THE ROLE OF MAMMALIAN TARGET OF RAPAMYCIN (MTOR) IN SKIN CARCINOGENESIS

A Dissertation in
Physiology
by
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ABSTRACT

Ultraviolet (UV) radiation is the major risk factor for developing skin cancer, the most prevalent cancer worldwide. Several studies indicate mammalian target of rapamycin (mTOR) signaling is activated by UVB and may play an important role in skin tumorigenesis. mTOR exists in two functionally and compositionally distinct protein complexes: the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-resistant mTOR complex 2 (mTORC2). Several studies indicate that mTORC1 and mTORC2 signaling are activated in response to UVB in keratinocyte cell lines and primary keratinocytes. However, the role of mTOR-dependent pathways in normal keratinocyte function and transformation has not been investigated.

These studies were designed to test the hypothesis that the two mTOR complexes play distinct roles in keratinocyte proliferation and pro-survival signaling in response to UVB. To better understand the role of mTORC1 and mTORC2 signaling in response to UVB we utilized the pharmacological inhibitor of mTORC1, rapamycin, and the mTOR kinase inhibitor, Torin2. We also generated inducible mTOR-deficient (K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl}) mice that allow epidermal-specific disruption of mTOR following topical treatment with 4-hydroxytamoxifen (4OHT). Rapamycin blocked UVB-induced phosphorylation of S6K1 (S6 kinase 1), the downstream target of mTORC1, and significantly reduced UVB-stimulated epidermal proliferation and cell cycle progression, but had no effect on cell death. In contrast, mTOR deletion, which attenuated UVB-induced phosphorylation of both S6K and the mTORC2 target AKT\textsuperscript{Ser473}, significantly increased apoptosis both \textit{in vivo} and in keratinocyte cultures, in addition to reducing hyperproliferation following UVB irradiation. Torin2 also sensitized keratinocytes to UVB-
induced apoptosis. The role of mTORC2 in UVB-induced pro-survival signaling was verified in Rictor<sup>−/−</sup> MEFs (mouse embryonic fibroblasts), which lack functional mTORC2 and were more sensitive to UVB-induced apoptosis than controls. Additionally, Torin2 was able to sensitize wild-type but not Rictor<sup>−/−</sup> MEFs to UVB-induced cell death. These studies show for the first time that mTORC1 and mTORC2 play unique but complementary roles in controlling proliferation and apoptosis in the skin. Our results support a model where UVB-induced activation of mTORC1 mediates hyperproliferation while mTORC2 activates pro-survival signaling. This work provides new insight into the molecular mechanisms involved in UVB-induced skin cell damage leading to skin aging and skin cancer and underscores the importance of both mTOR complexes in mediating UVB-induced signaling in keratinocytes.

Previous studies utilizing chemical carcinogenesis have shown that inhibition of mTORC1 with rapamycin attenuates skin tumor formation. However, the role of mTORC2 in skin tumor development remains largely unexamined. Because our UVB results suggested that inhibition of mTORC2 signaling may prevent the formation of skin tumors through inhibiting cell survival, we utilized an inducible Rictor-deficient mouse model (K14CreER<sup>T</sup>;Rictor<sup>fl/fl</sup>) to investigate the role of mTORC2 signaling in tumor formation and maintenance. Rictor ablation within the basal layer of the epidermis significantly reduced tumor incidence and multiplicity in a chemical carcinogenesis model. Additionally, we demonstrated that inducing rictor deletion in pre-existing tumors inhibited further tumor growth and induced tumor regression. These data provide the first demonstration that Rictor expression is necessary for both skin tumor development and maintenance of established tumors. Moreover, our results provide new insight into the
pathogenesis of skin cancer and highlight mTORC2 as a possible target for treatment and prevention of cutaneous SCC (squamous cell carcinoma).
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<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>4EBP1</td>
<td>eukaryotic translation initiation factor 4E binding protein</td>
</tr>
<tr>
<td>4OHT</td>
<td>4-hydroxy-tamoxifen</td>
</tr>
<tr>
<td>6-4PP</td>
<td>(6-4) photoproducts</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>cellular FLICE-inhibitory protein</td>
</tr>
<tr>
<td>CC3</td>
<td>cleaved caspase-3</td>
</tr>
<tr>
<td>CPD</td>
<td>cyclobutane pyrimidine dimers</td>
</tr>
<tr>
<td>Cre</td>
<td>Causes recombination (recombinase)</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>D:A</td>
<td>DMSO:Acetone [1:9]</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channels</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal regulated kinase 1/2</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506-binding protein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>i.p.</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>K14</td>
<td>keratin 14</td>
</tr>
<tr>
<td>K5</td>
<td>keratin 5</td>
</tr>
<tr>
<td>KSC</td>
<td>keratinocyte stem cells</td>
</tr>
<tr>
<td>LKB1</td>
<td>liver kinase B 1 (STK11)</td>
</tr>
<tr>
<td>MCL</td>
<td>mantel cell lymphoma</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>mLST8</td>
<td>mammalian lethal with sec-13 protein 8 (also known as GβL)</td>
</tr>
<tr>
<td>mSin1</td>
<td>mammalian stress-activated map kinase-interacting protein 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin (mechanistic target of rapamycin)</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NMSC</td>
<td>Non-melanoma skin cancer</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide dependent kinase-1</td>
</tr>
<tr>
<td>PI</td>
<td>proliferation index</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PRAS40</td>
<td>proline-rich AKT substrate 40kDa</td>
</tr>
<tr>
<td>protor1/2</td>
<td>protein observed with rictor 1 and 2</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog deleted on chromosome 10</td>
</tr>
<tr>
<td>Raptor</td>
<td>regulatory-associated protein of mTOR</td>
</tr>
<tr>
<td>RCC</td>
<td>renal cell carcinoma</td>
</tr>
<tr>
<td>RHEB</td>
<td>Ras homolog enriched in the brain</td>
</tr>
<tr>
<td>Rictor</td>
<td>rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>S6K1</td>
<td>S6 kinase 1</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SGK1</td>
<td>serum- and glucocorticoid-induced protein kinase 1</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TPA</td>
<td>tetradecanoyl phorbol acetate</td>
</tr>
<tr>
<td>TS</td>
<td>tuberous sclerosis</td>
</tr>
<tr>
<td>TSC1</td>
<td>tuberous sclerosis complex 1 (hamartin)</td>
</tr>
<tr>
<td>TSC1</td>
<td>tuberous sclerosis complex 2 (tuberin)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
</tr>
<tr>
<td>wt</td>
<td>weight</td>
</tr>
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</table>
XP  xeroderma pigmentosum
XPC  xeroderma pigmentosum C protein
Chapter 1

Introduction

1.1 mTOR

Mammalian target of rapamycin (mTOR or mechanistic target of rapamycin) is a highly conserved and ubiquitously expressed serine/threonine kinase that belongs to the phosphatidylinositol kinase-related protein kinase family [1]. In response to a variety of stimuli, including growth factors, nutrients and cellular stress, mTOR regulates cell growth, cell cycle progression, protein translation, differentiation and activates pro-survival/anti-apoptotic signaling [1]. Because mTOR dysregulation has been implicated in a wide variety of human disease, including cancer, obesity, diabetes and neurodegeneration, (recently reviewed in [2]), it has become the focus of intense study.

mTOR was identified as the molecular target of the macrolide rapamycin (sirolimus) in 1994 [3,4], after first being discovered in yeast as TOR1 and TOR2 [5,6]. mTOR acts as the catalytic subunit of at least two functionally and compositionally distinct protein-signaling complexes: the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-resistant mTOR complex 2 (mTORC2) (See Figure 1-1A). Both complexes contain mTOR, mLST8 (mammalian lethal with sec-13 protein 8) [7,8] and DEPTOR (DEP domain-containing TOR interacting protein) [9]. mTORC1 also contains Raptor (regulatory associated protein of mTOR), a positive regulator of mTOR1, acting as an adaptor protein that recruits mTOR substrates [10,11,12,13] and PRAS40, an mTOR1 inhibitor (proline-rich AKT substrate 40kDa) [14,15]. mTORC2
also includes Rictor (rapamycin-insensitive companion of mTOR), mSIn1 (mammalian stress-activated map kinase-interacting protein 1) and protor1/2 (protein observed with rictor 1 and 2) [8,15,16,17,18,19].

1.1.1 mTORC1

mTORC1 is the better characterized of the two mTOR complexes. It can be activated by a diverse number of upstream signals, including growth factors, stress, energy status, oxygen, and amino acids that in turn alter a wide variety of cellular functions (See Figure 1-1B) (reviewed in [1]). Increased levels of nutrients under conditions of sufficient oxygen increase the production of ATP, which prevents the inhibitory effect of AMPK (AMP-activated kinase) on mTORC1. Growth factors, including EGF (epidermal growth factor), TGF (transforming growth factor), VEGF (vascular endothelial growth factor) and IGF-1 (insulin-like growth factor) activate mTORC1 through binding to RTKs (receptor tyrosine kinase) (See Figure 1-2). Activation of RTK in turn stimulates the PI3K (phosphatidylinositol-3-kinase) and Ras pathways. The respective effectors of these pathways, AKT (protein kinase B) and ERK1/2 (extracellular signal regulated kinase 1/2), directly phosphorylate and inactivate the TSC1/TSC2 (tuberous sclerosis complex 1/tuberous sclerosis complex 2) complex. TSC1/TSC2 is a GTPase-activating protein (GAP) for the GTPase RHEB (Ras homolog enriched in the brain). When GTP is bound, RHEB directly interacts with and stimulates mTORC1 kinase activity. TSC1/2 inhibits mTORC1 activity by converting RHEB to its inactive GDP-bound form [20].
4EBP1 (eukaryotic translation initiation factor 4E binding protein) and S6K1 (S6 kinase 1) are the best defined substrates directly phosphorylated by mTORC1 (reviewed in [21]) and they in turn regulate protein synthesis. Unphosphorylated 4EBP1 represses translation, however mTORC1 phosphorylation of 4EBP1 induces its release of eIF4E (eukaryotic translation initiation factor 4E) [22], allowing translation initiation of cap-dependent mRNAs, including those involved in proliferation (c-myc, cyclin D1 and ODC) [23] and anti-apoptotic signaling (Bcl-xL and Bcl-2) [23]. Phosphorylation of S6K leads to increased ribosome biogenesis, translation initiation and elongation [24,25,26,27]. Additionally, S6K1 plays a role in apoptotic resistance by phosphorylating and inactivating the pro-apoptotic protein Bad [28]. Phosphorylation of S6K and 4EBP1 are often used as readouts of mTORC1 activity. There are other mTORC1 substrates, including STAT3, LIPIN, CLIP-170 [29,30]. However, their role in mTORC1 signaling is not completely defined.

Most of our current understanding of mTORC1 and its role in cellular functions stems from studies using rapamycin (sirolimus), which was originally found in the soil bacterium *Streptomyces hygroscopicus* discovered on Easter Island, Rapa Nui [31]. Rapamycin inhibits mTORC1 through binding FKBP12 (FK506-binding protein), which then binds to mTOR [32]. Inhibition of mTORC1 by rapamycin is not complete; recent studies demonstrated that phosphorylation of both 4EBP1 and S6K are initially blocked with rapamycin, however in some cell types, 4EBP1 becomes rephosphorylated and can mediate ‘rapamycin-resistant’ mTORC1 activities [33]. Additionally, it has been shown that prolonged rapamycin treatment can also inhibit mTORC2 assembly in some but not all cells [34,35], making the situation more complex.
1.1.2 mTORC2

While the function of mTORC2 is less well understood, it has been shown that growth factors activate mTORC2 through a poorly defined mechanism that requires PI3K [17,18,36]. One study suggests PI3K signaling increases mTORC2 activation by regulating its intracellular location [37]. Additionally, it has recently been shown that mTORC2 binds to ribosomes in a PI3K-dependent manner [38], suggesting that ribosomes could play a role in mTORC2 activation.

The absence of mTORC2 specific inhibitors has complicated the study of mTORC2 downstream effects. However, various genetic techniques have demonstrated that mTORC2 plays an important role in apoptosis, proliferation and cytoskeleton organization (See Figure 1-2) [16,18,36,37,39]. It has been shown that mTORC2 phosphorylates AKT at Ser473, thus promoting cell survival and proliferation [16,36,39,40,41,42]. Complete activation of AKT first requires phosphorylation at Thr308 within the activation loop by PDK1 (phosphoinositide dependent kinase-1), which is activated by PI3K signaling. mTORC2 phosphorylates Ser473 of the AKT hydrophobic motif and pAKT^{Ser473} is often used as a readout of mTORC2 activity [18,39,43]. Ser473 phosphorylation is necessary and sufficient for AKT to phosphorylate FoxO1/3a [18]. In the absence of phosphorylation, FoxO translocates to the nucleus and increases transcription of genes regulating cell cycle arrest (p21, p27) and apoptosis (FasL, TRAIL) [44]. However, mTORC2-dependent phosphorylation of FoxO by AKT results in cytoplasmic retention and inhibition of FoxO transcriptional activity. Inactivation of FoxO transcription factors may explain mTORC2's role in cellular proliferation and survival. Interestingly, phosphorylation at Thr308 is sufficient for AKT-induced phosphorylation of
TSC2 and activation of mTORC1 [18], suggesting that mTORC2 activity may not be required for mTORC1 activation.

In addition to activating AKT, mTORC2 has also been shown to directly phosphorylate and activate SGK1 (serum- and glucocorticoid-induced protein kinase 1), which controls ion transport [45]. SGK1, like AKT, phosphorylates FoxO1/3a, providing another manner for mTORC2 to block apoptosis and cell cycle arrest. Additionally, mTORC2 can phosphorylate PKC-α and alter cytoskeleton organization through RhoA and Rac1 [8,16,40,41]. The downstream effectors of mTORC2 continue to be characterized. However, this endeavor is complicated by the differences observed in various cell types and genetic techniques used to inhibit mTORC2. While studies using siRNA have shown that acute downregulation of mTOR, rictor, or mLST8 results in altered cytoskeleton organization [8,16], MEFs with rictor, mSin1, or mLST8 deleted do not have cytoskeletal defects [18,39,43]. These conflicting results suggest that compensatory mechanisms might come into play with prolonged inhibition of mTORC2 and illustrate the need for further studies to better define the actions of mTORC2.

1.2 mTOR and Cancer

Hyperactivation of mTOR signaling is found in most human cancers and occurs either through mutational activation or amplification of upstream signals: growth factor receptors, the catalytic subunit of PI3K (PI3KCA), AKT, Ras, or by inactivation or deletion of the negative regulators of these pathways: PTEN (phosphatase and tensin homolog deleted on chromosome 10), TSC1/2, or p53 (reviewed in [46]). Recently, the first activating mutations of mTOR in human cancers were identified in renal cell
carcinomas and large intestinal adenocarcinomas [47]. Hyperactivation of mTOR signaling has been linked to increased proliferation and reduced sensitivity to apoptotic stimuli in a wide variety of cancer cell lines [1,48,49,50] through a number of downstream effectors (See Figure 1-3). Because mTOR activation is seen in so many types of cancer, clinical trials examining the therapeutic use of rapamycin analogs or ‘rapalogs’ (temsirolimus, everolimus, ridaforolimus), which have the same mechanisms of action as rapamycin but better pharmacokinetic properties [51], are underway (reviewed in [52]). However, the results from many of these trials have been variable. For example, while approximately 40% of patients with mantle cell lymphoma showed slowing of progression [53,54], less than 10% of glioblastoma patients responded [55,56]. This may be explained by enhancement of AKT signaling due to relief of an S6K-dependent negative feedback loop targeting PI3-kinase [8,16,57,58,59], which has been documented in the presence of rapamycin. Loss of this feedback inhibition in cancer cells may have the effect of enhancing cell survival and proliferation via induction of mTORC2.

Though mTORC2’s role in cancer has not been explored as thoroughly as mTORC1, recent studies suggest that mTORC2 activity is essential for transformation in a number of cancer cell types (reviewed in [60]). Multiple agents utilizing combined targeting of both mTOR complexes or alternatively mTORC1 and AKT are in various stages of preclinical and clinical development with the hope to improve the efficacy and alleviate feedback complications seen with rapalogs alone [61]. To fully exploit the clinical potential of targeting the mTOR pathway, it is important to better understand the distinct roles of the two TOR complexes in tumor development.
1.2.1 mTOR and Familial Tumor Syndromes

The first evidence suggesting a link between mTOR activation and human cancer came from familial cancer syndromes. Many of these syndromes develop because of germline mutations and inactivation of negative regulators of mTORC1 signaling, resulting in hyperactivation of mTOR. For example, germline inactivating mutations in TSC1 or TSC2 cause tuberous sclerosis (TS) in humans, which is characterized by nonmalignant hamartomas in the brain, kidney and other organs [62]. Slow growing subependymal giant-cell astrocytomas are seen in nearly 20% of TS patients, typically in the Foramen of Monro and cannot always be resected surgically [63]. A recent trial with everolimus in these patients revealed a reduction in tumor size of greater than 30% [64]. In 2010, the FDA approved the use of everolimus in TS patients with subependymal giant-cell astrocytomas who are not candidates for surgical resection.

LKB1 (liver kinase B1), which normally functions to inhibit mTORC1 through activation of AMPK [65], is mutated in Peutz-Jegher syndrome [66]. Patients with Peutz-Jegher syndrome develop gastrointestinal hamartomas in addition to gastrointestinal and lung carcinomas [67]. Other hamartoma syndromes linking hyperactivation of mTOR signaling to the development of benign tumors include neurofibromatosis and lymphangioleiomyomatosis. Additionally, patients with Cowden disease have mutations in PTEN, which antagonizes the activity of PI3K (See Figure 1-2). These patients are predisposed to both hamartomas and malignant cancer, particularly early onset breast cancer, and such tumors show hyperactivation of both mTORC1 and mTORC2 signaling [68].
1.2.2 mTOR and Hematologic Cancers

Mantel Cell Lymphoma (MCL) has the poorest prognosis of all of the non-Hodgkin’s lymphomas [69] and is characterized by overexpression of cyclin D1 [70]. Cyclin D1 is known to be regulated by mTORC1 and multiple drug trials have shown that rapalogs increase the survival of these patients [71,72]. *In vitro* studies show that rapamycin induces cell cycle arrest in MCL cells [73].

Patient-derived AML (acute myeloid leukemia) cells also showed hyperactivation of the mTOR pathway [74,75]. In fact nearly 40% of AML samples have point mutations in the class III RTKs, c-KIT or FLT3, which are upstream of both AKT and Ras [76]. Interestingly, rapalogs inhibit both mTORC1 and mTORC2 in AML cells *in vitro* [35], suggesting the effectiveness of the treatment might be attributed to the concurrent inhibition of mTORC2 rather than mTORC1 alone.

1.2.3 mTOR and Renal Cell Carcinoma

mTORC1 signaling is hyperactivated in most renal cell carcinomas (RCC) [77], likely because 80% of clear cell renal carcinomas have deletion or inactivating mutations of both VHL (Von Hippel-Lindau) alleles [78]. VHL is a negative regulator of HIF1α [79] and loss of VHL in kidney cells results in increased HIF1α [80]. HIFα transcriptionally activates a number of genes including VEGF, EGFR and IGFR [81,82,83] producing subsequent activation of the PI3K/AKT/mTOR pathway [84]. Additionally, HIFα is translated in a mTOR-dependent manner [85] resulting in a positive feed-forward loop [86]. Studies show rapamycin blunts VHL-null induced elevation of HIF1α levels in
kidney cells [80]. In fact, the FDA approved the rapalogs: temsirolimus and everolimus for treatment of metastatic RCC after randomized drug trials showed increased patient survival [87,88].

1.2.4 mTOR and Lung Cancer

Activation of mTOR signaling is commonly found in lung cancer. Activating mutations of the EGF receptor (EGFR) are found in nearly 75% of non-small cell lung cancers (NSCLC) [89]. Cancer transcriptome database mining, revealed that amplification of the region on chromosome 7 containing the RHEB gene locus is common in lung cancer, as well as liver and bladder cancers [90]. LKB1 mutations/deletions and subsequent mTORC1 activation are also found in nearly 30% of lung cancers [91,92]. Additional studies have shown that LKB1 mutations strongly correlate with a history of heavy cigarette smoking [93]. mTORC1 activation has also been shown in lung cancer cell lines [94]. However, although evidence suggests that mTORC1 would likely be a good therapeutic target, everolimus showed only limited improvement in patient survival in patients with stage IIIb-IV NSCLC previously treated with conventional chemotherapy [95]. Whether these disappointing outcomes were due to incomplete inhibition of 4EBP1 phosphorylation, increased mTORC2 activation and phosphorylation of AKT through the loss of negative feedback loops, or activation of alternative oncogenic pathways remains to be explored and illustrate the need for a better understanding of the distinct roles of the two mTOR complexes in tumor development and growth.
1.2.5 mTOR and Gastric Cancer

mTOR has also been shown to be phosphorylated and its signaling hyperactivated in tumor samples from patients with gastric cancer [96]. Additionally, mTOR hyperactivation strongly correlated with tumor progression, increased metastasis and poor patient survival [97,98]. Nearly a third of patients previously unresponsive to treatment showed improvement when rapalogs were used in combination with traditional chemotherapeutic agents [99], indicating that inhibiting mTOR in combination with other agents may be effective in these malignancies.

1.2.6 mTOR and Breast Cancer

One study indicating the importance of mTOR signaling in breast cancer found an association between RHEB upregulation and poor prognosis in breast (independent of HER2 or PTEN mutations) and head and neck cancers [90]. Targeting mTOR in breast cancer has the potential to help ameliorate resistance to some current therapies. For example, in vitro studies have shown that mTOR activation in breast cancer cells is at least partially responsible for resistance to estrogen receptor antagonist treatment [100] and that mTOR inhibition with rapamycin restored responsiveness to hormonal interventions [101]. Additional studies also suggest that mTORC2 activation plays a role in breast cancer development. Rictor protein levels are elevated in breast tumors relative to surrounding tissue and higher levels were associated with increased metastasis [102]. Moreover, inhibition of mTORC2 but not mTORC1 induced apoptosis in breast cancer cells [103], suggesting that targeting mTORC2 signaling may prove to be an effective strategy for treatment of breast cancer.
1.2.7 mTOR and Prostate Cancer

Hyperactivation of mTOR signaling is very common in prostate cancer. Rheb and PIK3CA are frequently amplified in prostate cancer [104,105]. PTEN is often deleted in prostate cancer and in prostate cancer cell lines [106,107,108,109] and is also correlated with poor prognosis [107]. The role of mTOR in prostate cancer is complicated by cross-talk with the androgen receptor (AR) signaling and mTOR signaling. mTOR activation can induce ligand independent activation of the AR and might be a mechanism for resistance to current therapies [110,111]. In addition, rapamycin treatment induces increased transcription of the AR in some prostate cancer cell lines [112,113]. Trials are currently underway exploring the efficacy of inhibiting both mTOR and AR signaling pathways (reviewed in [46]).

1.2.8 Summary: Targeting mTOR in cancers

mTOR hyperactivation has been attributed to tumorigenesis due to a variety of mutations in a high percentage of solid tumors (reviewed in [114]). More than 30% of all solid tumors have point mutations or amplifications in PIK3CA [115], an upstream activator of mTOR signaling. Hyperactivation of the mTOR pathway has also been linked to poor patient prognosis in a number of cancer types. Furthermore, many cancer cell lines treated with rapamycin show decreased proliferation and increased apoptosis. Collectively, these studies highlight the importance of mTOR in tumor development. However, though many studies have suggested that mTORC1 inhibition offers much promise as a therapeutic strategy, in practice, the use of rapamycin derivatives as chemotherapeutic agents has had limited success.
Studies have also shown upregulation of mTORC2 signaling and overexpression of the mTORC2 component Rictor in a variety of solid human tumors and cancer cell lines [102,116,117]. Inhibition of mTORC2, through siRNA targeting rictor, induces apoptosis in cancer cell lines derived from a variety of tissues [37,103,116,118]. However, currently much is still unknown about the impact of mTORC2 activation in cancer development. Although mTORC2 may also prove to be a valuable target for cancer prevention and treatment, a better understanding of the contribution of each TOR complex to tumor development is needed.

### 1.3 Skin Cancer

The incidence of skin cancer, including melanoma and nonmelanoma skin cancer (NMSC), is higher than all other cancers combined [119]. NMSC, which comprises 96% of all skin cancer [119], represent a significant health care fiscal burden. Statistics indicate that NMSC treatment represents 4.5% of all Medicare expenditures [120]. NMSC is of particular concern because its rate is increasing [121] and primary prevention strategies have had limited success at reducing skin cancer incidence [122].

#### 1.3.1 NMSC

NMSC, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), represents the largest percentage of all cancers diagnosed in the United States [119]. It is estimated that over 3.5 million cases were diagnosed in the US in 2012 [119]. The actual incidence is likely higher than these numbers represent, because NMSCs are
not reported to cancer registries and current estimates are based on health care claims data [123]. Approximately 80% of NMSC are BCCs in the United States [124], although the ratio of BCC:SCC declines in older populations [125] or in regions closer to the equator [124]. The overall lifetime risk for developing SCC is approximately 10%, while BCC is 30% [126]. Additionally, while the incidence of both BCC and SCC are increasing, the age-adjusted rate of NMSC increased by nearly 80% from 1992 to 2006 [127] and the incidence of SCC is increasing at a rate nearly 4 times that of BCC [128,129,130]. Importantly, the rate of SCC diagnosed in younger populations (less than 35 years of age) is also increasing [131]. This trend is particularly frightening, because SCCs have a greater capacity for invasion and metastasis than BCCs and are responsible for the majority of NMSC mortality [132], including 3,010 deaths in 2012 [119]. Collectively, these studies emphasize the urgent need to develop new strategies for prevention and treatment of NMSC. Increased knowledge of the signaling pathways involved in the development of NMSC could be used to identify novel targets and mechanism-based strategies for chemoprevention.

