Title

Knockdown of PTCH1 in human keratinocytes reveals that GLI1 is activated by nuclear SMO, a novel mechanism that is unresponsive to anti-SMO compounds

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Abstract

Basal cell carcinoma (BCC) of the skin is predominantly associated with mutational inactivation of PTCH1 resulting in constitutive activation of the Hedgehog (HH) developmental pathway. Tumourigenesis is linked to induced GLI transcription factors via a pathway requiring the transmembrane protein SMOOTHENED (SMO); indeed, targeting SMO has proven effective in treating some BCCs while others are poorly responsive. To understand how reduced PTCH1 function contributes to BCC, shRNA was employed to suppress PTCH1 in immortalised keratinocytes. Compared to control shCON cells, shPTCH1 cells displayed a compact, holoclone-like morphology with increased expression of GLI1 and SMO. While SMO siRNA effectively suppressed SMO expression and downstream GLI1 activity in shPTCH1 cells, the latter was not influenced by SMO pharmacological inhibition. Immunohistochemistry revealed increased nuclear SMO localisation in shPTCH1 cells and a putative SMO nuclear/nucleolar localisation signal (N(o)LS) was identified targeting EGFP to the nucleus/nucleolus. Mutational inactivation of the N(o)LS inhibited nuclear EGFP-SMO and suppressed GLI1 induction compared to wildtype protein. *In vivo*, nuclear SMO was observed in human and mouse BCCs although the pattern was heterogeneous between tumours. DNA microarray profiling revealed that over 80% of differentially expressed genes (DEGs) identified in shPTCH1 cells (>2-fold, $p<0.01$) were unaffected by SMO inhibitors including the cancer stem cell marker THY1 and chemokine CXCL11 which is over-expressed in BCC. Taken together, these data indicate that SMO regulates GLI1 through a nuclear mechanism unresponsive to anti-SMO inhibitors. Moreover, the loss of PTCH1 may result in aberrant regulation of SMO-independent mechanisms which are also important for BCC biology.

Introduction

Basal cell carcinoma (BCC) is the most common skin cancer with several clinical subtypes. BCC commonly manifests as a non-aggressive and slow-growing tumour histologically categorised as nodular or superficial. Micronodular and infiltrative subtypes are more aggressive and may be locally destructive if left untreated (1). BCC incidence is associated with exposure to ultraviolet radiation, commonly developing in elderly people especially on the head and neck (1). Hedgehog (HH) signalling was first linked to BCC due to loss of function mutations in the *Ptch1* gene in patients with nevoid BCC syndrome (NBCCS) or Gorlin syndrome, an autosomal dominant disorder predisposing sufferers to early onset BCC amongst other developmental defects (2-4). Loss of PTCH1 function leads to constitutive

activation of the GLI1 and GLI2 transcription factors via a pathway requiring the transmembrane protein SMOOTHENED (SMO) (1). Up to 70-80% of sporadic BCCs have loss of function mutations in PTCH1, 6-21% harbour activating mutations in SMO and GLI1 and GLI2 are rarely mutated (3, 5, 6).

Our knowledge of HH signalling in BCC stems mainly from mouse models but this is constrained as *Ptch1*$^{-/-}$ mice are embryonic lethal. However, *Ptch1*$^{+/-}$ mice develop follicular hamartomas with BCCs arising after ultraviolet irradiation (7) while conditional *Ptch1* knockout mice develop BCC-like lesions with tumours displaying nuclear GLI1 expression, indicative of HH pathway activation (8). Additionally, overexpression of *Gli1* leads to BCC-like epidermal tumour formation and targeted expression of an active mutant of *Gli2* to the basal layer of murine epidermis is sufficient to sustain BCC-like lesions (9, 10). Regarding SMO, conditional expression of an active SMO mutant (M2) induces BCC-like tumours when activated in the murine basal layer (11). Intriguingly, reports show that SMO-M2 induced follicular hamartomas and not BCC-like lesions concluding that, by comparison to the *K5-GLI2ΔN* mouse model (10), GLI1 and GLI2 expression was insufficiently high to initiate the more malignant tumour form (12). To our knowledge, GLI1 and GLI2 expression levels have not been compared between SMO-M2 follicular hamartomas and irradiated *Ptch1*+/- or conditional *Ptch1*-/- BCC-like tumours, but although it is difficult to compare different mouse studies, if loss of PTCH1 alone is sufficient to induce BCC-like lesions (8) while ectopic SMO-M2 is not (albeit at low levels) (12), this suggests that SMO-independent mechanisms may contribute to carcinogenesis in tumours associated with loss of PTCH1 function. Indeed, such a hypothesis was proposed to explain why, unlike irradiated *Ptch1*+/- mice, SMO-M2 tumours do not arise from the hair follicle (13). However, the same study also concluded that tumours may not arise in ears or tails of irradiated *Ptch1*+/- mice because of low SMO expression and thus the canonical signal cannot be transduced.

