, Cyp24a1 and transglutaminase 1 (Tgm1) were 5’-GCAAGGTCAAGACTGAGGCTGA-3’/5’-GCTGCTCCTGTGTGTGCTATCTCTTT-3’; 5’-AGAACATGTGCTGCTCATGGC-3’/5’-TCATCTTGGCGTAGAGCTGGTGGCT-3’; 5’- GTGTGGCAAGCGCACACGCT-3’/5’-CCGTGACAGCAGCGTACAGT-3’ and 5’-GCAGTGGTGTAAATGCAGCTGG-3’/5’-ATGAGGAGCTCAAGGGCAATGC-3’, respectively.

Amplification of 18S rRNA was performed as an endogenous control for the normalization of target gene expression. The amount of target and endogenous reference was determined using the relative standard curve method. Each sample was measured in triplicates. Graphs represent the mean value of all measurements.

Glycerol-3-phosphate dehydrogenase (mGapd), wildtype (wt) Ptch transcripts (derived from the non-recombined Ptch<sub>fl</sub>ox locus) and Ptch<sub>del</sub> transcripts were detected by RT-PCR as described in (13, 24).

Protein extraction and Western blot
*P*tc*h*\(^{-/-}\) fibroblasts were transfected with *Vdr*-specific siRNA or control siRNA as described. After 72 h the cells were harvested. Nuclear fractions of the transfected cells and of *Vdr*\(^{-/-}\) fibroblasts were isolated using the kit NE-PER Nuclear and Cytoplasmatic Extraction Reagents (Thermo scientific, Rockford, IL, USA) according to the manufacturer’s instructions. Protein concentrations were measured by Pierce BCA Protein Assay Kit (Thermo scientific) and 16.5 µg of each protein sample of the nuclear fraction were analysed by western blot using a rabbit anti-Vdr antibody (1:100; clone 9A7; Abcam, UK) and a mouse anti-heat shock 70 kDa protein 8 (HSC-70) (1:10,000; Santa Cruz, Germany) in combination with horseradish peroxidase (HRP)-conjugated goat anti rat IgG (1:10000; Thermo scientific) and rabbit anti-mouse IgG (1:5000; GE Healthcare, Germany), respectively. Signals were detected by using ECL+ reagent (GE Healthcare).

**Statistics**

Mann-Whitney-U testing was performed to determine the significance of the results.
Results

Calcitriol activates the Vdr-pathway, suppresses Hh-signaling and inhibits proliferation of cultured Ptch mutant BCC cells

As human BCC (5, 25-27), BCC from Ptch\textsuperscript{flax/flax}ERT2\textsuperscript{+/−} mice express elevated Vdr levels compared to normal murine skin (Fig. 1A).

To test the response of Ptch deficient murine BCC cells to calcitriol \textit{in vitro}, BCC-bearing skin punches were collected 30, 40 or 60 days after BCC initiation in Ptch\textsuperscript{flax/flax}ERT2\textsuperscript{+/−} mice (see material and methods). The punches were cultured with 10 nM calcitriol or with 10 µM cyclopamine for 10 days (the structures of cyclopamine and calcitriol are provided in Fig. 1B). Gli1 expression levels served to monitor Hh-signaling activity. Cyp24a1 (24-hydroxylase) transcripts were measured to estimate activation of Vdr.

Calcitriol led to a significant induction of Cyp24a1 transcription (Fig. 1C), which was consistent with the presence of Vdr in BCC (Fig 1A). In addition, calcitriol decreased Gli1 expression, thus indicating an inhibition of the Hh-signaling pathway. As expected, cyclopamine did not induce Cyp24a1 transcription but repressed Gli1 expression. The repressive effect was more pronounced than that achieved with calcitriol (Fig. 1C).

Next, we assessed the antiproliferative effects of calcitriol. As revealed by anti-Ki67 antibody staining, calcitriol inhibited proliferation of tumor cells in BCC-bearing skin punches (Fig. 1D). Most interestingly, the antiproliferative effect of calcitriol was more pronounced than that achieved with the Hh-specific inhibitor cyclopamine (Fig. 1D).