1.3.1.1 Risks of NMSC

Ultraviolet (UV) radiation has been implicated as the main cause of NMSC. Most SCCs occur in sun-exposed areas of the body and have been associated with chronic sun exposure [133]. The risk of SCC is increased in fair-skinned patients, those with increased cumulative sun exposure, those living in geographic locations closer to the equator and older populations (reviewed in [134]). Additionally, the 3-year risk of SCC in patients previously diagnosed with SCC is more than 10 times the rate in the general
Patients previously diagnosed with an actinic keratosis (AK) are also at increased risk for SCC [136]. AKs are intra-epidermal malignancies that exist on a continuum with SCC. However, not all AK progress to SCC, the 10-year transformation rate is estimated to be only 16% [137]. Why some AK advance to SCC while others do not remains unknown, however, a better understanding of the transduction pathways activated during tumor development could provide valuable clues.

In addition to the increasing overall rate of NMSC, there are also specific groups who are at particularly high risk for developing SCC. Xeroderma pigmentosum (XP) is an autosomal recessive genetic disease of the DNA damage repair machinery [138]. These patients have hypersensitivity to UV light and significantly increased (2000-fold higher) risk of SCC, BCC and melanoma [138,139,140].

Another population at risk for NMSC includes patients who have taken photosensitizing medications for prolonged periods of time. A recent large case-control study, found an increased risk for developing SCC and BCC associated with photosensitizing medications, particularly antimicrobials used for skin conditions and thiazide diuretics [141]. Organ transplant recipients taking immunosuppressive drugs to prevent rejection are also at a higher cancer risk, with SCC being the most common neoplasm [142]. The incidence of SCC is 60-100-fold greater in organ transplant recipients than the in general population [143] and frequently patients are diagnosed with multiple SCCs [144]. These high-risk populations not only provide valuable information about the pathways involved in NMSC development, but also highlight the need to develop prevention strategies.
1.3.1.2 Ultraviolet Radiation

Human skin is continuously exposed to environmental influences, including ultraviolet (UV) irradiation, the leading cause of skin cell damage and skin cancer [145]. The UV spectrum is divided into UVC (200-280nm), UVB (280-320nm) and UVA (320-400nm). Of these, UVB wavelengths are the most energetic and account for the majority of the biologically damaging effects of sun exposure [146]. UVB is absorbed by macromolecules, inducing DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) [147,148]. Failure to repair these DNA lesions results in C→T and CC→TT transitions during DNA replication (reviewed in [149]).

Mutations in p53, a transcription factor induced by DNA damage and involved in regulating cell cycle and apoptosis, are the most common mutation in SCC [150]. Mutation at the dipyrimidine hot spot areas of p53 (codons 177, 196, 278, 294, or 342) are found in 80% of AKs and greater than 90% SCC (>90%) [151,152]. The importance of p53 in preventing skin cancer is demonstrated by studies using p53 mutant mice, which are significantly more susceptible to UVB carcinogenesis and chemical carcinogenesis [153,154]. Interestingly, loss of p53 function also promotes mTORC1 activation [155]. Additionally, activating mutations in ras are also commonly found in UVB-induced SCC [156,157].

In addition to inducing DNA damage and mutation, UVB activates multiple signal transduction pathways that induce expression of specific genes linked to skin cancer [122]. Although the mechanism for the tumor-promoting effects of UVB is not well understood, UVB-induced activation of signal transduction pathways, independent of its DNA-damaging effects, are believed to be responsible. Studies have shown that UVB
induces phosphorylation and activation of a variety of cell surface receptors (EGF receptor, PDGF receptor, FGF receptor, insulin receptor) [158,159,160,161,162,163]. Induction of cascades downstream of these receptors may promote the survival and proliferation of damaged and potentially cancerous cells and provides a possible target for chemoprevention and therapeutic intervention. Previous work in mouse models has shown that targeting pro-survival pathways, such as by knocking out the anti-apoptotic gene Bcl-xL, increased the sensitivity of DNA-damaged keratinocytes to apoptotic signaling and was sufficient to inhibit skin carcinogenesis [164].

NMSC pathogenesis is characterized by both enhancement of cell proliferation and inhibition of apoptosis. However, the molecular mechanisms involved in the pathogenesis of NMSC are not completely understood. Therefore, a better understanding of the pathogenesis of NMSC and the signaling pathways induced by UVB might allow earlier intervention and prevent formation of these tumors.

1.3.2 mTOR and human NMSC

Several reports indicate that mTOR signaling may play a critical role in NMSC development. Immunohistochemical analysis of human epidermal tumors showed that mTOR and its downstream effectors 4EBP1, S6K and AKTSer473 are phosphorylated at much higher levels in SCCs and precancerous AKs than in normal skin [165]. More recently, reverse phase protein microarray analysis of SCC, AK and normal skin revealed aberrantly activated mTORC1 and mTORC2 pathways in the precancerous and transformed tissues [166].
Clinical studies suggest that transplant patients taking rapalogs have a reduction in cutaneous carcinogenesis by 50% or more compared with patients on alternative immunosuppressive agents [167,168,169,170]. Data compiled from over 30,000 kidney transplant recipients found the use of mTOR inhibitors as maintenance immunosuppressive therapy produced a remarkable reduction in NMSC incidence compared to the calcineurin inhibitor Cyclosporine A (CsA) [171]. A dramatic decrease in the incidence of skin malignancies was also observed in transplant patients who were converted to mTOR inhibitors after three months of treatment with CsA (the CONVERT study) [172]. The multicenter TUMORAPA (Efficacy of Rapamycin in Secondary Prevention of Skin Cancer in Kidney Transplant Recipients) trial, found that switching transplant patients, who had at least one previous cutaneous SCC, from calcineurin inhibitors to sirolimus significantly prolonged their cutaneous SCC-free survival. New carcinomas developed in 22% of the sirolimus group and 39% of the calcineurin inhibitor group [173]. Collectively, these studies suggest that targeting mTOR could significantly impact prevention and treatment of NMSC in the general population as well.

1.3.2.1 mTOR and UV

The link between mTOR and the development of NMSC is supported by studies showing that UVB activates the PI3K/AKT survival pathway via the EGF receptor in human skin in vivo [162]. In vitro studies show UVB induces activation of both mTORC1, as measured by p-4EBP1 and p-S6K , and mTORC2 as measured by p-AKT<sup>Ser473</sup> in human and murine primary keratinocytes and cell lines [162,174,175,176,177,178]. Photocarcinogenesis is characterized by impaired apoptosis and enhanced cell
proliferation. However, the specific roles of mTORC1-dependent and mTORC2-dependent pathways in UVB-induced proliferation and apoptosis have not been investigated. This knowledge could provide useful insight for the prevention and management of skin cancer.

1.4 Mouse models

Since over 95% of the mouse genome is similar to the human genome [179], research using mice as a model organism has provided valuable insights into human biology and diseases. Additionally, the ease of genetic manipulation in mice provides an invaluable tool to decipher the molecular signaling pathways important to a diverse spectrum of diseases. The use of innovative genetic strategies in mouse models allows researchers to address mechanistic questions in a physiologically relevant setting to allow better understanding of disease pathophysiology and increased ability to identify therapeutic targets for human diseases.

1.4.1 Mouse models of skin carcinogenesis

The mouse skin chemical carcinogenesis model has been widely utilized to identify key molecular steps relevant to tumor formation in the skin and the information acquired has been applied more generally to identify signaling pathways required for cellular transformation and tumor progression in other tissues (reviewed in [180]). The mouse multistage model of carcinogenesis is frequently used in chemoprevention studies because it is an in vivo model that allows one to visually follow the progression of
tumors at different stages of development. Carcinogenesis in epithelial tissues, including the skin, is generally divided into three stages: initiation, promotion and progression [181] (See Figure 1-4). The initiation stage of carcinogenesis is an irreversible event in which carcinogens damage DNA, inducing mutations in critical genes within target stem cells. In skin experimental models, this is accomplished with a single topical application of 7,12-dimethylbenz[a]anthracene (DMBA), which results in an activating mutation at codon 61 of the H-Ras gene resulting in an A\rightarrow T transversion [182,183]. During the promotion stage, clonal expansion of initiated cells results in the formation of benign papillomas. Multiple applications of a tumor-promoting agent such as the phorbol ester tetradecanoyl phorbol acetate (TPA) are used to induce promotion. Progression is defined as the conversion of benign papillomas into invasive carcinomas. The rate of spontaneous progression in mice is dependent on the genetic background used. For example the C57BL/6 strain of mice are very resistant to conversion, while 50% of the FVB/N mice develop SCC with the DMBA/TPA protocol [184,185].

Alternatively, UVB has been shown to act as both a tumor initiator inducing mutations in cells and a tumor promoter, with a single exposure inducing epidermal proliferation [139]. Repeated progressively increasing doses of UVB will induce tumors in mice, although the tumor latency is significantly longer compared to the chemical carcinogenesis protocol [164]. Like the chemical carcinogenesis model, UVB-induced tumors in mice also frequently have mutations in Ras, however, the mutation often occurs in codon 12 [186]. Interestingly, although Ras is not commonly mutated in human SCC of the general population (3-30%) [187,188], patients treated for melanoma with the B-Raf inhibitor Vemurafenib are frequently diagnosed with SCC after beginning treatment and the majority (60%) of these SCC have Ras mutations [189].
As mentioned previously, p53 is the most commonly mutated gene in human SCC. Similar to human SCC, p53 mutations do occur in UVB-induced SCC in mice; however, the mutations are most often found in codon 270 (C→T). This codon corresponds with human codon 273, but there is no dipyrimidine sequence at this site in the human gene [190]. Additionally, at least one copy of p53 is mutated in 30% of DMBA-induced SCC in mice [191].

1.4.2 Cre-LoxP mouse models

The use of gene knockout models in mice is often limited because deletion of essential genes results in embryonic lethality, preventing the study of those genes in adult animals. This issue has been overcome using Cre-LoxP mediated conditional knockout strategies to delete essential genes in only a specific tissue, often bypassing the lethality of whole-body knockout. Cre-LoxP strategies require two different genetically engineered mice (See Figure 1-5A). The first strain contains the gene targeted for deletion flanked by two LoxP sites. LoxP sites are a 34-base pair sequence identified first in bacteriophages and not native to the mouse genome [192]. The second mouse expresses Cre (causes recombination) recombinase driven by a tissue specific promoter. In tissues where Cre is expressed, the DNA segment flanked by the LoxP sites will be excised and the target gene silenced. In tissues where Cre is not expressed, the ‘floxed’ (flanked by LoxP) gene will remain active. Though the Cre-LoxP system is extremely useful, the expression of a constitutively active Cre, even in a tissue-specific manner, can be problematic if deletion of the floxed gene alters development. To overcome this problem, regulatable or inducible Cre mice are
commonly employed (reviewed in [193]). One example of an inducible Cre involves expressing the Cre enzyme fused to a modified estrogen receptor (See Figure 1-5B). The Cre fusion protein is expressed only in the target tissue, but is trapped in the cytoplasm of the cell until the animal is treated with tamoxifen. The type of system allows both spatial (tissue-specific promoter) and temporal (tamoxifen treatment) control of floxed gene deletion (reviewed in [194]).

1.4.2.1 Cre-LoxP mouse models in the skin

The use of transgenic mice has been particularly useful in the study of skin physiology and disease pathology. The human keratin 14 (K14) and keratin 5 (K5) promoters target the expression of transgenes to the mitotically active cells of the basal layer of the epidermis, the outer root sheath and bulge region of the hair follicle where keratinocyte stem cells (KSC) reside (See Figure 1-6), the oral epithelia, the esophagus and the mammary gland [195,196,197]. The use of keratin promoters allows the expression of particular genes to be limited to specific cell populations within the skin. The inducible Cre-LoxP system has been particularly useful allowing one to examine the consequence of deleting (or expressing) a gene of interest in the adult epidermis, thus circumventing any problems caused if a particular gene plays an essential role in epidermal development. In addition, the use of the K5-CreER or K14-CreER transgenic animals is advantageous because it permits one to delete a gene at different points in the carcinogenesis protocol, allowing examination of the preventative or therapeutic effect of targeting a specific gene. Tamoxifen can be applied topically, providing another layer of tissue specificity [198]. This type of ligand-inducible Cre system facilitates
detailed analysis of gene function and has been used to assess the roles of various genes in different stages of skin carcinogenesis [199,200,201,202].

1.4.2.2 Cre-LoxP mouse models of mTOR signaling

Global deletion of mTOR or raptor in mice result in early embryonic lethality [39,203,204], while deletion of rictor, mSin, or mLST8 causes lethality at embryonic day 10.5 [18,39,43,205]. A number of publications have successfully used Cre-LoxP models to delete mTOR, raptor and rictor specifically in skeletal muscle, adipose or liver to explore the specific roles of mTORC1 and mTORC2 in whole-body metabolism and tissue development [206,207,208,209,210,211,212]. Interestingly, but not unexpectedly, the effects of tissue-specific deletion of raptor and rictor are significantly different. Additionally, the importance of mTORC1 versus mTORC2 signaling is different depending on the tissue examined. For example, in skeletal muscle it has been shown that mTORC1 is essential for normal function [208,209], while rictor deletion has minimal impact [209,210]. On the other hand, adipose-specific deletion of rictor revealed that mTORC2 function in fat is involved in whole organism energy sensing; experimental animals had defective insulin signaling, glucose uptake and altered non-adipose organ size [212]. It is important to note that there were distinct differences in glucose homeostasis when Rictor was deleted in the liver using a constitutive Cre compared to Rictor deletion in adult animals using an inducible Cre (CreER) [213] (See Figure 1-5). This study illustrates that caution must be used when conclusions are drawn from studies that delete components of mTOR early in development rather than in adult
animals and emphasizes the appeal of utilizing an inducible system when investigating the role of mTOR.

1.4.3 mTOR and mouse models of cancer

*In vitro* studies have shown that genetically targeting *mTOR*, *raptor* or *rictor* in cancer cell lines often causes decreased proliferation and increased apoptosis \[116,118,214,215,216\]. Additionally, there have been numerous studies illustrating the utility of targeting mTORC1 signaling in mouse models of a variety of cancers (reviewed in \[1\]). However, it was only recently that genetic mouse models were used to examine the role of mTORC2 in the development of cancer. In Guertin *et al.*, the effects of rictor or raptor ablation were compared in the setting of PTEN-deficient prostate cancer \[37\]. Ablation of raptor had little effect on cancer development, but deletion of one or both copies of rictor significantly reduced the development of prostate cancer. Similar effects were seen when comparing the efficacy of mTOR deletion to rapamycin treatment in PTEN-deficient prostate cancer \[217\]. These studies suggest the less explored mTORC2 could prove to play a critical role in tumor development. Additionally, these studies showed no deleterious effects from ablation of mTOR signaling in the normal prostate epithelium, emphasizing the attractiveness of targeting mTORC2 signaling in cancer prevention and treatment.
1.4.3.1 mTOR and mouse models of skin cancer

A considerable amount of evidence using mouse models indicates that mTOR is an attractive target for chemoprevention of NMSC. Although most studies have focused on mTORC1, emerging evidence indicates the potential of targeting mTORC2. For example, heterozygous Ptch1 mice (Ptch1+/−) are commonly used as a murine model of UVB-induced BCC because inactivating mutations of Ptch1 are found in hereditary nevus basal cell carcinoma syndrome as well as sporadic BCCs [218]. Western blot analysis of a panel of UVB-induced BCC in Ptch1+/− mice showed increased levels of markers of both mTORC1 activity (p-4EBP1 and p-S6K) and mTORC2 activity (p-AKT) compared to adjacent skin [219].

A number of studies have indicated that mTORC1 is likely playing an important role in SCC tumor promotion (Table 1-1). Initial studies showed that caloric restriction in mice reduced susceptibility to two-stage tumorigenesis protocols [220], by inhibiting TPA-induced activation of epidermal PI3K [220, 221]. The same group was able to mirror this effect with topical application of the mTORC1 inhibitor, rapamycin [222]. Additional groups have successfully used rapamycin to induce tumor regression in UVB- and chemically induced tumors [223, 224]. The importance of mTORC1 in SCC tumor development is also supported by mouse models that have activated mTORC1 signaling in the epidermis [90, 225]. These mice show increased sensitivity to TPA-induced epidermal hyperproliferation and chemically-induced tumors. Studies using transgenic mice that overexpress or knock-out AKT show more dramatic effects than those targeting mTORC1 signaling [226, 227], suggesting that AKT and mTORC2 might also be important in SCC tumor development. Additionally, a single exposure of UVB or single
treatment of TPA induces phosphorylation and activation of mTOR and down stream effectors of both mTORC1 (S6K) and mTORC2 (AKT$_{\text{Ser}473}$) in the epidermis of mice [228].

Collectively, these results suggest that mTOR signaling plays a critical role in the development of many types of cancer, including skin SCC. Multiple studies using mice suggest that mTORC1 contributes to SCC tumor formation. However, the contribution of mTORC2 has not been examined. Additionally, although studies have shown that both mTOR complexes are activated by UVB, the primary carcinogen of NMSC, the downstream results of this activation, particularly the effects on proliferation and apoptosis, is currently unknown. The work described in this thesis sought to establish a better understanding of the signal transduction pathways induced by UVB in keratinocytes by investigating the specific roles of mTORC1 and mTORC2 using both pharmacological inhibitors and genetic ablation techniques. Our results support a model whereby both mTOR complexes play independent but complimentary roles in UVB-induced skin tumor development. Further studies have illustrated the essential role of mTORC2 in the development and maintenance of papillomas. Our studies provide new insight into the molecular mechanisms involved in UVB-induced skin cell damage leading to skin aging and skin cancer and highlight mTORC2 as a possible target for treatment and prevention of cutaneous SCC.
Figure 1-1: mTORC1 and mTORC2 complexes. A, mTOR acts as the catalytic subunit of two distinct protein-signaling complexes: mTORC1 and mTORC2. Both complexes contain mTOR, mLST8 and DEPTOR. mTORC1 also contains Raptor and PRAS40. mTORC2 includes Rictor, mSin1 and protor1/2. B, Both mTOR complexes are activated by a number of upstream factors and control a broad nexus of cellular functions. (1-1B is from [2]).
Figure 1-2: mTOR signaling cascade. Both mTORC1 and mTORC2 can be activated by a number of upstream factors. mTORC1 phosphorylates 4EBP1 and S6K, while mTORC2 phosphorylates AKT at Ser473. (adapted from [50]).
Figure 1-3: mTOR signaling in cancer. Oncogene activation (red) and tumor suppressor gene loss (green) alter mTOR signaling and promote tumorigenesis. (from [2]).
Figure 1-4: *Multistage mouse skin carcinogenesis model.* Initiation occurs via DMBA or UVB causing genetic mutation. Promotion occurs through repeated exposure to TPA or UVB, which induces clonal expansion of initiated cells and generalized epidermal hyperproliferation. With further genetic mutations some papillomas progress to carcinomas (adapted from [229]).
Figure 1-5: **Cre-LoxP System.** A, Using a transgene that expresses Cre under a tissue specific promoter allows deletion of ‘floxed’ genes only in the specific tissue where Cre is expressed. B, Using an inducible Cre allows the user to temporally control the activation of the recombinase activity. In the example shown, the modified Cre is trapped in the cytoplasm because of the presence of the estrogen receptor (ER). Tamoxifen treatment allows the enzyme to translocate into the nucleus and delete the ‘floxed’ gene.
Figure 1-6: Keratin promoters. Human keratin promoters are often used to drive the expression of transgenes within the mouse epidermis. Keratin 5 (K5) and keratin 14 (K14) are expressed in the basal layer of the epidermis and the bulge region of the hair follicle (adapted from [229]).
<table>
<thead>
<tr>
<th>Mouse System</th>
<th>Treatment</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1 KO</td>
<td>DMBA/TPA (skin papilloma)</td>
<td>KO mice had reduced tumor number and size</td>
<td>[226]</td>
</tr>
<tr>
<td>K5-AKT and K5-myrAKT</td>
<td>DMBA/TPA (skin papilloma &amp; SCC)</td>
<td>Epidermal hyperplasia; increased papilloma and carcinoma formation; small number of mice also developed papillomas spontaneously, or with DMBA or TPA only</td>
<td>[227]</td>
</tr>
<tr>
<td>K14-Rheb</td>
<td>DMBA (skin papilloma)</td>
<td>Epidermal hyperplasia and tumor formation; small number of mice also developed spontaneous papillomas</td>
<td>[90]</td>
</tr>
<tr>
<td>LKB1&lt;sup&gt;+/−&lt;/sup&gt; and K14-Cre; LKB1&lt;sup&gt;LoxP/LoxP&lt;/sup&gt;</td>
<td>DMBA [pups] (skin papilloma &amp; SCC)</td>
<td>LKB1&lt;sup&gt;+/−&lt;/sup&gt; and K14-Cre/LKB1&lt;sup&gt;LoxP/LoxP&lt;/sup&gt; had increased number of papillomas and carcinomas; K14-Cre/LKB1&lt;sup&gt;LoxP/LoxP&lt;/sup&gt; also had delayed hair growth and a wavy hair phenotype.</td>
<td>[225]</td>
</tr>
<tr>
<td>FVB/N + rapamycin</td>
<td>DMBA/TPA (skin papilloma)</td>
<td>i.p. rapamycin induced apoptosis and inhibited proliferation inducing tumor regression</td>
<td>[223]</td>
</tr>
<tr>
<td>FVB/N + rapamycin</td>
<td>DMBA/TPA (skin papilloma)</td>
<td>Topical rapamycin reduced tumor incidence and multiplicity; Topical rapamycin induced tumor regression</td>
<td>[222]</td>
</tr>
<tr>
<td>SKH-1 + sirolimus</td>
<td>UVB (skin papilloma)</td>
<td>i.p. sirolimus reduced tumor multiplicity and progression (also showed CsA increased tumor multiplicity, which sirolimus also blocked)</td>
<td>[230]</td>
</tr>
<tr>
<td>SKH-1 + rapamycin</td>
<td>UVB (skin papilloma)</td>
<td>Dietary rapamycin reduced multiplicity and increased latency</td>
<td>[224]</td>
</tr>
<tr>
<td>K14-CreER&lt;sup&gt;tam&lt;/sup&gt;; LSL-K-ras&lt;sup&gt;G12D&lt;/sup&gt;; p53&lt;sup&gt;LoxP/LoxP&lt;/sup&gt; + rapamycin</td>
<td>Oral tamoxifen (oral mucosa papillomas &amp; tongue SCC)</td>
<td>i.p. rapamycin reduced tumor formation</td>
<td>[231]</td>
</tr>
<tr>
<td>nu/nu + rapamycin</td>
<td>HNSCC tumor xenograph</td>
<td>i.p. rapamycin induced apoptosis and inhibited proliferation, inducing tumor regression</td>
<td>[232]</td>
</tr>
<tr>
<td>C3H/HeJ + everolimus</td>
<td>intradermal injection of SCC VII cells (spontaneous murine SCC cell-line)</td>
<td>Gavage everolimus inhibited tumor growth and metastasis</td>
<td>[233]</td>
</tr>
</tbody>
</table>

Table 1-1: **Mouse models of mTOR in skin carcinogenesis.** Previously published mouse models linking mTOR signaling and skin carcinogenesis.
Hypothesis and Specific Aims

Hypothesis: Both mTORC1 and mTORC2 have critical but divergent roles in the development of SCC. Inhibition of mTOR signaling will suppress the expansion of cells by promoting apoptosis through inhibition of mTORC2 signaling and attenuate cell proliferation through mTORC1 inhibition, thus preventing the formation of NMSC (Figure 1-7).

Specific Aim 1: Examine the contribution of mTORC1 to UVB- and TPA- induced effects in keratinocytes using rapamycin.

Specific Aim 2: Characterize the effects of inhibiting both mTOR complexes in the keratinocyte response to UVB treatment.

Specific Aim 3: Assess the specific role of mTORC2 in mediating UVB-induced pro-survival signals.

Specific Aim 4: Examine the effect of mTORC2 ablation on tumor development and maintenance using a two-stage chemical carcinogenesis model.
Figure 1-7: Possible role of mTOR in skin SCC development. Both mTORC1 and mTORC2 are known to play roles in cell proliferation and survival in some cell types. Additionally, both UVB and TPA activate mTORC1 and mTORC2 signaling. We hypothesize that mTOR activation in response to UVB activates pro-survival signaling, inhibiting apoptosis of cells that could possibly harbor mutations. Additionally, we theorize that mTOR activation is partially responsible for mediating UVB- and TPA-induced epidermal hyperproliferation. Targeting mTOR should inhibit the survival and proliferation of keratinocytes following UVB or chemical exposure and prevent the formation of papillomas.
Chapter 2

Rapamycin attenuates UVB- and TPA- induced cell cycle progression and proliferation

2.1 Introduction and Rationale

In photocarcinogenesis, UVB-stimulated activation of serine/threonine and tyrosine kinase signaling pathways and transcription factors mediate a number of pathologic changes in the skin. The resulting keratinocyte proliferation, epidermal hyperplasia and tumor promotion [139] are similar to the effects seen with topical TPA treatment [181]. Characterization of signaling activation during tumor promotion can generate a better understanding of the development of NMSC, hopefully leading to the identification of targets for chemoprevention.

