**p53 Regulates Stemness in Mucoepidermoid Carcinoma**

by

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A dissertation submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy
(Cancer Biology)
in The University of Michigan
2020

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## DEDICATION

To my parents, Rosa Ramirez and Ismael Rodriguez, and to my sister, Idalia Rodriguez, for their unconditional love and support through my journey. Although thousands of miles separate us, you always made sure to let me know I was not alone. I would have not been able to reach my dreams if I didn't have you cheering me on every step of the way.

And to all the patients that have been affected with salivary gland mucoepidermoid carcinoma. You are not forgotten. This work is for you with the hope that better treatment options are developed to help you fight this disease and to give you better quality lives.

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis advisor Dr. Jacques E. Nör for his investment in my education and development as a scientist. As his student I have grown both as an independent scientist and as a person. With much patience, he has given me the space to grow and mature. He encouraged me from early on to take ownership of my work, to be confident of my science, and to look for creative solutions to the questions we study. Importantly, he made sure I never forgot the goal of our research, to help patients with salivary gland cancers. For his guidance, I am forever grateful. In addition, I would like to thank my thesis committee members: David Beer, Mats Ljungman, Peter Polverini, and Max Wicha for being an integral part of my development and for giving me a welcoming space to exchange ideas about science and help guide my project. I feel extremely blessed to have such amazing scientists rooting for my success.

I would also like to thank the past and current members of the Nör lab for providing me with advice and mentorship throughout these five years working in the lab. Their assistance, guidance, and encouragement helped me achieve the work I present here today and for that I am forever grateful.

To the Cancer Biology Program faculty, staff, and students, for providing a well-rounded graduate experience and challenging me to think about science in a critical way. I especially thank Elizabeth Lawlor, David Lombard, Zarinah Aquil, and Dawn Storball whose dedication to my success and the success of all Cancer Biology students has gone above and beyond. And to the Program in Biomedical Sciences and the Rackham

Graduate School for their investment in graduate education and the many opportunities they provided to help me grow outside of my research and help shape my views and interests. To the Diversity, Equity, and Inclusion (DEI) Certificate Program for expanding my personal, societal, and world views and giving me tools to better myself and help me be more conscientious of those around me. To the Translational Research Education Certificate Program for satisfying my desire of connecting basic science to applicable research that can change patient lives.

To the family I got to choose in Michigan, to my sisters: Deema Totah, Lianette Rivera, and Rose Figueroa. I thank them for their unconditional love and support and for being a source of strength when I needed it the most. Also, to the amazing group of friends I have made throughout the years in Michigan who have always lent me an ear, a hand, and have made my time in Michigan just brighter: Alyssa, Jess, Lorena, and Rodrigo.

Finally, to my family. I am forever grateful for their endless support in helping me reach my career and personal goals. To my parents, this Ph.D. was possible because of all the sacrifices they made throughout their life for me to be here and finish my dream of becoming a scientist. To my sister, my biggest cheerleader and my biggest role model. To my brother, for his unconditional love. To my grandparents, who passed away during a very difficult time in my graduate training and whom I miss dearly but will forever be in my heart. I couldn’t have asked to be born into a better family. With their support I know I can do anything I set my heart to. It has been hard being away from them for so long, but they taught me to never give up and to continue moving forward. I thank them for being

the fuel I need in my life to step up to any challenge and to be the best version of myself. I love them with all my heart, and I hope I can make them proud.

Last but definitely not least, to my little bundle of joy, Lila. I would have not survived all the hardships during this time if I didn't have her by my side. She was my oasis in my darkest times. Taking care of her, forced me to take care of myself and gave me the strength and hope I needed to move past the difficulties and continue forward in this journey.

## FINANCIAL ACKNOWLEDGEMENTS

I would like to acknowledge all of the funding resources that have made this work possible. Thank you to the Rackham graduate school for awarding me the Rackham Merit Fellowship upon my acceptance into the program. A very large thank you goes to the Cancer Biology program training grant (T32 CA140044) which provided support for my second year in the lab. To Yale Ciencia Academy, for providing me a one-year career development fellowship. To the NIH/NIDCR R01-DE021139S1 (CRR) for providing me support for the following 2 years of my research. I would also like to acknowledge the funding supporting my project in the Nör lab: R01-DE021139 and R01-DE23220 (JEN) from the NIH/NIDCR.

