Murine basal cell carcinoma leads to tumor-mediated alterations in endocrine Igf1 signaling

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Abstract
The intrinsic properties underlying cancer development are extensively studied while the effect of a cancer on the host is often overlooked. Activation of the Hedgehog (Hh) signaling pathway underlies a number of types of common human cancers, yet little is known concerning endocrine signaling in such tumors. Here, we investigated endocrine signaling in a murine model of basal cell carcinoma (BCC) of the skin, the most common cancer. BCCs were generated by the activation of Hh signaling resulting from the specific deletion of the Ptch1 gene in the developing epidermis. Subsequently, a severe growth deficiency was observed in the murine BCC model, and we identified a deficiency of circulating Igf1 (Igf1). We demonstrate that Hh pathway activation in murine BCC induces Igf binding proteins, thereby regulating Igf1 sequestration into the skin and skewing Igf endocrine signaling. Significantly, these results show that Hh-induced tumors can have endocrine effects on normal tissues that in turn can greatly impact the host. This study not only identifies that Igf is important in Hh-associated skin tumors but also exemplifies the need to consider endocrine signaling when interpreting complex in vivo tumor models.

Key Words
- Patched1
- skin
- IgF
- BCC

Introduction
It is becoming increasingly recognized that the interaction between solid tumors and their supporting stroma is a key determinant of tumor initiation and growth. While the molecular basis of tumor initiation and growth within the tumor itself is studied comprehensively in a number of systems, much less is known of the manner or mechanism in which a tumor influences its immediate environment, and the host as a whole. Hedgehog (Hh) signaling is central to the development of many tumor types, including basal cell carcinoma of the skin (BCC; Hahn et al. 1996, Diepgen & Mahler 2002, Adolphe et al. 2006), the most common cancer in humans. In a number of systems, Hh signal-induced tumors have stromal alterations that are critical for tumor development, including prostate (Fan et al. 2004, Shaw et al. 2010), brain (Yauch et al. 2008), pancreatic (Nakamura et al. 2010), and skin tumors such as BCC (Marsh et al. 2008). It has also been reported that Hh signal activation regulates or requires many paracrine and endocrine signaling factors including EGF (Palma & Ruiz i Altaba 2004), FGF (Fogarty et al. 2007), VEGF-A (Chen et al. 2011), and Igf (Rao et al. 2004). Despite these advances, much remains to be determined regarding how Hh signal activation influences the surrounding normal and tumor stroma.
The majority of BCC arise from mutation of Patched1 (Ptch1; Gailani et al. 1996, Wicking & Bale 1997). Ptch1 is a transmembrane receptor that maintains the Hh signal in an inactive state (Chen & Struhl 1998) but direct binding of Hh ligands to Ptch1 causes the Hh pathway to become active through disrupting the interaction between Ptch1 and another member of the signaling complex, Smoothened. Thus, the Hh pathway can be activated in a paracrine fashion by Hh ligands, or cell autonomously activated by loss of Ptch1 function. The net result of Hh pathway stimulation is an increase in activated Gli transcription factors, which promote target gene expression. In BCC, mutation of Ptch1 results in inappropriately activated Hh signaling, increased Gli transcription factors, and expression of whole cell genes cyclin D1 and Myc (Adolphe et al. 2006, Nieuwenhuis et al. 2007). Aberrant cell cycle regulation causes uncontrolled proliferation within the epidermal basal cell compartment, ultimately resulting in BCC.

In some contexts, the Hh pathway can regulate growth factors known for both endocrine and paracrine signaling and often associated with cancer, including IGF (Igf; Hahn et al. 2000, Bigelow et al. 2005, de Bont et al. 2008). Igf signaling is of particular importance as it coordinates growth and proliferation throughout the body. Igf1 is the primary mediator of GH-induced growth and coordinates postnatal growth of nearly every tissue (Baker 2008). Igf signaling is of particular importance as it coordinates growth and proliferation throughout the body. Igf1 is the primary mediator of GH-induced growth and coordinates postnatal growth of nearly every tissue (Baker et al. 1993). Igf signaling has also been implicated in Hh-induced medulloblastoma and rhabdomyosarcoma as it appears that Igf2 synergizes with Hh signaling to promote tumorigenesis (Hahn et al. 2000). We have also previously demonstrated Hh signal regulation of Igf signal components including Igf2 in mesenchymal cells in vitro and Igfbp2 in Hh-induced BCC (Ingram et al. 2002, Villani et al. 2010). However, despite multiple reports of Hh regulation of Igf, much remains to be investigated regarding the Hh and Igf interaction in the initiation, development, and metastasis of cancer.