The importance of HH signalling in BCC development is supported by approval of the anti-SMO compounds vismodegib and sonidegib to treat advanced/metastatic BCC. Complete responses have been documented and the overall response rate for locally advanced and metastatic BCC is around 50% (14). However, this also suggests that in the absence of a drug bioavailability issue, many BCCs are driven by SMO-independent mechanisms or that some tumours are heterogeneous such that their progression is not solely driven by SMO-dependent signalling.

To address these issues and to investigate the HH signalling pathway further in BCC, we analysed the effects of suppressing the PTCH1 gene in immortalised human keratinocytes. Our results show that the majority of transcriptome changes observed upon PTCH1 suppression are independent of SMO activity. In addition, we present evidence that the increase of GLI1 transcription in PTCH1 knockdown cells is associated with nuclear SMO function, a mechanism insensitive to pharmacological inhibition. Nuclear SMO was also observed in human and notably mouse BCCs although the pattern of expression was heterogeneous. In summary, this study identifies a novel mode of SMO-GLI1 signalling as well as revealing the presence of SMO-independent signalling mechanisms downstream of PTCH1 that may be relevant to BCC biology.

Materials and methods

Retroviral transduction of immortalised keratinocytes and cell culture

Two immortalised keratinocyte cell lines were used for this study: NEB1 and N/Tert cells. Cell were cultured in Alpha MEM and 10% (v/v) FBS (Lonza), 2 nM L-glutamine, 2% (v/v) penicillin streptomycin (PAA Laboratories) and keratinocyte growth medium (KGM) supplement consisting of 10 ng/ml epidermal growth factor (EGF), 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, $1.8x10^{-4}$ M adenine and $1x10^{-10}$ M cholera toxin (Sigma). SMO inhibitors KAAD-Cyc and SANT-1 (Merck) were used at 100 nM and 1 µM concentrations from 24 to 96 hours. Cells were negative for mycoplasma, tested using MycoAlert (Lonza). Cells were retrovirally transduced with PTCH1 small hairpin RNAs (shRNA) targeting exons 3 (AAGGTGCTAATGTCCTGACCA) and exon 24 (AAGGAAGGATGTAAAGTGGT). A non-targeting control sequence was used (GCGCGATATATAGAATACG). The sequences were cloned into the pSUPERIOR.retro.puro vector (Oligoengine). Retrovirally transduced cells were then selected with 1 µg/ml Puromycin (Sigma) for a week and cultured normally thereafter from which clonal cell lines were derived.

EGFP-SMO/M2, EGFP-NLS-SMO and GLI1-Luciferase reporter vectors

To generate the EGFP-SMO/M2 constructs, SMO/M2 was excised from the PRK-SMO vector (6). SMO-M2 contains an activating point mutation of W535L (6). Purified DNA products were cloned into pEGFP-C2 (Clontech Laboratories). To generate the EGFP-NLS-SMO construct, the predicted N(o)LS sequence of SMO was cloned into pEGFP-C2. SMO-NLS-F-5' GAGGCAGAGATCTCCCCAGA 3' and SMO-NLS-R-5' ATATGGATCCGTGTGGAGGAAGAAGGAG 3' oligonucleotides were used. The purified SMO N(o)LS DNA was ligated into pEGFP-C2. Site directed mutagenesis using the QuickChange II XL SDM kit (Agilent) was utilised to modify the N(o)LS of SMO according to the manufacturer's instructions. Mutagenesis primers used were as follows: ESMOmNLS-F-5' CTTCTTCCGGGCCAGGGCCGCCTGCAGTCGAGATCTGA 3'

and ESMOmNLS-R-5' TCAGATCTCGACTGCAGGCGGCCCTGGCCCGGAAGAAGA 3' that mutate K679A and K680A respectively (Supplementary B). GLI1 firefly luciferase reporter pGL3-6GBS (15) combined with a pCMV-Renilla luciferase normalisation vector were transfected into keratinocytes using Fugene6. Cells were harvested 24 hours post-transfection and RNA extracted for qPCR analysis.

