

Exploiting the origins of Ras mediated squamous cell carcinoma to develop novel therapeutic interventions

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The small GTPase Ras is activated in a high proportion of human cancers. Attempts to clinically block Ras activity through pharmacological means has proven largely ineffective thus far. We employed an inducible mouse model of squamous cell carcinoma (SCC) to study the effect of Ras activation and show that hair follicle stem cells (HFSCs) are a cell of origin for SCC, whereas their more restricted progeny cannot serve as cancer cells of origin and are refractory to Ras activation. We propose that by identifying the unique mechanisms by which HFSCs are mobilized to initiate Ras mediated tumorigenesis, the molecular process behind SCC can be more completely elucidated and context dependent activities for Ras more clearly defined. Here, we summarize our recent results and point to future experiments designed to create novel therapeutics by exploiting the differential sensitivities of various cells within the epidermis to Ras activation.

Introduction

Small GTPases in skin cancers. Squamous cell carcinoma (SCC) of the skin is a highly prevalent cancer with a predisposition to metastasize. Risk factors for this cancer include high lifetime UVB radiation due to sun exposure and a compromised immune system.^{1,2} Specifically, organ transfer recipients with chronically suppressed immune systems have a 65–250-fold increase in SCC risk.³ The high prevalence and significant morbidity of SCC demonstrates a need for orally or topically administered therapies to prevent

or halt progression of this cancer. Previous work on murine SCC suggested that activating mutations in the small G-protein Ras are able to drive SCC.^{4,5} In fact, Ras is mutated in 30% of all human cancers including a small percentage of SCC.^{6–9} The high mutation rate for Ras has led to significant effort to develop small molecule inhibitors, with little success thus far. Instead, many groups are now focusing on developing inhibitors for effectors of Ras signaling, such as the Raf family of small GTPases.¹⁰ However, a new high-risk group for cutaneous SCC has emerged in individuals taking the B-Raf inhibitor PLX4032 as a treatment for melanoma, a cancer which is thought to be unrelated to SCC despite sharing an anatomical target.¹¹ As of now, it is unclear why this drug sensitizes patients to SCC or promotes its initiation, but there is evidence for crosstalk and aberrant feedback within the Ras/Raf circuit.^{12–14}

Numerous questions remain about how events downstream of Ras/Raf activation initiate various types of tumors, and how manipulation of these events could be exploited to treat cancer. Several groups, including our own, take advantage of murine models of various cancers initiated by constitutive Ras activity. Recently, we used Ras activation in specific cell types in the epidermis to identify cancer cells of origin, and to determine whether different types of cells exhibit unique molecular responses to Ras activation.^{15,16}

To initiate tumorigenesis in vivo, we bred mice harboring the widely-used *LSL-Kras^{G12D}* knock-in allele to animals containing a floxed version of the *p53* tumor suppressor gene.^{17,18} The *LSL-Kras^{G12D}*

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allele, when combined with Cre recombinase, creates a Ras isoform that lacks GTPase activity, and is therefore continuously able to stimulate downstream pathways. To target expression of Cre to a skin stem cell population, the *K15-CrePR* allele was used for expression in the hair follicle (HF) stem cell niche, and the *Shh-CreER* knock-in allele was used to target the immediate descendants of the hair follicle stem cells, the matrix transit amplifying cells.^{19,20} Comparison of the same tumorigenic insult to these two stages of the same lineage led to the conclusion that hair follicle stem cells were able to generate SCCs, whereas their *Shh*-expressing transit-amplifying descendants could not. From this in vivo, inducible, genetically engineered mouse model, the natural history of this cancer can be traced from its origins to its end-stage. This model provides the ability to analyze a wide variety of time-points that can be assessed for responses to Ras activation and for the development of potential interventions specific to cancer cells of origin. This model system can be also exploited as a pre-clinical testing ground for target validation of prospective SCC therapeutics.

Prevention of SCC Initiation

Targeting signaling pathways downstream of Ras. Given that patients taking PLX4032 and similar B-Raf inhibitors as a treatment for melanoma often develop SCCs, targeted therapeutics are needed in order to prevent this unfortunate side effect. Our mouse model provides a means to understand the intrinsic factors unique to HF stem cells that are necessary for SCC initiation, and thereby allows for the determination of potential targets downstream of Ras/Raf signaling for chemoprevention.

To screen for signaling pathways downstream of Ras that could be activated during the initiation of hyperplasia and/or during epithelial to mesenchymal transition (EMT) in *K15-CrePR; Kras^{G12D}* and *K15-CrePR; Kras^{G12D}; p53^{fl}* mice, candidates were selected based on known downstream effectors (Fig. 1). We examined several signaling pathways downstream of Ras, including Map Kinases (Erk and p38) and Akt.