Here, we investigated endocrine signaling in a Hh-activated tumor model in order to better understand secondary growth phenotypes commonly associated with the development of cancer. We show that mice with the Hh pathway activated in Keratin 14 expressing cells develop BCC and also manifest a significant postnatal growth defect associated with low circulating Igf1 levels. The Hh-activated epidermis has dysregulated local production of Igfbps and sequester-free Igf1. The sequestration of Igf1 in Hh-activated skin provides a link between lower serum Igf1 levels evident in the growth-retarded, BCC-prone mice. These data not only indicate a pathological link between Hh and Igf1 signaling but also illustrate a novel mechanism whereby even a highly localized tumor can have profound systemic endocrine effects.

Materials and methods

Mice

Mice were bred via crossing Ptch1 conditional mice with a K14-Cre recombinase line, genotyped via PCR as previously published (Jonkers et al. 2001, Ellis et al. 2003). Cre recombinase function was tested via crossing K14-Cre mice to a Z/AP indicator line (K14-Cre:Z/AP) (Lobe et al. 1999). Mice were housed in light-controlled facility and work performed according to institutional ethics requirements. Mice were weighed daily and at collection to determine growth curve. Blood was collected via cardiac puncture after IP of xylazine hydrochloride, 13 mg/kg (Ilium Xylazil-20), and tiletamine–zolazepam, 33 mg/kg (Zoletil-50), diluted to 5% (v/v) solution in saline. Samples were centrifuged at 6153 g for 15 min at 4 °C, serum decanted, and then at 15 350 g for 10 min at 4 °C. Pathology was recorded upon dissection and all organs were inspected. Organs were weighed and percentages from whole body weight were calculated.

Histology

Hematoxylin and eosin staining was performed as previously published (Villani et al. 2010). K14-Cre:Z/AP tissues were embedded in OCT compound (Tissue-Tek 4583) for sectioning or fixed in 0.2% glutaraldehyde for whole mount, all other histology was performed on paraffin-embedded tissue. Alkaline phosphatase (AP) staining performed post-PBS wash with AP fix (0.2% glutaraldehyde, 50 mM EGTA, 100 mM MgCl2, 0.02% NP-40, 0.01% Na-deoxycholate, and 2 mM MgCl2, in PBS), incubation at 70 °C for 30 min in PBS, and 2× PBS washes. Slides were then washed 10 min in AP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 10 mM MgCl2 in H2O) followed by color incubation with BM purple AP substrate (Roche 11442074001) at 4 °C for 0.5–36 h. Slides were then washed with 0.1% Tween 20, 2 mM MgCl2 in PBS, dehydrated, and mounted with Entellan. Whole mount AP staining was performed similarly but samples were stored at 4 °C in AP buffer.

Hormone and nutrient analysis

Nutrient values were determined using whole blood. GH levels were assayed using Linco rat GH RIA kit,
RGH-45HK, as per manufacturer’s instructions. GH receptor (GHR) activation was assayed after administering 4 μg/g GH IP to p19 K14-Cre:Ptch1lox/lox mice and control littermates followed by liver collection 15 min post-injection. JAK2 and STAT5 were assayed via immunoprecipitation (IP) from whole liver protein and the tyrosine-phosphorylated fraction determined. IP was performed on whole fresh protein extract in IP lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM Na vanadate, 10 mM NaF, and 0.6% Triton-X-100). Protein A (40 μl) (Sigma P3391) or protein G (Sigma P3296) fast flow sepharose was blocked in 1% BSA in TBS for 2–4 h. Beads washed twice with lysis buffer were then incubated with 2 μg of antibody Jak2 (Santa Cruz sc-278) or Stat5 (Santa Cruz sc-835) in lysis buffer at 4 °C rotating overnight. Protein (40 mg) was then incubated rotating 2 h at 4 °C. Samples were centrifuged, washed twice with PBS, heated for 5 min at 100 °C, and were then extracted in Laemelli sample buffer. Samples were separated via polyacrylamide electrophoresis and probed for phosphorylated and total JAK2 and STAT5.