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| | |
|---|---|
| ACC | adenoid cystic carcinoma |
| ALDH | aldehyde dehydrogenase |
| ANOVA | analysis of variance |
| Bmi-1 | B cell-specific Moloney murine leukemia virus integration site 1 |
| CSC | cancer stem cell |
| DMSO | dimethyl sulfoxide |
| EGFR | epidermal growth factor receptor |
| EMT | epithelial to mesenchymal transition |
| GA | genetic alteration |
| HER2 | human epidermal growth factor 2 |
| HNSCC | head and neck squamous cell carcinoma |
| HPV | human papilloma virus |
| $IC_{50}$ | half maximal inhibitory concentration |
| iPSC | induced pluripotent stem cell |
| MDM2 | murine double minute 2 |
| MEC | mucoepidermoid carcinoma |
| NES | nuclear export signal |
| NLS | nuclear localization signal |
| ns | not significant |

| | |
|---|---|
| OD | optical density |
| Pan-CK | pan-cytokeratin |
| PcG | polycomb group |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| SGC | salivary gland carcinoma |
| SD | Standard Deviation |
| shRNA | short hairpin RNA |
| μM | Micro-molar |

## ABSTRACT

Salivary gland carcinomas are rare and heterogenous malignancies that present diagnostic and treatment challenges for clinicians. Amongst them, mucoepidermoid carcinoma (MEC) is the most common salivary gland cancer. Little is known about the pathobiology of MEC, partly due to limited preclinical research models. Our group and others have shown that cancer stem cells (CSC), $ALDH^{high}CD44^{high}$ cells, drive MEC tumorigenesis. We have found that treatment of human MEC cell lines (UM-HMC-1, UM-HMC-3A, UM-HMC-3B) with sub-lethal doses of MI-773, an MDM2 inhibitor that results in accumulation of p53 and activation of downstream signaling, significantly decreases the CSC fraction both *in vitro* and in xenograft tumors. Furthermore, upon p53 activation there is a decrease in Bmi-1 protein levels. Bmi-1 has been widely studied as a marker of stem cell and CSC self-renewal. We hypothesized that the p53 pathway regulates MEC CSC maintenance. **In this thesis we aim to: 1) determine the role of p53 in MEC CSC fate and 2) investigate the therapeutic potential of MDM2 inhibitors in murine models of MEC.** We found that while activation of p53 signaling does not cause apoptosis of MEC CSCs, it changes their cell cycle profile through p21. Furthermore, p53 activation causes a decrease in the sphere forming ability and self-renewal of MEC CSCs and in their Bmi-1 protein levels, independent of p21 signaling. Additionally, we found an induction of pan-cytokeratin expression, indicating differentiation of the CSCs. In contrast, shRNA downregulation of p53 resulted in increased Bmi-1 protein levels, enhanced CSC

self-renewal, and an expansion of the stem cell pool *in vivo*. While no changes in Bmi-1 mRNA levels were detected after treatment with MDM2 inhibitors, we found a decrease in Bmi-1 protein stability. Importantly, we found that therapeutic activation of p53 using MI-773 prevented tumor recurrence in mice. Further characterization of the regulation of Bmi-1 by p53 still needs to be evaluated. These results indicate p53 signaling regulates the cell cycle and self-renewal of MEC CSC through p21 and Bmi-1 signaling axes. This body of work enhances our understanding of the role of p53 in MEC CSC biology and gives insight into the potential therapeutic benefits of MDM2 inhibitors or other p53-activating therapies for MEC.

# CHAPTER 1. Introduction: Thesis Overview, Background, and Aims*[1]

## 1.1 Thesis Overview

This dissertation focuses on determining the role of p53 in regulating the cell fate of mucoepidermoid carcinoma (MEC) cancer stem cells (CSCs). Using tools such as MDM2 inhibitors and genetic silencing of p53, we studied the effects of p53 signaling in the regulation of MEC CSC maintenance. Particularly, we focused on studying how the p53 pathway regulates the self-renewal and differentiation potential of MEC CSCs. We had previously found the MEC CSC population decreases with the use of inhibitors of the MDM2-p53 protein interaction.[1] CSCs, like normal adult stem cells, are defined by their ability to self-renew and produce cells that can differentiate. The tumor suppressor p53 is primarily known for its role in maintaining genome integrity by halting the cell cycle or inducing apoptosis of cells with damaged DNA. However, research in stem cell biology has shown that p53 can also regulate processes such as stem cell self-renewal.[2-5] To carefully assess the effects of p53 activation on the loss of the CSC pool, we looked at p53-dependent cell cycle arrest and apoptosis and distinguished these from the stemness properties of self-renewal and differentiation. Furthermore, we assessed the therapeutic potential of activating p53 using MDM2 inhibitors and explored the potential of sensitizing