Similar results were obtained, when the BCC-derived cell line ASZ001 was incubated with 10 nM calcitriol or with 10 µM cyclopamine for 48 h. As shown in figure 1E both substances efficiently inhibited Hh-signaling. However, only calcitriol significantly inhibited BrdU-incorporation in these cells (Fig. 1F). Inhibition of proliferation apparently was not
accompanied by an increase in apoptosis, as calcitriol had no effect on caspase 3/7 activity (Fig. 1G).

Together, these results show that calcitriol efficiently inhibits the Hh-signaling pathway and activates Vdr-signaling in BCC. In contrast to cyclopamine only calcitriol mediated antiproliferative effects in BCC, even though cyclopamine more efficiently inhibits Hh-signaling.

**Calcitriol treatment of Ptch mutant mice inhibits the Hh-signaling pathway and growth of BCC and stimulates BCC differentiation**

Next the *in vivo* antitumoral effects of calcitriol were tested in the *Ptch*\(^{\text{flx/flx}}\) ERT2\(^{+/-}\) mouse model for BCC. In this model, where all mice develop full-blown BCC 90 days after activation of ERT2 recombinase by tamoxifen (13), treatment can be commenced at a specified time after tumor induction and at a defined age of the animals.

Preliminary studies, in which mice were treated daily with 40 ng/kg calcitriol starting at the day of tamoxifen injection for 90 subsequent days (days 0-90), did not result in an induction of Vdr-signaling or differentiation (data not shown). Therefore, we increased the daily calcitriol dose to 100 ng/kg. Calcitriol treatment was started either immediately (day 0) or 60 days after BCC initiation (\(n_{60-90d}=6; n_{0-90d}=4\)). Vehicle treated animals (\(n_{\text{vehicle}}=17\)) served as controls. The treatment was continued until day 90, when all mice were sacrificed (see Fig. 2A).

Calcitriol therapy led to a significant increase in serum calcium concentrations (Fig. 2A) without causing weight loss, hypercalcemia-driven kidney damage or signs of nephrocalcinosis (data not shown). This indicates that the treatment induced calcitriol-specific systemic effects without causing toxicity. Tumor areas on H&E-stained skin sections in mice treated on days 0-90, but not on days 60-90, were significantly decreased when compared to
the vehicle treated group (Fig. 2B, C). Furthermore, calcitriol inhibited tumor cell proliferation as measured by Ki67+ cells in tumors treated on days 0-90 (Fig. 2D). This was accompanied by a significant decreased expression of Hh-pathway target genes Gli1 and Gli2 (28-30) in BCC treated for days 0-90 (Fig. 2E). Consistent with the results from our in vitro studies no significant increase of caspase 3 positive BCC cells were observed (data not shown). Moreover, calcitriol treatment resulted in a substantial activation of Vdr-signaling as revealed by an enhanced expression of the immediate Vdr-target gene Cyp24a1 (Fig. 2E). Finally, a significantly increased expression of the keratinocyte differentiation markers and Vdr-target genes Tgm1 and K10 were detected (Fig. 2F). Whereas both differentiation markers can be induced by active Vdr-signaling, K10 expression also depends on Hh-signaling activity (10, 29, 31-34).

In summary, our data demonstrate that calcitriol significantly inhibits proliferation and induces cellular differentiation of Ptch-associated BCC in vivo. Moreover, this response is accompanied by activation of Vdr- and inactivation of the Hh-signaling pathways.

**Calcitriol inhibits Hh-signaling downstream of Ptch but upstream of Gli1**

Vitamin D3 has been shown to inhibit Hh-signaling at the level of Smo (12, 35, 36). In order to investigate whether the mechanism of Hh-pathway inhibition mediated by calcitriol is similar to that of Vitamin D3 we made use of PtchfloxfloxERT2+/- and Smo-/- fibroblasts. PtchfloxfloxERT2+/- fibroblasts normally express wt Ptch transcripts from the Ptchfloxfloxfloc allele. Upon tamoxifen-induced activation of ERT2 recombinase the exons 8 and 9 of the floxed Ptch alleles are excised. This results in the expression of an aberrant, non-functional Ptch transcript (Fig. 3A).

