12-17 weeks after *Ptch1* deletion, we observed spontaneous regression of HF-derived tumors. This occurred in all mice, regardless of *Ptch2* status (FIG 5.2D,F). HF tumors in our model derive from the bulge and SHG, both of which undergo extensive remodeling throughout the hair cycle. Because of this, we predict that regression in this context could be hair cycle dependent. However, carefully timed follow-up experiments would be necessary to confirm this hypothesis. In contrast to HF-derived tumors, TD tumors did not undergo regression (FIG 5.3A-B). Instead, these tumors progressed throughout our experimental time-points. These tumors invaded the underlying dermis, and resemble human BCC tumors. In addition, a subset of tumors displayed changes in pigmentation (FIG 5.3B), which is also reported in some human BCCs [41-44].

Altogether, our results suggest that loss of *Ptch2* does not affect BCC cell of origin, tumor growth, or HH activation in the absence of *Ptch1*. This study confirms our previous observation that the IFE is resistant to BCC formation, while the HF/TD readily forms tumors [23]. Loss of *Ptch2* also does not enhance BCC formation in the HF/TD compartments. We observed that HF derived tumors regress 12-17 weeks after *Ptch1* deletion, while TD tumors do not. We suspect this regression is hair cycle dependent, although further experiments are necessary to confirm this hypothesis.

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5.7 Author Contributions

S.C.P. and S.Y.W. conceived and performed experiments, wrote the manuscript and secured funding. A.A.D. provided expertise, technical support and feedback.
Figure 5.1 The IFE resists BCC formation

A. Schematic showing areas of activity (blue) for K14-Cre\textsuperscript{ERT}.

B. Mice were induced with 3x IP injections of tamoxifen (TAM) 1mg/40g, biopsies were taken 5 and 10 weeks post TAM.

C. qPCR showing that \textit{Ptch2} expression is absent in \textit{K14;Ptch1;Ptch2}\textsuperscript{+/-} skin (left), \textit{Gli1} expression is not altered by \textit{Ptch2} deletion (right).

D. H&E showing that loss of \textit{Ptch2} does not affect BCC formation in \textit{K14;Ptch1;Ptch2} mice. IHC showing no difference in K17 (red) expression in \textit{K14;Ptch1;Ptch2}.

E. H&E showing that loss of \textit{Ptch2} does not affect BCC formation in ear or tail skin.
Figure 5.2 HF-derived BCC is not altered by loss of \textit{Ptch2}

A. Schematic showing areas of activity (blue) for Gli1-Cre\textsuperscript{ERT2}.

B. Mice were induced with 1x IP injection of tamoxifen (TAM) 5mg/40g, biopsies were taken 5, 12, and 17 weeks post TAM.
C. qPCR showing that \textit{Ptch2} expression is absent in \textit{Gli1;Ptch1;Ptch2}⁻/⁻ skin (left), \textit{Gli1} expression is not altered by \textit{Ptch2} deletion (right).

D. H&E showing that loss of \textit{Ptch2} does not affect BCC formation or regression \textit{Gli1;Ptch1;Ptch2} mice.

E. IHC showing that loss of \textit{Ptch2} does not affect BCC marker expression.

F. Hair follicle tumor area 5, 12, and 17 weeks post TAM induction in \textit{Gli1;Ptch1;Ptch2}⁺/⁻ (green) and \textit{Gli1;Ptch1;Ptch2}⁻/⁻ (red) mice. (5 weeks, n=6/6, p=0.79, 12 weeks, n=5/5, p=0.15, 17 weeks, n=4/3, p=0.68). Data are represented as mean ±SEM.
Figure 5.3 TD-derived BCCs resist regression
A. (Left) H&E showing that loss of Ptch2 does not affect TD-derived tumor (dashed line) formation/progression in Gli1;Ptch1;Ptch2 mice. (Right) IHC showing loss of Ptch2 does not affect TD marker expression, K17 (red) or K8 (green).
B. (Left) Bright field image showing pigmentation of TD-derived tumors 17 weeks post TAM. (Right) H&E showing advanced TD-derived tumors 17 weeks post TAM.
5.9 Reference List


Chapter VI – Discussion and Future Directions

6.1 Understanding BCC cell of origin

In chapter III, I sought to clarify conflicting data on the cell of origin of BCC. Previous studies employed broad expression of SmoM2 [1] or deletion of Ptch1 [2, 3] throughout the epidermis. In contrast, I generated mice with targeted Ptch1 deletion in several distinct epidermal compartments using four different Cre recombinase drivers. I believe this model more closely mimics human disease, as spontaneous tumors likely arise from one or a few cells which acquire oncogenic mutations within a non-mutated population. This model also allows me to observe tumor initiation and growth within a microenvironment that mimics the human disease state.