Several reports suggest that mTORC1 activation may contribute to the epidermal hyperplasia seen during tumor promotion. AKT signaling is activated in the epidermis by a variety of tumor promoting agents, including TPA and UVB [228]. Mice expressing a constitutively active form of AKT in the epidermis show spontaneous epidermal hyperplasia, similar to the effect seen with repeated tumor promoter applications [227]. Interestingly, this phenotype is nearly identical to that seen in mice overexpressing Rheb, the immediate upstream activator of mTORC1 [90]. These results suggest that mTORC1 is responsible for mediating the epidermal hyperplasia induced by AKT activation.

Activation of AKT signaling contributes to increased survival and proliferation in a variety of tumorigenesis models [48,234]. However to date, the contribution of mTORC1 to cell proliferation and survival during tumor promotion in the skin has not been fully
explored. Although studies in mice suggest that mTOR could be a potential chemotherapeutic or preventative target in SCC [222,223,224,230], these studies did not explore the contribution of the two mTOR complexes to skin tumor development or the downstream effects of mTOR activation on proliferation and apoptosis in the skin.

In this study we sought to better delineate the pathogenesis of SCC by examining the specific contribution of mTORC1 to UVB- and TPA-induced proliferation and survival in keratinocytes (Figure 2-1). We utilized rapamycin for in vitro studies in both HaCaT cells, a human keratinocyte cell line and murine primary keratinocytes, to examine the role of mTORC1 in UVB-induced cell cycle progression and survival. Additionally, we applied rapamycin topically to mice prior to UVB and TPA exposure to investigate the effects of mTORC1 inhibition in an in vivo setting.

2.2 Materials and Methods

2.2.1 Cell culture

HaCaT a spontaneous immortalized nontumorigenic human keratinocyte cell line [235], (obtained from The German Cancer Research Center) were maintained in High-Glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biological), 110 mg/L sodium-pyruvate (Sigma) and 25 mg/L Gentamicin (Invitrogen), trypsinized using 0.05% Trypsin plus EDTA (Invitrogen) and split 1:10. Cells were cultured for less than 20 passages and incubated in 5% CO₂ at 37°C. Cells were seeded in 60-mm² plates at a density of
150,000 – 300,000 per plate and in 96-well plates at 7,500-10,000 cells per well. All experiments were done when plates had reached approximately 80% confluence.

Primary mouse keratinocytes were isolated from 1-3d old pups as described previously [236]. Briefly, full thickness skin was floated overnight at 4°C in Ca^{2+}-free 0.25% Trypsin without EDTA (Cellgro) to separate the epidermis from the dermis. Epidermal sheets were minced and the cell suspension was strained (100-µm nylon filter, Fisher) and plated at a density of one-half mouse equivalent (ME) per 60mm² plate, or approximately 2 ME for a 96-well plate (1/40 ME per well). Cells were grown in keratinocyte growth medium [calcium-free MEM Eagle with Earle's BSS, glutamine and non-essential amino acids (Lonza) supplemented with 8% chelexed FBS (Atlanta Biological), 1% glutamine (Invitrogen) and 25 μg/mL penicillin/streptomycin (Invitrogen)]. Cells were maintained in 7% CO₂ at 36°C and medium was changed every other day.

Rapamycin (Developmental Therapeutics Program, National Cancer Institute) or vehicle (DMSO) was added to the medium at a 1:1000 dilution 1 h prior to UVB treatment. For cell cycle experiments (low-dose UVB), medium was replaced with 0.1% fetal bovine serum (FBS) for 24 h prior to UVB treatment. Cells were washed twice with PBS, then in a minimal volume of PBS exposed to UVB (FS20 UVB bulbs, National Biological) emitting UV light between 290-320nm. The irradiation intensity was monitored using a UVB 500C meter (National Biological). After irradiation, PBS was removed and conditioned medium with drug treatments was added back.
2.2.2 Mouse studies

All experiments involving mice were carried out in compliance with the Guide for the Care and Use of Laboratory Animals and protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. The dorsal surface of FVB/N mice (6-8 weeks) was shaved with electrical clippers and mice were allowed to rest for 24-48 h prior to all experiments; only mice in the resting stage of the hair cycle were used. For UVB studies, 2-4 mice were used for each treatment group and housed together. Mice were treated topically with rapamycin (25, 100, 200 nmol in 100 µL DMSO:Acetone [1:9], D:A) or vehicle 1 h prior to exposure to UVB irradiation from UVB lamps (FS20 UVB bulbs, National Biological) at a dose of 120 mJ/cm². Bulb intensity was measured at the beginning of each experiment. For TPA studies, 4 mice were treated with 100 nmol rapamycin or vehicle 1 h prior to TPA (6.8 nmol Calbiochem) or acetone (200 µL). The treatment regimen was repeated every three days for a total of four treatments.

2.2.3 Western blotting

For in vitro studies, cells were washed twice with cold PBS and harvested by scraping into RIPA buffer [20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na₃VO₄, 1 µg/mL leupeptin, 1mM PMSF, 1X protease inhibitor cocktail] (Santa Cruz Biotechnology), centrifuged and supernatants collected. In selected experiments examining apoptosis, detached cells were collected via centrifugation of
medium and combined with the attached cells in RIPA buffer. For in vivo studies, animals were euthanized 6 h after UVB or TPA and dorsal skin was treated with a depilatory agent for 3 min, washed, excised and underlying fat and connective tissue were removed. Epidermal samples were collected by scraping the surface of excised skin with a razor blade and placed into ice-cold RIPA buffer. Following sonication, samples were centrifuged and supernatants collected. Whole skin samples were flash-frozen and later processed in RIPA buffer by homogenizing for 30 sec on ice using a Polytron homogenizer, centrifuged at 30,000 x g for 30 min at 4°C and supernatants collected. Protein concentrations were determined using the BioRad assay. Equal amounts of protein were subjected to electrophoresis. Western blotting was performed as described previously [237]. Antibodies used include AKT, p-AKT<sup>Ser473</sup>, S6K, p-S6K<sup>Thr389</sup>, Caspase-3, 4EBP1, (all from Cell Signaling, 1:1000) and GAPDH (Proteintech, 1:2000).

2.2.4 Flow cytometry

For cell cycle analysis, cells were trypsinized and collected 18 h after UVB. Cells were fixed with ice-cold ethanol overnight. Samples were washed twice with ice-cold PBS and then incubated with propidium iodide (20 µg/mL) containing RNase A (Sigma). The DNA content was determined using a FACSCalibur cytometer (Beckman Coulter) and analyzed with Modfit LT software (Verify Software).
2.2.5 Histology and Immunohistochemistry

Tissue sections were collected from mice at 24 or 48 h after UVB treatment (24 h was used for mock animals) or at 6 h after the final TPA/vehicle treatment. Mice received intraperitoneal (i.p.) injections of 5-bromo-2-deoxyuridine (BrdU, Sigma) at 100 µg/g body wt in NaCl 1 h prior to euthanasia. Dorsal skin was treated with a depilatory agent for 3 min, washed, excised and fixed for 6h in 10% neutral buffered formalin. Skin was embedded in paraffin and 5 µm sections were cut for immunohistochemistry. The effect of rapamycin on UVB- and TPA-induced epidermal hyperplasia was studied by histopathological examination of hematoxylin and eosin (H&E) stained tissue sections. Epidermal thickness was measured at 5 locations in four different sections for each mouse. Epidermal proliferation was assessed in vivo using BrdU incorporation. Sections were deparaffinized, rehydrated and stained with an anti-BrdU antibody as described previously [238]. Epidermal proliferation index (PI) was determined by calculating the percentage of basal cells positive for BrdU. A minimum of 1000 continuous basal cells were counted for each mouse at 3 different locations.

2.2.6 ODC activity assay

The ODC enzymatic activity was measured in epidermis and dermis samples collected 6 h after final TPA treatment. An excised portion of skin was placed in ice-cold water for 3 min, 55°C for 30 sec and returned to ice-cold water for 3 min. Connective tissue was removed from the samples. Epidermis samples were collected by scraping the surface of excised skin with a razor blade. Remaining tissue after scrapping was
collected as the dermis. Epidermis and dermis samples were flash frozen. Later samples were suspended in ODC harvest buffer [10mM Tris-HCl pH 7.5, 2.5mM dithiothreitol, 0.1mM EDTA]. Following sonication (epidermis) and homogenization (dermis) samples were centrifuged and supernatants collected. ODC activity was measured by the release of $^{14}$CO$_2$ from L-[1-14C] Ornithine as described previously [239].

2.2.7 Cell viability

The cell viability at 24 h after UVB exposure was determined colorimetrically by MTS assay (CellTiter 96 Aqueous Proliferation Assay, Promega) according to the manufacturer's instructions and monitored at 490nm using a model 3550-UV plate reader (BioRad). Each condition was plated in triplicate and the experiments were repeated at least three times.

2.2.8 Statistical analysis

Data are expressed as the mean of at least three independent experiments analyzed by 2-sided Student’s t-test. A p-value of <0.05 was considered significant.

2.3 Results

2.3.1 Rapamycin inhibits UVB-induced cell cycle progression in HaCaT cells

Previous work showed that sub-apoptotic doses of UVB (2.5 to 10 mJ/cm$^2$) induce G1-S cell cycle progression in serum-deprived HaCaT cells [240]. Interestingly,
the same study found that these doses of UVB also activate EGFR/PI3K/AKT signaling [240]. Other work has shown that mTORC1 activation induces the increased translation of cyclinD1, a cell cycle protein necessary for G1-S cell cycle progression [241]. We hypothesized that the UVB-induced cell cycle progression seen in HaCaT cells is mediated by mTORC1 signaling. Initial studies were first performed to determine whether mTOR signaling is activated in response to low dose UVB in this system.

HaCaT cells were serum-starved to synchronize them in G0 and then subjected to 0, 2.5, 5 and 10 mJ/cm² UVB. Low dose UVB activates both mTORC1 and mTORC2 signaling pathways as measured by phosphorylation of S6K and AKT<sup>Ser473</sup> respectively (Figure 2-2). At the 0h time point and in mock-UVB treated cells, the levels of p-S6K and p-AKT<sup>Ser473</sup> are nearly undetectable. There is a dramatic increase in the levels of phosphorylation of both markers of mTOR signaling at 30 min and these levels remain high at 2h. Further studies were performed to verify that rapamycin treatment suppressed UVB-induced activation of mTORC1 using the 2h time point. Rapamycin (50nM) suppressed UVB-induced S6K phosphorylation, but did not alter phosphorylation of AKT<sup>Ser473</sup> (Figure 2-3), indicating that in this system rapamycin inhibition is specific to mTORC1 and does not alter UVB-induced activation of mTORC2 signaling. To determine whether mTORC1 activation contributes to UVB-mediated cell cycle progression, cell cycle analysis was performed on vehicle and rapamycin treated cells 18h after UVB exposure. The percentage of vehicle-treated cells in S phase was significantly increased with UVB treatment, indicating UVB does induce G1-S cell cycle progression in our system (Figure 2-4). The portion of cells in S-phase after UVB was significantly attenuated in rapamycin treated cells compared to vehicle control, indicating that UVB-induced cell cycle progression is mediated in part by mTORC1 activation.
2.3.2 Topical Rapamycin inhibits UVB-induced epidermal hyperproliferation

Our results show that mTORC1 mediates UVB-induced cell cycle progression in an *in vitro* system. Because cellular proliferation in response to UVB is a key feature of photo-carcinogenesis, we next sought to explore the role of mTORC1 in UVB-mediated responses *in vivo*. Wild-type FVB/N adult mice were treated topically with rapamycin (100nmol) 1 h prior to irradiation with a single dose of UVB (120 mJ/cm²), a level considerably lower than the minimal erythemic dose in FVB/N mice (750 mJ/cm² [242]), but known to induce epidermal hyperproliferation [243]. Immunoblot analysis of whole-skin samples at 6 h post UVB was used to assess the levels of mTOR activation. The level of p-S6K was too low in mock-irradiated samples to discern any noticeable inhibitory effect of rapamycin (Figure 2-5). However, there was a dramatic increase in p-S6K and p-AKT^Ser473^ levels in vehicle-treated skin after UVB irradiation. Rapamycin pretreatment prevented UVB-stimulated phosphorylation of S6K. Interestingly, unlike our *in vitro* model, rapamycin appears to induce mTORC2 activation in murine skin, likely due to release of an S6K-dependent negative feedback loop targeting PI3-kinase [57,58].

The effect of mTORC1 inhibition on UVB-induced epidermal hyperplasia was assessed by examining the thickness of the epidermis in H&E skin sections at 24 and 48 h after UVB exposure (Figure 2-6). Rapamycin treatment showed no effects on epidermal thickness in mock irradiated mice. There was noticeable epidermal thickening in vehicle-treated animals at 24 and 48 h following UVB exposure, but this effect was significantly reduced in animals treated with rapamycin. Additional studies, using 50 nmol and 200 nmol doses of rapamycin show that the inhibition of epidermal thickening
Proliferation was measured by assessing BrdU incorporation into keratinocytes in the basal layer of the epidermis. Vehicle- treated mice showed a significant increase in BrdU-positive cells at 48 h post UVB and this effect was significantly reduced by rapamycin treatment (Figure 2-8). Although rapamycin treatment did not completely block UVB-induced proliferation, the attenuation was significant, suggesting that mTORC1 is at least partially responsible for epidermal hyperproliferation following UVB exposure.

2.3.3 Topical Rapamycin inhibits TPA-induced epidermal thickening and ODC activation

To further validate the role of mTORC1 in tumor promotion, we examined the effect of rapamycin treatment of TPA-induced effects in the epidermis. TPA, a phorbol ester, is the tumor promoter routinely used to induce proliferation during two stage chemical carcinogenesis. Animals were topically treated with rapamycin 1 h prior to TPA. Treatment regimen was repeated every three days for a total of four treatments. Immunoblot analysis of whole-skin harvested 8h after the final TPA treatment showed that TPA induced hyperphosphorylation of 4EBP1 and topical rapamycin treatment blocked phosphorylation, increasing levels of hypo-phosphorylated 4EBP1 (Figure 2-9). The effect of rapamycin (25 and 100nmol) on TPA-induced hyperplasia was explored by examining the epidermal thickness in H&E sections (Figure 2-10). TPA treatments resulted in a dramatic increase in epidermal thickness. Similar to the results seen in our UVB experiments, rapamycin blocked TPA-induced epidermal thickening in a dose-dependent manner.
Ornithine decarboxylase (ODC) activity of our TPA-treated samples was also examined in the presence of rapamycin. ODC is the rate limiting enzyme in biosynthesis of polyamines and is up-regulated during TPA-induced tumor promotion [244]. Previous work in our lab has shown that the translation of ODC is mTOR dependent [245]. Consistent with these previous findings, there was a dose-dependent decrease in ODC activity in epidermal samples from rapamycin treated mice (Figure 2-11), although the changes were not statistically significant due to small sample size.

2.3.4 Rapamycin does not alter UVB-induced apoptosis

Because SCC pathogenesis is characterized by both enhancement of cell proliferation and inhibition of apoptosis, and previous work has shown that UVB-induced activation of AKT promotes the survival of epidermal keratinocytes [246], we next sought to explore if mTORC1 plays a role in keratinocyte survival following UVB exposure. Preliminary experiments verified that the higher doses of UVB that induce apoptosis, also activate mTOR signaling. The phosphorylation levels of S6K and AKT after 50 mJ/cm² UVB exposure in murine primary keratinocytes were examined by western blot analysis (Figure 2-12). Enhanced signaling though both mTORC1 and mTORC2 was indicated by increased levels of p-S6K and p-AKT^{Ser473} at 30 min after 50 mJ/cm² UVB and remained up-regulated at 2 h. Treatment of cells with rapamycin completely blocked UVB-induced activation of mTORC1 signaling (p-S6K), but had little if any effect on mTORC2 activity (p-AKT^{Ser473}) (Figure 2-13). To determine if inhibition of mTORC1 could sensitize keratinocytes to cell death, cells cultured with various rapamycin concentrations were harvested 24 h after UVB exposure and cell viability was assessed.
using MTS assay. Treatment with a wide range of rapamycin concentrations did not alter cell viability in mock-irradiated cells (Figure 2-14A). Exposure to 50 mJ/cm² UVB induced an obvious decrease in cell viability (54% ± 3.6%). However, rapamycin did not enhance UVB-mediated cell death in wild-type primary keratinocytes (Figure 2-14A), and this same result was seen in HaCaT cells (Figure 2-15A). Additionally, increasing the pretreatment timing with rapamycin from 1 h to 24 h did not enhance UVB-mediated cell death (Figure 2-15B,C). Western blot analysis of cleaved Caspase-3 verified that there was no increase in UVB-mediated apoptosis with rapamycin treatment (Figure 2-14B), indicating that mTORC1 does not play a critical role in mediating keratinocyte survival following UVB exposure.

2.4 Discussion

The data described here provide compelling evidence that mTORC1 is a critical mediator of cell proliferation during tumor promotion. Our in vitro studies utilizing keratinocyte cell lines and primary keratinocytes clearly show that inhibition of mTORC1 with rapamycin attenuates UVB-induced cell cycle progression (Figure 2-4) but does not sensitize or inhibit keratinocytes to UVB-induced cell death (Figure 2-14 and 2-15). Although studies in mice suggest that targeting mTORC1 with rapamycin blocks chemically- and UVB-induced tumor formation [222,223,224,230], our studies show for the first time that rapamycin is acting to block UVB-induced hyperproliferation (Figure 2-6 and 2-8).

Activation of proliferation and survival signals are important mechanisms that contribute to tumor formation. Although our studies provide strong supporting evidence
of the critical role of mTORC1 in tumor promotion, we did not fully explore the downstream mechanism of mTORC1 responsible for mediating UVB-induced cell proliferation. We did provide some evidence indicating that rapamycin treatment blocks upregulation of ODC by TPA. Previous work has shown that upregulation of ODC is both necessary and sufficient for tumor promotion [247, 248, 249]. However cyclinD1, an essential cell cycle regulator protein is also translated in a mTORC1-dependent manner [241] and could provide an additional mechanism for mTORC1-mediated cell proliferation. It is also possible that mTOR plays a role in tumor promotion-associated inflammation. Chronic inflammation is a risk factor for various human cancers [250, 251] and anti-inflammatory drug usage has been linked to a reduction in the incidence of several human cancers [250, 252]. Interestingly, topical rapamycin has been shown to decrease the infiltration of macrophages, T cells, neutrophils, and mast cells into the dermis of mice following TPA treatment [222]. Future efforts to examine the effects of rapamycin on immune cells and inflammation in our UVB-treated mice, may provide insight into why we see a reduction in UVB-mediated epidermal thickening at 24 h when we do not observe any difference in proliferation at this timepoint (Figure 2-6 and 2-8). It is possible that rapamycin’s effect on immune cell infiltration is contributing to the inhibitory phenotype that we saw following UVB treatment.

Collectively, we provide several lines of evidence supporting a mechanism of tumor formation in the skin where mTORC1 signaling is induced by UVB or TPA and acts as a critical mediator of tumor promotion through enhanced proliferation. Rapamycin attenuates UVB-induced cell cycle progression in an *in vitro* system and these results were confirmed *in vivo*. Additionally, our studies conclusively demonstrated that rapamycin treatment does not sensitize keratinocytes to UVB-induced apoptosis,
indicating that mTORC1 signaling does not contribute to the increased pro-survival signaling seen following UVB exposure (Figure 2-16). It is important to note that recent studies have shown that rapamycin is not completely effective at inhibiting mTORC1 activity and that rapamycin-resistant mTORC1 function mediated through 4EBP1 might play a significant role in cellular proliferation [253,254,255,256]. Future experiments utilizing genetic techniques targeting raptor to completely inhibit mTORC1 activity, will allow us to fully explore the role of mTORC1 in UVB signaling cascades. The role of mTORC2 in UVB-mediated phenotypes, which is addressed in detail in chapters 3 and 4 of this thesis, must also be considered. The effect of rapamycin on mTORC2 signaling seems to differ depending on the system used. Rapamycin inhibited mTORC1 signaling, as measured by p-S6K in all of the model systems that we utilized, and our in vitro data suggest that rapamycin treatment had little effect on mTORC2 induced activation of AKT. However, we saw enhanced phosphorylation of AKT\textsuperscript{Ser473} when rapamycin was used in vivo. This effect is likely due to relief of an S6K-dependent negative feedback loop targeting PI3-kinase [57,58]. Studies utilizing Cre-LoxP animal models and the recently developed mTOR kinase inhibitors that target both mTORC1 and mTORC2 (Chapter 3 and 4) explore the possibility that mTORC2 and/or rapamycin-resistant mTORC1 functions are contributing to NMSC formation.
Figure 2-1: Possible roles of mTORC1 in mediating UVB- and TPA-induced effects. mTORC1 is known to play a role in cell proliferation and survival in some cell types. Additionally, both UVB and TPA are known to induce mTORC1 signaling. We hypothesize that mTORC1 signaling is responsible for mediating UVB- and TPA-induced epidermal hyperproliferation. It is also possible that mTORC1 is playing a role in stimulating cell survival signaling.
Figure 2-2: Low-dose UVB induces mTORC1 and mTORC2 activation in HaCaT cells. Serum-starved HaCaT cells were treated with low dose UVB (2.5, 5, or 10 mJ/cm²) or mock irradiated. Whole cell lysate was harvested in RIPA buffer at indicated times. Data are representative of 3 independent experiments (only 2 for 2.5 mJ/cm²).
Figure 2-3: Rapamycin blocks UVB-induced activation of mTORC1 in HaCaT cells. Serum-starved HaCaT cells were treated with rapamycin (50 nM) or DMSO for 1 h prior to low dose UVB (2.5, 5, or 10 mJ/cm²) or mock irradiation. Whole cell lysate was harvested in RIPA buffer at 2h post-irradiation. Data are representative of 3 independent experiments.
Figure 2-4: *Rapamycin attenuates UVB-induced G1 → S cell cycle progression in HaCaT cells.* Serum starved HaCaT cells were treated with rapamycin (50nM) or DMSO 1 h prior to UVB exposure. 18 h following irradiation cells were trypsinized for propidium iodide flow cytometry analysis. A, All cells. B, S-phase only (mean ± SEM). # p < 0.01 for UVB vs mock, * p < 0.05 and ** p < 0.01 for rapamycin vs vehicle; Data are representative of 5 independent samples, 2 experiments.
Figure 2-5: *Rapamycin blocks UVB-induced activation of mTORC1 in whole-skin extracts.* 7-week old FVB/N mice were shaved 24h prior to topical treatment with rapamycin (100 nmol) or vehicle (100 µL DMSO:Acetone (1:9) mixture). 1 h after treatment mice were exposed to 120 mJ/cm² UVB and whole skin was harvest 6 h after UVB. Data are representative of 3 independent experiments.
Figure 2-6: **Rapamycin attenuates UVB-induced epidermal thickening.** FVB/N at 7-weeks of age were treated topically with rapamycin (100 nmol) or vehicle 1 h prior to being exposed to 120 mJ/cm^2 or mock UVB irradiation. A, Representative H&E images of skin sections (scale bar = 50 μm). B, Quantification of epidermal thickness (mean ± SEM) for 3-4 mice/group; # p < 0.05 for UVB vs mock, * p < 0.05 for rapamycin vs vehicle.
Figure 2-7: Rapamycin attenuation of UVB-induced epidermal thickening is dose dependent. FVB/N at 7-weeks of age were treated topically with rapamycin (0, 25, 100, 200 nmol) 1 h prior to being exposed to 120 mJ/cm² or mock UVB irradiation. A, Representative H&E images of skin sections (scale bar = 50 µm). B, Quantification of epidermal thickness (mean ± SEM) for 2-4 mice/group;
Figure 2-8: **Rapamycin inhibits UVB-induced epidermal hyperproliferation.** FVB/N at 7-weeks of age were treated topically with rapamycin (100 nmol) or vehicle 1 h prior to being exposed to 120 mJ/cm² or mock UVB irradiation. A, Representative BrdU staining images (scale bar = 50 µm). B, Quantification of BrdU Proliferation Index (PI) (mean ± SEM) for 3-4 mice/group; # p < 0.05 for UVB vs mock, * p < 0.05 for rapamycin vs vehicle.
Figure 2-9: **Rapamycin attenuates TPA-induced activation of mTORC1 in whole-skin extracts.** FVB/N mice at 7 weeks of age were shaved 24 h prior to the start of the experiment. Mice were treated topically with rapamycin (100 nmol) or vehicle (D:A 100 µL) 1 h prior to TPA (6.8 nmol) or vehicle (acetone 200 µL). Treatment regimen was repeated every 3 days for a total of 4 treatments. Tissue specimens were collected 8 h after final TPA treatment. Data are representative of 2 independent experiments.
Figure 2-10: Rapamycin attenuates TPA-induced epidermal thickening. FVB/N mice at 7 weeks of age were shaved 24 h prior to the start of the experiment. Mice were treated topically with rapamycin (25 nmol or 100 nmol) or vehicle (D:A 100 µL) 1 h prior to TPA (6.8 nmol) or vehicle (acetone 200 µL). Treatment regimen was repeated every 3 days for a total of 4 treatments. Tissue specimens were collected 6 h after final TPA treatment. A, Representative H&E images from skin sections. B, Quantification of epidermal thickness (mean ± SEM) for 4 mice/group; # p < 0.01 relative to control, * p < 0.05 relative to TPA.
Figure 2-11: Rapamycin inhibits TPA-induced increase in ODC activity in the epidermis. FVB/N mice at 7 weeks of age were shaved 24 h prior to the start of the experiment. Mice were treated topically with rapamycin (25 nmol or 100 nmol) or vehicle (D:A 100 µL) 1 h prior to TPA (6.8 nmol) or vehicle (acetone 200 µL). Treatment regimen was repeated every 3 days for a total of 4 treatments. Tissue specimens were collected 8 h after final TPA treatment using 3 mice/group. Data are representative of 2 independent experiments.
Figure 2-12: **UVB induces mTORC1 and mTORC2 activation in murine primary keratinocytes.** Wild-type primary keratinocytes were harvested from 1-3 day-old FVB/N pups and plated in low-calcium media. Confluent cells were exposed to 50 mJ/cm² UVB irradiation, protein was harvested at the indicated times and subjected to immunoblot analysis of mTORC1 and mTORC2 activation markers. Data are representative of 2 independent experiments.
Figure 2-13: **Rapamycin blocks UVB-induced activation of mTORC1 in primary keratinocytes.** Wild-type primary keratinocytes were harvested from 1-3 day-old pups and plated in low-calcium media. When confluent, cells were treated with rapamycin for 1 h and exposed to 50 mJ/cm² UVB irradiation. Immunoblot analysis of mTOR activation markers at 2 h post-irradiation of cells pretreated with various doses of rapamycin. Data are representative of 2 independent experiments.
Figure 2-14: *Rapamycin does not sensitize keratinocytes to UVB-induced cell death.* Wild-type primary keratinocytes were harvested from 1-3 day-old FVB/N pups and plated in low-calcium media. Confluent cells were treated with rapamycin for 1 h and exposed to 50 mJ/cm² UVB irradiation. A, MTS cell viability was determined 24 h post UVB exposure. B, Immunoblot analysis of cleaved caspase-3, a marker of apoptosis, at 9 h post UVB. All data are representative of 3-5 independent experiments.
Figure 2-15: Prolonged rapamycin treatment does not sensitize keratinocytes to UVB-induced cell death. A, HaCaT cells were treated with rapamycin for 1 h and exposed to 50 mJ/cm² UVB irradiation. MTS cell viability was determined 24 h post UVB exposure. B, Wild-type primary keratinocytes cells were treated with rapamycin for 24 h and exposed to 50 mJ/cm² UVB irradiation. MTS cell viability was determined 24 h post UVB exposure. C, HaCaT cells were treated with rapamycin for 24 h and exposed to 50 mJ/cm² UVB irradiation. MTS cell viability was determined 24 h post UVB exposure. Data are representative of 3-4 independent experiments.
Figure 2-16: Role of mTORC1 in mediating UVB- and TPA-induced effects. Our studies demonstrate that mTORC1 signaling is in part responsible for mediating UVB- and TPA-induced epidermal hyperproliferation, but does not appear to play a role in stimulating UVB-induced cell survival signaling.
Chapter 3
Inhibition of mTORC1 and mTORC2 block UVB-induced proliferation and sensitize keratinocytes to apoptosis