**Igf and Igfbp analysis**

Skin segments were homogenized in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% Na-deoxycholate, 1% NP-40, and 0.1% SDS) containing protease inhibitor (Roche 1 697 489) and PhosphoSTOP phosphatase inhibitor (Roche 04906837001). Samples were sonicated 2×30 s and centrifuged at 13 000 g for 30 min at 4 °C. Igf binding proteins (Igfbps) were removed using Bioclone in assay acid–ethanol extraction before determination of Igf levels via RIA (Bioclone cat number IGF50) or ELISA (R&D assay acid–ethanol extraction before determination of Igf binding proteins (Igfbps) were removed using Bioclone in assay acid–ethanol extraction before determination of Igf levels via RIA (Bioclone cat number IGF50) or ELISA (R&D assay acid–ethanol extraction before determination of Igf levels via RIA (Bioclone cat number IGF50) or ELISA (R&D). Samples were centrifuged (40 000 g) and were then extracted in Laemelli sample buffer. Samples were separated via polyacrylamide electrophoresis and probed for phosphorylated and total JAK2 and STAT5.

**RNA analysis**

RNA was prepared from whole skin using QIAgen RNAeasy fibrotic kit (QIAgen 74704). cDNA was prepared from 500 ng RNA and RT was performed using M-MLV reverse transcriptase (Invitrogen 28025-013). Taqman inventoried assays were purchased from Applied Biosystems (Mm00833447, Mm00492632m1, Mm00515156m1, Mm00494922m1, Mm00516037, and Mm00599696m1) and real-time PCRs were performed on 7000 and 7500 RealTime and analysis was performed using ABI prism 7000 SDS software.

**Explant culture**

Explant culture was performed on p19 skin as previously published (Villani et al. 2010) with biotinylated Igf1 (Gropep, Adelaide, SA, Australia; AQU050) added to explants at isolation. Biotin was then detected on sections after 30-min ABC treatment (Vector Labs, Burlingame, CA, US; PK-6101) with DAB peroxidase substrate (Vector Labs KK-4100).

**Statistical analysis**

Statistical analyses were performed using Word Excel and ABI prism statistical software and analyses performed are indicated in figure text.

**Results**

**K14-Cre recombinase-induced deletion of Ptch1 results in severe growth deficiency**

Epidermal deletion of a Ptch1 conditional allele via K14-Cre recombinase (K14-Cre:Ptch1lox/lox mice) results in the development of BCC lesions before 4 weeks of age (Villani et al. 2010). Concomitant with BCC development, K14-Cre:Ptch1lox/lox mice present with a severe growth deficiency and K14-Cre:Ptch1lox/lox mice (13.0 g ± 0.54, n = 34) do not progress beyond 49% of control littermate weight (26.6 g ± 0.60, n = 67) (Fig. 1A). Weight differences are equivalent between sexes (Fig. 1B) and significant differences are first observed around postnatal day 19 (Fig. 1C), the same age as hyperproliferative lesions were observed to first develop in the skin (Supplementary Figure 1, see section on supplementary data given at the end of this article). Organ weight percentages of total body weight show that K14-Cre:Ptch1lox/lox mice are proportionally smaller than control littermates and do not identify a candidate tissue mediating a growth defect in K14-Cre:Ptch1lox/lox mice (Fig. 1D).

**K14-Cre:Ptch1lox/lox mice have abnormalities of the skin and thymus**

Whole body growth can be influenced by many factors from a number of different tissues. K14-Cre-mediated Ptch1 mutation may also occur in stratified epithelia...
beyond the epidermis (Jonkers et al. 2001). To further characterize K14-Cre function, K14-Cre mice were bred with a Z/AP allele (Lobe et al. 1999), such that alkaline phosphatase (Alk. Phos.) staining of K14-Cre:Z/AP tissue indicates Cre recombinase function. Alk. Phos. staining identified K14-Cre-induced recombination in the skin (Fig. 2B), thymus (Fig. 2A and E), testes (Fig. 2F), and bladder lining (Fig. 2D) but not in many endogenous K14 expressing tissues including the pituitary, lung (Fig. 2C), nasal septum, and esophagus (Fig. 2H). No phenotypic differences between K14-Cre:Ptch1lox/lox mice and littermates were identified in the esophagus (Fig. 3A and B), stomach (Fig. 3C and D), colon (Fig. 3E and F), kidney, and liver (data not shown). In addition to the skin, K14-Cre:Ptch1lox/lox mice were found to have a hypoplastic thymus (Fig. 3G), which we have previously characterized as an alteration in T-cell maturation (Siggins et al. 2009). K14-Cre:Ptch1lox/lox mice do not breed successfully, indicating that K14-Cre:Ptch1lox/lox mice may also have a gonadal phenotype. Finally, analyses of serum nutrients (Fig. 2F) revealed no significant differences between K14-Cre:Ptch1lox/lox and littermate controls.