RNA, cDNA extraction and quantitative PCR (qPCR) analysis

RNA was extracted from cells using RNeasy Mini Kit (Qiagen). cDNA was then synthesised using the Superscript VILO cDNA synthesis kit (Invitrogen). 100 ng of cDNA per reaction was used for qPCR analysis using the Rotor-Gene SYBR green PCR kit (Qiagen). The Rotorgene 2000 machine (Qiagen) was used for the analyses with GAPDH the reference gene. Primer sequences for qPCR were: AGR2: F-5' GGGATGGAGAAAATTCCAGTG 3', R-5' GGGTACAATTCAGTCTTCAG 3'; AMOT: F-5' CATGGAGGGCAGGATTAAGA 3', R-5' TCGTCTCGCTTTTCTTCCAT 3'; CXCL11: F-5' GTGCTACAGTTGTTCAAGGC 3', R-5' CTAGGTTTTTCAGATGCTCT 3'; FBN2: F-5' AATGTGGGTCTCAACCTTCG 3', R-5' CTGTAGCCACCCAGGATGTT 3'; GAPDH F-5' GTGAACCATGAGAAGTATGACA 3', R-5' CATGAGTCCTTCCACGATACC 3'; GFP: F-5' TATATCATGGCCGACAAGCA 3', R-5' GAACTCCAGCAGGACCATGT 3'; GLI1: F-5' GAAGACCTCTCCAGCTTGGA 3', R-5' GGCTGACAGTATAGGCAGAG 3'; GLI2: F-5' TGGCCGCTTCAGATGACAGATGTTG 3', R-5' CGTTAGCCGAATGTCAGCCGTGAAG 3'; Luciferase: F-5' AGTGCTCATCATCGGGAATC 3', R-5' CATCCAACATTTTCGTGTCG 3'; MMP2: F-5' AGGGCACATCCTATGACAGC 3', R-5' ATTTGTTGCCCAGGAAAGTG 3'; MMP9: F-5' TTGACAGCGACAAGAAGTGG 3', R-5' TCACGTCGTCCTTATGCAAG 3'; PTCH1: F-5' ACTCGCCAGAAGATTGGAGA 3', R-5' TCCAATTTCCACTGCCTGTT 3'; SMO: F-5' GGCTGCTGAGTGAGAAG 3', R-5' CTGGTTGAAGAAGTCGTAGAAG 3'; SNAI1: F-5' TTTACCTTCCAGCAGCCCTA 3', R-5' CCCACTGTCCTCATCTGACA 3'; THY1: F-5' CCCAGTGAAGATGCAGGTTT 3', R-5' GACAGCCTGAGAGGGTCTTG 3'; VIM: F-5' CCCTCACCTGTGAAGTGGAT 3', R-5' CAACCAGAGGGAGTGAATCC 3'.

Immunocytochemistry and ImageJ staining quantification

15,000 cells per well were seeded in 12 well plates on 18 mm diameter glass cover slips (VWR International), for three days. Cells were fixed with 4% (w/v) paraformaldehyde and permeabilised with 0.1% (w/v) Triton X-100 (Sigma). 3% (w/v) BSA (Fisher Scientific) was used for blocking and primary antibodies diluted to 1:1000 in 3% (w/v) BSA. Primary antibodies used for this study were Fibrillarin-C13C3 (Cell Signaling), GLI1-C18, GLI1-H300, PTCH1-C20, SMO-C17, SMO-N19 (Santa Cruz) and SMO-ab72130 (Abcam). Fluorescence dye labelled Alexa 488/568 (Invitrogen) secondary antibodies were diluted to 1:800 in PBS. Cell nuclei were stained with 0.1 µg/ml of DAPI (Sigma). Coverslips were mounted onto microscope slides using Vectashield mounting medium (Vector Laboratories). Images were taken on the Zeiss LSM 510 Meta Confocal Microscope and quantified using Image J software.

Immunohistochemistry

SMO protein expression was examined in 29 biopsies of human BCC, 14 invasive and 15 non-invasive samples. *Ptch1*$^{+/-}$ γ-irradiated mice tumours were kindly provided by Anna Saran (ENEA, Rome). 4 µm tissue sections were cut from paraffin blocks and transferred to microscope slides. Slides were run on the Ventana discovery platform (Ventana Molecular Discovery Systems, Roche Diagnostics) with an automated protocol, including de-paraffinisation, antibody incubations, DAB application, haematoxylin and bluing reagent counterstaining and finally slide cleaning. Following dehydration in a series of solvents, the slides were mounted with coverslips.

Gene expression microarray

Gene expression microarray profiling was performed on NEB1-shCON and NEB1-shPTCH1 cells lines using Human Gene 1.1 ST Array Strip (Affymetrix). Each sample was repeated in triplicate and the data normalised using the MetaCore pathway analysis software (GeneGo). Three Affymetrix chips were used for each sample and the raw data was processed using the multi array average (RMA) method. Probabilities of gene expression between the experimental and control group were generated using Wilcoxon's Signed Rank test. Turkey's Biweight method was used to obtain log ratios that are then anti-logged to generate fold change values comparing genes between samples. Genes with a P-value at or below 5% and a fold change greater than +/- 2 fold are considered as differentially expressed genes (DEGs). DEGs were analysed in MetaCore using its GeneGo database of cellular pathways and process networks.

Results

PTCH1 gene suppression leads to increased GLI1 activity

To understand role of PTCH1 in keratinocyte biology and BCC formation, we used shRNA to suppress PTCH1. Human skin keratinocyte cell lines (NEB1 and N/Tert) were retrovirally transduced with PTCH1 shRNA targeting exons 3 or 24 and normalised against a non-targeting control vector (shCON). Clonal cell lines were generated from heterogeneous populations of the PTCH1 knockdown cells and validated by qPCR; exon 24 clone 1 (C1) of NEB1 cells displayed the

strongest level of PTCH1 suppression and the highest level of GLI1 mRNA expression (Supplementary A) which was confirmed at the protein level as well as by increased GLI reporter activity (Figures 1A and 1C). PTCH1 protein expression was predominantly nuclear in shCON cells which may represent the C-terminal fraction previously described (21). In addition to the upregulation of GLI1, suppression of PTCH1 reduced SHH and IHH mRNA levels whereas SMO was increased but there was no significant effect upon GLI2 expression (Figure 1B). To confirm that increased GLI1 resulted directly from reduced PTCH1 activity, we made use of the fact that the shPTCH1 construct targets downstream of the STOP codon at the end of exon 23. Ectopic expression of the coding sequence of only the PTCH1-1B ($PTCH1_L$) isoform suppressed increased GLI1 mRNA expression by ~50% in NEB1-shPTCH1 cells (Figure 1D); higher suppression was probably hampered by PTCH1-1B appearing to induce an apoptotic response in some cells (unpublished observations) as described in 293T cells (16). Morphologically, shPTCH1 cells formed holoclone-like colonies, characteristic of nodular/micronodular BCC tumour islands with smaller nuclei compared to shCON cells also containing more nucleoli (Figures 1E and 1F).