3.1 Introduction and Rationale

As described in Chapter 2, UVB activation of mTORC1 induces cell cycle progression and epidermal hyperproliferation. However, activation of mTORC1 does not appear to affect UVB-induced apoptosis. These data support the hypothesis that mTORC1 inhibition with rapamycin prevents NMSC by attenuating tumor promotion but does not play a role in tumor initiation. Although this hypothesis is corroborated by a number of studies showing that rapamycin has the same effect in mouse models [222,223,224,230], the clinical anticancer activity of rapalogs has been disappointing. One possible explanation for the poor response seen clinically is that inhibition of mTORC1 relieves a known negative feedback loop where phosphorylation of S6K inhibits PI3K. This results in stimulation of PI3K-AKT anti-apoptotic responses when mTORC1 is inhibited [58]. We did observe some activation of AKT with a single dose of rapamycin (Figure 2-5), and this finding was more pronounced in studies where topical rapamycin was continued for longer periods of time [222]. Additionally, in keratinocytes, rapamycin is largely ineffective at inhibiting mTORC2 (Figure 2-13), which plays a role in the PI3K-AKT anti-apoptotic signaling cascade.

NMSC pathogenesis is characterized by enhanced cell proliferation and inhibition of apoptosis [257]. We hypothesize that targeting both mTORC1 and mTORC2 could be
more effective in the prevention of NMSC, by inhibiting mTORC1 driven UVB-induced proliferation and inhibiting possible mTORC2-induced pro-survival/anti-apoptotic signaling (Figure 3-1). In support of this hypothesis, Nardella et al. found that genetically ablating mTOR was more effective than rapamycin in preventing prostate cancer in PTEN deficient mouse models [217].

Another drawback of rapalogs and a possible explanation for their disappointing clinical applications is that rapamycin is an incomplete inhibitor of mTORC1. Although phosphorylation of both 4EBP1 and S6K are initially blocked with rapamycin, in some cell types 4EBP1 becomes rephosphorylated [33]. The incomplete inhibition of 4EBP1 phosphorylation by rapamycin is particularly important because a number of studies have illustrated the significance of 4EBP1 in tumorigenesis [258,259,260,261,262]. Petroulakis et al. showed tumorigenesis is increased in p53 knockout mice that lack both 4EBP1 and 4EBP2 [259], while other studies have shown that expressing a non-phosphorylated form of 4EBP1 is capable of suppressing tumorigenesis [261,262]. The importance of 4EBP1 in mediating ‘rapamycin-resistant’ mTORC1 function has recently been seen in studies utilizing the newly generated mTOR kinase inhibitors [253,256].

mTOR kinase inhibitors are ATP analogs that inhibit mTOR activity by competing for the kinase domain of mTOR, inhibiting both mTORC1 (S6K and 4EBP1) and mTORC2 (AKT<sup>Ser473</sup>) signaling (reviewed in [61]). Studies using these inhibitors in mTORC2 deficient cells (mSin or Rictor knock-out) show that they are more effective than rapamycin at inhibiting cell proliferation [253,256], illustrating the importance of rapamycin-resistant mTORC1 function in cell proliferation. It is also possible that rapamycin-resistant mTORC1 activation of proliferation plays a role in cancer. In fact, it was recently shown that PP242, a mTOR kinase inhibitor, is more effective than
rapamycin at inhibiting proliferation in leukemia cells [263]. Interestingly, PP242 also caused apoptosis in both human and mouse leukemia cells, while rapamycin had no effect on cell death. However, whether these effects were mediated by inhibiting mTORC2 or rapamycin-resistant mTORC1 activity was not examined.

Additional studies utilizing inhibitors targeting PI3K signaling support our hypothesis that targeting both mTOR complexes will more effectively block UVB-induced changes associated with NMSC development. Recently, the PI3K and AKT inhibitor perifosine was shown to be effective against a number of human SCC cells in xenograph models [264]. In vitro studies utilizing perifosine in HaCaT cells revealed inhibition of UVB-induced activation of mTORC1 and mTORC2, in addition to UVB-mediated activation of other kinases such as ERK [265]. This inhibition sensitized cells to UVB-mediated reduction in cell viability. Although perifosine inhibits a number of pathways, the studies reported here support the hypothesis that these effects are mediated through inhibition of mTOR signaling.

Our studies described in Chapter 2 indicate that mTORC1 plays a role in UVB-induced proliferation, but not survival. However, the roles of mTORC2 or rapamycin-resistant mTORC1 function were not explored. We hypothesize that inhibiting both mTOR complexes should sensitize keratinocytes to UVB-induced apoptosis (Figure 3-1). In order to inhibit both mTORC1 and mTORC2 signaling, we utilized a genetic model of conditional mTOR deletion in the epidermis. We also employed the mTOR kinase inhibitor Torin2 to pharmacologically inhibit the two TOR complexes. mTOR deficient mice showed decreased sensitivity to UVB-induced hyperproliferation in the epidermis. Additionally, inhibition of both mTOR complexes sensitized keratinocytes to UVB-induced apoptosis, in contrast to our results with rapamycin (Chapter 2). Taken together
with the studies reported in Chapter 2, we show here for the first time that mTOR plays a critical role in both UVB-induced proliferation and pro-survival signaling. We propose from these studies that targeting both mTOR complexes could be an effective chemopreventative approach in NMSC, whereby inhibition of mTORC2 sensitizes keratinocytes to UVB-induced apoptosis and inhibition of mTORC1 attenuates UVB-enhanced proliferation, thus blocking two critical steps in photocarcinogenesis.

3.2 Materials and Methods

3.2.1 Mouse studies

All experiments involving mice were carried out in compliance with the Guide for the Care and Use of Laboratory Animals and protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. We utilized an inducible Cre-LoxP mouse model to ablate mTOR in the epidermis. Floxed mTOR mice (mTOR^{fl/fl}) (generous gift of Drs. CJ Lynch and SK Bronson, Dept. of Cell and Molecular Physiology, Penn State College of Medicine), contain LoxP sites flanking exons 49 and 50 of the mTOR gene (Figure 3-2) [266]; therefore, recombination results in a frame-shift mutation and loss of the essential kinase domain [267]. K5-CreERT^{2} mice [199] express a tamoxifen-activated Cre recombinase fused to a modified estrogen receptor in the basal layer of the epidermis under the control of the keratin 5 promoter. The CreER fusion protein is inactive in the cytoplasm of cells until binding of 4-hydroxy-tamoxifen (4OHT) allows its translocation into the nucleus where Cre induces recombination.
All animals were backcrossed for at least 9 generations onto the FVB/N background. K5-CreER\textsuperscript{T2} mice were bred with mTOR\textsuperscript{fl/fl} mice to ultimately generate mice hemizygous for the K5Cre-ER\textsuperscript{T2} transgene and homozygous for the mTOR floxed allele (K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl}). The dorsal surface of the mice, aged 6-8 weeks, was shaved with electrical clippers and mice were allowed to rest for 24-48 h prior to all experiments; only mice in the resting stage of the hair cycle were used. K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} and mTOR\textsuperscript{fl/fl} controls were treated topically with 1 mg 4OHT (Sigma) in 100µL or vehicle (DMSO:Acetone [1:9]; D:A) daily for 5 consecutive days. For UVB studies, 3-5 mice were used for each treatment group and housed together. Mice were exposed to UVB irradiation from UVB lamps (FS20 UVB bulbs, National Biological) emitting UV light between 290-320 nm at a dose of 120 mJ/cm\textsuperscript{2}. Bulb intensity was measured at the beginning of each experiment using a UVB 500C meter (National Biological).

### 3.2.2 Cell culture

Primary mouse keratinocytes were isolated from 1-3d old pups as described previously [236]. Briefly, full thickness skin was floated overnight at 4°C in Ca\textsuperscript{2+}-free 0.25% Trypsin without EDTA (Cellgro) to separate the epidermis from the dermis. Epidermal sheets were minced and the cell suspension was strained (100-µm nylon filter, Fisher) and plated at a density of one-half mouse equivalent (ME) per 60mm\textsuperscript{2} plate, or approximately 2 ME for a 96-well plate (1/40 ME per well). Cells were grown in keratinocyte growth medium [calcium-free MEM Eagle with Earle's BSS, glutamine and non-essential amino acids (Lonza) supplemented with 8% chelexed FBS (Gibco), 1% Glutamine (Invitrogen) and 25 µg/mL penicillin/streptomycin (Invitrogen)]. Cells were
maintained in 7% CO₂ at 36°C, and medium was changed every other day. Primary keratinocyte cultures from transgenic animals (K5-CreERT₂;mTOR<sup>fl/fl</sup> and mTOR<sup>fl/fl</sup>) were supplemented with 5 nM 4OHT (1:1000 in DMSO) for 4 days to induce recombination.

HaCaT keratinocytes were maintained in High-Glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biological), 110 mg/L sodium-pyruvate (Sigma) and 25 mg/L gentamicin (Invitrogen), trypsinized using 0.05% Trypsin plus EDTA (Invitrogen), and split 1:10. Cells were cultured for less than 20 passages and incubated in 5% CO₂ at 37°C. Cells were seeded in 60-mm<sup>2</sup> plates at a density of 150,000 – 300,000 per plate and in 96-well plates at 7,500-10,000 cells per well. All experiments were done when plates had reached approximately 80% confluence.

Torin2 (Tocris), rapamycin (Developmental Therapeutics Program, National Cancer Institute), or vehicle (DMSO) was added to HaCaT or primary keratinocyte medium at a 1:1000 dilution 1 h prior to UVB treatment. Cells were washed twice with PBS, then in a minimal volume of PBS exposed to UVB (FS20 UVB bulbs, National Biological) emitting UV light between 290-320 nm. After irradiation, PBS was removed and conditioned medium with drug treatments was added back. The irradiation intensity was monitored prior to each experiment.

### 3.2.3 PCR Genotyping

Mouse tail and keratinocyte DNA was isolated using REDExtract-N-Amp PCR Kit (Sigma). Information on primer sequences and PCR programs can be found in Appendix A.
3.2.4 Western blotting

For *in vivo* studies, animals were euthanized 6 h after UVB exposure and dorsal skin was treated with a depilatory agent for 3 min, washed, excised and underlying fat and connective tissue were removed. Epidermal samples were collected by scraping the surface of excised skin with a razor blade and placed into ice-cold RIPA buffer. Following sonication, samples were centrifuged, and supernatants collected. Whole skin samples were flash-frozen and later processed in RIPA buffer by homogenizing for 30 sec on ice using a Polytron homogenizer, centrifuged at 30,000 x g for 30 min at 4°C, and supernatants collected. For *in vitro* studies, cells were washed twice with cold PBS, harvested by scraping into RIPA buffer (Santa Cruz Biotechnology), centrifuged, and supernatants collected. In selected experiments examining apoptosis, detached cells were collected via centrifugation of medium and combined with the attached cells in RIPA buffer. Protein concentrations were determined using the BioRad assay. Equal amounts of protein were subjected to electrophoresis. Western blotting was performed as described previously [237]. Antibodies used include AKT, p-AKT<sup>Ser473</sup>, S6K, p-S6K<sup>Thr389</sup>, Caspase-3, p-4EBP1<sup>Ser65</sup>, 4EBP1 (all from Cell Signaling, 1:1000) and GAPDH (Proteintech, 1:2000).

3.2.5 Histology and Immunohistochemistry

Tissue sections were collected from mice at 24 or 48 h after UVB treatment (24h was used for mock animals). Mice received intraperitoneal (i.p.) injections of 5-bromo-2-deoxyuridine (BrdU, Sigma) at 100 µg/g body wt in NaCl 1 h prior to euthanasia. Dorsal
skin was treated with a depilatory agent for 3 min, washed, excised, and fixed for 6h in 10% neutral buffered formalin. Skin was embedded in paraffin and 5 µm sections were cut for immunohistochemistry. The effect of rapamycin on UVB- and TPA-induced epidermal hyperplasia was studied by histopathological examination of hematoxylin and eosin (H&E) stained tissue sections. Epidermal thickness was measured at 5 locations in four different sections for each mouse. Epidermal proliferation was assessed in vivo using BrdU incorporation. Sections were deparaffinized, rehydrated, and stained with an anti-BrdU antibody as described previously [238]. Epidermal proliferation index (PI) was determined by calculating the percentage of basal cells positive for BrdU. A minimum of 1000 continuous basal cells were counted for each mouse at 3 different locations. Apoptosis was assessed using immunohistochemical staining with cleaved caspase-3 antibody (Cell Signaling). Intrafollicular apoptotic keratinocytes were counted microscopically in at least 5 nonoverlapping low power fields using 3-4 mice for each condition.

### 3.2.6 Cell viability

The cell viability after UVB exposure was determined colorimetrically by MTS assay (CellTiter 96 Aqueous Proliferation Assay, Promega) according to the manufacturer’s instructions and monitored at 490 nm using a model 3550-UV plate reader (BioRad). Each condition was plated in triplicate and the experiments were repeated at least three times. Cell viability was calculated by setting the mean vehicle-treated mock UVB viability to 100%. Relative change in viability was calculated taking the ratio of cell viability of UVB to mock for each drug regimen.
3.2.7 Statistical analysis

Data are expressed as the mean of at least three independent experiments analyzed by 2-sided Student’s t-test. A p-value of <0.05 was considered significant.

3.3 Results

3.3.1 Deletion of mTOR in the epidermis of mice attenuates UVB-induced hyperproliferation

In order to evaluate the contribution of both mTOR-signaling complexes in UVB-mediated responses in keratinocytes, we first explored the consequences of deleting mTOR in the basal layer of the skin, in order to disrupt both mTORC1 and mTORC2 signaling. Since homozygous deletion of mTOR is lethal in utero [203], we used a transgenic system with 4OHT-inducible deletion of mTOR in the epidermis (K5-CreERT2;mTORfl/fl). We verified that topical treatment with 4OHT, but not vehicle, leads to CreERT2-dependent recombination of the mTOR allele (ΔLoxP) by PCR analysis using DNA from the dorsal epidermis harvested from mice 1 week after the final 4OHT treatment (Figure 3-3A). Western blot analysis confirmed reduction of mTOR protein levels in epidermal extracts of 4OHT-treated K5-CreERT2;mTORfl/fl animals relative to controls (Figure 3-3B).

To verify that mTORC1 and mTORC2 signaling was inhibited in our genetic model, we examined the phosphorylation of S6K and AKT^{Ser473} in whole skin protein extracts. In the absence of UVB stimulation of mTOR pathways, there was no apparent difference between the vehicle-treated and 4OHT-treated animals (Figure 3-4). However,
p-S6K and p-AKT<sup>Ser473</sup> levels were dramatically increased at 6 h after UVB (120 mJ/cm<sup>2</sup>) in vehicle-treated mice and this effect was significantly attenuated upon treatment of K5-CreERT<sup>2</sup>; mTOR<sup>fl/fl</sup> animals with 4OHT, confirming that 4OHT treatment in K5-CreERT<sup>2</sup>; mTOR<sup>fl/fl</sup> mice was sufficient to block UVB-induced activation of both mTORC1 and mTORC2. Gross examination and histological evaluation of H&E stained skin sections in mock-irradiated animals revealed no obvious differences between vehicle-treated and 4OHT-treated animals (Figure 3-5A), suggesting that mTOR is not essential for epidermal homeostasis in adult animals. UVB irradiation caused a significant increase in epidermal thickness in vehicle-treated K5-CreERT<sup>2</sup>; mTOR<sup>fl/fl</sup> mice at 24 h and 48 h, and this effect was significantly reduced in mice treated with 4OHT (Figure 3-5). Additional control animals, including mTOR<sup>fl/fl</sup> mice treated with 4OHT and mTOR<sup>fl/fl</sup> mice treated with vehicle, showed no recombination of the LoxP allele and responded similarly to K5-CreERT<sup>2</sup>; mTOR<sup>fl/fl</sup> mice treated with vehicle at all timepoints (Figure 3-6).

UVB-induced proliferation was quantified by examining the BrdU proliferation index by immunohistochemistry. K5-CreERT<sup>2</sup>; mTOR<sup>fl/fl</sup> mice treated with 4OHT showed a significant reduction in proliferation index at 48 h after UVB treatment compared to vehicle-treated controls (Figure 3-7). As shown in Figure 3-8, the relative reduction in UVB-induced epidermal thickening and proliferation caused by mTOR deletion did not differ significantly from the effects seen in Chapter 2 with topical rapamycin treatment, suggesting the proliferation response to UVB is mediated by mTORC1 but not mTORC2.
### 3.3.2 mTOR deletion sensitizes keratinocytes to UVB-induced apoptosis

Since we did not see a difference in UVB-induced apoptosis with rapamycin treatment (Chapter 2), we sought to determine the effects UVB on apoptosis in our mTOR deletion model. We first utilized primary keratinocytes isolated from K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> mice. PCR and western blot analysis verified recombination of the mTOR allele (ΔLoxP) and mTOR deletion in cells cultured with 4OHT, but not vehicle (Figure 3-9). Western blot analysis of mTOR activation markers revealed that there was no obvious difference in mTORC1 and mTORC2 activities in 4OHT-treated keratinocytes not exposed to UVB (0 min) (Figure 3-10). However, when keratinocytes were exposed to UVB (50 mJ/cm<sup>2</sup>) to induce activation of both mTOR complexes, a dramatic reduction in phosphorylation of both S6K and AKT<sup>Ser473</sup> was observed in 4OHT-treated cells compared to vehicle, indicating downregulation of both mTORC1 and mTORC2 signaling (Figure 3-10). In contrast to inhibition of mTORC1 with rapamycin (Chapter 2), deletion of mTOR enhanced UVB-induced cell death (Figure 3-11A). Although there was no difference in cell viability in mock irradiated cells, K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> keratinocyte cultures treated with 4OHT contained significantly fewer viable cells 24 h after UVB exposure compared to both vehicle-treated K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> and 4OHT-treated mTOR<sup>fl/fl</sup> cells (Figure 3-11A). To determine whether the significant decrease in cell viability was due to apoptosis, immunoblot analysis of cleaved caspase-3 was examined (Figure 3-11B). Cell extracts harvested at 9 h after UVB showed significantly higher levels of cleaved caspase-3 in mTOR-ablated keratinocytes than was observed in vehicle-treated K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> cells.

We further investigated the effects of mTOR deficiency on UVB-mediated apoptosis in vivo using our K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> mice. UVB irradiation increased the
number of epidermal cleaved caspase-3 (CC3) positive cells 24 h and 48 h after irradiation in K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} mice treated with vehicle. The number of apoptotic cells was significantly increased in the epidermis of mTOR-deficient mice (4OHT treated) compared to vehicle controls at 24 h (Figure 3-12).

3.3.3 Torin2 sensitizes HaCaT cells and wild-type primary keratinocytes to UVB-induced apoptosis

To complement our studies using a genetic approach to inhibit the two mTOR complexes, we also utilized the mTOR kinase inhibitor Torin2. We first evaluated the effects of Torin2 on mTOR signaling following UVB exposure in both HaCaT cells and wild-type primary keratinocytes. Western blot analysis revealed that a minimum concentration of 10 nM of Torin2 was sufficient to inhibit both mTORC1 and mTORC2 signaling (Figure 3-13) in both cell types. Interestingly, unlike rapamycin, Torin2 was able to completely ablate UVB-induced phosphorylation of 4EBP1, demonstrating Torin2’s ability to inhibit rapamycin-resistant mTORC1 functions.

MTS assays were performed to examine the effect of Torin2 on UVB-mediated reduction of cell viability. Unlike the results we saw with rapamycin in Chapter 2, Torin2 significantly reduced viability in the mock-irradiated cells (Figure 3-14A), suggesting that Torin2 is more effective at inhibiting proliferation in keratinocytes than rapamycin. UVB exposure resulted in a significant decrease in cell viability of both HaCaT cells and primary keratinocytes, and Torin2 treatment significantly enhanced this loss in cell viability (Figure 3-14B). In order to determine whether the loss in cell viability observed with Torin2 pretreatment was due to apoptotic cell death, we performed western blots
examining the levels of cleaved and total caspase-3 (Figure 3-15). There were noticeably higher levels of cleaved-caspase-3 in Torin2 treated cells after UVB exposure, indicating that like genetic deletion of mTOR, inhibition of the two mTOR complexes with Torin2 sensitizes keratinocytes to UVB-induced apoptosis.

3.4 Discussion

The data described here show for the first time that simultaneous downregulation of both mTORC1 and mTORC2 signaling pathways sensitizes keratinocytes to UVB-induced apoptosis. Utilizing a LoxP-Cre model system, we were able to induce genetic ablation of mTOR in primary keratinocytes and in the epidermal basal layer of adult mouse skin. Deletion of mTOR prevented UVB-induced phosphorylation of S6K and AKT$^{\text{Ser473}}$, markers of mTORC1 and mTORC2 activity.

While mTOR deletion did inhibit UVB-induced epidermal hyperproliferation, the inhibitory effect was not significantly enhanced compared to topical rapamycin ($p=0.11$). These results suggest that neither mTORC2 nor rapamycin-resistant mTORC1 functions play a role in UVB-induced cell cycle progression and proliferation. However, it is difficult to compare pharmacologic inhibition to genetic ablation. The effects of mTOR deletion may be dampened because it is unlikely that recombination and deletion occurred in all keratinocytes within the epidermis. In vivo studies utilizing Torin2 could provide valuable information concerning a possible role for mTORC2 or rapamycin-resistant mTORC1 in UVB-mediated proliferation. Interestingly, treatment with the mTOR kinase inhibitor Torin2, significantly decreased cell viability in mock-irradiated cells. This effect was not seen with rapamycin treatment and was likely due to inhibition of
proliferation in normal cells. However, more studies, possibly utilizing genetic techniques targeting raptor, rictor, mSin1, or 4EBP1, are needed to determine if mTORC2 or rapamycin-resistant mTORC1 signals contribute to UVB-induced and normal keratinocyte cell cycle progression and proliferation.