**Growth deficit is associated with decreased circulating Igf1**

The proportional growth deficit observed in K14-Cre:Ptch1lox/lox mice, in the absence of any clear organ defect, is suggestive of a hormonal alteration. First, we examined GH as a possible cause of the observed growth deficiency,
but this appears unlikely as plasma GH levels (Fig. 4A) and STAT5/JAK2 (Fig. 4B and C) signal activation are normal in K14-Cre:Ptch1lox/lox mice. Due to similarity between growth curves in K14-Cre:Ptch1lox/lox and that observed for Igf1 null mice (Baker et al. 1993), we then examined Igf1 serum levels. Serum Igf1 levels are markedly reduced in K14-Cre:Ptch1lox/lox mice (Fig. 4D) and consequently may be involved in the observed growth alteration. While adecrease in Igf1 transcript levels in the liver was observed (Fig. 4E), no gross morphological abnormalities nor evidence of Hh signal alteration in the liver of K14-Cre:Ptch1lox/lox mice were observed. Furthermore, Igf level alterations do not correlate with altered serum Igfbp levels, as Igfbp1, Igfbp2, and Igfbp5 do not change in K14-Cre:Ptch1lox/lox serum (Fig. 4F). Accordingly, we examined the possibility that the observed levels of circulating Igf1 are not due to phenotypic alterations in K14-Cre:Ptch1lox/lox liver but are controlled from another tissue. Due to the severity of the growth phenotype and correlated development of BCC and growth defects in K14-Cre:Ptch1lox/lox mice, the skin was investigated for a possible role in the regulation of Igf1 levels.

Hh signaling activation in the skin alters Igfbp expression and results in Igf1 sequestration

Igfbps are modulators of Igf signaling and we have previously defined members as Hh signaling targets (Ingram et al. 2002). Accordingly, we next investigated the hypothesis that aberrant regulation of Igfbps in the skin of K14-Cre:Ptch1lox/lox mice could contribute to the low serum Igf1 levels in these mice. Analysis of skin Igfbp transcript levels revealed alterations in Igfbp2, Igfbp4, and Igfbp5 after epidermal Ptch1 deletion (Fig. 5). Igfbp1 and Igfbp6 were not detectable in skin and Igfbp3 showed no alterations after Ptch1 deletion (Fig. 5). As previously published, Igfbp2 appeared positively associated with BCC development (Fig. 5B) and was significantly increased in adult Ptch1-deleted skin (Villani et al. 2010) while Igfbp4 was decreased in adult Ptch1-deleted skin (Fig. 5F). Igfbp5 was decreased significantly in 10-day-old K14-Cre:Ptch1lox/lox skin compared with controls (Fig. 5G); however, this was no longer statistically significant at 6 weeks of age. These results support the possibility that Igfbp alteration in K14-Cre:Ptch1lox/lox skin may affect circulating Igf1 levels, thereby contributing to the observed reduced serum Igf1 levels. In order to test this hypothesis, we analyzed the ability of K14-Cre:Ptch1lox/lox skin to recruit biotinylated Igf1 (B-Igf1) ligand in explant culture. B-Igf1 could not be detected in control skin
explants or non-B-Igf-treated cultures, despite a healthy proliferating appearance (Fig. 6). By contrast, B-Igf could be detected histochemically in epidermal cells of K14-Cre:Ptch1lox/lox skin explants (Fig. 6). These data suggest that K14-Cre:Ptch1lox/lox skin can recruit Igf1 into the skin, indicating that these mice are likely growth deficient due to sequestration of Igf1 hormone into the skin and away from the majority of other growing systems.

Discussion

This study has identified a link between Igf signaling and Hh signaling in skin cancer due to a severe growth deficiency identified during investigations into BCC development in K14-Cre:Ptch1lox/lox mice. Endocrine regulation appears to be far more important in this system than one would expect, and this study clearly shows how important it is to consider not just local but also systemic effects in complex murine models of cancer.