In PtchfloxfloxERT2+/- fibroblasts both, the treatment with Shh-N-CM or the tamoxifen-induced Ptch mutation result in activation of Hh-signaling as revealed by induction of Gli1 expression (Fig. 3B). Co-incubation of Shh- or tamoxifen-treated PtchfloxfloxERT2+/- fibroblasts with
calcitriol significantly inhibited Gli1 expression (Fig. 3B). Similar results were obtained with the validated Smo inhibitor cyclopamine (Fig. 3B). These data show that similar to cyclopamine, calcitriol inhibits Hh-signaling downstream of Ptch.

The effects of calcitriol were also tested in Smo−/− cells, which express basal Gli1 levels. Due to lack of Smo, Hh-signaling cannot be activated in these cells by incubation with Shh-conditioned medium (37). However, in these cells Hh-pathway activity can be restored by transfection with a hSMO expression plasmid.

As shown in figure 3C, calcitriol did not influence basal Gli1 expression levels in Smo−/− cells (Fig. 3C). However, calcitriol efficiently inhibited Hh-signaling after restoration of Hh-pathway activity upon transfection with hSMO. These results are similar to those obtained with cyclopamine (Fig. 3C) and show that calcitriol normally inhibits Gli1 expression at the level of Smo.

**Calcitriol inhibits Hh-signaling in a Vdr-independent manner**

Next, we tested whether calcitriol-mediated inhibition of the Hh-signaling pathway is independent of Vdr-signaling. For this purpose we used Vdr−/− fibroblast (21). In these cells Vdr-signaling is completely abrogated, since incubation with calcitriol does not result in the induction of the Vdr-target Cyp24a1 (Fig. 4A). As revealed by Gli1 expression, incubation of the cells with Shh-N-CM resulted in induction of Hh-pathway activity (Fig. 4A), which was significantly inhibited by addition of calcitriol or by the control substance cyclopamine (Fig. 4A). Similar results were achieved in Ptc−/− or Shh-N-CM-treated Ptc−/− ERT2 +/+ cells after siRNA-mediated Vdr knockdown. Efficient downregulation of Vdr expression was verified by western blot (Fig. 4B), qRT-PCR and by a significantly lower Cyp24a1 expression after calcitriol treatment in comparison to the controls (Fig. 4C, D). Whereas Vdr knockdown per se did not significantly attenuate Hh-pathway activity, treatment with calcitriol resulted in
a downregulation of *Gli1* expression (Fig. 4C, D). These data show that calcitriol-mediated inhibition of the Hh-signaling pathway occurs independently of the Vdr. Taken together, these data demonstrate that calcitriol inhibits Hh-pathway activity downstream of *Ptch* at the level of Smo in a Vdr-independent manner.
Discussion

The active form of vitamin D₃, calcitriol, is rapidly gaining importance in oncology due to its antiproliferative and differentiation-inducing effects combined with its low toxicity (5). Our work extends the range of calcitriol tumor targets to those induced by an abnormal activity of the Hh-signaling pathway (summarized in Fig. 5). Specifically, we show that calcitriol inhibits growth of BCC induced by deletion of the Hh receptor Ptch. This is accompanied by the expression of relevant cell differentiation markers. Our data suggest that calcitriol could be a valuable supplement or even alternative to the established treatments of BCC, the most common tumor in humans, associated with aberrant Hh-pathway activity.

As assessed by reduced Gli1 transcription, calcitriol inhibits canonical Hh-signaling independently of Vdr-signaling and downstream of Ptc (Fig. 5). An obvious molecular target of this Vdr-independent effect of calcitriol is Smo, since Smo-deficient cells (unlike those reconstituted with Smo or Ptc-deficient ones) show no decreased Gli1 transcription in response to this substance. A similar observation has been made for the inactive form of calcitriol, vitamin D₃ (12). Nevertheless, whether calcitriol directly binds to Smo should be addressed in future studies.