TARGETING BOTH mTOR COMPLEXES, THROUGH EITHER GENETIC ABLATION OF mTOR OR TREATMENT WITH Torin2, SIGNIFICANTLY ENHANCE THE LEVELS OF UVB-INDUCED APOPTOSIS both in vivo and in cell culture experiments. These data are novel, because prior to these studies, skin carcinogenesis studies have focused on the possible role of mTOR in tumor promotion, and have concentrated on inhibiting mTORC1 using rapamycin. Our results clearly demonstrate that mTOR, either through mTORC2 or rapamycin-resistant mTORC1 signaling, enhances keratinocyte survival after UVB-exposure, and suggest that complete inhibition of mTOR might improve the therapeutic efficacy.

NMSC pathogenesis is characterized by both enhancement of cell proliferation and inhibition of apoptosis. Induction of apoptosis following DNA damage is an essential protective mechanism, ensuring the removal of damaged cells that may harbor oncogenic mutations. However, in addition to causing DNA damage, UVB also activates signaling cascades that promote the survival of these potentially cancerous cells. Previous work in other mouse models has shown that targeting pro-survival pathways induced by UVB increases the sensitivity of DNA-damaged keratinocytes to apoptotic signaling, and is sufficient to inhibit skin carcinogenesis [164]. The studies reported here verify that activation of mTOR by UVB leads to increased keratinocyte pro-survival signaling, suggesting that the combined targeting of both mTOR complexes may be an effective chemoprevention strategy against photocarcinogenesis. The studies presented in this and the previous chapter highlight the differences seen between rapamycin
treatment and complete inhibition of the two mTOR complexes. Whether the differences in UVB-induced apoptosis were due to inhibition of mTORC2 or rapamycin resistant mTORC1 function will be addressed through the data presented in the following chapter.
Figure 3-1: Possible role of mTOR in mediating UVB-induced effects. Our studies in Chapter 2 with rapamycin indicate that mTORC1 plays a role in UVB-induced proliferation, but not survival. However, the roles of mTORC2 or rapamycin-resistant mTORC1 function have not yet been explored. We hypothesize that inhibiting both mTOR complexes, through Cre-LoxP deletion of mTOR or use of mTOR kinase inhibitors, will sensitize keratinocytes to UVB-induced apoptosis.
Figure 3-2: *Diagram of mTOR LoxP allele.* Diagram of mTOR alleles. LoxP sites (triangles) flank exons 49 and 50 of the kinase domain. A tissue specific promoter drives Cre recombinase expression. In tissues where Cre is expressed, the DNA segment flanked by the LoxP sites will be excised, and the mTOR gene silenced. In tissues where Cre is not expressed, the floxed mTOR gene will remain active.
Figure 3-3: **Topical application of 4OHT induces LoxP recombination and mTOR deletion in the epidermis of K5-CreERT2; mTOR<sup>fl/fl</sup> mice.** K5-CreERT2; mTOR<sup>fl/fl</sup> mice at 7-weeks of age were treated topically with 4OHT (1 mg) or vehicle (D:A DMSO:Acetone [1:9]) daily for 5 days. A, PCR analysis of epidermal DNA harvested 7 d after final 4OHT treatment. ∆LoxP denotes primers specific to the recombined mTOR allele. B, Immunoblot analysis of mTOR in epidermis extracts harvested at 7 and 14 d after final 4OHT treatment. Data are representative of 2-4 independent experiments.
Figure 3-4: mTOR deletion in the epidermis blocks UVB induction of mTORC1 and mTORC2 signaling in vivo. K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> mice at 7-weeks of age were treated topically with 4OHT (1mg) or vehicle daily for 5 days. Immunoblot analysis of mTORC1 and mTORC2 activation markers was performed in whole-skin extract harvested 6h following UVB (120 mJ/cm<sup>2</sup>) radiation exposure. Data are representative of 2 experiments.
**Figure 3-5**: *mTOR deletion attenuates UVB-induced epidermal thickening.* K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup> mice at 7-weeks of age were treated topically with 4OHT (1 mg) or vehicle daily for 5 days. 14d after final treatment mice were exposed to UVB (120 mJ/cm<sup>2</sup>). **A**, Representative H& E images of skin sections (scale bar = 50 µm) **B**, Quantification of epidermal thickness (mean ± SEM) for 3-4 mice/group; * p < 0.05, *** p < 0.005 for 4OHT vs vehicle; # p < 0.005 for UVB vs mock.
Figure 3-6: Vehicle-treated K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl}, 4OHT-treated mTOR\textsuperscript{fl/fl}, and vehicle-treated mTOR\textsuperscript{fl/fl} mice do not differ. mTOR\textsuperscript{fl/fl}, and K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} mice, at 7-weeks of age were treated topically with 4OHT (1mg) or vehicle (D:A) daily for 5 days. A, PCR analysis of epidermal DNA harvested 14 d after final 4OHT treatment. $\Delta$LoxP denotes primers specific to the recombined mTOR allele. B, Representative H&E images (scale bar = 50$\mu$m) of skin sections from additional control mice (vehicle-treated mTOR\textsuperscript{fl/fl} and 4OHT-treated mTOR\textsuperscript{fl/fl}). C, Quantification of epidermal thickness (mean ± SEM) for 3-4 mice/group; includes data from Figure 3-4 for comparison; †: p < 0.05 for Cre/mTOR-4OHT vs. mTOR-vehicle, vs. mTOR-4OHT, and vs. Cre/mTOR-4OHT; ‡: p < 0.05 for Cre/mTOR-4OHT vs. mTOR-vehicle, vs. mTOR-4OHT, and vs. Cre/mTOR-4OHT.
Figure 3-7: mTOR deletion attenuates UVB-induced epidermal proliferation. K5-CreER12;mTORfl/fl mice at 7-weeks of age were treated topically with 4OHT (1 mg) or vehicle daily for 5 days. 14d after final treatment mice were exposed to UVB (120 mJ/cm²) radiation exposure. Mice received i.p. BrdU injections 1 h prior to harvesting tissues. A, Representative BrdU staining images of skin sections (scale bar = 50 µm). B, Quantification of BrdU Proliferation Index (PI: % BrdU positive cells) (mean ± SEM) for 3-4 mice/group; ** p < 0.01 for 4OHT vs vehicle; # p < 0.005 for UVB vs mock
Figure 3-8: The effect of mTOR deletion on UVB-induced epidermal thickening and proliferation did not differ significantly from rapamycin results. The percent change in (A) epidermal thickening (B) and BrdU proliferation did not differ significantly between Rapamycin treatment (results from Chapter 2) and CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} mice. Controls for both experimental groups (WT-vehicle and Cre/mTOR-vehicle) were set to 100% at 24 and 48h. (ns – not significant)
Figure 3-9: 4OHT induces LoxP recombination and mTOR deletion in the primary keratinocytes from K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} mice. K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} keratinocytes were harvested from 1-3 day-old pups and cultured in 4OHT (5 nM) or vehicle for 3 days. A, PCR analysis of K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} primary keratinocyte DNA harvested 24 h after final 4OHT treatment. ∆LoxP denotes primers specific to the recombined mTOR allele. B, Immunoblot analysis of mTOR in primary keratinocyte whole-cell lysate. Data are representative of 3-4 independent experiments.
**Figure 3-10: mTOR deletion in primary keratinocytes blocks UVB induction of mTORC1 and mTORC2 signaling.** K5-CreER\(^{T2}\);mTOR\(^{fl/fl}\) keratinocytes were harvested from 1-3 day-old pups and cultured in 4OHT (5 nM) or vehicle for 3 days. Immunoblot analysis of mTORC1 and mTORC2 activation markers in K5-CreER\(^{T2}\);mTOR\(^{fl/fl}\) keratinocyte extracts harvested at the indicated time following UVB (50 mJ/cm\(^2\)) exposure. Data are representative of 3 independent experiments.
Figure 3-11: mTOR deletion in mouse primary keratinocytes sensitizes cells to UVB-induced apoptosis. K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} and mTOR\textsuperscript{fl/fl} keratinocytes were harvested from 1-3 day-old pups and cultured in 4OHT (5 nM) or vehicle for 3 days prior to UVB (50 mJ/cm\textsuperscript{2}) exposure. A, MTS cell viability (mean ± SEM) of mTOR\textsuperscript{fl/fl} and K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} keratinocytes at 24 h post UVB; *** p < 0.005. B, Immunoblot analysis of K5-CreER\textsuperscript{T2};mTOR\textsuperscript{fl/fl} primary keratinocytes probed for caspase-3 and quantification (mean ± SEM) at 9-h post UVB; * p<0.05. Data are representative of 3-4 independent experiments.
Figure 3-12: mTOR deletion in the epidermis sensitizes keratinocytes to UVB-induced apoptosis. K5-CreER^{T2};mTOR^{fl/fl} mice at 7-weeks of age were treated topically with 4OHT (1mg in 100 μL) or vehicle daily for 5 day. 14d after final treatment mice were exposed to UVB (120 mJ/cm²). A, Representative cleaved Caspase-3 staining images of skin sections (scale bar = 200 μm) from K5-CreER^{T2};mTOR^{fl/fl} mice treated topically with 4OHT and exposed to UVB (120 mJ/cm²). B, Quantification of cleaved capsase-3 (CC3) staining (mean ± stdev) for 3-5 mice/group; ** p < 0.01 for 4OHT vs DMSO; # p < 0.05 for UVB vs mock (0h).
Figure 3-13: Torin2 blocks UVB stimulation of mTORC1 and mTORC2 signaling. Cells were pretreated with vehicle (DMSO), rapamycin (50 nM), or various doses of Torin2 (nM) for 1 h and exposed to 25 mJ/cm² UVB irradiation. Whole cell lysate was harvested in RIPA buffer at 2h post-irradiation and subjected to immunoblot analysis of mTORC1 and mTORC2 activation markers. A, HaCaT cells B, wild-type primary keratinocytes. Data are representative of 2-4 independent experiments.
Figure 3-14: Torin2 sensitizes cells to UVB-induced cell death. Human HaCaT cells and mouse primary keratinocytes were treated with vehicle (DMSO), rapamycin (50 nM), or various doses of Torin2 (nM) for 1 h and exposed to 25 mJ/cm² UVB irradiation. A, MTS cell viability (mean ± SEM) of HaCaT cells (left) and wild-type primary keratinocytes (right) at 24 h post UVB. B, MTS relative change in cell viability (mock of each condition set to 100%). * p < 0.05; ** p < 0.01; *** p < 0.005. Data are representative of 3 independent experiments.
Figure 3-15: Torin2 sensitizes cells to UVB-induced apoptosis. A, Human HaCaT cells and B, mouse primary keratinocytes were treated with vehicle (DMSO), rapamycin (50 nM), or various doses of Torin2 (nM) for 1 h and exposed to 25 mJ/cm² UVB irradiation. Whole cell extracts were collected at A, 12 h and B, 24 h post-UVB and subject to immunoblot analysis. Data are representative of 2 independent experiments.
Chapter 4
mTORC2 specific inhibition sensitizes cells to UVB-induced apoptosis

4.1 Introduction and Rationale

Our previous studies in Chapter 3 demonstrate that inhibiting both mTOR complexes sensitizes keratinocytes to UVB-induced apoptosis. However, whether those effects were mediated through inhibition of mTORC2 signaling or rapamycin-resistant mTORC1 function was not explored. The studies reported here investigate whether inhibition of mTORC2 signaling, through targeting the mTORC2 component rictor, reproduces the effects seen when both mTOR complexes are targeted. AKT activation is known to induce pro-survival signaling in many cell types. Because mTORC2 activates AKT through phosphorylation at Ser473, we hypothesize that mTORC2 signaling stimulates UVB-mediated cell survival signaling through this pathway. Therefore, inhibiting mTORC2 signaling by deletion of rictor should sensitize cells to UVB-induced apoptosis (Figure 4-1).

Although the role of mTORC2 in cancer has not been explored as thoroughly as mTORC1, a number of studies suggest that mTORC2 signaling plays a pivotal role in cancer development. Guertin et al. found that genetically ablating mTORC2 activity, by deleting rictor, was sufficient to block the development of PTEN-deficient prostate cancer in mice [37]. Importantly, they also found that rictor deletion had no adverse effects in
normal prostate epithelium, suggesting mTORC2 is not essential for adult prostate epithelium homeostasis. Other studies show silencing rictor through RNA interference (RNAi) induces apoptosis in several cancer cell lines [37,116,118] and it appears that sensitivity or resistance to rapamycin-induced cell death in colorectal cancer cells is dependent on the ability of rapamycin to inhibit mTORC2-mediate phosphorylation of AKT$^{\text{Ser473}}$ [215]. Studies have shown that mTORC2 signaling is upregulated in human actinic keratosis and SCC [165,166] and Rictor protein levels are increased in UVB-induced SCC in p53$^{+/^-}$ SKH-1 mice [268]. However, very little information is available on the possible influence of mTORC2 signaling on the development of NMSC skin cancer.

Although no specific mTORC2 inhibitors exist, many studies investigating the specific role of mTORC2 use a genetic approach that targets either rictor or mSin1 because the structural integrity of mTORC2 requires both proteins [16,18]. However, there have been challenges utilizing these techniques. Transient depletion of mTOR, rictor, or mLST8 with siRNA results in altered cytoskeleton organization [8,16]. However, MEFs with rictor, mSin1, or mLST8 deleted do not have cytoskeletal defects [18,39,43]. Additionally, siRNA knockdown of rictor blocks phosphorylation of AKT at both Ser473 and Thr308, while there is no inhibition of p-AKT$^{\text{Thr308}}$ in Rictor$^{-/-}$ MEFs [36,43], indicating that compensatory mechanisms might come into play with prolonged inhibition of mTORC2. In order to overcome any potential issues observed between acute and prolonged deletion of mTORC2, we utilized both rictor knock-out MEFs and primary keratinocytes with inducible rictor deletion to investigate the ability of mTORC2 to suppress apoptosis after UVB exposure (Figure 4-1). Initial studies performed in rictor knock-out MEFs (Rictor$^{-/-}$) [43], verified that targeting rictor completely blocked UVB-induced activation of mTORC2. Studies utilizing these cells and the mTOR kinase
inhibitor Torin2, allowed us to differentiate between rapamycin-resistant mTORC1 effects and those mediated through mTORC2. We further explored the role of mTORC2 in UVB-mediated keratinocyte cell death by employing primary keratinocytes harvested from K14-CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> mice. Our results provide evidence for the first time that UVB activation of mTORC2 stimulates pro-survival signaling, and suggest that mTORC2 could prove to be a promising target for prevention of NMSC.

4.2 Materials and Methods

4.2.1 Cell culture

Control (Rictor<sup>Ex3cond/wt</sup>) and rictor-null (Rictor<sup>Ex3del/Ex3del</sup>) mouse embryo fibroblasts (MEFs), abbreviated Rictor<sup>+/+</sup> and Rictor<sup>-/-</sup>, were a generous gift from Dr. Mark Magnuson, Vanderbilt University [43]. They were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% FBS (Atlanta Biological) and 100 μg/mL Penicillin/Streptomycin (Invitrogen) and incubated in 5% CO<sub>2</sub> at 37°C. Cells were seeded in 60-mm<sup>2</sup> plates at a density of 80,000-150,000 for Rictor<sup>+/+</sup> cells and 125,000-250,000 for Rictor<sup>-/-</sup> per plate and in 96-well plates at 4,000-7,000 for Rictor<sup>+/+</sup> cells and 7,500-10,000 for Rictor<sup>-/-</sup> per well. All experiments were done when cells had reached approximately 80% confluence. Torin2 (Tocris), rapamycin (Developmental Therapeutics Program, National Cancer Institute), or vehicle (DMSO) was added to the cell culture medium at a 1:1000 dilution 1 h prior to UVB treatment.
4.2.2 K14-CreER\textsuperscript{T}:Rictor\textsuperscript{fl/fl} and K14-CreER\textsuperscript{T}:Raptor\textsuperscript{fl/fl} primary keratinocytes

We utilized Cre-LoxP mouse models to created primary keratinocyte cultures with inducible deletion of rictor or raptor. Floxed rictor mice (Rictor\textsuperscript{fl/fl}) contain LoxP sites flanking exons 4 and 5 of the rictor gene \cite{209}, while floxed Raptor mice (Raptor\textsuperscript{fl/fl}) contain LoxP sites flanking exons 6 and 7 of the Raptor gene \cite{206} (Figure 4-2). These mice were crossed with K14-CreER\textsuperscript{T} mice \cite{198}, which express a tamoxifen-activated Cre recombinase fused to a modified estrogen receptor in the basal layer of the epidermis under the control of the keratin 14 promoter, which is also expressed in cultured primary keratinocytes. The CreER fusion protein remains inactive in the cytoplasm of the cells until 4OHT binds and allows translocation into the nucleus where Cre induces recombination. All animals were backcrossed for at least 10 generations onto the C57BL/6 background. All experiments involving mice were carried out in compliance with the Guide for the Care and Use of Laboratory Animals and protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine.

Primary mouse keratinocytes were isolated from 1-3 d old pups as described previously \cite{236}. Briefly, full thickness skin was floated overnight at 4°C in Ca\textsuperscript{2+}-free 0.25% Trypsin without EDTA (Cellgro) to separate the epidermis from the dermis. Epidermal sheets were minced and the cell suspension was strained (100-\mu m nylon filter, Fisher) and plated at a density of one-half mouse equivalent (ME) per 60 mm\textsuperscript{2} plate, or approximately 2 ME for a 96-well plate (1/40 ME per well). Cells were grown in keratinocyte growth medium [calcium-free MEM Eagle with Earle’s BSS, glutamine and non-essential amino acids (Lonza) supplemented with 8% chelexed FBS (Atlanta
Biological), 1% Glutamine (Invitrogen) and 25 μg/mL Penicillin/Streptomycin (Invitrogen)]. Cells were maintained in 7% CO₂ at 36°C, and medium was changed every other day. Primary keratinocyte cultures from transgenic animals (K14-CreER<sup>T</sup>;Rictor<sup>fl/fl</sup>, Rictor<sup>fl/fl</sup>, K14-CreER<sup>T</sup>;Raptor<sup>fl/fl</sup>, and Raptor<sup>fl/fl</sup>) were supplemented with 2 nM 4OHT (Sigma) (1:1000 in DMSO) for 4 days to induce recombination. Mouse tail and keratinocyte DNA was isolated using REDExtract-N-Amp PCR Kit (Sigma). Information on primer sequences and PCR programs can be found in Appendix A.

### 4.2.3 UVB Treatment

Cells were washed twice with PBS, then in a minimal volume of PBS exposed to UVB (FS20 UVB bulbs, National Biological) at doses indicated (25-50 mJ/cm<sup>2</sup>). Bulb intensity was measured at the beginning of each experiment using a UVB 500C meter (National Biological). After irradiation, PBS was removed and conditioned medium with drug treatments was added back.

### 4.2.4 Western blotting

Cells were washed twice with cold PBS, harvested by scraping into RIPA buffer (Santa Cruz Biotechnology), centrifuged, and supernatants collected. In selected experiments examining apoptosis, detached cells were collected via centrifugation of medium and combined with the attached cells in RIPA buffer. Protein concentrations were determined using BioRad assay. Equal amounts of protein were subjected to electrophoresis. Western blotting was performed as described previously [237].
Antibodies used include AKT, p-AKT\textsuperscript{Ser473}, S6K, p-S6K\textsuperscript{Thr389}, Caspase-3, p-4EBP\textsuperscript{1 Ser65}, 4EBP1 (all from Cell Signaling, 1:1000) and GAPDH (Proteintech, 1:2000).

### 4.2.5 Cell viability and apoptosis

Cell viability after UVB exposure was determined colorimetrically by MTS assay (CellTiter 96 Aqueous Proliferation Assay, Promega) according to the manufacturer’s instructions and monitored at 490 nm using a model 3550-UV plate reader (BioRad). Cells for each condition were plated in triplicate and the experiments were repeated at least three times. Cell viability was calculated by setting the mean vehicle-treated mock UVB viability to 100%. Relative change in viability was calculated taking the ratio of cell viability of UVB to mock for each drug regimen.

Apoptosis was assessed with flow cytometry using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions. The percentage of apoptotic cells was determined using a FACSCalibur cytometer (Beckman Coulter) and analyzed with Modfit LT software (Verify Software).

### 4.2.6 Statistical analysis

Data are expressed as the mean of at least three independent experiments analyzed by 2-sided Student’s t-test. A p-value of <0.05 was considered significant.
4.3 Results

4.3.1 Rictor deficient MEFs are more sensitive to UVB-induced apoptosis

In order to evaluate the specific role of mTORC2 in UVB-induced apoptosis, we first utilized rictor-null MEFs (Rictor\textsuperscript{-/-}). Initial studies were performed to characterize these cells. Immunoblot analysis revealed that p-AKT\textsuperscript{Ser473} was undetectable in Rictor\textsuperscript{-/-} cells (Figure 4-3). Further studies examined the effect of UVB-induced activation of mTOR signaling in these cells. UVB (50 mJ/cm\textsuperscript{2}) caused increased phosphorylation of S6K and AKT\textsuperscript{Ser473} in wild-type MEFs (Rictor\textsuperscript{+/-}) at 30 min and the levels remained elevated at 2 h (Figure 4-4). UVB stimulated p-S6K in a similar manner in Rictor\textsuperscript{-/-} cells, but p-AKT\textsuperscript{Ser473} was completely absent, illustrating the loss of mTORC2 signaling (Figure 4-4). To determine the role of mTORC2 in UVB-induced cell death, MTS assays were utilized. There were significantly fewer viable rictor-null cells compared to wild-type cells at 24 h after UVB exposure (Figure 4-5A). To verify whether this represents an increased sensitivity to apoptosis in the absence of mTORC2 signaling, cells were analyzed using Annexin V by flow cytometry. There was a significant increase in the percentage of apoptotic Rictor\textsuperscript{-/-} cells following UVB compared to wild-type cells (Figure 4-5B). The marked increase in apoptosis was verified by Western blot analysis of caspase-3. The results show that cleaved caspase-3 begins to accumulate in Rictor\textsuperscript{-/-} cells by 6 h and continues to increase up to 12 h after UVB exposure (Figure 4-5C). In contrast, wild-type MEFs show considerably less caspase-3 cleavage over the same time course. These data are consistent with the hypothesis that downregulation of mTORC2 signaling sensitizes cells to UVB-induced apoptosis.
4.3.2 Torin2 sensitizes wild-type MEFs but not Rictor deficient MEFs to UVB-induced apoptosis

In Chapter 3, we showed that genetic ablation of *mTOR* or treatment with the mTOR kinase inhibitor Torin2 sensitize keratinocytes to UVB-induced apoptosis. In contrast, we demonstrate in Chapter 2 that rapamycin does not alter UVB-induced cell death. In order to differentiate whether the sensitization of cells to UVB-induced apoptosis is mediated solely through mTORC2 inhibition rather than a combination of mTORC2 and rapamycin-resistant mTORC1 inhibition, we examined the effects of Torin2 on UVB-induced cell death in *rictor*-null and wild-type MEFs. First we tested the ability of Torin2 to inhibit UVB-induced activation of mTOR signaling. Western blot analysis revealed that 10 nM and higher concentrations of Torin2 completely block UVB-induced phosphorylation of S6K, our marker of mTORC1 activity (Figure 4-6). There is some inhibition of AKT phosphorylation with 10 nM, however, complete inhibition of mTORC2 signaling in wild-type MEFs required 50 nM or higher concentrations. AKT phosphorylation was not measurable in the *rictor*-/MEFs. The MTS results showed that Torin2 reduces cell viability of mock irradiated cells in both the wild-type and *rictor*-/groups, indicating that the reduction of cell viability seen with Torin2 is mediated through inhibition of rapamycin-resistant mTORC1 function (Figure 4-7A). In contrast, when we examined the relative change in cell viability following UVB exposure, we noted that although Torin2 concentrations of 50 nM and higher significantly increased the UVB-mediated cell death in wild-type MEFs, Torin2 had no effect in *rictor*-/cells (Figure 4-7B). Interestingly, Torin2 decreased cell viability following UVB in wild-type MEFs to the levels comparable to those seen in *rictor*-/MEFs. Caspase-3 western blots verified that the decreased viability is due to enhanced apoptosis (Figure 4-7C). Collectively, these
results suggest that Torin2 enhances sensitivity to UVB-induced apoptosis solely through inhibition of mTORC2 activity.