In this study, I found that the hair follicle bulge is highly susceptible to Ptch1-driven BCC formation, consistent with previous studies [2, 3]. However, I also found additional BCC-susceptible compartments. The isthmus, which maintains the hair canal or infundibulum (INF), efficiently forms BCC tumors upon Ptch1 deletion. I also identified a novel “hot spot” for BCC tumorigenesis in the mechanosensory touch dome (TD). Surprisingly, I found that the interfollicular epidermis (IFE) is resistant to BCC formation. This is also consistent with previous studies using Ptch1 loss-of-function models of BCC [3]. However, this directly conflicts with models of BCC that employ SmoM2 or Gli2ΔN expression [1, 4].
This differential response to *Ptch1* deletion highlights a potential role for the micro-environment including nerves, blood vessels, immune cells, and dermal fibroblasts in mediating BCC tumorigenesis. Additionally, understanding the mechanisms employed by the IFE to resist tumor formation, could give insight into potential avenues for BCC treatment.

6.2 Neural promotion of BCC

6.2.1 Sensory nerves promote TD-derived BCC

Based on the results of our cell of origin study, I looked to the micro-environment to understand why some epidermal cells resist tumor formation. One obvious difference between the IFE and hair follicle (HF)/TD compartments is the presence of innervation. Nerves have been implicated in several cancer types and diseases including psoriasis [5], prostate [6], gastric [7], and pancreatic cancer [8], and therefore present a potential explanation for our observations. In addition, the sensory nerves that innervate the HF and TD secrete HH ligand [9], which is the pathway affected in BCC.

In this study, I found that surgical denervation slows BCC tumor growth specifically in tumors derived from the TD (chapter III). This was the first evidence for nerves as a potential therapeutic target for BCC tumors. Surprisingly, there was little effect on HF-derived tumors. However, the HF isthmus niche supports BCC tumorigenesis, despite its lack of innervation. Therefore, other aspects of the HF niche appear able to compensate for loss of nerves.

Because cutaneous sensory nerves secrete HH ligand, I hypothesized that nerve-derived HH promotes BCC. I attempted to test this using neutralizing antibodies for SHH ligand. While I observed a minor reduction in both TD and HF tumor growth after two weeks of treatment, the differences were not statistically significant. However, these experiments were limited by the
duration of antibody treatment, as sustained treatment was cost prohibitive. Perhaps a longer
treatment period would have yielded more conclusive results.

When the TD is surgically denervated, it quickly loses its identity and reverts to an IFE-
like phenotype (chapter II, [10]). It loses HH activation, expression of Keratin 17, columnar
morphology, and association with Merkel cells (MCs). Denervation also reduces the ability of
the TD to form BCC tumors (chapter III). Based on these results, I predicted that if loss of
nerves confers BCC-resistance to the TD, then perhaps ectopic innervation imparts BCC-
susceptibility onto the IFE. The TD requires expression of the nerve growth factor
Neurotrophin-3 (NT-3) to maintain innervation and identity [11]. Overexpression of NT-3
throughout the epidermis leads to ectopic innervation of the IFE and increased innervation of the
HF/TD [12].