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[1] A part of this chapter was taken from work originally published in *Critical Reviews™ in Oncogenesis.* Christie Rodriguez-Ramirez & Jacques E. Nör. p53 and Cell Fate: Sensitizing Head and Neck Cancer Stem Cells to Chemotherapy. *Crit Rev Oncog* **23**, 173-187 (2018). © Begell House Inc.

these highly resistant cells to conventional chemotherapy upon p53 pathway activation. This chapter provides an overview of concepts important for this dissertation work, introduces the development of a new *in vivo* model of MEC, and concludes with our aims.

### 1.2 The CSC Hypothesis

The CSC hypothesis states that a small subset of cancer cells are endowed with tumor initiating, self-renewal, and multi-lineage differentiation potential.[6] This hypothesis is based on evidence that: (1) only a small population of cancer cells within a tumor are capable of initiating and propagating tumors in immunodeficient mice; (2) tumors resulting from CSCs contain a mixture of tumorigenic and nontumorigenic cells that recapitulate original tumor heterogeneity; and (3) tumors generated by CSCs can be serially transplanted, demonstrating self-renewal capabilities. On the other hand, the bulk of a tumor is composed of transit-amplifying cells and post-mitotic differentiated cells that do not contribute to tumor initiation.

Rather than proposing CSCs are equal to physiological stem cells, the CSC hypothesis attempts to explain that CSCs share similar characteristics to adult stem cells. The first studies supporting the CSC hypothesis showed that tumors have a distinct hierarchy of cells, similar to that observed in adult tissues.[7] Within a tumor, cells can be distinguished by the expression of different cell lineage markers. This hierarchical organization was first evident in leukemic cells and later proven in breast cancer and other solid malignancies.[8-11] Furthermore, CSCs maintain plasticity between epithelial and mesenchymal cell stages, and molecular and environmental cues can shift between acquiring or loosing these stem-like characteristics.[12-15]

The frequency and prevalence of CSCs can vary by tumor type and amongst patients with the same type of cancer.[16-18] Studies have shown that CSCs can be used as predictive and prognostic indicators for patient survival.[16-18] However, there are no CSC-specific markers that enable isolation of these cells with high specificity. Instead, existing markers can only enrich for CSC populations.[19] This complicates the study of CSCs and presents challenges for using CSC-markers to develop targeted therapies.

### 1.3 Bmi-1 and Self-renewal

The *Bmi-1* gene (B cell-specific Moloney murine leukemia virus integration site 1) was originally identified as an oncogene that cooperates with *c-Myc* during lymphomagenesis.[20-22] *Bmi-1* is a core member of the Polycomb Repressive Complex 1 (PRC1) required for mono-ubiquitination of histone 2A.[23] It usually functions as an epigenetic silencer controlling development by regulating genes involved in cell growth and differentiation.[24] *Bmi-1* knockout mice suffer from skeletal malformations, abnormalities in the hematopoietic and nervous systems, and growth retardation.[25,26] These effects are partially due to de-repression of the *Ink4a/Arf* locus.[27,28] Using alternative reading frames, this locus codes for two proteins, $p16^{Ink4a}$ and $p19^{Arf}$, that are important regulators of the retinoblastoma and p53 pathways.[29] In-kyung Park and colleagues showed that the absence of Bmi-1 caused defects in hematopoietic stem cell self-renewal.[30] Afterwards, Iwama and collaborators went on to show that Bmi-1 is the only component from the PcG family that is critical for hematopoietic stem cell self-renewal where forced expression of Bmi-1 significantly increased symmetric stem cell divisions.[31] Since then, many groups have shown the importance of Bmi-1 in self-renewal

of adult stem cells and CSCs.[32-34] Importantly, they have shown that Bmi-1 regulates self-renewal partly through the *Ink4a/Arf* locus.[28,32]