4.3.3 4OHT induced rictor deletion and decreased mTORC2 signaling in K14CreER\textsuperscript{T2}/Rictor\textsuperscript{fl/fl} primary keratinocytes

We next examined the role of mTORC2 signaling and UVB-induced cell death in a keratinocyte model. We utilized primary keratinocytes isolated from K14-CreER\textsuperscript{T2};Rictor\textsuperscript{fl/fl} mice. PCR and western blot analysis verified recombination of the rictor allele (\textDelta\text{LoxP}) and rictor deletion in cells cultured with 4OHT, but not vehicle (Figure 4-8). Additionally, preliminary studies with K14-CreER\textsuperscript{T2};Raptor\textsuperscript{fl/fl} primary keratinocytes verified recombination and Raptor protein knock-down (Figure 4-9). However, because our results using Torin2 in Rictor\textsuperscript{\textminus}\textminus MEFs (Figure 4-7) demonstrated that inhibition of rapamycin-resistant mTORC1 function did not sensitize cells to UVB-mediated apoptosis, we chose to concentrate on rictor knock-down primary keratinocytes. Western blot analysis of mTOR activation markers of mock-irradiated K14-CreER\textsuperscript{T2};Rictor\textsuperscript{fl/fl} primary keratinocytes revealed complete loss of pAKT\textsuperscript{Ser473} levels (Figure 4-10). Phosphorylation of S6K was dramatically induced when rictor was knocked out via 4OHT treatment, suggesting that when mTORC2 is specifically inhibited there is an acute activation of mTORC1 signaling. We are currently examining the sensitivity of these rictor-deficient primary keratinocytes to UVB-induced apoptosis using MTS assays and western blot analysis.
4.4 Discussion

Apoptosis following UVB exposure is an essential tumor prevention mechanism, as it removes DNA damaged cells that could harbor mutations in critical oncogenes [269]. However, previous studies have shown that UVB-induced activation of the EGFR/PI3K/AKT signaling cascade induces cell survival [163,174]. Increased survival of damaged cells leads to tumor initiation. Therefore, targeting these pro-survival pathways should attenuate tumor formation by inhibiting keratinocyte transformation. We previously demonstrated in Chapter 3 that inhibiting both mTOR complexes, by either genetically targeting mTOR or utilizing the mTOR kinase inhibitor Torin2, sensitized keratinocytes to apoptosis following UVB exposure. Here we utilized rictor knock-out MEFs and primary keratinocytes with inducible rictor deletion to investigate the role of mTORC2 signaling in UVB treated cells. The studies presented here demonstrate for the first time that inhibition of mTORC2 sensitizes cells to UVB-induced apoptosis and strongly support the hypothesis that UVB activation of pro-survival signaling is mediated through activation of mTORC2 (Figure 4-11).

In both our fibroblast and keratinocyte systems, rictor deletion blocked UVB-mediated activation of p-AKT^{Ser473}, a known downstream target of mTORC2. However, recently it has been shown that Rictor has functions independent of mTORC2 through interactions with a number of proteins, including integrin-linked kinase (ILK) [270], Cullin-1 [271], Myo1C [272], and PKCζ [273]. Rictor’s interaction with ILK is especially important to note because ILK has been shown to play a role in ovarian and breast cancer cell proliferation and survival [274,275] and can also phosphorylate AKT at Ser473 [276]. It is possible we see attenuation of UVB-induced phosphorylation of
AKT<sup>Ser473</sup> in our Rictor ablated cells due to inhibition of ILK function, rather than inhibition of mTORC2. McDonald <i>et al.</i> found that Rictor-ILK is primarily responsible for AKT<sup>Ser473</sup> phosphorylation in a variety of breast cancer cell lines and that siRNA targeting <i>ILK</i> or <i>rictor</i> but not <i>mTOR</i> reduced p-AKT<sup>Ser473</sup> levels [270]. However, UVB-induced p-AKT<sup>Ser473</sup> levels were reduced in <i>rictor</i> deficient MEFs and primary keratinocytes, and in <i>mTOR</i> deficient primary keratinocytes (Chapter 3), supporting the theory that phosphorylation of AKT following UVB is mediated through mTORC2. Future experiments utilizing siRNA targeting of ILK could be employed to rule out the possibility that UVB-induced phosphorylation of AKT<sup>Ser473</sup> is mediated by Rictor-ILK.

Reduced Rictor protein levels sensitized both primary keratinocytes and MEFs to UVB-induced apoptosis. Additionally, we show that the mTOR kinase inhibitor Torin2 sensitizes wild-type but not Rictor<sup>−/−</sup> MEFs to UVB-induced apoptosis. These results provide additional evidence supporting our hypothesis that mTORC2 activates pro-survival signaling following UVB exposure. Our data presented here combined with the results from Chapter 3, support a hypothesis that UVB-induction of mTORC2 initiates pro-survival signaling cascades. Future studies repeating our experiments in mSin1 knock-out MEFs [18], which lack mTORC2 activity but have intact Rictor, could verify that the effects we see are attributed to mTORC2 inhibition. However, mSin1 also contains a Ras-binding domain and may also have functions independent of mTORC2 [277].

Torin2 did not sensitize Rictor<sup>−/−</sup> MEFs to UVB-induced apoptosis, indicating that the pro-survival signaling downstream of mTOR is mediated solely through mTORC2 and not a rapamycin-resistant mTORC1 function (Figure 4-7). However, Torin2 did significantly reduce cell viability in mock-irradiated cells independent of functional
mTORC2, suggesting that rapamycin-resistant mTORC1 functions contribute to cell viability and potentially play a role in keratinocyte cell proliferation. Future work examining the effects of Torin2 and siRNA targeting raptor on UVB-induced cell cycle progression in HaCaT cells (similar to those done in Chapter 2) and in vivo studies in our Raptor LoxP mice will be used to verify a role for rapamycin-resistant mTORC1 function in UVB-induced cell cycle progression and proliferation.

The mechanism downstream of mTORC2 responsible for the pro-survival signals seen following UVB exposure remains to be defined. AKT relays survival signals through a number of mechanisms including phosphorylation and inactivation of the pro-apoptotic protein Bad [278] and phosphorylation inhibition of FoxO1/3a [18], which blocks the transcription of pro-apoptotic genes such as FasL and TRAIL [44]. Further studies are needed to identify the downstream effector(s) of mTORC2 mediating the pro-survival effects observed. It is also possible that proteins other than AKT mediate the pro-survival signals downstream of mTORC2. SGK1, another protein activated by mTORC2 [45], can also phosphorylate FoxO1/3a [279]. Recent work has shown that down-regulation of mTORC2 signaling results in decreased levels of cellular FLICE-inhibitory protein (c-FLIP) [280], which normally functions to suppress apoptosis by blocking cleavage and activation of capsase-8 [281]. However, transfection of cells with an activated form of AKT did not reverse the c-FLIP levels, indicating that the observed effects were mediated through mTORC2 but not AKT [280].

As shown in Figure 4-10, ablation of rictor in primary keratinocytes resulted in increased phosphorylation of S6K. The mechanism is unknown, it was recently shown that Rictor is directly phosphorylated at Thr 1135 by S6K1 [282]. Phosphorylation at this site does not alter mTORC2 kinase activity, but it is possible that knocking out rictor
activates S6K1 through relief of a yet undefined feedback mechanism involving this phosphorylation site. This effect of increase S6K phosphorylation raises the concern that targeted inhibition of mTORC2 induces activation of mTORC1. We have previously shown that activation of mTORC1 plays a critical role in UVB-induced keratinocyte proliferation (Chapter 2). Further study is needed, but it is possible that mTORC1 activation could blunt the therapeutic effects of mTORC2 inhibition.

In summary, we utilized a variety of techniques to investigate the role of mTORC2 in mediating UVB-induced pro-survival signaling. We show here for the first time that selectively inhibiting mTORC2 sensitizes cells to UVB-induced apoptosis. Apoptosis following UVB-exposure represents a defense mechanism because failure to remove damaged cells can lead to aberrant regulation of cell signaling and tumor formation. Though future work is needed to verify that the cells undergoing apoptosis are indeed those possessing DNA damage, our results described here suggested that inhibition of mTORC2 signaling will be effective at preventing the formation of skin tumors through inhibiting cell survival. The data presented in the following chapter address this theory with in vivo studies using Rictor LoxP mice.
Figure 4-1: Hypothesized role of mTORC2 in mediating UVB-induced effects. AKT activation is known to induce pro-survival signaling in many cell types. Because mTORC2 is known to activate AKT through phosphorylation at Ser473, we hypothesize that mTORC2 stimulates UVB-mediated cell survival signaling. Inhibiting mTORC2 signaling through deletion of rictor should sensitize cells to UVB-induced apoptosis.
Figure 4-2: Diagram of Raptor and Rictor LoxP alleles. Diagram of Raptor and Rictor alleles. LoxP sites (triangles) flank exons 6 and 7 of Raptor and exons 4 and 5 of Rictor. A tissue specific promoter drives Cre recombinase expression. In tissues where Cre is expressed, the DNA segment flanked by the LoxP sites will be excised, and the *raptor* or *rictor* gene silenced. In tissues where Cre is not expressed, the floxed gene will remain active.
Figure 4-3: Characterization of mTOR signaling in Rictor deficient MEFs. Rictor wild-type (+/+) and knock-out (-/-) MEFs were grown to approximately 70-80% confluence. Whole cell lysate was harvested in RIPA buffer and examined using immunoblot analysis. Data are representative from 2 independent experiments.
Figure 4-4: Rictor deficient MEFs exhibit reduced UVB-induced activation of mTORC2 but normal activation of mTORC1. Rictor wild-type (+/+) and knock-out (-/-) MEFs were exposed to UVB (50 mJ/cm²) at 70-80% confluency. Whole cell lysate was harvested in RIPA buffer at the indicated times after UVB and subjected to immunoblot analysis of mTORC1 and mTORC2 activation markers. Data are representative of 3 independent experiments.
**Figure 4-5**: *Rictor deficient MEFs are more sensitive to UVB-induced apoptosis.*

Rictor wild-type (+/+) and knock-out (-/-) MEFs were exposed to UVB (50 mJ/cm²). A, MTS cell viability (mean ± SEM) at 24 h post UVB exposure; ** p<0.01. B, Annexin-V flow cytometry (mean ± SEM) at 24 h post UVB exposure. C, Immunoblot analysis of caspase-3 and quantification (mean ± SEM); * p<0.05. All data are representative of 2-4 independent experiments.
Figure 4-6: Effects of rapamycin and Torin2 on UVB-induction of mTOR signaling in Rictor deficient MEFs. Rictor wild-type (+/+) and knock-out (-/-) MEFs were treated with rapamycin (50 nM), various doses of Torin2 (nM) or DMSO for 1 h prior to UVB (25 mJ/cm²) exposure. Whole cell lysates were harvested in RIPA buffer at 2 h post-UVB and subjected to Immunoblot analysis of mTORC1 and mTORC2 activation markers. Data are representative of 2 independent experiments.
Figure 4-7: Torin2 sensitizes wild-type but not Rictor deficient MEFs to UVB-induced apoptosis. Rictor wild-type (+/+) and knock-out (-/-) MEFs were treated with rapamycin (50 nM), various doses of Torin2 (nM) or DMSO for 1 h prior exposed to UVB (25 mJ/cm²). A, MTS cell viability at 12 h post UVB exposure showing % viable cells. B, MTS cell viability at 12 h post UVB exposure showing % change in viability with UVB; * p<0.05, ** p<0.01 C, Immunoblot analysis of cleaved caspase-3, a marker of apoptosis, at 12-h post UVB. Data are representative of 2-3 independent experiments.
Figure 4-8: 4OHT induces LoxP recombination and Rictor deletion in K14-CreERT2;Rictorflo/fl primary keratinocytes. K14-CreERT2;Rictorflo/fl and Rictorflo/fl keratinocytes were harvested from 1-3 day-old pups and cultured in 4OHT (2 nM) or vehicle for 3 days. A, PCR analysis of primary keratinocyte DNA harvested 24 after final 4OHT treatment. ∆LoxP denotes primers specific to the recombined Rictor allele. B, Immunoblot analysis of Rictor in primary keratinocyte whole-cell lysate. Data are representative of 2-3 independent experiments.
**Figure 4-9: 4OHT induces LoxP recombination and Raptor deletion in K14-CreER\textsuperscript{T};Raptor\textsuperscript{fl/fl} primary keratinocytes.** K14-CreER\textsuperscript{T};Raptor\textsuperscript{fl/fl} and Raptor\textsuperscript{fl/fl} keratinocytes were harvested from 1-3 day-old pups and cultured in 4OHT (2 nM) or vehicle for 3 days. A, PCR analysis of primary keratinocyte DNA harvested 24 h after final 4OHT treatment. ΔLoxP denotes primers specific to the recombined Raptor allele. B, Immunoblot analysis of Raptor in primary keratinocyte whole-cell lysate. Data are representative of 2 independent experiments.
Figure 4-10: Rictor deletion in the primary keratinocytes blocks UVB induction of mTORC2 signaling but enhances mTORC1 signaling. K14-CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} and Rictor\textsuperscript{fl/fl} keratinocytes were harvested from 1-3 day-old pups and cultured in 4OHT (2 nM) or vehicle for 3 days. Immunoblot analysis of mTORC1 and mTORC2 activation markers in primary keratinocyte extracts harvested 2 h following UVB (25 mJ/cm\textsuperscript{2}) exposure. Data are representative of 2 independent experiments.
Figure 4-11: Role of mTORC1 and mTORC2 in mediating UVB-induced effects in keratinocytes. Our studies demonstrate that both mTORC1 and mTORC2 signaling are induced by UVB. While mTORC1 is in part responsible for stimulating epidermal proliferation, mTORC2 mediates pro-survival signaling.
Chapter 5

Targeted disruption of Rictor inhibits chemically-induced skin carcinogenesis

5.1 Introduction and Rationale

Recent studies in cancer biology suggest that mTORC2 could be a promising therapeutic target because its activity is essential for transformation and viability in a number of cancer cell types (reviewed in [60]). In addition to studies showing upregulation of mTORC2 signaling in a variety of human cancer types, several reports have shown that the mTORC2 component Rictor is overexpressed in a number of tumors and cancer cell lines [102,116,117]. In particular, Rictor levels are significantly higher in breast tumors relative to surrounding tissue and higher levels were associated with increased metastasis [102]. Additionally, targeted inhibition of mTORC2 but not mTORC1 induced apoptosis in breast cancer cell cultures and xenograph models [103]. Other studies have shown similar results in cancer cell lines derived from a variety of tissues [37,116,118]. Guertin et al. found that genetically ablating mTORC2 activity by deleting rictor, blocked tumor development in a PTEN-deletion induced mouse model of prostate cancer. Other studies have shown that mTORC2 signaling is upregulated in human SCC [165,166] and that Rictor protein levels are increased in UVB-induced mouse models of SCC [268], suggesting that targeted inhibition of mTORC2 signaling may inhibit tumor development. However, very little information is available on the possible influence of mTORC2 signaling on the development of skin cancer.
The multistage model of mouse skin carcinogenesis is one of the best defined experimental models of tumor development and has been used extensively to better understand the development of epithelial cancers (reviewed in [283]). In particular this model has provided an invaluable tool to identify the underlying molecular mechanism associated with the various stages of epithelial carcinogenesis. In this model tumor development occurs in three stages: initiation, promotion, and progression (Figure 5-1). Tumor initiation by a single topical dose of the carcinogen DMBA induces mutations in critical genes, typically activating mutation at codon 61 of the \( H-Ras \) gene \([182,183,283]\).

Following initiation, tumor promotion is stimulated by repeated applications of the non-mutagenic tumor promoter TPA, which stimulates epidermal cell proliferation and hyperplasia. During promotion, initiated cells undergo clonal expansion, resulting in the development of premalignant papillomas. The progression of papillomas into malignant squamous cell carcinomas (SCCs) occurs stochastically; there is an increased probability of additional genetic alterations as the cell population expands.

Our lab has previously used the two-stage mouse skin carcinogenesis model to investigate molecular mechanisms contributing to skin cancer development \([284,285]\). Previous studies utilizing DMBA/TPA chemical carcinogenesis have shown that inhibition of mTORC1 with rapamycin attenuates tumor formation \([222]\). In Chapter 2, we demonstrate that rapamycin acts by blocking TPA-induced proliferation. Collectively, these studies demonstrate that targeting mTORC1 inhibits skin carcinogenesis. However, the role of mTORC2 in tumor development remains largely unexamined. In this chapter, we investigated the role of mTORC2 in two-stage chemical carcinogenesis. We have previously shown that mTORC2 plays a critical role in pro-survival/anti-apoptotic signaling in keratinocytes following exposure to the complete carcinogen UVB (Chapter
3 and 4). We hypothesize that mTORC2-induced activation of pro-survival signaling could contribute to tumorigenesis at a number of stages during tumor development (Figure 5-1). At initiation, we propose that mTORC2 is required for keratinocyte stem cell survival following carcinogen-induced (DMBA) DNA damage. At the promotion stage, mTORC2 is proposed to mediate cell survival necessary for clonal expansion. Finally, mTORC2 could play a role in tumor maintenance and growth by preventing apoptosis. Utilizing an inducible LoxP-Cre mouse model (K14CreER^T;Rictor^flo/flo^) and topical 4OHT to temporally control deletion of Rictor specifically in the epidermis, we investigated the role of mTORC2 signaling in tumor formation and maintenance. We show that inhibition of mTORC2 signaling through rictor ablation within the basal layer of the epidermis confers resistance to tumor development. Moreover, we demonstrate that inducing rictor deletion in pre-existing tumor inhibited further tumor growth. These data provide the first demonstration that Rictor expression is necessary for both skin tumor development and maintenance of established tumors.

5.2 Materials and Methods

5.2.1 Generation of K14-CreER^T;Rictor^flo/flo^ mice

All experiments involving mice were carried out in compliance with the Guide for the Care and Use of Laboratory Animals and protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. We utilized an inducible Cre-LoxP mouse model to ablate Rictor in the epidermis. Floxed Rictor mice (Rictor^flo/flo^) contain LoxP sites flanking exons 4 and 5 of the rictor gene (Figure
These mice were crossed with K14-CreER\textsuperscript{T} mice \cite{198}, which express a tamoxifen-activated Cre recombinase fused to a modified estrogen receptor under the control of the keratin 14 promoter. The CreER fusion protein remains inactive in the cytoplasm of the cells until 4OHT binds and allows translocation into the nucleus where Cre induces recombination in the basal layer of the epidermis. All animals were backcrossed for at least 10 generations onto the C57BL/6 background. K14-CreER\textsuperscript{T} mice were bred with Rictor\textsuperscript{fl/fl} mice to ultimately generate mice hemizygous for the K14-CreER\textsuperscript{T} transgene and homozygous for the Rictor floxed allele (K14-CreER\textsuperscript{T}; Rictor\textsuperscript{fl/fl}). Mouse tail DNA was isolated using REDExtract-N-Amp PCR Kit (Sigma). Information on primer sequences and PCR programs can be found in Appendix A. The dorsal surface of the mice (6-8 weeks) was shaved with electrical clippers and mice were allowed to rest for 24-48 h prior to all experiments; only mice in the resting stage of the hair cycle were used for the following experiments.

### 3.2.4 Western blotting

K14-CreER\textsuperscript{T}; Rictor\textsuperscript{fl/fl} and Rictor\textsuperscript{fl/fl} controls were treated topically with 1 mg 4OHT (Sigma) in 100\textmu L or vehicle (DMSO:Acetone [1:9]; D:A) daily for 5 consecutive days. Animals were euthanized 14 days after the final treatment. Skin was treated with a depilatory agent for 3 min, washed, excised and underlying fat and connective tissue were removed. Epidermal samples were collected by scraping the surface of excised skin with a razor blade and placed into ice-cold RIPA buffer. Following sonication, samples were centrifuged, and supernatants collected. Protein concentrations were determined using the BioRad assay. Equal amounts of protein were subjected to
electrophoresis. Western blotting was performed as described previously [237]. Antibodies used include Rictor, Raptor (both from Cell Signaling, 1:1000) and GAPDH (Proteintech, 1:2000).

5.2.3 Disruption of Rictor signaling at initiation during two-stage carcinogenesis

Three groups of mice (control: K14-CreER^{T};Rictor^{fl/fl} + vehicle n=17; Rictor^{fl/fl} + 4OHT n=17; experimental: K14-CreER^{T};Rictor^{fl/fl} + 4OHT n=16) were subjected to two-stage skin carcinogenesis at 6-7 weeks of age. Animals were treated topically with 2 mg 4OHT (Sigma) in 100\(\mu\)L or vehicle (DMSO:Acetone [1:9]; D:A) daily for 5 consecutive days. 3 days after final 4OHT treatment mice were initiated with a single topical dose of DMBA (400 nmol; Sigma) in 200 \(\mu\)L acetone. One week after initiation, mice were treated with topical TPA (17 nmol; Calbiochem) in 200 \(\mu\)L acetone twice weekly for 21 weeks (Figure 5-2A). Mice were monitored weekly for tumor formation. The dimensions of skin tumors (if greater than 1 mm x 1 mm) were recorded. Surface area (S.A.) was calculated as \[\text{length} \times \text{width}\]. Volume was calculated as \[\text{length} \times (\text{width}^2)/2\]. Tumors were collected at the end of the study for evaluation.

5.2.4 Effect of Rictor deletion on continued growth of existing papillomas

K14-CreER^{T};Rictor^{fl/fl} and Rictor^{fl/fl} were subjected to two-stage skin carcinogenesis as described above to generate papillomas. When animals developed tumors of sufficient size (at least 3 mm x 2 mm), mice (K14-CreER^{T};Rictor^{fl/fl} and Rictor^{fl/fl}) were treated with 2 mg of 4OHT (in 100 \(\mu\)L DMSO:sunflowerseed oil [1:9]) i.p. for five
consecutive days and this regimen was repeated every two weeks. The change in tumor dimensions was monitored weekly for four weeks (Figure 5-2B). All mice continued to receive TPA treatment twice weekly for the remainder of the experiment.

5.2.5 Statistical analysis

The effect of Rictor deletion on tumor incidence was analyzed using log-rank analysis of Kaplan-Meier tumor-free survival curves. For tumor multiplicity, differences in the average number of papillomas per mouse were analyzed using 2-way ANOVA. Tumors were counted only if the length and width were both greater than 1 mm. Tumors in regression experiments were compared to their initial size using a paired student’s t-test. All other statistical comparisons utilized a two-tailed unpaired Student’s t-test.

5.3 Results

5.3.1 Analysis of Rictor deficiency in keratinocytes of K14CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> mice after 4OHT treatment

The early embryonic lethality caused by deletion of the rictor gene in mice [43] led us to use a 4OHT-inducible Cre-LoxP system to selectively ablate rictor in the epidermis of mice in order to investigate the role of mTORC2 in skin tumor development and maintenance. Initial experiments were conducted to validate the efficiency of rictor deletion in the epidermis of K14-CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> mice. For these experiments mice were treated topically with 2 mg 4OHT or vehicle (D:A 200 µL) daily for five days. PCR analysis using DNA from the dorsal epidermis harvested from mice 1 week after the final
4OHT treatment verified recombination only occurred in mice expressing the K14-CreER\textsuperscript{T} transgene and treated with 4OHT (Figure 5-3A). Immunoblot analysis of epidermal extracts of K14-CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} mice confirmed reduced levels of Rictor in mice treated with 4OHT, but not vehicle-treated mice (Figure 5-3B), illustrating the effectiveness of our model system.

### 5.3.2 Rictor disruption significantly reduces skin tumor development

To investigate the role of Rictor in skin carcinogenesis, Rictor deficient mice (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} + 4OHT n=16) and two control groups (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} + DMSO n=17, Rictor\textsuperscript{fl/fl} + 4OHT n=17) were subjected to a two-stage skin carcinogenesis protocol (Figure 5-2A). Skin-specific Rictor-deficient mice (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} + 4OHT) were resistant to tumorigenesis (Figure 5-4). After 21 weeks of promotion there was a striking reduction in tumor incidence (percentage of mice bearing at least one tumor) from 100% in controls to only 44% in Rictor deficient mice (p < 0.0001) (Figure 5-4A). The tumor multiplicity (number of tumors per mouse) relative to the control groups was also significantly decreased beginning at week 13 (Figure 5-4B). In addition, Rictor deficient mice had a dramatically lower tumor burden (as measured by both mean tumor surface area or volume per mouse) at 21 weeks (Figure 5-5 and 5-6). Further immunohistochemical analysis of the tumors is ongoing. We will examine the histologic grade of the tumors and the levels of p-AKT\textsuperscript{Ser473} as a marker of mTORC2 activation. Additionally, proliferation and apoptosis within the tumors will be studied using BrdU and cleaved-caspase-3 staining.
5.3.3 Deletion of Rictor in pre-existing papillomas inhibits tumor growth

To evaluate whether Rictor disruption effectively inhibited growth of pre-existing papillomas in the continued presence of tumor promotion, K14CreERT$_T$;Rictor$^{fl/fl}$ and Rictor$^{fl/fl}$ mice that had developed primary skin tumors (minimum size 3 mm x 2 mm) were treated with 4OHT by i.p. injection (Figure 5-2B) Twice weekly TPA treatment was continued throughout the experiment. 4OHT-induced Rictor disruption inhibited further growth of tumors (Figure 5-7A). The relative size of tumors in Rictor deficient mice was significantly lower than control mice ($p < 0.01$ at week 4). Additionally, while tumors in control mice increased significantly in size by the conclusion of the experiment ($p < 0.05$ relative to initial size), the tumor surface area in Rictor deficient mice decreased significantly ($p < 0.05$). At the conclusion of the experiment, among the 12 tumors from inducible Rictor-deficient mice treated with 4OHT, 83% underwent a reduction in tumor size (Figure 5-7B and Table 5-1), while all control tumors increased in size or remained constant (Figure 5-8). Whether the reduction in tumor growth caused by rictor deletion was due to inhibition of proliferation or increased apoptosis remains to be determined and these experiments are ongoing. However, these results clearly indicate that inhibition of mTORC2 signaling by inducible disruption of Rictor efficiently prevented further growth of skin papillomas.
5.4 Discussion

In the present study, we demonstrate that Rictor, an essential component of mTORC2, is critical for skin tumor development and maintenance. The ligand-inducible Cre recombinase, combined with topical 4OHT application, allows gene expression to be regulated temporally during carcinogenesis. Inducible Rictor-deficient mice (K14CreERT<sup>T</sup>;Rictor<sup>fl/fl</sup>) showed LoxP recombination and reduced levels of Rictor in the epidermis following topical applications of 4OHT (Figure 5-3). To evaluate the functional role of mTORC2 in tumor development, Rictor was temporally disrupted prior to initiation of two-stage chemical carcinogenesis. As shown in Figure 5-4, Rictor-deficient mice were highly resistant to chemical carcinogenesis. Tumor development was significantly delayed and the number of tumors was also significantly reduced. We also examined the effect of rictor deletion on pre-existing tumors. 4OHT-induced rictor deletion not only prevented further growth of tumors but also produced a significant reduction in tumor area relative to the initial size (Figure 5-7). The current results directly demonstrate for the first time a requirement for Rictor in both the development and continued growth of papillomas.