GLI1 is activated by nuclear SMO

In addition to the increase in SMO mRNA (Figure 1B), an increase of SMO protein expression was observed by immunocytochemistry in shPTCH1 cells compared to shCON using a commercial N-terminal antibody (Santa Cruz N-19). More specifically, SMO displayed cytoplasmic and nuclear localisation in shCON cells with a comparable but more intense fluorescent pattern in shPTCH1 cells (Figure 2A). A similar pattern of fluorescence was observed using another antibody targeting the C-terminus (Santa Cruz C-17), which provides further evidence for the presence of nuclear SMO in human keratinocytes (Figure 2A). SMO N-19 antibody specificity was confirmed by a reduction of the fluorescent signal in cells transfected with SMO siRNA (Figure 4D).

To our knowledge, nuclear SMO has not been described before however, in contrast to the *Drosophila* homologue, human and mouse SMO contain a C-terminal region predicted to harbour a monopartite nuclear/nucleolar localisation signal or N(o)LS (Supplementary B). Tagging EGFP with the putative N(o)LS sequence targeted the protein to the nucleolus based on fluorescent co-localisation with Fibrillarin. A full-length EGFP-SMO fusion protein also localised to the nucleus and occasionally to the nucleolus. Mutational inactivation of residues within the N(o)LS region mNLS2 (Supplementary B) localised EGFP-SMO exclusively to the cytoplasm (Figure 2B) and impaired its ability to increase GLI1 mRNA levels (Figure 2C).

In addition to the full length SMO above we also expressed the constitutively active SMO-M2 mutant which showed much higher levels if GLI1 expression (Supplementary C). Mutation of the NLS2 in SMO-M2 also significantly downregulated GLI1. Interestingly mutation at another site mNLS1 within the N(o)LS (Supplementary B did not influence the ability of EGFP-SMO or EGFP-SMO-M2 to activate GLI1 expression (thus providing an internal negative control). The fact that GLI1 levels were reduced with mNLS2 in both shCON and shPTCH1 cells and for both EGFP-SMO and EGFP-SMO-M2 provides very strong evidence for a specific role of nuclear SMO in GLI regulation (Supplementary C).

Nuclear SMO expression is observed in mammalian skin and BCCs

SMO protein expression was examined in a panel of BCCs to determine its subcellular localisation: the SMO-N19 antibody was employed having confirmed its specificity for SMO by immunocytochemistry (Figures 2A and 4D). In human BCCs, the staining pattern was predominantly cytoplasmic and membranous with some evidence of nuclear expression (Figure 3A). In contrast, mouse BCC tumours derived from *Ptch1*$^{+/-}$ γ-irradiated mice showed clear evidence of nuclear SMO although heterogeneous staining was also observed (Figure 3B).

GLI1 expression is unresponsive to anti-SMO inhibition in shPTCH1 cells

We assessed the canonical PTCH-SMO-GLI1 signalling axis in shPTCH1 cells by employing SMO pharmacological inhibitors. 24 hours post exposure, GLI1 protein and mRNA expression was strongly suppressed in shCON cells whereas no changes were observed in shPTCH1 cells treated with 100 nM KAAD-Cyc (Figure 4A, 4B). The same lack of response observed in shPTCH1 cells treated with 100 nM SANT-1 (Figure 4C); in addition, no change in GLI1 expression was observed upon prolonged exposure to either inhibitor (up to 96 hrs). To validate these results, other NEB1 and N/Tert-shPTCH1 clones were examined, targeting exon 3 and 24. Again, elevated GLI1 expression was not suppressed by KAAD-Cyc nor SANT-1 indicating that the increase of GLI1 observed upon PTCH1 suppression in NEB1 and N/Tert human keratinocytes is insensitive to SMO pharmacological inhibition (Supplementary D, E). To investigate the SMO-GLI1 signalling axis in more detail, shPTCH1 cells were treated with siRNA targeting SMO (siSMO). Intriguingly, GLI1 protein expression was reduced in both shCON and shPTCH1 cells suggesting that SMO is required for GLI1 signalling (Figure 4D). When comparing the effects of KAAD-Cyc and siSMO in shPTCH1 cells, GLI1 mRNA

expression was reduced by siSMO but not KAAD-Cyc while GLI2 expression was reduced by both methods (Figure 4E). Although GLI2 expression was not elevated in shPTCH1 cells, GLI2 levels were suppressed by KAAD-Cyc in both shCON and shPTCH1 cell lines thus supporting the concept of a canonical SMO-GLI2 signalling axis independent of PTCH1 control.