Bmi-1 levels are tightly regulated and display dosage sensitivities and tissue and cell-type specific requirements for cell proliferation and stem cell self-renewal.[27,28] The Ring-domain in Bmi-1 was shown to be important for its tumorigenic function in lymphomas.[22] Bmi-1 forms a heterodimeric Ring-Ring complex with Ring1b that enhances the E3 ligase activity of Ring1b.[35] This Ring-domain is important for the regulation of the *Ink4a/Arf* locus. Bmi-1 promotes proliferation by repressing the *Ink4a/Arf* locus encoding the CDK4/6 inhibitor $p16^{Ink4a}$ and the p53 activator $p19^{Arf}$. $p19^{Arf}$ controls p53 activity by sequestering MDM2 (Mouse double minute 2), an E3 ubiquitin ligase of p53 that targets p53 for proteasomal degradation. The $p16^{Ink4a}$ protein inhibits the binding of Cyclin D to CDK4/6 resulting in cell cycle arrest. By repressing $p16^{Ink4a}$, Bmi-1 induces cell proliferation. The absence of Bmi-1 can also directly regulate p53 protein stability. Bmi1 regulates p53 levels in an $Ink^{4a}$/ARF-independent manner by directly binding, together with Ring1A and Ring1B E3 ligases, to the p53 protein inducing its polyubiquitination and subsequent degradation.[36] On the other hand, lack of Bmi-1 expression causes premature entry into cellular senescence.[37] Bmi-1 can cooperate with other oncogenes in processes that can lead to cell transformation.[21,37] This is consistent with findings that the *Ink4a* locus is tumor suppressive.[38,39]

A study found that Bmi-1 mRNA is overexpressed in a subset of head and neck squamous cell carcinoma (HNSCC) patients.[40] They found that pharmacological inhibition of Bmi-1 by PTC-209 reduces tumorsphere formation and the percentage of $ALDH^+$ cancer stem cells in HNSCC cell lines. Importantly, they found that PTC-209 significantly

reduces tumor growth of HNSCC xenograft models. These results indicate that pharmacological inhibition of Bmi-1 is a potential therapeutic strategy for HNSCC patients, in particular those with aberrant Bmi-1 overexpression. Our group found that treatment of HNSCC cell lines with cisplatin induces Bmi-1 expression and enhances the fraction of CSCs.[41] Meanwhile, Chen and collaborators showed that targeting Bmi-1+ CSCs overcame chemoresistance and inhibited metastasis in HNSCC.[42] Through genetic lineage tracing they showed that Bmi-1 marks a population of slow cycling CSCs responsible for tumor initiation, progression, and cervical lymph node metastasis. They demonstrated that these cells are resistant to cisplatin treatment. Importantly, they found that combination treatment with cisplatin and PTC-209 had better therapeutic response than monotherapy alone. They also showed that recurrent tumors that became resistant to cisplatin, were sensitive to this combination therapy. These findings support the hypothesis that CSCs are chemoresistant and contribute to tumor metastasis and recurrence. Furthermore, their findings support the hypothesis that combination treatments that target both the CSCs and the bulk tumor cells are better at eradicating tumors.

### 1.4 Mucoepidermoid Carcinoma

MEC is the most common malignancy of the major and minor salivary glands, accounting for about 30-35% of all salivary gland carcinomas.[43,44] It is a heterogeneous cancer that consists of mucin-producing, epidermoid, and intermediate cells. MEC is histologically classified into low-, intermediate-, or high-grade disease. Clinical behavior can vary from slow growing and indolent to locally aggressive and highly metastatic tumors. High-grade MEC have a prominent epithelial/solid component and have an infiltrative border.

Although epidermoid cells predominate, intermediate and mucous cells are also present and distinguish these cancers from squamous cell carcinomas. Intermediate-grade MEC often present with demarcated borders and are mostly solid, with intermediate cells predominating over mucinous cells. Finally, low-grade MEC have a prominent cystic/mucous component and are well-demarcated, with pushing margins.