Previous studies from our lab have suggested that mTORC2-mediated induction of pro-survival signaling plays an important role in skin carcinogenesis. Targeted inhibition of mTORC2 sensitizes cells to UVB-induced apoptosis (Chapter 4). Consistent with our hypothesis that targeted inhibition of mTORC2 would prevent skin carcinogenesis, prior studies utilizing mice deficient in AKT1, a known downstream target of mTORC2, show increased tumor latency and reduced tumor number when exposed to DMBA/TPA carcinogenesis [226]. In this study, we show that Rictor
deficiency in mice inhibits tumor development and induces regression of pre-existing tumors. Ongoing studies examining the mechanism by which rictor deletion alters tumor development and growth are underway. Immunohistochemical analysis of tumors in our regression study will determine whether the reduction in tumor size in pre-existing tumors is mediated through enhanced apoptosis, decreased proliferation, or a combination. Immunohistochemical analysis of prostate tumors in Rictor-deficient mice showed that the tumors developed from cells that had failed to undergo Cre-induced recombination and retained Rictor and p-AKT\textsuperscript{Ser473} levels comparable to control animals [37]. Future studies will investigate the possibility that tumors from our Rictor deficient mice also developed from cells that failed to undergo recombination. Additional studies will examine the \textit{H-ras} mutation profile within these tumors to determine if the tumors in Rictor deficient mice arise from cells with different mutations than control animals.

The spatial and temporal targeting of \textit{rictor} also facilitates further studies to investigate the possible roles of Rictor at the distinct stages (initiation, promotion) of chemical carcinogenesis. Studies are underway to determine if \textit{rictor} deletion is acting to inhibit tumor formation during the initiation or promotion stages of tumorigenesis by examining the effects of \textit{rictor} deletion on TPA-induced proliferation and apoptosis following DMBA applications. After 4OHT-induced \textit{rictor} deletion, mice were treated with a single dose of DMBA, or four doses of TPA. We are currently examining apoptosis (cleaved caspase-3) and proliferation (BrdU) in the skin from these animals. Preliminary examination of H&E sections seems to indicate that \textit{rictor} deletion does not alter TPA-induced epidermal thickening (data not shown). Our mouse model could also be used to delete \textit{rictor} after initiation (DMBA treatment) but prior to promotion (TPA), which would definitely determine if Rictor plays a necessary role in promotion. Additionally, we are
backcrossing the Rictor\textsuperscript{fl/fl} mice onto the FVB/N background to be used in studies using UVB (similar to those in Chapter 3). Further work will address the possibility that the reduction in tumorigenesis we see with ablation of \textit{rictor} is due to mTORC2-independent rictor function [270,271,272].

The discovery that mTORC2 is responsible for phosphorylation of AKT at Ser473 [36], has led to speculation that mTORC2-specific inhibitors might be valuable cancer therapeutics. The possibility has been previously addressed by genetically ablating mTORC2 activity in a prostate model. Silencing of \textit{rictor} prevents PTEN-deletion induced prostate cancer [37]. The results from our study clearly illustrate that Rictor plays a critical role in skin tumor development and maintenance. Ongoing studies will investigate the signaling pathways downstream of Rictor and mTORC2 that contribute to tumor growth and development. Our studies emphasize the need to explore targeting mTORC2 in other cancer types in vivo, which can be accomplished by crossing Rictor\textsuperscript{fl/fl} mice with other tissue-specific Cre animals. Additionally, our work underscores the need to develop mTORC2-specific inhibitors as possible chemotherapeutics agents.
Figure 5-1:  

**Model for the proposed role of mTORC2 and Rictor in multi-stage skin carcinogenesis.** mTORC2 plays a critical role in pro-survival/anti-apoptotic signaling within the epidermis. Ablating Rictor will allow us to investigate the role mTORC2 signaling in tumor development and maintenance. At initiation, we propose that mTORC2 is required for keratinocyte stem cell survival following carcinogen-induced (DMBA) DNA damage. At the promotion stage, mTORC2 is proposed to mediate cell survival necessary for clonal expansion. Finally, mTORC2 could play a role in tumor maintenance and growth by preventing apoptosis. Our model system allows us to induce rictor deletion and inhibition of mTORC2 signaling at different stages in tumor development by varying the time when 4OHT is applied (adapted from [286]).
Figure 5-2: Chemical carcinogenesis protocols. Groups of mice were treated topically with 400 nmol DMBA in 200 µL acetone, followed by twice weekly applications of 17 nmol TPA in 200 µL acetone. A, Protocol utilized to examine the effect of Rictor deletion on tumor development. Rictor deletion was induced prior to DMBA initiation with topical application of 2 mg 4OHT daily for 5 treatments. B, Protocol utilized to examine the effect of Rictor deletion on tumor growth. When tumors reached a minimum size of 3 mm x 2 mm, Rictor deletion was induced with i.p. injections of 2 mg 4OHT. 4OHT treatment was repeated daily for five days and the regimen was repeated every 2 weeks. Twice weekly TPA treatments were continued and tumors were harvested 4 weeks after the initial 4OHT treatment.
Figure 5-3: Topical application of 4OHT induces LoxP recombination and Rictor deletion in the epidermis of K14-CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> mice. K14-CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> mice at 7-weeks of age were treated topically with 4OHT (2 mg) or vehicle daily for 5 days. A, PCR analysis of epidermal DNA harvested 7d after final 4OHT treatment. ΔLoxP denotes primers specific to the recombined Rictor allele. B, Immunoblot analysis of Rictor in epidermal extracts harvested at 7 d after final 4OHT treatment. Data are representative of 2 independent experiments.
Figure 5-4: Rictor deletion inhibits tumor formation. Rictor-deficient mice (K14CreER\textsuperscript{ERT};Rictor\textsuperscript{fl/fl} + 4OHT n=16) and two control groups (K14CreER\textsuperscript{ERT};Rictor\textsuperscript{fl/fl} + DMSO n=17, Rictor\textsuperscript{fl/fl} + 4OHT n=17) were subjected to a two-stage skin carcinogenesis protocol. Papillomas greater than 1 mm x 1 mm were counted weekly. A, Tumor incidence was calculated from the number of mice with at least one tumor. B, Tumor multiplicity (tumor burden), expressed as the average number of tumors per mouse ± SEM.
Figure 5-5: Tumor burden is decreased in Rictor-deficient mice. Rictor deficient mice (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} + 4OHT n=16) and two control groups (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} + DMSO n=17, Rictor\textsuperscript{fl/fl} + 4OHT n=17) were subjected to a two-stage skin carcinogenesis protocol after 4OHT or vehicle treatment. Dimensions of tumors greater than 1 mm x 1 mm were measured weekly. A, Average tumor surface area per mouse ± SEM, was calculated \([\text{length} \times \text{width}]\). B, Average tumor volume per mouse ± SEM, was calculated \([\text{length} \times (\text{width}^2)/2]\).
**Figure 5-6: Rictor is necessary for tumor formation.** Rictor deficient mice (K14CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> + 4OHT) and two control groups (Rictor<sup>fl/fl</sup> + 4OHT and K14CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> + D:A) were subjected to a two-stage skin carcinogenesis protocol. Images of mice in our first cohort of animals after 21 weeks of TPA treatment. Numbers indicate the numbers of papillomas found on the dorsal surface of each mouse. Arrows indicate the three tumors that developed in Rictor-deficient mice. K14CreER<sup>T</sup>;Rictor<sup>fl/fl</sup> and Rictor<sup>fl/fl</sup> are abbreviated as Cre/Ric and Ric.
**Figure 5-7: Rictor is essential for tumor maintenance and growth.** Dimensions of tumors (initial size > 3 mm x 2 mm) were measured prior to 4OHT treatment and weekly thereafter. A, Average percent change in surface area relative to initial size; mean±SEM. B, Waterfall graph showing the percent change in surface area for each individual tumor relative to its initial size.

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**Table 5-1: Effects of 4OHT-induced Rictor deletion on skin tumor surface area.** Dimensions of tumors were measured prior to 4OHT treatment and weekly thereafter. Percent change in surface area after 4 weeks was calculated relative to initial surface area for each tumor.
Figure 5-8: *Rictor deletion induces tumor regression*. Representative tumors from Rictor\(^{fl/fl}\) (Ric) and K14CreER\(^{T2}\);Rictor\(^{fl/fl}\) (Cre/Ric) mice before (Pre-Tx) and 4 weeks after (Post-Tx) i.p. 4OHT treatment to induce *rictor* deletion in mice expressing Cre.
**Chapter 6**

**Discussion**

**6.1 General summary and conclusions**

The main goal of this work was to investigate the role of mTOR in skin carcinogenesis. Because NMSC pathogenesis is characterized by enhanced cell proliferation and inhibition of apoptosis [257], we sought to differentiate the contributions of mTORC1 and mTORC2 on UVB-induced keratinocyte proliferation and survival. In Chapter 2, studies using rapamycin showed that mTORC1 inhibition blocked UVB-induced phosphorylation of S6K (Figures 2-3, 2-5 and 2-13) and significantly reduced UVB-stimulated epidermal proliferation and cell cycle progression (Figures 2-4, 2-6 and 2-8). However, rapamycin treatment had no effect on UVB-induced cell death (Figure 2-14). In Chapter 3, we inhibited both mTORC1 and mTORC2 utilizing the mTOR kinase inhibitor Torin2 and an inducible mTOR-deficient (K5-CreER<sup>T2</sup>;mTOR<sup>fl/fl</sup>) mouse model that allows epidermal-specific disruption of mTOR following topical treatment with 4-hydroxytamoxifen (4OHT). Inhibiting both mTOR complexes attenuated UVB-induced phosphorylation of both S6K and the mTORC2 target AKT<sup>Ser473</sup> (Figures 3-4, 3-10 and 3-13). In contrast to our rapamycin results, inhibiting both TOR complexes significantly increased apoptosis both in vivo (Figure 3-12) and in keratinocyte cultures following UVB (Figures 3-12, 3-14 and 3-15). mTOR ablation also reduced UVB-induced hyperproliferation (Figures 3-5 and 3-7), however, the effect was not significantly different from our rapamycin results (Figure 3-8). Collectively these studies suggest that
mTORC1 activation by UVB induces keratinocyte proliferation, while mTORC2 activates pro-survival signaling.

The role of mTORC2 in UVB-induced pro-survival signaling was verified in Chapter 4, utilizing Rictor\textsuperscript{−/−} MEFs, which lack functional mTORC2. Rictor\textsuperscript{−/−} MEFs showed a complete loss of AKT\textsuperscript{Ser473} phosphorylation, even when stimulated with UVB (Figure 4-4). Additionally, these cells were more sensitive to UVB-induced apoptosis than controls (Figure 4-5). Because recent studies have reported that mTORC1 can have rapamycin-resistant functions [253,256], we utilized Torin2 in our Rictor\textsuperscript{−/−} and control MEFs. While Torin2 did sensitize control MEFs to UVB-induced apoptosis, it had no effect on cell death in Rictor\textsuperscript{−/−} cells (Figure 4-7), indicating that the pro-survival signaling downstream of mTOR is mediated solely through mTORC2 and not a rapamycin-resistant mTORC1 function. It is also possible that the activation of pro-survival signaling could be mediated through mTORC2-independent Rictor signaling or off-target effects of Torin2, as discussed in Chapter 4. Though this remains a possibility, it seems less likely given that our experiments utilizing Torin2 treatment, Rictor\textsuperscript{−/−} cells, and mTOR\textsuperscript{−/−} all show similar effects.

The studies reported here show for the first time that mTORC1 and mTORC2 play unique but complementary roles in controlling proliferation and apoptosis in the skin following UVB exposure. Our results support a model where UVB-induced activation of mTORC1 mediates hyperproliferation while mTORC2 activates pro-survival signaling (Figure 6-1). Our studies provide new insight into the molecular mechanisms involved in UVB-induced cell damage and the pathogenesis of skin cancer and underscore the importance of both mTOR complexes in mediating UVB-induced signaling in keratinocytes.
Previous studies utilizing chemical carcinogenesis have shown that inhibition of mTORC1 with rapamycin attenuates skin tumor formation [222]. However, the role of mTORC2 in skin tumor development remains largely unexamined. Because our results from Chapters 2 and 3 suggested that inhibition of mTORC2 signaling may prevent the formation of skin tumors through inhibiting cell survival, we utilized an inducible Rictor-deficient mouse model (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl}) to investigate the role of mTORC2 signaling in tumor formation and maintenance. Rictor ablation within the basal layer of the epidermis significantly reduced tumor incidence and multiplicity (Figure 5-4). Additionally, we demonstrated that Cre-mediated rictor deletion in pre-existing tumors inhibited further tumor growth (Figure 5-7). These data provide the first demonstration that Rictor expression is necessary for both skin tumor development and maintenance of established tumors, providing exciting insight into future targets for cancer treatment.

6.2 Ongoing studies

6.2.1 Rictor Studies

Our prevention study in Rictor-deficient mice (K14CreER\textsuperscript{T};Rictor\textsuperscript{fl/fl} treated with 4OHT prior to DMBA/TPA; Figure 5-2A) showed a significant reduction in tumor formation. On-going studies will determine the histologic grade of the tumors in order to differentiate papillomas and carcinomas in our experimental and control groups. It is possible that the tumors in our ‘Rictor-deficient’ mice arose from cells that failed to undergo LoxP recombination and might retain one or both copies of the Rictor gene. Immunohistochemical staining of p-AKT\textsuperscript{Ser473} will help to identify whether tumors have
functional mTORC2 signaling. Additionally, proliferation and apoptosis within the tumors will be studied using BrdU and cleaved-caspase-3 staining. PCR-based analysis of the tumors will examine the *Ha-Ras* mutation profile to determine if the tumors in Rictor-deficient mice arise from cells with different mutations than control animals. For example, tumors in K6-ODC mice exhibit *K-Ras* mutations rather than the typical *Ha-Ras* [287]. In addition, immunohistochemical analysis of BrdU and cleaved caspase-3 in tumors from our regression study (4OHT treated after tumors formed; Figure 5-2B) will be completed in order to ascertain whether the reduction in tumor growth caused by rictor deletion was due to inhibition of proliferation or increased apoptosis.

In order to more directly investigate the role of mTORC2 in tumor promotion, Rictor-deficient mice were treated twice-weekly with TPA, for a total of four treatments. Skin samples were harvested 6 h after the final TPA treatment. Visual inspection of H&E sections suggests that mTORC2 inhibition does not attenuate TPA-induced hyperproliferation (data not shown). However, these results will be quantified and confirmed with BrdU staining. Skin sections from control and Rictor-deficient mice treated with a single dose of DMBA are also available. Cleaved-caspase-3 immunohistochemical staining will determine if Rictor-deficient mice are more sensitive to apoptosis following carcinogen treatment. The short-term *in vivo* experiments will allow us to more directly assess the role of Rictor in tumor promotion and initiation. The successful completion of these studies will elucidate the role of Rictor in skin tumor proliferation and survival and provide additional support for mTORC2 as an important target for cancer prevention.
6.2.2 Role of mTOR in UVB-induced DNA damage repair

Though our studies have shown that inhibition of mTORC2 sensitizes keratinocytes to UVB-induced apoptosis, further work is needed to verify that the cells undergoing apoptosis are those that have DNA damage. Because UVB induces DNA damage in the form of cyclobutane pyrimidine dimers (CPDs), we are currently optimizing an ELISA assay for CPD lesions. We will isolate DNA from living (attached) and dead (floating) cells at 0 and 24 h after 25 mJ/cm$^2$ UVB exposure using rapamycin and Torin2 treated HaCaT cells to verify that apoptosis selectively occurs in cells that have unrepaired DNA damage. Studies have shown that at 24 h post-UVB, ~50% of the initial CPD lesions remain in HaCaT cells, but less than 10% remain in cells pre-treated with the PI3K inhibitor LY294002 [288], which indicates that PI3K inhibition enhances UVB-induced apoptosis preferentially in cells that have DNA damage. Use of Torin2 and rapamycin will determine if this phenomenon is mediated through mTOR. Similar experiments utilizing Torin2 in Rictor$^{-/-}$ MEFs, will investigate the possible influences of rapamycin-resistant mTORC1 and mTORC2 signaling.

We will also utilize this assay to investigate a possible role for mTOR in DNA damage repair. UVB-induced DNA damage is repaired by nucleotide excision repair (NER) [289]. Studies have shown that loss of PTEN or AMPK enhances cell survival and reduces NER through decreased levels of XPC (xeroderma pigmentosum C protein), which is necessary for NER [290,291]. Because both AMPK and PTEN are negative regulators of mTOR, these studies suggest that mTOR activation might inhibit NER, providing an additional pathway where by mTOR activation enhances tumorigenesis (Figure 6-1). We plan to examine the levels of XPC after UVB-exposure in cells treated
with rapamycin and Torin2. Additionally, we will isolate DNA at 0, 6, 12 and 24h and perform CPD ELISA assays to monitor the rate of repair. For these studies we are using a lower dose of UVB (2.5 and 5 mJ/cm²), to prevent apoptosis. Our initial western blots confirmed that 50 nM Torin2 inhibits low-dose UVB-induced activation of AKT<sup>Ser473</sup>, S6K, and 4EBP1 (Figure 6-2). Future work will investigate the specific influences of the two mTOR complexes on CPD repair using primary keratinocytes from our Rictor- and Raptor-deficient mice.

6.3 Future Directions

Our studies provide exciting and novel insights on the role of mTOR in cancer development but also reveal valuable areas for future research. The experiments and applications reviewed here encompass the broader directions our current research could be extended toward.

6.3.1 Defining the UVB-mTOR pathway

6.3.1.1 Upstream of mTOR

Our studies have shown that UVB activates mTORC1 (p-S6K and p-4EBP1) and mTORC2 (p-AKT<sup>Ser473</sup>). However the upstream mediators of this activation remain unclear. Studies have shown that UV rapidly activates EGFR in keratinocytes through reactive oxygen species-mediated inactivation of protein tyrosine phosphatases [292,293]. EGFR<sup>−/−</sup> MEFs and wild-type MEFs treated with EGFR inhibitors have
attenuated UV-induced phosphorylation of S6K and AKT$^{\text{Ser473}}$ [163], suggesting that EGFR activation initiates the signaling cascades that activate mTOR. However, the exact signal pathways downstream of EGFR that activate mTOR remain unclear. Inhibition of PI3K but not AKT inhibits UV-induced phosphorylation of S6K [177], indicating mTORC1 activation is dependent on PI3K but not AKT. Additionally, treatment with p38 inhibitors or rapamycin, but not PI3K inhibitors, blocks UVB-induced phosphorylation of 4EBP1 in a mouse epidermal cell line [175]. Moreover, a recent study has shown that UVB induced downregulation of PTEN [246], providing another mechanism through which UVB could activate mTORC1 and mTORC2. The cellular- and animal-based systems characterized in this thesis provide valuable tools that can be utilized to dissect the pathways responsible for activation of mTORC1 and mTORC2.

In Chapter 4, we also show that p-S6K is elevated in our 4OHT-induced Rictor-deficient primary keratinocytes. Additional investigation utilizing mSin$^{-/-}$ cells rather than Rictor$^{-/-}$ cells could provide clues to elucidate the mechanism responsible for this upregulation.

### 6.3.1.2 Downstream Effectors of mTOR and UVB

Our studies in Chapters 2 and 3 demonstrate that mTORC1 activation mediates UVB-induced keratinocyte proliferation. However, we did not fully explore the downstream mechanism of mTORC1 responsible for mediating UVB-induced cell proliferation. We did provide evidence indicating that rapamycin treatment blocks upregulation of ODC by TPA. Previous work has shown that upregulation of ODC, which is the first enzyme in the polyamine biosynthetic pathway, is both necessary and
sufficient for tumor promotion [247,248,249]. However cyclinD1, an essential cell cycle regulatory protein is also translated in a mTORC1-dependent manner [241] and could provide an additional mechanism for mTORC1-mediated cell proliferation. Additionally, Torin2 significantly reduced cell viability in mock-irradiated cells independent of functional mTORC2 (Chapter 4), suggesting that rapamycin-resistant mTORC1 functions contribute to cell viability and potentially play a role in keratinocyte cell proliferation. Future work examining Torin2-treated or Raptor-deficient mice could elucidate the role of rapamycin-resistant mTORC1 functions in UVB-induced proliferation. Additional studies utilizing siRNA to knockdown 4EBP1 (Eif4ebp1/Eif4ebp2), S6K (Rps6kb1/Rps6kb2), or other less well-defined mTORC1 substrates (STAT3, LIPIN, CLIP-170) [29,30] in HaCaT cells could allow us to examine which downstream effectors of mTORC1 mediate UVB-induced cell cycle progression.

It is also possible that mTOR plays a role in tumor promoter-associated inflammation. Chronic inflammation is a risk factor for various human cancers [250,251] and anti-inflammatory drug usage has been linked to a reduction in the incidence of several human cancers [250,252]. Interestingly, topical rapamycin has been shown to decrease the infiltration of macrophages, T cells, neutrophils and mast cells into the dermis of mice following TPA treatment [222]. In our rapamycin experiments we saw a reduction in UVB-induced epidermal thickening at 24 h (Figure 2-6), but did not see a difference in epidermal proliferation until 48 h (Figure 2-8). Though more investigation is needed, we hypothesize that this reduction is due to decreased immune cell infiltration and inflammation. We have also noted the same phenotype in mTOR-deficient mice (Figures 3-5 and 3-7), indicating that this phenomenon is keratinocyte-mediated rather than through inhibition of mTORC1 in immune cells.
In Chapter 5, we showed that *rictor* deletion inhibits UVB-induced activation of mTORC2 and sensitized cells to UVB-induced apoptosis. However, further studies are needed to isolate the downstream signaling mechanism. For example, studies have shown that mTORC2 phosphorylates AKT at Ser473, promoting cell survival through a number of downstream effectors [16,36,39,40,41,42]. Ser473 phosphorylation is sufficient for AKT to phosphorylate FoxO1/3a [18]. In the absence of phosphorylation, FoxO translocates to the nucleus and increases transcription of apoptosis-inducing genes such as FasL and TRAIL [44]. AKT can also relay survival signals through phosphorylation and inactivation of the pro-apoptotic protein Bad [278,294]. Activated AKT also inhibits the release of the apoptotic activator cytochrome c from the mitochondria [295] and phosphorylates and inhibits the pro-apoptotic activity of Bim [296]. Future studies examining the downstream effectors of mTORC2 responsible for mediating pro-survival signaling could provide additional potential targets for NMSC prevention strategies.

It is possible that proteins other than AKT mediate the pro-survival signals downstream of mTORC2. SGK1, another protein activated by mTORC2 [45], can also phosphorylate FoxO1/3a [279]. Recent work has shown that down-regulation of mTORC2 signaling results in decreased levels of cellular FLICE-inhibitory protein (c-FLIP) [280], which functions to suppress apoptosis by blocking cleavage and activation of caspase-8 [281]. Additionally, Rictor has functions independent of mTORC2 through interactions with a number of proteins, including integrin-linked kinase (ILK) [270], Cullin-1 [271], Myo1C [272] and PKCζ [273]. It is possible that inhibition of mTORC2-independent Rictor signaling contributes to the increased apoptosis and reduced sensitivity to carcinogenesis seen in Rictor-deficient cells and mice. Further studies are
needed to identify the downstream effector(s) of mTORC2 that mediate the observed effects.

6.3.2 mTOR and cancer: Beyond proliferation and apoptosis

6.3.2.1 mTOR and migration/metastasis

Intensive studies have focused on the role of mTOR in controlling cancer cell proliferation and survival. However, mTOR may also play a critical role in other facets of cancer development including advanced disease and metastasis. Studies have shown that both mTORC1 and mTORC2 are involved in epithelial-mesenchymal transition (EMT), migration and metastasis in colorectal cancer [297]. The molecular mechanisms by which mTOR regulates metastatic disease are not fully understood. However, it is proposed that mTOR’s role in controlling cytoskeletal organization contributes to the mechanism. A number of studies suggest that mTORC1 plays a role in cellular migration and cytoskeletal organization. mTORC1 pathways control translation of the proteins involved in cellular motility, including the small GTPases RhoA and Rac1 [298]. Additionally, downstream from mTORC1, S6K1 can phosphorylate the focal adhesion protein paxillin and alter F-actin organization [299,300]. Studies show that rapamycin inhibits cell motility and migration in cancer cell lines from various tissues of origin [301].

Recent studies however suggest that mTORC2 activation plays a larger role in cancer metastasis. The mTOR kinase inhibitor PP242 and siRNA knock-down of mTOR or rictor inhibited migration in breast cancer cells, while rapamycin and knock-down of raptor had no effect [103]. Additionally, mTORC2 inhibition reduces migration of breast
cancer and glioma cells [102,116]. Though the precise mechanism of how mTORC2 controls cytoskeletal organization remains unknown, studies implicate PKC, RhoA, and Rac [8,16,297]. Recent studies also suggest that Rictor might play a mTORC2-independent role in cellular migration. Rictor was found to interact with PKCζ and mediate metastasis in breast cancer cells [102]. Additionally, Rictor can associate with Myo1c and regulate cortical actin remodeling [272]. Rictor levels are elevated in a number of cancer cells and could regulate actin cytoskeleton and cellular migration independent of mTORC2. Future work to elucidate the roles of mTORC1 and mTORC2 in metastatic disease and cancer cell migration could be achieved using the cellular systems that we have already optimized.