The global effects of PTCH1 suppression are largely insensitive to SMO inhibition

To characterise the global effects of PTCH1 suppression, shCON and shPTCH1 cells were subject to cDNA microarray profiling. Furthermore, to determine if the effects of PTCH1 suppression are sensitive to pharmacological SMO inhibition, shPTCH1 cells were additionally exposed to 100 nM KAAD-Cyc for 24 hrs prior to RNA harvesting. In comparison to shCON cells, expression levels of 213 transcripts were altered ≥2-fold (137 increased and 76 decreased, $p<0.01$) in shPTCH1 cells (Table 1). However, of the 76 downregulated genes the expression of 41 genes was further reduced by KAAD-Cyc treatment while 47 of the 137 upregulated genes was further increased (Figure 5A). Exposure to KAAD-Cyc failed to normalise the expression of the majority of transcripts to basal levels with the inhibitor often exacerbating the effects of PTCH1 suppression. Only two upregulated DEGs CXCL10 and SNORA38B, were reduced by 50% upon KAAD-Cyc treatment.

Table 1 contains transcripts associated with tumour biology including the cancer stem cell marker THY1 (↑6.3-fold), EMT markers VIM (↑3.6-fold) and SNAI1 (↑2.4-fold), and matrix metalloproteases MMP7 (↑9.5-fold), MMP2 (↑4.1-fold) and MMP9 (↑2.2-fold). In addition, the chemokines CXCL10 (↑8.2-fold) and CXCL11 (↑4.9-fold) proved of interest as both are known to be elevated in BCC (17). Of the suppressed transcripts FBN2 (↓10.2-fold), AMOT (↓3.7-fold) and AGR2 (↓3.3-fold) have been associated with tumour biology. Excluding MMP7 and CXL10 (not detectable with two primer sets each), the differential expression of the transcripts listed above was confirmed by qPCR in shPTCH1 cells (Figure 5B). However, elevated SNAI1 expression was partially suppressed by KAAD-Cyc whereas there was a potent (albeit potentially undesirable) increase of MMP9. Finally, a similar but reduced pattern of differential expression was observed for these transcripts in the shPTCH1-ex3 clone as well as in one N/Tert-shPTCH1 clonal cell line (Supplementary F).

Discussion

The purpose of this study was to identify novel targets downstream of PTCH1 relevant to BCC biology and regulated by SMO-dependent and SMO-independent mechanisms. PTCH1 was stably suppressed in immortalised human keratinocytes and clonal cell lines were generated. The fact that clonal cell lines with strong PTCH1 suppression adopt a holoclone-like morphology characteristic of BCC nodular islands supports the validity of the model. We have previously shown that retroviral GLI1 expression promotes tight N/Tert colony formation and endogenous GLI1 may help promote this phenotype in the shPTCH1 model (18). However, the level of ectopic GLI1 is considerably higher than that induced upon PTCH1 suppression and it is likely that additional factors regulate colony formation in shPTCH1 cells. Interestingly, whilst validating the shPTCH1 model with regards to canonical PTCH/SMO/GLI signalling, some observations do not correlate with current dogma i.e. PTCH1 suppression led to an increase of GLI1 but not GLI2 and an increase of GLI1 correlated with increased nuclear SMO which was unresponsive to anti-SMO pharmacological agents.

With regards to GLI2, it has been shown to activate GLI1 but this has utilised an artificial active mutant and not the wildtype protein (19). GLI2 mouse models of BCC also employed the active mutant (20) and although elevated GLI2 levels have been observed in human tumours, the role of the wildtype protein remains unclear. GLI2 was not increased in shPTCH1 cells (Figure 1B) which indicates that GLI2 expression is PTCH-independent. Despite this, GLI2 was suppressed upon KAAD-Cyc treatment revealing that it is controlled by SMO (Figure 4E). It is also possible that PTCH1 suppression permits GLI2 nuclear localisation and subsequent activation of GLI1 but this does not correlate with the fact that GLI2 was suppressed by anti-SMO inhibitors. Another consideration is the nuclear localisation of PTCH1 in NEB1-shCON cells (Figure 1A) which was detected using a C-terminal antibody. PTCH1 can be cleaved with the C-terminal fragment (PTCH1-C) residing in the nucleus to negatively regulate GLI1 (21). Based upon our immunohistochemistry data, it is likely that suppression of PTCH1-C permits the increase of endogenous GLI1 (Figures 1A-D). Irrespective of the role of GLI2 or PTCH1, SMO control of GLI1 was confirmed by a reduction of GLI1 in the presence of SMO siRNA in both shCON and shPTCH1 cells (Figure 4D, 4E). By contrast, anti-SMO inhibitors only suppressed GLI1 levels in shCON cells. It is unlikely that failure to suppress GLI1 was due to the increase of SMO in shPTCH1 cells as GLI1 levels remained elevated in the presence of high drug concentrations and therefore we hypothesise that nuclear SMO regulates GLI1 through a mechanism that is unresponsive to anti-SMO inhibition.