6.2.2 Limitations of NT-3 mediated ectopic innervation

In chapter IV, I generated NT-3 overexpressing mice in combination with Ptch1 deletion,
to test whether ectopic innervation confers BCC susceptibility. Despite the presence of ectopic
innervation in NT-3 expressing mice, I surprisingly did not observe ectopic HH activation or
BCC induction in the IFE. There are a few potential explanations for this result. Firstly,
although NT-3 primarily signals through the TrkC receptor, it also can bind to TrkA and TrkB
present on different types of nerve fibers [13]. As such, it is possible that the ectopic nerves
observed in the IFE of NT-3 expressing mice are non TrkC positive mechanosensory nerves, like
those that innervate the TD. The wildtype IFE does contain free nerve endings which are
nociceptive and are comprised of C-fibers and Aδ fibers [14]. NT-3 expression could also
enhance the branching/density of those fibers, which may not have the ability to secrete HH
ligand, or support tumor growth. Secondly, even if the ectopic fibers present in NT-3 mice are TrkC positive, it is possible that the micro-environment of the IFE cannot support maturation of the nerve ending. For example, in the TD, nerves contact Merkel cells. Whether Merkel cells are required for the neural secretion of HH ligand is unclear. However, perhaps the absence of Merkel cells in the IFE, prevents ectopic fibers from secreting HH to activate signaling in the IFE.

Moving forward, there are several experiments I would propose. First, to characterize the nerve fiber type, sections from NT-3 expressing mice can be stained with antibodies for TrkA/B and C [13]. This would classify ectopic IFE fibers as mechnosensory or nociceptive. An alternative approach for characterizing these fibers would be to assess the myelination status, as different fibers vary in myelination [15, 16]. In addition, crossing NT-3 mice with a Shh-Cre inducible reporter could address whether ectopic nerve fibers express HH ligand [9]. This method was used previously by Brownell et al., to confirm the expression of Shh in sensory nerves [9].

In contrast to the IFE, ectopic innervation of the hair canal or infundibulum (INF) promotes ectopic HH activation. This suggests that ectopic nerves can secrete HH in certain contexts. Perhaps, unlike the IFE, the ectopic nerves which innervate the INF are TrkC positive and able to secrete HH ligand. In addition, while Merkel cells are a part of the TD microenvironment, they are not present in the innervated region of the hair follicle, which also receives nerve-derived HH. As such, the INF micro-environment may be sufficient to support HH secretion from ectopic fibers. I also observed efficient BCC induction from the INF upon Ptch1 deletion. This was not specific to ectopic innervation, as previous experiments targeting the INF also resulted in BCC tumors (see chapter III).
For future studies, it would be interesting to compare INF-derived BCC tumor growth in hyper-innervated (NT-3) vs. control animals. For these studies, I would cross \( Lrig1;Ptc\)h1 mice (used in chapter III) with our NT-3 animals and compare the rate of INF-derived tumor induction and progression. This would address whether ectopic INF innervation promotes enhanced tumorigenesis.

My preliminary results suggest that \( Gli1;Ptc\)h1;NT-3 animals display enhanced tumor initiation in both the lower HF and TD compartments compared to \( Gli1;Ptc\)h1 animals. However, these results could be due to increased recombination, as the driver (\( Gli1-Cre^{ERT2} \)) is HH responsive and NT-3 animals have increased HH signaling. In order to confirm this result, it is necessary to carefully quantify the recombination efficiency of \( Gli1-Cre^{ERT2} \) in NT-3 expressing mice compared to wildtype. If NT-3 animals display increased recombination, it would be necessary to repeat the tumor experiments using HH-independent Cre-drivers.

6.2.3 Residual \textit{Ptch1} expression in the tumor micro-environment may protect against BCC

Because my attempts to impart BCC susceptibility onto the IFE via hyper-innervation were unsuccessful, I sought to identify a factor that could be protecting the IFE from BCC, and/or mediating nerve-derived HH signaling in the HF/TD. Previous studies have highlighted the ability of PTCH2 to compensate for loss of PTCH1 in several tissues [17-20]. I thus hypothesized that PTCH2 could be suppressing BCC in the IFE, while PTCH2 in the HF/TD is inhibited by nerve-derived HH ligand. In chapter V, I generated mice lacking both \textit{Ptc\( h1 \)} and \textit{Ptch2} and assessed BCC in several epidermal compartments. However, I found that additional loss of \textit{Ptch2} had no effect on BCC in the IFE, HF, or TD.
These results were surprising, as dual deletion of PTCH receptors in embryonic skin yields a much more severe phenotype than loss of either *Ptch1* or *Ptch2* [17, 18]. Compared to adult skin, embryonic skin is more proliferative, and undergoing major morphological changes as hair follicles develop, which could account for the differences in response to *Ptch1/2* deletion. However, another important distinction between embryonic studies and our adult model is the efficiency of *Ptch1* deletion. In their embryonic studies, Adolphe et al., utilized *K5-Cre* to delete *Ptch1* on a *Ptch2* null background. This driver induces efficient recombination in all epidermal cells [18]. In addition, embryonic deletion of *Ptch1/Ptch2* caused inflammation and a severe barrier defect, which could account for a more severe phenotype [18].