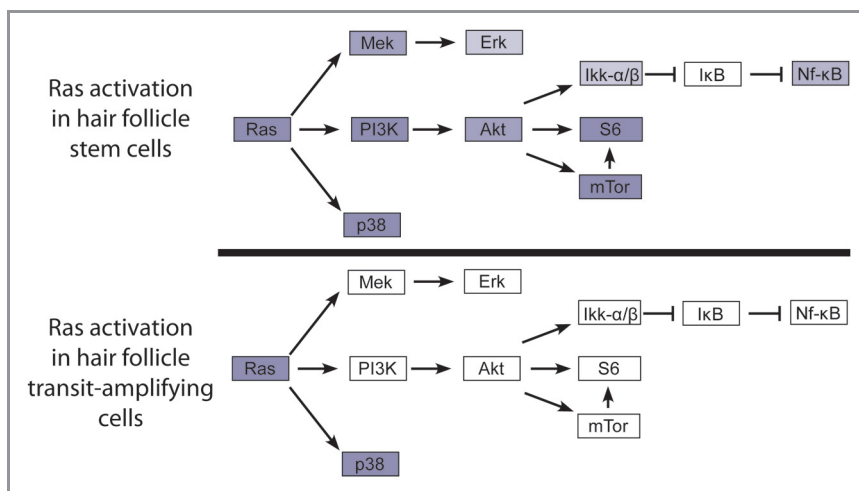


Figure 1. Ras signaling pathways examined in SCC prone *Kras^{G12D}* induced hair follicle stem cells and in SCC resistant *Kras^{G12D}* induced hair follicle transit amplifying cells. P-Erk, p-Akt, p-S6 and p-p38 stained hair follicle hyperplasia and cyst structures at high levels when *Kras^{G12D}* was induced in hair follicle stem cells. P-mTor, p-Ikk α/β and p-NF κ B were also present, albeit at lower levels. In contrast, only p-p38 was found in the *Shh*-expressing hair follicle transit amplifying cells following *Kras^{G12D}* induction. Darker shadings of gray represent higher activity levels. (Image adapted from White et al.¹⁵).

First, Erk1/2 activation was examined by IHC for phosphorylated Erk (p-Erk). This signaling effector of the Ras pathway was found at high levels in hyperplastic hair follicles and the basal cells of the epidermal cyst structures of skin with *Kras^{G12D}* expression originating from HF stem cells. This indicates that administration of an inhibitor of MEK, an upstream regulator of Erk1/2 activity, might provide a preventative response to *Kras^{G12D}* induced tumorigenesis. AZD6244 is one such inhibitor that may prove useful.²¹ This potential target is further supported by previous transgenic animal studies that manipulated MEK activity.^{22,23} We also examined the p38 arm of the Ras signaling by p-p38 staining. Though this marker was detected during hyperplasia and in epidermal cysts, it was also found throughout the hair follicle in control skin. This indicates that attempting to inhibit this pathway may not be useful, as it may affect normal skin homeostasis.

Second, we examined the Akt arm of Ras signaling. Using IHC for p-Akt, it was determined that Akt signaling was indeed found in some hyperplastic hair follicles and in epidermal cyst structures. Further downstream of Akt, we examined both phosphorylated mTor and phosphorylated NF κ B. Phospho-mTor was evident in hyperplastic hair follicles and

cyst structures at low levels compared with the robust activity of p-Akt. Rapamycin, a potent inhibitor of mTor signaling, has been suggested as a potential chemopreventative agent by studies in head and neck squamous cell carcinomas and murine chemical carcinogenesis.²⁴⁻²⁶ Rapamycin, or a similar analog, may have some preventative effect in the initial stages in tumorigenesis in our model and in patients with *Kras*-inducing SCC. To examine another output of Akt signaling, we examined p-NF κ B. NF κ B signaling has been implicated in a wide range of tumorigenesis processes, including EMT and inflammation.²⁷ p-NF κ B was also detected during tumorigenesis initiation in this model system. Bortezomib and Bay-117082, inhibitors of NF κ B signaling, have recently been shown to be effective in inhibiting tumorigenesis in a model of lung cancer.²⁸ Notably, this lung cancer model utilizes the same genetic insults we used in our mouse model system. Additionally, bortezomib has been shown to have some limited effect on human cases of head and neck squamous cell carcinomas.²⁹ This suggests that these inhibitors may also be useful in our model of cutaneous SCC.