Here, we observed that epidermal loss of Ptch1 in K14-Cre:Ptch1lox/lox mice leads rapidly to both BCC development and growth retardation (3–4 weeks post-deletion). Coincident with these phenotypes, circulating Igf1 levels drop and its absence during the postnatal growth spurt (around 20 days old in mice) is a likely cause of the growth deficiency. We propose that BCC

Figure 4
Growth defects in K14-Cre:Ptch1lox/lox mice are associated with systemic Igf1 deficiency. GH levels (A) do not differ between controls and K14-Cre:Ptch1lox/lox mice and GH induces JAK2 (B) and STATS (C) in K14-Cre:Ptch1lox/lox mice similar to controls. Igf hormone levels are greatly decreased in K14-Cre:Ptch1lox/lox plasma compared with controls (D) and Igf transcript levels are also decreased in the liver (E). Densitometry of Igf ligand blots shows that circulating Igf binding protein levels are similar between controls and K14-Cre:Ptch1lox/lox mice (F). P values were calculated by two-tailed Student’s t-test.
development is causal in reducing circulating Igf1 levels, which results in the observed growth deficiency. In addition to an epidermal-based model, many alternative causes for growth retardation in K14-Cre:Ptch1lox/lox mice were investigated here. However, no clear extra-epidermal mechanism for the growth defect could be found. For example, we found no evidence for impaired food absorption, as serum nutrients and gut morphology were not altered (Fig. 3F), or altered liver phenotype (data not shown). While a decrease in the transcript level of Igf1 was observed in the liver, we propose that this is not causal for the growth deficiency as liver-specific Igf1 deletion resulting in complete ablation of liver Igf1 production does not result in growth deficiency; therefore, it is unlikely that the small changes in transcript level in K14-Cre:Ptch1lox/lox mice cause the observed growth defect (Yakar et al. 1999).

The epithelial specific nature of Ptch1 deletion in this model indicates that an epithelial source of growth retardation is most likely. Hh signaling has been shown to interact with Igf signaling in other models. For example, in the cerebellar tumor medulloblastoma, Hh signaling promotes Igf2 signaling and thereby recruits Igf2 into the tumor from circulation (Rao et al. 2004).
tissue as large as the skin, and considering the amplification in cell number that occurs as a result of BCC in K14-Cre:Ptch1lox/lox mice, it is likely that a similar Igf recruitment processes would mediate large overall changes in hormone levels. As Igfbps are known to regulate serum levels of Igf1, we hypothesized that Igfbp production in Ptch1-deleted epidermis modulates circulating Igf levels. This contention was supported by the upregulation of Igfbps with a high Hh signal and the demonstration of the ability of K14-Cre:Ptch1lox/lox skin to recruit Igf1 ligand. The data do not support the possibility that the observed low levels of serum Igf1 is due to Igfbp release into the circulation as circulating Igfbp levels do not appear to be altered (Fig. 3). On the basis of these data, we propose that Igf1 is being recruited into the skin where it may facilitate BCC proliferation and consequently lead to decreased circulating Igf1, which manifests as a secondary growth defect.

The data presented here indicate that Ptch1 mutation is modulating the Igf axis in the skin. Igfbp alteration may reflect an altered cell-type composition in the skin and suggest that Igfbps mediate Hh regulation of skin homeostasis. Igfbp5 regulates hair follicle growth (Schlake 2005), and therefore, Igfbp5 downregulation in K14-Cre:Ptch1lox/lox skin most likely reflects the hair loss that occurs concomitant with BCC development in K14-Cre:Ptch1lox/lox skin. We have previously demonstrated that Igfbp2 is a key regulator of progenitor cell regulation and BCC development in K14-Cre:Ptch1lox/lox skin (Villani et al. 2010). The data presented in this study indicate that Igfbp4 is negatively associated with BCC and this observation remains to be further investigated. Igfbps are known to be targets of Hh signaling in tissues other than skin (Ingram et al. 2002, Allan et al. 2003, Lipinski et al. 2005, Villani et al. 2010), which in combination with these results suggest that Igfbps in general may represent important tissue-specific mediators of Hh signaling.

Interestingly, Hh signal alterations are associated with as yet unexplained body size phenotypes. Murine models of Hh signal alteration have been described as having a growth alteration (Milenkovic et al. 1999) and increased body size is seen in the human syndrome nevoid BCC syndrome in which patients carry a heterozygote Ptch1 mutation (Gorlin 2004). Gorlin syndrome patients’ carry whole body heterozygote Ptch1 mutation, quite different from the BCC model discussed within this work, which has epithelial specific homozygous Ptch1 mutation. In combination with other published data showing Igfbps are common, tissue specific Ptch1 targets this indicates that Igf signaling should be considered as a likely contributor to Gorlin syndrome and other Hh related growth phenotypes and therefore Igf action may be of broader relevance to the many human disease conditions caused by Hh signal modulation.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0307.

**Declaration of interest**

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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