In contrast, the inducible *K14-CreERT* driver utilized to delete *Ptch1* in my study drives recombination in ~15% of IFE cells (see chapter III). As such, in my model, *Ptch1* expression is maintained in non-recombined cells. A recent study from Roberts et al., suggests that in addition to inhibiting HH via ligand sequestration, expression of PTCH receptors in cells adjacent to those lacking *Ptch1/2* can inhibit Smoothened (SMO) in a non-cell autonomous manner [21]. Based on these results, I hypothesize that the IFE employs several cell autonomous and non-cell autonomous mechanisms for resisting BCC. For example, in cells which lack *Ptch1* only, HH signaling is mediated by expression of *Ptch2*. This was shown previously by Adolphe et al., where in the embryonic epidermis complete loss of *Ptch1* is partially compensated by expression of *Ptch2* [17, 18]. Since I did not observe any significant differences between our *K14;Ptch1;Ptch2HET* mice and our *K14;Ptch1;Ptch2KO* mice, my data suggests that in the absence of *Ptch1* and *Ptch2*, *Ptch1* expression in neighboring cells can partially inhibit HH activation, thus preventing BCC induction. In contrast, in environments where HH ligand is
present such as the TD, neighboring cells have active signaling, and thus PTCH1 is unable to inhibit SMO cell autonomously or non-cell autonomously.

Based on this hypothesis, I expect that BCC initiation in the IFE requires either 1) efficient loss of both PTCH receptors in a large continuous population, such that neighboring cells (with retained Ptch1 expression) are unable to compensate or 2) activating mutations in Smo, such that both PTCH receptors are unable to autonomously or non-autonomously inhibit activation. The second is consistent with mouse models of SMO-driven BCC, which are reported to arise from the IFE [1]. The first point can be explored further in our model system by increasing the efficiency of Ptch1 deletion in our Ptch2 null mice. This can be achieved by maintaining K14;Ptch1;Ptch2KO and K14;Ptch1;Ptch2HET mice on tamoxifen chow instead of using a single IP injection to induce recombination. Based on my hypothesis, I would expect the Ptch2KO mice to have a more severe BCC-like phenotype. By increasing the efficiency of Ptch1 deletion in the epidermis, Ptch1 expression in non-recombined cells would be diminished. This would more clearly address the role of Ptch2 in adult BCC.

6.3 Are nerves a target for BCC treatment?

My studies suggest neural promotion of BCC; however, additional studies will be necessary to evaluate whether sensory nerves are a realistic therapeutic target. Further investigation into the mechanism of neural promotion of BCC is essential. I predict this promotion is HH dependent. However, it is possible that other nerve-derived factors are responsible for this phenomenon.

Investigating the effect of blocking sensory function on epidermal homeostasis and tumor growth could highlight potential factors involved in this process. In addition to HH ligand,
sensory nerves secrete Substance P (SP) and Calcitonin-gene related peptide (CGRP), which facilitate neurotransmitter release [22, 23]. Both SP and CGRP have been implicated in promoting acanthosis and inflammation in psoriasis [5], and can be inhibited by botulinum toxin A (BoNT-A) [24]. BoNT-A treatment also reduces gastric tumor growth [7]. Whether BoNT-A affects neural HH secretion is unknown, however treatment of BCC models with BoNT-A or other neurotoxins could assess whether SP or CGRP are required for nerve promotion of BCC.