Indeed, the most intriguing aspect of the shPTCH1 model concerns the localisation of SMO. SMO expression for both RNA and protein was increased. The reliability of anti-SMO antibodies has been much debated in the Hedgehog field; however, a similar expression pattern was observed with three antibodies (SMO-N19, C-17 and ab72130 (data not shown)) which was suppressed upon SMO siRNA treatment (Figure 4D). We are unaware of any studies that have tested anti-SMO antibody specificity using siRNA but nuclear SMO expression was recently described in cancers of unknown primary origin and this significantly correlated with nuclear GLI1 and nuclear β-catenin (22). Strong SMO expression was observed in nodular BCC which was predominantly cytoplasmic but with clear areas of nuclear staining (Figure 3A, 3B). SMO expression was reduced in morphoeic tumours and although this may appear paradoxical it correlates with data showing that GLI1 expression is reduced in morphoeic BCC (23). Nuclear SMO was most prevalent in mouse BCC-like nodular tumours which may reflect the more homogenous nature of the system but, as for human tumours, expression was also reduced in morphoeic-like BCC (Figure 3B).

BCC tumours which become resistant to vismodegib most commonly acquire point mutations affecting their ability to bind SMO (24). How and if nuclear SMO expression influences drug effectiveness remains to be determined but the overall response to anti-SMO compounds in clinical trials was ~50% (14). As such, many BCCs are classified as SMO-independent because they are dependent upon other mechanisms which correlates with the lack of SMO expression observed in some tumours (Figure 3B). Alternatively, some tumours may still be SMO-dependent but an anti-SMO compound lacks efficacy because this is influenced by SMO subcellular localisation. Indeed, it has been postulated that more selective targeting of subcellular compartments may improve G-protein-coupled-receptor (GPCR) drug selectivity (25).

SMO is a seven-pass transmembrane protein closely related to the Frizzled family of GPCR proteins. Drosophila and vertebrate SMO share considerable homology although the former has a longer C-terminal tail. The C-terminal has been studied comprehensively, particularly the region that is phosphorylated and determines the extent of pathway activation (26). However, unlike human SMO the Drosophila homologue does not contain a putative NLS (homology with dSMO extends to residue 632 for human SMO) and although such a region has not been described for SMO, NLS domains have been recognised in other GPCRs including the Bradykinin and Apelin receptors (25). Nuclear translocation of GPCRs is mediated by importins and can be constitutive or agonist-induced; for example, Frizzled2 is cleaved upon Wingless stimulation with the C-terminus entering the nucleus to regulate postsynaptic neuron development in Drosophila (27). Future work will determine if SMO is similarly cleaved but the fact that nuclear SMO is detected with an N-terminal antibody suggests that this is not the case and not all GPCRs are cleaved before entering the nucleus. Other proteins that regulate GPCR signalling in the nucleus and harbour NLS sequences include β-arrestins and the G-protein-coupled-receptor kinases (GRK) (28, 29). Therefore, the identification of an NLS in SMO as a GPCR family member is not without precedent and represents a mode of signalling already established for these proteins.

With regards to the NLS sequences, the SMO region predicted to be an N(o)LS targets EGFP to the nucleolus (Figure 2B). Indeed, whilst NoLS (nucleolar) are normally distinct to NLS (nuclear) they may occur in the same region which can make it difficult to delineate the role of specific subdomains. Studies suggest that shared motifs create genetic stability and that a combined N(o)LS region may facilitate active nuclear import (30). We have not functionally delineated the SMO N(o)LS region but determined that it is functional and that nuclear import is important for GLI1 regulation (Figure 3C). Whether SMO nucleolar localisation regulates GLI1 requires further investigation but to our knowledge nucleolar function has not been described for other GPCRs. The nucleolus is emerging as a target for cancer therapy and is regulated by oncogenic signalling pathways including RAS/ERK and PI3K/AKT/mTOR which influence GLI activity (31). Nucleoli are often more abundant in tumours compared to normal cells and this has been observed in BCC (32). Interestingly, PTCH1 suppression results in an increase in the number of nucleoli (Figure 1F). It has also been observed that the number of nucleoli increased in fibroblasts after X-Ray treatment in patients with Gorlin syndrome (33).

Regarding a nuclear role for GPCRs, control of gene regulation has been reported for various proteins which may represent signalling from the nuclear membrane or more directly; for example, it was shown that F2rl1 forms part of a transcriptional complex with Sp1 that modulates Vegfa expression (34). Delineation of the N(o)LS region may help determine if SMO localises to the nuclear membrane and regarding DNA binding, it is intriguing that SMO has a putative leucine zipper region from residues 405-426 that was not previously predicted. Microarray data highlights the downregulation of zinc-finger proteins commonly localised to the nucleus/nucleolus with more genes downregulated upon KAAD-Cyc treatment in shPTCH1 cells (Supplementary Table 1). In particular, ZNF750 is known to be involved in psoriasis, squamous cell carcinoma, oesophageal, lung and cervical cancers (35). The role that SMO plays in promoting

nucleolar changes requires further investigation as well as the consequences of reduced zinc-finger protein expression in shPTCH1 cells.