This examination of Ras signaling indicates that the inhibition downstream of

Kras at the level of MEK, mTor or NFκB individually, or in combination, may represent a chemopreventative therapeutic regimen that can be administered simultaneous with tumor initiation in our mouse model of SCC. If successful, this may represent a potentially useful method to inhibit SCC formation in patients taking B-Raf inhibitors or in patients with compromised immune systems. However, activation of ERK, AKT and mTor (p-Erk1/2, p-Akt and p-mTor) was not detected in cells of the bona fide SCC, suggesting that these downstream pathways are not required to sustain the dedifferentiated state. This striking observation indicates that inhibition of these pathways may not be therapeutically useful following the onset of SCC in our model or in patients presenting with high grade Kras-derived SCCs. How the cancer cells evolve to shed the necessity for activity of pathways downstream of Ras or utilize alternate Ras signaling pathways warrants further investigation.

Identifying the molecular basis of sensitivity to Ras activity. In an alternate approach, since the direct descendants of the hair follicle stem cells are completely refractory to *Kras*^{G12D} and *Kras*^{G12D}; *p53*KO induced tumorigenesis, a molecular comparison between transit amplifying cells and the parental stem cells could point toward new targets for tumorigenesis prevention. Though very closely related in hierarchy, the intrinsic properties that facilitate tumorigenesis have been lost during the transition to the transit amplifying cell type. To reveal the nature of these intrinsic properties, cell populations purified just following induction of *Kras*^{G12D} expression alone and/or with *p53*KO could be compared in detail on the genetic, epigenetic, transcriptome and

proteome levels. Novel or known mediators of *Kras* signaling not found in the transit amplifying population could provide targeting candidates for further exploration.

Targeting EMT to SCC progression. The *K15-CrePR*; *Kras*^{G12D} and *K15-CrePR*; *Kras*^{G12D}; *p53*^{ff} mouse models are excellent systems to study *Kras*^{G12D} induced epithelial to mesenchymal transition (EMT). EMT is thought to be a necessary precursor to invasiveness and metastasis, and this process results in the spindle shaped cells of the SCC produced in *K15-CrePR*; *Kras*^{G12D}; *p53*^{ff} mice.³⁰ In both *K15-CrePR*; *Kras*^{G12D} and *K15-CrePR*; *Kras*^{G12D}; *p53*^{ff} skin, hair follicle stem cells undergo EMT following a brief phase of hyperplasia. This has been concluded by antibody staining of ectopic Tenascin-C, high levels of Vimentin, ectopic Keratin 8 and more recently, by ectopic N-cam staining (unpublished data). By purifying these cells from initiation of hyperplasia through induction of EMT, a detailed transcriptome and proteome profile can be generated. Since our model system can also incorporate a *LSL-Yfp* allele that generates YFP expression exclusively in *Kras*^{G12D} expressing cells, we can be confident that cells expressing these markers were once epithelial cells. This is important in order to distinguish them from nearby cancer associated fibroblasts, which express many of the same markers. These data could yield a wealth of information from an in vivo cancer that is undergoing crosstalk with its naturally occurring microenvironment, which contrasts to traditional xenograft studies, which creates an unnatural environment with crosstalk cues that may or may not be truly representative. Theoretically, if EMT can be pharmacologically blocked, the tumor cells may revert to a more keratinocyte-like nature,

which could thus be redirected from the path toward squamous cell carcinoma and instead become terminally differentiated skin cells.

Finally, though EMT is found in *K15-CrePR*; *Kras*^{G12D} skin, these cells do not proliferate into bona fide SCC. Only in *K15-CrePR*; *Kras*^{G12D}; *p53*^{ff} skin do transformed cells undergo a switch to high proliferation and then to SCC development. A comparison between these two mouse models may inform on how this switch occurs. The nature of the pathways induced by *Kras*^{G12D} in the context of *p53*KO in hair follicle stem cells may further provide novel targets for reversion back to a non-proliferating cell.

Conclusion

The methodologies outlined here provide a basis for determining potential therapeutic interventions through the detailed molecular understanding of the events that occur in squamous cell carcinoma from genesis to end-point. Squamous cell carcinomas found in patients fall under numerous sub-types, and determination of the human sub-type that most closely resembles that found in *K15-CrePR*; *Kras*^{G12D} and *K15-CrePR*; *Kras*^{G12D}; *p53*^{ff} mice will be necessary, so that promising therapeutic strategies developed in this preclinical model translate more precisely to the clinic.

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