6.3.2.2 mTOR and angiogenesis

Angiogenesis is a critical step needed for the development of cancer and anti-angiogenic therapies are being employed to slow the progression of disease. In addition to rapamycin’s role in inhibiting proliferation in cancer cells, it has also been shown to suppress angiogenesis in primary tumors [302]. mTORC1 regulates the translation of HIF1α, which in turn acts to increase the levels of VEGF, a potent activator of angiogenesis [303]. Rapalogs have been particularly successful for the treatment of Kaposi’s sarcoma [304]. These tumors are highly vascularized and have high levels of VEGF. It is likely that some of the success of rapalog treatment can be attributed to their ability to inhibit angiogenesis through inhibition of mTOR signaling in endothelial cells. Interestingly, in endothelial cells, prolonged rapamycin treatment inhibits mTORC2 and phosphorylation of AKT at Ser473 [34]. Additionally, studies indicate that hyperactivation
of AKT is necessary for pathological angiogenesis [305] and AKT1 activity is also required for migration of endothelial cells [306]. Collectively, these studies suggest that mTORC2 plays an important role in angiogenesis. Studies utilizing siRNA to knockdown raptor and rictor in endothelial cells indicate that both mTOR complexes function in hypoxia-induced proliferation [307]. Our Rictor−/− and Raptor−/− cells can be utilized to differentiate the importance of the two TOR complexes in mediating angiogenesis in cancer.

### 6.3.2.3 mTOR and autophagy/senescence

mTOR has also been implicated in autophagy, a catabolic process where cellular proteins and organelles are digested and recycled to sustain cellular metabolism under conditions of starvation. Autophagy is believed to have a dual role in cancer; it acts as a tumor suppressor by preventing the accumulation of damaged cellular components. However, in established tumors, cancer cells activate autophagy to enhance cell survival in response to increased metabolic damage or stress. Transgenic mice with faulty autophagy machinery have accelerated rates of spontaneous tumor development (reviewed in [308]). mTORC1 negatively regulates autophagy though phosphorylation and suppression of ULK1/Atg13/FIP200, a kinase complex required for autophagy initiation [309,310,311]. mTORC1 inhibition has been shown to activate autophagy and promote cancer cell survival (reviewed in [312]). Additionally, studies demonstrate that mTOR kinase inhibitors induce autophagy to a greater extent than rapamycin due to inhibition of rapamycin-resistant mTORC1 function [253,313]. Interestingly, the pro-apoptotic effects of mTOR kinase inhibitors in leukemia cells were enhanced by
chloroquine, an autophagy inhibitor [314]. These studies suggest that inhibition of mTORC1 in established tumors could enhance autophagy, providing a mechanism for increased survival of cancer cells. The potential role of mTORC2 in autophagy has not been fully explored. However, a recent study showed that downregulation of Rictor attenuates autophagy in an epidermoid carcinoma cell line [268]. Although further investigation is needed, these studies do suggest that development of a mTORC2-specific inhibitor could overcome the pro-survival effects mediated through autophagy when mTORC1 is inhibited.

In addition to autophagy, mTOR has also been implicated in oncogene-induced senescence, irreversible growth arrest induced by oncogene activation. Oncogene-induced senescence is a tumor suppressing defense mechanism. It was reported that constitutively active AKT induces senescence in primary endothelial cells through activation of FOXO3 [315]. It was also recently shown that mTORC1 but not mTORC2 disrupts and delays senescence [316,317,318], providing a possible rational for the failure of rapalogs to preform as successfully as expected in clinical trials.

The above studies emphasize that mTOR plays a role in multiple processes that contribute to cancer development and progression. Additionally, they imply that inhibition of mTORC1 could promote survival of pre-cancerous cells and suggest that mTORC2 might prove to be a better target for cancer treatment and prevention. In order to maximize targeting of mTORC1 and mTORC2 in cancer, a greater understanding of which cellular processes are regulated by mTORC1 and/or mTORC2 in cancer is needed. Additionally, it is likely that many of these roles are cell type specific. Combinations of Rictor\textsuperscript{fl/fl} and Raptor\textsuperscript{fl/fl} mice with different tissue-specific Cre transgenic
animals, in addition to studies utilizing siRNA, will allow a better understanding of the distinct functions of the two mTOR complexes.

6.3.3 mTOR and epidermal homeostasis

Although the majority of our research has focused on the role of mTOR in skin cancer development, many of our experimental systems could be applied to investigate other roles of mTOR in the skin. Studies suggest that mTOR may play a role in keratinocyte differentiation. Primary keratinocytes infected with a constitutively active form of PI3K were resistant to calcium-induced differentiation, while dominant-negative PI3K infected cells showed accelerated differentiation [319]. Additionally, human skin explants showed decreased levels of differentiation markers when cultured with rapamycin [320]. Future studies could investigate the role of mTOR signaling in keratinocyte differentiation utilizing Rictor- and Raptor-deficient primary keratinocytes.

Clinical evidence suggests that prolonged rapamycin treatment may delay wound healing [321,322]. Moreover, studies in mice with epithelial-specific ablation of Tsc1, the upstream repressor of mTORC1 demonstrated a direct link between mTOR and wound healing. Tsc1-deficient mice display increased epithelial cell proliferation, migration, and wound healing [323]. While collectively these studies raise concerns of possible side-effects when utilizing rapalogs for cancer treatment and prevention, they also suggest that topical rapalogs and mTOR kinase inhibitors could be utilized for the treatment of skin diseases that are characterized by increased proliferation and altered keratinocyte differentiation, particularly psoriasis. Psoriasis is characterized by cutaneous inflammation, increased epidermal proliferation, hyperkeratosis, angiogenesis, and
abnormal keratinization. Recently, immunohistochemical analysis of biopsies from psoriatic patients showed enhanced activation of mTORC1 [324]. Interestingly, elevated levels of phosphorylated mTOR were seen in both lesional and non-lesional skin, suggesting that pathological activation of mTOR might be an early event in psoriasis pathogenesis. Additional studies are needed to explore the possible role of mTOR signaling in the pathogenesis of psoriasis and whether targeting mTOR alone or in combination with UV-light treatments could be beneficial to these patients.

A number of recent reports suggest that mTOR signaling may play a role in keratinocyte stem cells (KSC). Like other tissue-specific stem cells, KSC have the unique ability to self-renew and to differentiate into multiple cell types within their tissue of origin. Recent evidence has indicated that the slow-cycling cells with high proliferative potential within the bulge region of the hair follicles are epidermal stem cells (reviewed in [325]). Immunohistochemical analysis has shown that mTORC1 and mTORC2 are activated in the bulge region of the hair follicle [326]. While studies have shown that mTOR signaling plays a role in stem cell maintenance and differentiation in a variety of tissues [327,328,329], only a few studies have examined the role of mTOR signaling in the keratinocyte stem cell population. Persistent expression of Wnt in the epidermis induces hyperproliferation of epithelial stem cells, ultimately exhausting the stem cell population [330]. Rapamycin treatment of these animals prevents both the hyperproliferation and stem cell depletion. Other studies have shown that expression of constitutively active AKT in the epidermis induces expansion of the keratinocyte stem cell population and proliferation of epidermal progenitor cells [331]. Additionally, Akt1 and Akt2 double knock-out mice have impaired skin development and die soon after birth [332]. Although we did not see any obvious hair or skin alterations in our mTOR- or
Rictor-deficient mice, future studies can explore the role of mTOR signaling in the maintenance and expansion of the epidermal stem cell population. Examples include studies to examine the expression of putative epidermal stem cell markers, K15 and CD34 [333,334] by immunohistochemistry, the use of BrdU label-retaining techniques [335,336] or fluorescence-activated cell sorting (FACS) to examine the population of cells expressing α6-integrin and CD34 [337,338]. These techniques can more accurately evaluate possible alterations in the epidermal stem cell population within our mice. Studies to examine the relationship between mTOR signaling and KSC could utilize our Rictor\textsuperscript{fl/fl} and Raptor\textsuperscript{fl/fl} crossed with the K15-CrePR1 mice, which express a RU486-inducible Cre only in the bulge region of the hair follicle [339]. This system will allow targeted disruption of mTOR components in the bulge region KSC, while our current studies utilizing the K5 and K14 promoters are expressed both in the bulge region, but also the basal layer of the epidermis.

It is important to note that considerable evidence has implicated KSC in epidermal tumor formation [340,341,342,343,344,345]. It is estimated that 3-5 individual genetic mutations are necessary in humans for a normal cell to transform into a cancer cell [346]. Therefore, only cells that reside in the epidermis for prolonged periods of time have the ability to acquire the number of genetic ‘hits’ necessary to undergo transformation. Accordingly, the long-lived KSCs seem the likely candidate. Further investigation the role of mTOR in KSC maintenance, expansion, and senescence could provide valuable information to clarify the mechanism by which mTOR inhibition attenuates tumor development and growth.
6.3.4 Clinical considerations

Our studies clearly support mTOR targeting for cancer prevention and treatment. However, there are inherent risks of mTOR inhibition. A recent meta-analysis of phase II and III clinical trials showed that patients taking rapalogs have a 2.6-fold increased risk of developing high-grade infections during treatment with mTOR inhibitors [347]. As mentioned previously, prolonged rapamycin may also delay wound healing [321,322]. This issue was highlighted in a recent case report [348]. A heart-transplant patient developing greater than 20 squamous cell carcinomas was initially switched from CsA to the rapalog, everolimus, to slow carcinogenesis. Though everolimus slowed the rate of SCC to only six annually, the patient was diabetic and developed limb-threatening skin fistulas and had to be switched back to CsA.

Rapamycin also induces glucose intolerance and insulin resistance in both mice and humans [349]. However, a recent study utilizing liver-specific ablation of rictor suggests that impaired insulin sensitivity induced by rapamycin is mediated through mTORC2 inhibition [213]. mTORC2, acting through SGK, activates epithelial sodium channel (ENaC)-dependent transport in kidney epithelial cells [350], suggesting that mTOR kinase inhibitors used for cancer treatment could have diuretic effects. While mTOR kinase inhibitors may inhibit mTORC1 and mTORC2 activity, activation of PI3K through relief of feedback inhibition of mTORC1 may limit their therapeutic potential. Studies have shown that mTOR kinase inhibitors can induce activation of AKT<sup>Thr308</sup> [9], which could theoretically activate mTOR-independent survival and proliferation pathways. Dual PI3K/mTOR inhibitors, which target the structurally related kinase domains of both mTOR and PI3K (reviewed in [351]) could potentially overcome the
feedback mechanism. By targeting PI3K, these inhibitors prevent feedback inhibition and are more effective than rapamycin at inhibiting cancer cell proliferation [352]. Interestingly, these inhibitors are effective in mouse cancer models with PI3K-driven tumors, but not K-Ras-driven tumors [353]. Other studies indicate that such broad inhibition of signaling cascades could be toxic in normal cells and these dual inhibitors have much narrower therapeutic windows [263]. These compounds are also associated with hyperlipidemia and hyperglycemia [354].

Given the limitations of current inhibitors, development of a mTORC2-specific inhibitor could prove to be a valuable tool for cancer treatment. Such an inhibitor should eliminate S6K-IRS feedback-induced activation of PI3K. However, the development of a mTORC2-specific inhibitor is a daunting task because the two complexes share the same catalytic domain. Small molecules that disrupt the protein-protein interactions specific to mTORC2 or that target the substrate binding interface [355] could potentially selectively inhibit mTORC2. Additionally, a recent report suggests that aloe-emodin, a natural compound found in aloe, selectively binds to and inhibits mTORC2 [356], although the mechanism remains unknown.

While a number of clinical trials utilizing mTOR kinase inhibitors and dual PI3K/mTOR inhibitors are currently in progress (reviewed in [357]), many questions about the future application of mTOR inhibitors in the clinic remain. Reliable biomarkers that predict which patients will respond to mTOR inhibitors are currently unavailable. Identification of biomarkers/predictors of efficacy require a better understanding of the molecular mechanisms involved when different aspects of the mTORC1 and mTORC2 pathways are inhibited in different cell types. Despite the concerns and need for future studies, mTOR remains a promising target for cancer prevention and treatment, as
evidenced by our dramatic tumor prevention and regression studies in Rictor-deficient mice (Figures 5-4 and 5-7).

6.4 Overall conclusions

NMSC is a significant public health concern with more than 3.5 million cases of NMSC being diagnosed in the US in 2012 [119]. NMSC also presents a considerable health care fiscal burden; treatment represents 4.5% of all Medicare expenditures [120]. Additionally, the rate of NMSC is increasing both in the general population [127], and in patients younger than 35 [131]. These staggering reports in conjunction with the limited success of primary prevention strategies [122], emphasize the urgent need to develop new strategies for prevention and treatment of NMSC. The elucidation of signaling pathways activated by UVB results in a better understanding of the biology and pathogenesis of NMSC and can be used to identify novel targets and mechanism-based strategies for chemoprevention.

NMSC pathogenesis is characterized by both enhancement of cell proliferation and inhibition of apoptosis. Induction of apoptosis following DNA damage is an essential protective mechanism, ensuring the removal of damaged cells that may harbor oncogenic mutations. However, UVB also activates signaling cascades that promote the survival of these potentially cancerous cells. The studies reported here investigated whether activation of mTORC1 and mTORC2 downstream effectors by UVB led to changes in keratinocyte pro-survival signaling. The effects of mTORC1 inhibition on apoptosis vary greatly depending on the system employed. Enhancement of AKT signaling can occur in the presence of rapamycin, due to relief of an S6K-dependent
negative feedback loop targeting PI3-kinase [57,58]. Loss of this feedback inhibition is thought to be responsible for increased mTORC2/AKT activation and decreased sensitivity to apoptotic stimuli in certain malignancies treated with rapamycin [58]. On the other hand, there are a number of studies that report enhancement of apoptosis by rapamycin [358,359,360,361,362,363]. It has been shown that prolonged rapamycin treatment reduces mTORC2 complex assembly and AKT activation in approximately 20% of cancer cell lines [34], which could have a direct effect on apoptosis pathways. It is thus possible that the pro-apoptotic effects of rapamycin seen in some previous studies are the result of mTORC2 inhibition rather than a direct effect on mTORC1. This rationale is supported by our results. We see no effect of rapamycin on mTORC2-dependent pathways in our system, and rapamycin treatment does not result in enhanced activation of apoptosis in UVB-treated cells. In contrast, 4OHT-induced mTOR deletion resulted in a significant increase in apoptosis following UVB in both keratinocytes culture and mouse epidermis. The mTOR kinase inhibitor, Torin2, also sensitized cells to UVB-induced apoptosis. Furthermore, rictor-null cells were more sensitive to UVB-induced apoptosis than their wild-type counterparts. Additionally, our studies using Rictor<sup>fl/fl</sup> mice directly demonstrated that the mTORC2 component, Rictor, plays a critical role in skin tumor development and maintenance. Collectively, these results indicate for the first time that mTORC2 activation by UVB plays a critical role in mediating pathways that control keratinocyte survival and suggest that mTORC2 is an attractive target for prevention and treatment of NMSC.

To our knowledge, this is the first study to report that mTORC1- and mTORC2-dependent pathways are both activated by UVB, and play unique roles in controlling proliferation and apoptosis in the skin. These results emphasize the need to further
elucidate the roles of mTORC1 and mTORC2 in photocarcinogenesis and their links to cell proliferation, apoptosis, DNA repair, and tumor development. A better understanding of the signal transduction pathways activated by UVB in keratinocytes is essential for effective prevention of skin cancer to reduce disease burden. Our data provide compelling evidence that both mTORC1 and mTORC2 act as critical mediators of UVB-activated signal transduction in keratinocytes, and suggest that the combined targeting of both mTOR complexes, or alternatively mTORC1 and AKT, will be an effective chemoprevention strategy to targeting both survival and proliferation of keratinocytes during skin carcinogenesis.

Our studies emphasize the importance of mTORC2 in tumor development. Recent publications have highlighted the drawbacks of mTORC1 inhibition, including activation of mTORC2-AKT pro-survival pathways and inhibition of cancer defense mechanisms like autophagy and senescence. These unintended effects could explain why previous clinical trials utilizing rapalogs have not provided the dramatic effects originally predicted. Our data suggests targeting both mTOR complexes or specifically inhibiting mTORC2 could overcome many of these barriers and feedback mechanisms, providing an attractive target for cancer therapies.
Figure 6-1: Role of mTORC1 and mTORC2 in mediating UVB-induced effects. Our studies indicate that mTORC1 plays a role in UVB-induced proliferation, but not survival. Additionally, our work demonstrates that mTORC2 signaling is in part responsible for stimulating UVB-mediated cell survival signaling. Previous work has shown that inhibition of mTORC1 with rapamycin inhibits tumor formation. We show that inhibiting mTORC2 signaling through deletion of rictor also inhibits tumor formation. Current research is investigating the possible role of mTORC1 and mTORC2 in UVB-induced DNA damage repair.
Figure 6-2: Torin2 blocks low-dose UVB stimulation of mTORC1 and mTORC2 signaling. HaCaT cells were serum deprived overnight and treated with vehicle (DMSO), rapamycin (50 nM), or various doses of Torin2 for 1h and exposed to 2.5 and 5 mJ/cm² UVB irradiation. Whole cell lysate was harvested in RIPA buffer at 2 h post-irradiation and subjected to immunoblot analysis of mTORC1 and mTORC2 activation markers. Data is representative from 2 independent experiments.
Appendix A

PCR Primers

K5-CreER<sup>T2</sup> and K14-CreER<sup>T1</sup>

- CARR16 (fw Cre): 5'-TGCTGTTTCACTGGTTATGCGG-3'
- CARR17 (rv Cre): 5'-TGCCCCCTGTTTCACTATCCAG-3'

Program: CRE1617
Band = 700bp

mTOR: WT, LoxP, ΔLoxP

- CARR03 (rv ΔLoxP): 5’-CCACGCATGGCCCCACTGTCTTT-3’
- CARR04 (rv WT and LoxP): 5’-GCAAGAAGCGAGACTGCTT-3’
- CARR05 (fw WT, LoxP, and ΔLoxP): 5’-GTCCACCAACTCCGTGCACGCA-3’

Program: MTOR345
WT Band = 355bp  (CARR04 and CARR05)
LoxP Band = 425bp (CARR04 and CARR05)
ΔLoxP Band = 460bp (CARR03 and CARR05)

Raptor: WT, LoxP, ΔLoxP

- CARR10 (fw WT, LoxP, and ΔLoxP): 5’-ATGGTAGCAGGCACACTCTTCATG-3’
- CARR11 (rv WT and LoxP): 5’-GCTAAACATTCAGTCCCTAATC-3’
- CARR12 (rv WT and ΔLoxP): 5’-CTCAGAGAACTGCAGTGCTGAAGG-3’
- CARR18 (fw WT): 5’-CTGTGTGTATTCTGAGAGTGGATGG-3’

Program: RAPRIC
WT Band = 141bp  (CARR10 and CARR11)
LoxP Band = 228bp (CARR10 and CARR11)
WT Band = 254bp  (CARR10, CARR12, and CARR18)
ΔLoxP Band = 204bp (CARR10, CARR12, and CARR18)

Rictor: WT, LoxP, ΔLoxP

- CARR13 (fw WT, LoxP, and ΔLoxP): 5’-TTATTAACCTGTGTTGCGTGCA-3’
- CARR14 (rv WT and LoxP): 5’-CGTCTTGAAGCGTTGCTGCAT-3’
- CARR15 (rv WT and ΔLoxP): 5’-CAGATCAAGCATGCTCACAAG-3’
- CARR19 (fw WT): 5’-CTGTGTGTATTCTGAGAGTGGATGG-3’

Program: RAPRIC
WT Band = 197bp  (CARR13 and CARR14)
LoxP Band = 295bp (CARR13 and CARR14)
WT Band = 368bp  (CARR13, CARR15, and CARR19)
ΔLoxP Band = 280bp (CARR13, CARR15, and CARR19)
Appendix B

Polyamine Data

B.1 Abstract

Transgenic mice provide a valuable system to study the development of skin tumorigenesis. Previously it has been shown that mice overexpressing constitutively active MEK in the skin (K14-MEK mice) exhibit epidermal hyperplasia and spontaneous skin tumor development. These tumors were also shown to express high levels of ornithine decarboxylase (ODC), the critical regulatory enzyme in the polyamine biosynthetic pathway. Polyamines are required for normal cell growth and differentiation, however, elevated levels of polyamines and ODC activity have been associated with neoplastic transformation and tumor promotion in a variety of tissues. Antizyme (AZ) suppresses ODC activity by targeting ODC for degradation. Thus, mice overexpressing AZ in the skin (K5-AZ mice) showed decreased tumor development in both the classical mouse skin chemical carcinogenesis model (DMBA/TPA) and transgenic models. We hypothesize that the upregulation of ODC through overexpressing constitutively active MEK in the skin will result in expansion of epidermal stem cells with decreased keratinocyte differentiation, in addition to the epidermal hyperplasia and tumorigenesis already seen. Overexpression of AZ should decrease ODC activity and reverse these phenotypes. Adult stem cells have the unique ability to self-renew and to differentiate into multiple cell types within their tissue of origin. Cell division in epidermal stem cells is asymmetrical, providing one replacement stem cell and a second transient amplifying (TA) cell. TA cells reside within the interfollicular epidermis (IFE) and after several rounds of division, give rise to terminally differentiated cells in the suprabasal layers of the epidermis expressing early differentiation markers K1/K10 and late differentiation markers loricrin and involucrin. To determine the effect of MEK and AZ expression on stem cell expansion, new born pups and adult mice were injected with BrdU using different wash out periods to label TA cells and epidermal stem cells. Our results showed that skin from K14-MEK mice had a significant increase in the number of TA cells within the IFE and hair follicle, while mice expressing both the K14-MEK and K5-AZ transgenes showed no difference in number of TA cells compared to wild-type littermates. Additionally, K14-MEK mice had a significant increase in the number of stem cells in the bulge region of the follicle, while AZ repressed the MEK-mediated increase in this cell population. Examination of primary keratinocyte cultures from the four genotypes showed that MEK keratinocytes had decreased levels of both early and late differentiation markers compared to wild-type and AZ. AZ/MEK keratinocytes expressed similar levels of differentiation markers as WT and AZ, indicating that AZ was able to attenuate MEK-induced suppression of differentiation. Our results suggest that ODC and polyamines are key regulators of epidermal stem cell expansion and proliferation/differentiation of keratinocyte progenitor cells.
B.2 Data

Figure B-1: Overexpression of AZ decreases MEK-induced epidermal hyperplasia. (A) Dorsal skin samples from 8-week old mice (C57BL/6 background) were fixed overnight, embedded in paraffin, cut into 5μm sections, and stained with H&E. (B) Epidermal thickness was calculated from the average thickness at four different location for each mouse, using 4 mice for each genotype.
Figure B-2: **Expression of constitutively active MEK results in an increase in the number of IFE TAs.** Overexpression of AZ represses the MEK-mediated increase in TAs. 8-week old mice were i.p. injected with 50µg/g BrdU and sacrificed 2 hours later. (A) Paraffin sections were stained for BrdU. (B) IFE TAs were quantified as the number of BrdU-positive cells per 10 IFE sections (3 different skin sections were tabulated per mouse), using 3 mice for each genotype.
Figure B-3: Expression of constitutively active MEK results in an increase in the number of Follicular TAs. Overexpression of AZ represses the MEK-mediated increase in TAs. 8-week old mice were i.p. injected with 50µg/g BrdU and sacrificed 2 hours later. (A) Paraffin sections were stained for BrdU. (B) Follicular TA cells were quantified as the number of BrdU-positive cells per hair follicle (10 follicles/mouse), using 3 mice for each genotype.
Figure B-4: Expression of constitutively active MEK results in an increase in the number of LRCs in the bulge region of the hair follicles. Overexpression of AZ partially represses the MEK-mediated increase in LRC. Pups were s.c. injected with 50μg/g BrdU every 12 hours on post-natal days 3-5 and sacrificed when mice were 8-weeks old. (A) Paraffin sections were stained for BrdU. (B) Follicular LRCs were quantified as the number of BrdU-positive cells per 10 hair follicles (50 follicles per mouse), using 3 mice for each genotype.
Figure B-5: **Constitutively active MEK suppresses expression of keratinocyte differentiation markers but overexpression of AZ attenuates this effect.** Primary keratinocytes from 1-3d. old pups were cultured for 7 days in low Ca\(^{2+}\) (0.05mM) media and harvested in RIPA buffer. The induction of differentiation markers was identified by Western blot analysis.
Figure B-6: Expression of constitutively active MEK suppresses calcium induced keratinocyte differentiation. Overexpression of AZ attenuates the MEK-mediated inhibition of differentiation. Primary keratinocytes from 1-3d. old pups were cultured for in low Ca\(^{2+}\) (0.05mM) media or high Ca\(^{2+}\) (1.0mM) media for 12h and harvested in RIPA buffer. The induction of differentiation markers was identified by Western blot analysis.
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