Besides inhibition of Hh-signaling pathway, calcitriol inhibits proliferation and increases the expression of skin differentiation marker in BCC. The latter effects are also induced by calcitriol in skin of patients with hyperproliferative skin diseases such as psoriasis (6-9, 34, 38). Calcitriol treatment specifically results in inhibition of proliferation of psoriatic skin and induction of differentiation of keratinocytes (9, 39, 40). Similarly to BCC, psoriatic skin also expresses high levels of the Hh-target gene Gli1 (41). These facts raise the question whether calcitriol mediates its antiproliferative effects and differentiation stimuli via activation of Vdr-signaling or rather via inhibition of the Hh-signaling pathway. A clue might come from our
present study: Our *in vitro* study shows that calcitriol has a significantly stronger antiproliferative effect on BCC than the pure Smo inhibitor cyclopamine, even though the latter substance inhibits Hh-signaling more efficiently (see Fig. 1D and 1F). Together with the fact that both calcitriol (present study) and cyclopamine (42) inhibit BCC proliferation *in vivo*, it is possible that calcitriol exerts its antiproliferative effects via both signaling pathways (e.g. by inhibition or activation of Hh- or Vdr-signaling, respectively). On the other hand, differentiation of calcitriol-treated BCC is probably unrelated to inhibition of the canonical Hh-signaling pathway because cylopamine never has been reported to induce the expression of keratinocyte differentiation markers in BCC. More likely, calcitriol induces BCC differentiation via Vdr-signaling, which is supported by the increased expression of the Vdr-target genes *Cyp24a1* and *Tgm1* (see Fig. 2F).

Antiproliferative and Hh-signaling inhibitory properties have also been described for the inactive form of calcitriol, vitamin D₃, on murine BCC *in vitro* and *in vivo* (35). In contrast to calcitriol, vitamin D₃ did not affect differentiation in BCC, although it was topically applied at high concentrations. Supposedly the treatment period using vitamin D₃ (i.e. 30 days) may have been too short to induce Vdr-signaling and thus a differentiation response. This suggestion is based on a comparison with our study, in which a 30 days calcitriol application also had no significant effects on these processes (see BCC treated for days 60-90, Fig. 2B-E).

Finally, a new model of tumorigenesis driven by *Ptch*-deficiency may emerge from our study. According to a recent work, *Ptch* might function as an efflux pump for vitamin D₃-related compounds with Hh-inhibitory properties (12). A deficiency of this compound due to *Ptch* inactivation would pathologically activate Hh-pathway and reduce Vdr-signaling. *Vice versa* application of this compound should result in inhibition of Hh-pathway and activation of Vdr-
signaling (see Fig. 5). Whether this vitamin D₃-related compound is calcitriol remains to be analyzed in the future.

Taken together the application of calcitriol holds promises as an effective anti-cancer drug in the treatment of BCC. Due to its dual effects on both Vdr- and Hh-signaling it may be superior to substances which solely target the Hh-signaling pathway. Calcitriol treatment may also be superior to application of vitamin D₃, which has to be metabolized before activating Vdr-signaling. The benefits of topical application of calcitriol in treatment of BCC have to be tested in the future.
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Figure legends

Figure 1: Calcitriol inhibits proliferation and the Hh-signaling pathway of BCC-bearing skin punches and of the BCC cell line ASZ001.
(A) Vdr expression levels of BCC (n=3) of Ptchflox/flaxERT2+/− mice 90 days after tumor induction compared to normal skin (NS; n=3). (B) Chemical structures of calcitriol and cyclopamine. (C) Gli1 and Cyp21a1 expression levels of cultured BCC-bearing skin punches isolated from Ptchflox/flaxERT2+/− mice 30, 40 or 60 days after BCC induction. (D) Ki67+ BCC cells of the respective punches. (E) Gli1 and Cyp21a1 expression levels and (F) BrdU-incorporation in ASZ001 cells. (G) Caspase 3/7 activities of ASZ001 cells. Cells treated with 500 nM staurosporin served as positive controls. The punches and the ASZ001 cells were incubated with vehicle (ethanol, EtOH), calcitriol or cyclopamine (CP) as indicated in the text. Gli1 expression and caspase 3/7 activities are shown in relation to the respective vehicle treated controls. Ki67+ BCC cells and BrdU-incorporation are represented as percentage of respective vehicle treated controls. Asterisks: p<0.05; error bars: mean+/−SD.