We also aimed to identify novel targets downstream of PTCH1 that may be important for BCC biology, particularly those that are SMO-independent. Microarray analysis identified 213 transcripts that were altered ≥2-fold in shPTCH1 cells versus the control (137 increased and 76 decreased). Of these, less than 20% returned to basal levels in the presence of KAAD-Cyc revealing that the majority of DEGs are SMO-independent in shPTCH1 cells or that if they are SMO-dependent, their mechanism of control is unresponsive to conventional anti-SMO inhibition.

CXCL11 is reported to increase proliferation of and enrich for BCC-derived epithelial cells as well as providing immunoprotection by increasing indoleamine 2,3-dioxygenase (IDO) expression through its receptor CXCR3 which is itself implicated in tumour biology (17). MMPs are associated with tumour invasion and metastasis by degradation of the extracellular matrix. MMP2 and MMP9 degrade collagen IV and the levels of MMP2 and MMP9 mRNA are significantly higher in nodular and infiltrative BCCs compared to normal adjacent tissue (36). Additionally, MMP9 expression was significantly higher in infiltrative compared to nodular tumours. MMP7 expression has also been described in BCC for aggressive and recurrent forms although we could not confirm the microarray result by qPCR (37).

THY1 (CD90) is a GPI-anchored cell surface protein expressed in various tissues and cancers. Although it is a putative cancer stem cell marker associated with multiple oncogenic processes including proliferation and metastasis, there is evidence supporting its role as a tumour suppressor (38) therefore, due to the cell-specific role of CD90, it is difficult to speculate about its role in BCC.

Increased expression of SNAI1 and VIM were of interest as both are associated with the cancer stem cell phenotype including epithelial-mesenchymal transition (EMT) (39). SNAI1 mRNA has been described in BCC and shown to be a target of GLI1 in the murine epidermis (40). Although we found no evidence that SNAI1 or VIM mRNA are direct GLI1 targets in human keratinocytes, both proteins were more highly expressed in GLI keratinocytes that survived genotoxic insult (18, 41). Indeed, VIM expression is associated with a more aggressive myofibroblast phenotype in BCC (42).

We have not examined the migratory/invasive potential of shPTCH1 cells *in vitro* but although BCCs may be locally invasive they are rarely metastatic and this correlates with the holoclone-like colonies observed in culture (Figure 1E). How PTCH1 regulates cell-cell adhesion warrants investigation but AMOT (angiomotin) positively regulates cell migration and increased expression has been described in metastatic breast cancer (43). In addition, AGR2 is associated with tumour progression and metastasis (particularly hormone-dependent cancers) and its suppression impairs the migration of NSCLC cells (44). As such, AMOT and AGR2 suppression may represent a negative feedback loop to limit tumourigenesis and the spread of transformed cells. It is likely that exposure to ultraviolet irradiation or other form of genetic insult will be required to transform shPTCH1 cells and the complexity by which BCCs arise and progress is highlighted by the extent of genomic aberrations identified in these common tumours (45).

Finally, FBN2 was the most potently suppressed gene in shPTCH1 cells. FBN2 is a glycoprotein that forms part of connective tissue microfibrils in the extracellular matrix, often with low expression in tumours (46). How reduced FBN2 expression influences tumour biology is unclear but it was hypothesised that, as for FBN1, this may relate to increased TGF-β signalling (47). Indeed, it has previously reported that integrin-mediated TGF-β activation modulates the stromal microenvironment in morphoeic BCC (23, 48).

Conclusion

Since the discovery of PTCH1 in BCC (2-5), several models have been created attempting to identify how HH signalling contributes to BCC tumourigenesis. To our knowledge, this is the first *in vitro* model to sustain PTCH1 suppression in immortalised human keratinocytes. The model enables us to show that PTCH1 regulates SMO expression and that SMO, as for other GPCRs, localises to the nucleus where it regulates GLI1 expression through a mechanism that is the subject of ongoing research. Whether or not nuclear SMO influences primary or acquired resistance to anti-SMO compounds warrants further investigation but these data suggest that drugs should be tested for efficacy against nuclear SMO. In addition, this model compliments other studies implying that PTCH1 regulates pathways independently of SMO (49) which may involve transmembrane as well as nuclear control. Future studies aim to further characterise these SMO-independent signalling pathways to help realise new targets for treating BCC.

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Figure legends

Figure 1: PTCH1 gene suppression leads to increased GLI1 activity
[**A**] NEB1-shPTCH1 cells show reduced PTCH1 and increased GLI1 protein expression, both PTCH1-C20 and GLI1-H300 antibodies display a nuclear staining pattern. [**B**] Suppression of PTCH1 reduces mRNA expression of SHH and IHH ligands with increased GLI1 and SMO but no significant change in GLI2 levels. [**C**] NEB1-shPTCH1 transfected with a 6x GLI binding site Firefly Luciferase reporter construct (6xGLI-BS-Firefly) and EGFP as a transfection control, confirm increased GLI1 activity based on a 3-fold increase in Luciferase mRNA expression. [**D**] Ectopic expression of the PTCH1B isoform (shRNA targets exon 24 beyond the STOP codon in exon 23) suppressed the increased GLI1 mRNA expression in NEB1-shPTCH1 cells. [**E**] NEB1-shPTCH1 cells form densely packed colonies similar in appearance to nodular or micronodular BCC tumours. [**F**] Due to tight packing of NEB1-shPTCH1 cells, the size of the nuclei appear smaller with a greater average number of nucleoli present (average taken from 50 cells). *P≤0.05 and **P≤0.01 (as calculated by Student's *t* test), error bars represent standard deviation, n=6.