While surgical denervation is not practical for BCC treatment, perhaps neurotoxins such as BoNT-A could work alone to mitigate tumor growth in resistant tumors, or in concert with HH-inhibitors such as vismodegib. In addition, because non-neural niches appear to resist BCC initiation, perhaps treating Gorlin patients, who continuously develop BCCs, with pharmaceutical nerve blockers could be preventative. Although the exact mechanism of neural promotion of BCC is still unclear, the possibility for novel therapeutic targets highlights the importance of this interaction in the future of BCC treatment.

6.4 Spontaneous regression: system limitation or potential therapeutic target?

In all long-term tumor studies, I observed almost complete regression of BCC tumors derived from the lower HF (chapter IV-V). Although, histological evidence of partial spontaneous regression has been observed in some human BCC tumors [25, 26], complete regression is very rare. In addition, human BCC regression is believed to be immune mediated [26, 27], while our tumors showed no histological evidence of immune infiltration. This observation presents a potentially troubling limitation of our model system. However, there are several explanations for this phenomenon which highlight distinctions between human and
mouse epidermal biology. Understanding how these differences may affect BCC progression is essential to effectively translate results to improve human BCC outcomes.

I hypothesize that regression in our model is hair cycle dependent. In mice, hair follicles cycle in synchrony. This is controlled by strong waves of BMP signaling from the dermal macro-environment, opposed by epidermal-derived Noggin expression and Wnt activation [28-31]. Mouse follicles cycle frequently, which accounts for hair shedding and short fur length. In contrast, human follicles cycle independently of each other. Human follicles have variable cycle length, with the anagen phase of scalp follicles lasting up to 6 years [32, 33].

During cycling, the lower portion of the follicle undergoes drastic remodeling, with widespread apoptosis during the catagen, or regression, phase of the cycle. Because tumors in my study derive from cells which normally undergo regression during cycling, I predict that despite oncogenic signaling, they are unable to resist the powerful dermal signaling that mediates cycling in mice. Human follicles have a drastically longer anagen phase, and are not subject to dermal BMP waves, as cycling is asynchronous [32, 33]. This could explain why regression is so rare in human BCC. An alternative explanation could be that either the cell of origin for human tumors is not within the lower follicle, or that lower follicle tumors require additional mutations to resist regression. In our model, tumors derived from the upper follicle and TD in our model resist regression (chapters IV-V). Based on this observation, perhaps these tumors provide a more accurate model for human BCC. Overall, this observation has given key insight into the limitations of our model system which will need to be considered in future studies.

For future studies, there are several methods to test whether the hair cycle mediates HF-derived BCC regression. Cyclosporine A treatment has been shown to inhibit the anagen to catagen transition [34]. If tumor regression occurs during this transition, I hypothesize that
animals treated with Cyclosporine A would resist tumor regression. Alternatively, dexamethasone treatment induces pre-mature catagen [34]. Based on my hypothesis, I predict that treating Gli1;Ptch1 mice with dexamethasone could enhance regression.

In addition to pharmaceutical modulation of the hair cycle, genetic manipulation can also manipulate cycle timing. For example, deletion of Twist1, a basic helix loop helix transcription factor expressed in the dermal papilla (DP), in adult mice causes increased hair growth and prolonged anagen [35]. Perhaps, combining Twist1 deletion with Ptch1 models of BCC could prevent tumor regression. These experiments would involve generating Gli1-CreERT2;Ptch1flo/flo;Twist1flo/flo (Gli1;Ptch1;Twist1) mice. Because Gli1 is expressed in the HF and the DP, this model would promote both Ptch1 and Twist1 deletion in the DP and HF. If HF-derived tumor regression is catagen mediated, I would expect Gli1;Ptch1;Twist1 mice to display prolonged anagen and delayed regression. Loss of Twist1 in the HF should not confound these results, as Twist1 is normally only expressed in a subset of cells outside the outer root-sheath above the hair follicle bulge [35], which is not targeted by Gli1-CreERT2 [9].

The results from these studies could highlight the hair cycle as a potentially powerful player in mediating HF-derived BCC tumor growth. Perhaps, this understanding could be translated into a therapeutic target, where pharmaceutical induction of catagen promotes BCC regression.
6.5 Reference List