Figure 2: Calcitriol treatment of BCC-bearing mice inhibits tumor growth and the Hh-signaling pathway, and induces expression of differentiation markers. (A) Treatment scheme and calcium serum levels of BCC-bearing Ptchflox/flaxERT2+/− mice. BCC were induced in Ptchflox/flaxERT2+/− mice by tamoxifen. Treatment with 100 ng/kg/d calcitriol started either directly (0-90) or 60 (60-90) days after tumor induction and was continued until day 90 when all mice were sacrificed. Serum calcium levels of the respective BCC-bearing Ptchflox/flaxERT2+/− mice were measured before (day 0) and during therapy. Serum calcium levels of vehicle-treated mice served as controls (nvehicle=17; n60-90d=6; n0-90d=4). (B) H&E stainings, (C) relative tumor areas of H&E stained BCC and (D) percentage of Ki67+ BCC cells of vehicle- or calcitriol-treated mice. (E) Gli1, Gli2, Cyp24a1 and (F) Tgm1 and K10 expression levels from vehicle or calcitriol-treated BCC-bearing mice. Values of vehicle-treated controls...
for Gli1, Cyp24a1, Tgm1 and K10 expression were set to 1. One Asterisk: p=0.06; two Asterisks: p<0.05; error bars: mean+/-SD.

Figure 3: Calcitriol inhibits the Hh-signaling pathway downstream of Ptch but upstream of Gli1. (A) Ptch ablation in tamoxifen-treated Ptch\textsuperscript{flax/flax} ERT2\textsuperscript{+/−} fibroblasts after incubation with 10 µM tamoxifen for 48 h. Wt Ptch transcripts (derived from the non-recombined Ptch\textsuperscript{flax} locus) and Ptch\textsuperscript{del} transcripts were detected by PCR using the primer pair mPtc11/mPtc7R as described in (13, 24). cDNA derived from 12.5 days old wild-type mouse embryos (E12.5 Ptch\textsuperscript{+/+}) was used as a control. (B) and (C) Gli1 and Cyp24a1 expression levels of (B) Ptch\textsuperscript{flax/flax} ERT2\textsuperscript{+/−} and (C) Smo\textsuperscript{−/−} fibroblasts after treatment with vehicle (EtOH), calcitriol, or cyclopamine (CP). Ptch\textsuperscript{flax/flax} ERT2\textsuperscript{+/−} fibroblasts were cultured in either Shh-N-conditioned medium (Shh-N-CM) or control medium (CoM) with or without 10 µM tamoxifen as indicated. Smo\textsuperscript{−/−} fibroblasts were either transfected with a hSMO expressing plasmid or with empty vector as indicated. Asterisks: p<0.05; error bars: mean+/-SD; ntc: no template control.

Figure 4: Calcitriol inhibits the Hh-signaling pathway in a Vdr-independent manner. (A) Gli and Cyp24a1 expression of Vdr\textsuperscript{−/−} fibroblasts after treatment with vehicle (EtOH), calcitriol, or cyclopamine (CP) and Shh-N-conditioned medium (Shh-N-CM) or control medium (CoM). (B) Analyses of the Vdr protein level of nuclear extracts of si-Vdr or si-control transfected Ptch\textsuperscript{−/−} and Vdr\textsuperscript{−/−} fibroblasts by western blot. Detection of HSC-70 protein served as control. (C) and (D) Gli1, Cyp24a1 and Vdr expression of Ptch\textsuperscript{−/−} and Shh-stimulated Ptch\textsuperscript{flax/flax} ERT2\textsuperscript{+/−} fibroblasts after si-Vdr or si-control transfection and calcitriol treatment. Gli1 expression of Ptch\textsuperscript{−/−} fibroblasts and Vdr expression of Ptch\textsuperscript{−/−} and Ptch\textsuperscript{flax/flax} ERT2 cells are shown in relation to the respective vehicle-treated control. Asterisks: p<0.05; error bars: mean+/-SD.
Figure 5: Model for the dual function of calcitriol in *Ptc*-associated BCC. Normally Ptch inhibits its signaling partner Smo, thereby regulating the activity of the Hh-signaling pathway (normal cell). Mutations of *Ptc* lead to a constitutive activation of the Gli transcription factors, which results in cell proliferation and tumor formation (BCC cell). The known Smo-inhibitor cyclopamine inhibits Hh-pathway in the *Ptc*-mutant cells and thus Hh-related processes involved in tumor growth (cyclopamine-treated BCC cell). In contrast, calcitriol inhibits Hh-pathway activity and additionally activates Vdr-signaling (calcitriol-treated BCC cell). Consequently, calcitriol not only inhibits tumor-relevant processes mediated by Hh signaling, but also induces anti-proliferative effects and differentiation processes via the Vdr-signaling pathway.
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