Figure 2: GLI1 is activated by nuclear SMO
[**A**] Staining using two SMO antibodies (N and C terminal protein) revealed distinct nuclear localisation of SMO in shPTCH1 cells with a higher percentage of nuclear SMO in shPTCH1 cells (calculated by ImageJ, n=20). [**B**] NEB1 cells transfected with EFP containing the predicted N(o)LS sequence from SMO directs GFP to the nucleolus (Supplementary C). EGFP-SMO fusion protein is expressed in both the nucleus and nucleolus and by mutating the N(o)LS, nucleolar GFP expression is lost and the protein is retained in the cytoplasm. [**C**] shCON cells and to a greater extent shPTCH1 cells transfected with EGFP-SMO show upregulation of GLI1 mRNA expression that is then reduced to below basal levels upon mutation of N(o)LS sequences. *P≤0.05 and **P≤0.01 (as calculated by Student's *t* test), error bars represent standard deviation, n=6.

Figure 3: Nuclear SMO expression is observed in mammalian skin and BCCs
[**A**] SMO-N19 staining of human morphoeic BCCs show cytoplasmic expression and variable staining intensities while nodular BCCs also express cytoplasmic SMO; certain tumour nodules also express nuclear SMO protein (marked by arrows). [**B**] Mouse BCC tumours derived from *Ptch1*$^{+/-}$ γ-irradiated mice show distinct nuclear SMO expression although there is heterogeneity between tumours.

Figure 4: PTCH1-suppressed cells are unresponsive to anti-SMO inhibitors
24 hours of 100 nM KAAD-Cyc is able to suppress GLI1 [**A**] protein and [**B**] mRNA expression in NEB1-shCON cells however, NEB1-shPTCH1 cells show no GLI1 reduction. [**C**] The same lack of response in NEB1-shPTCH1 cells was seen with 100 nM SANT-1, also with extended treatment up to 96 hours where GLI1 protein remained constant. [**D**] Although shPTCH1 cells did not respond to SMO inhibitors, SMO siRNA (siSMO) did suppress GLI1 protein expression compared to control siRNA (siCON) suggesting that SMO is required for GLI1 signalling. [**E**] qPCR confirms that GLI1 expression is reduced by siSMO but not KAAD-Cyc in shPTCH1 cells while GLI2 expression is suppressed by both siSMO and KAAD-Cyc. *P≤0.05 and **P≤0.01 (as calculated by Student's *t* test), error bars represent standard deviation, n=6.

Figure 5: The global effects of PTCH1 suppression are largely insensitive to SMO inhibition
[**Table 1**] List of top and bottom DEGs (≥2 fold change, p>0.01) comparing NEB1-shPTCH1 and NEB1-shPTCH1 treated with 100 nM KAAD-Cyc to NEB1-shCON cells reveals various genes reported to be involved in BCC biology are differentially expressed. [**A**] Diagram of identified DEGs and the proportion of upregulated and downregulated genes further altered by KAAD-Cyc treatment. [**B**] qPCR on NEB1-shPTCH1 cells was performed to validate the results from the microarray with a number of cancer related genes all strongly upregulated in NEB1-shPTCH1 and also in cells treated with 100 nM KAAD-Cyc. *P≤0.05 and **P≤0.01 (as calculated by Student's *t* test), error bars represent standard deviation, n=6.

Supplementary:
[**A**] Various exon 24 clones generated from heterogeneous populations of PTCH1 suppressed NEB1 cells show reduced PTCH1 and increased GLI1 mRNA expression. [**B**] Both human and mouse SMO protein sequences contain predicted nuclear and nucleolar localisation signals located within the C-terminal region, non-matching amino acids are highlighted in red. Site directed mutagenesis was employed to alter the N(o)LS by changing each amino acid to alanine for the mNLS construct, mNLS2 was used for Figures 2B and 2C. [**C**] NEB1-shCON cells and to a greater extent NEB1-shPTCH1 cells transfected with EGFP-SMO and EGFP-SMO-M2 show upregulation of GLI1 mRNA expression that is then reduced to below basal levels upon mutation of N(o)LS sequences, particularly mNLS2. qPCR analysis for GLI1 expression in [**D**] NEB1 and [**E**] N/Tert exon 3 and exon 24 shPTCH1 clones treated with both KAAD-Cyc and SANT-1 show all shPTCH1 clones are unresponsive to SMO pharmacological inhibitors. [**F**] qPCR on NEB1-shPTCH1 exon 3 clone and N/Tert-shPTCH1 cells to validate the results for cancer related genes obtained from the microarray analysis. [**Table 1**] List of differentially expressed zinc-finger protein genes. *P≤0.05 and **P≤0.01 (as calculated by Student's *t* test), error bars represent standard deviation, n=6.