MEC tumors are thought to originate from stem cells in the excretory duct of the salivary gland.[45] Very little is known about their pathobiology, limiting the development of effective mechanism-based therapies. As such, as of May 2020 the FDA has not approved any systemic therapy for MEC patients. Their standard of care involves radical surgery and radiation therapy, resulting in high morbidity, facial disfigurement and poor survival, as many patients progress towards disseminated disease.

Over 80% of MEC cases contain a t(11;19)(q21-22;p13) translocation that results in a fusion between the *MAML2* and *CRCT1* or *CRCT3* genes.[46] In this fusion, the Notch-binding domain of *MAML2* is replaced by the CREB-binding domain of *CRCT1/3*.[47] The molecular and pathological consequences of this fusion are still being elucidated, but it is thought CREB dysregulation, mediated by this fusion, participates in tumorigenesis.[48] It has been suggested that the *CRCT1-MAML* fusion is an early event in MEC pathogenesis and that it can serve as a biomarker of MEC. As such, a sub-group of fusion-negative high-grade tumors are suspected of being a different class of carcinomas and that only fusion-positive tumors should be classified as MEC.[49]

Apart from the *CRCT-MAML* fusion, very few recurrent genetic alterations (GAs) have been found in MEC. Recent genomic studies have shown that *TP53* GAs can occur in 20-30% of MEC patients and that they positively correlate with high-grade disease.[46]

Additionally, gene amplification of human epidermal growth factor 2 (*HER2)* or increased copy number of either *HER2* or epidermal growth factor receptor (*EGFR*) are associated with high-grade MEC irrespective of *MAML2* fusion status, and with worse overall survival.[50,51]

### 1.5 *In Vitro* and *In Vivo* Models of MEC

Salivary gland cancers are understudied malignancies. The development of effective mechanism-based therapies for MEC has been hindered by a lack of good pre-clinical research models. To date, no genetic mouse models of MEC have been successfully generated. Additionally, few patient-derived xenograft models (PDX) exist. Until recently, there were no tumorigenic cell lines of MEC and most studies relied on retrospective analysis of paraffin-embedded tissues. Our laboratory is uniquely equipped for the study of MEC as we generated the first tumorigenic cell lines from MEC patients[52], allowing us to conduct both *in vitro* and *in vivo* mechanistic and therapeutic studies. Importantly, one of these cell lines came from a recurrent tumor while another from a lymph node metastasis in the same patient. These cell lines contain the *CRCT1-MAML* fusion characteristic of MEC (>80% of patients).

To enhance our ability to study MEC tumor biology, we developed a simple protocol for generating orthotopic xenograft tumors by injecting MEC cell lines into the submandibular glands (SMG) of mice. To do this, a small, 1-mm incision is made in the skin above the salivary gland area of immunodeficient mice. After identifying the SMGs, a small volume (<10μL) of a MEC cell suspension is injected into the SMG gland and the incision is closed using sutures (Figure 1.1, a). Growing tumors are easily palpable (Figure 1.1, b) and, similar to orthotopic breast xenograft tumors in the mammary fat pad

of mice, can be roughly measured with the use of calipers. More precise measurement for comparing tumor sizes among study groups can be done with the use of cell imaging techniques such as *in vivo* bioluminescence. Nevertheless, another way to reliably detect phenotypic differences in tumor growth is to weigh the salivary glands upon termination of the experiment. While both SMG can be certainly excised together and measured, these glands can be easily separated from each other. However, orthotopic xenograft tumors can be difficult to separate from nearby salivary glands as these tumors can readily invade the surrounding tissues.

We observed that the developing tumors recapitulate disease presentation in patients, allowing us to identify the three cell types found in MEC (*i.e.* epidermoid, mucin producing, and intermediate cells) (Figure 1.1, c). Xenograft tumors generated subcutaneously are primarily solid and undifferentiated with sparing presence of mucin-producing cells, measured by mucicarmine stain. However, we see a wider distribution of the three main cell types that make up MEC tumors in the orthotopic xenografts (Figure 1.1, c). Importantly, tumor growth is well tolerated in mice with no apparent weight loss before tumor endpoint (*i.e.* 2 $mm^3$). Of note, we found these tumors were also capable of forming micro-metastasis in the lungs of mice (Figure 1.1, d), which is not observed in mice harboring subcutaneous xenografts. One striking finding is that while the UM-HMC-1 cell line establishes tumors with low frequency in our subcutaneous xenograft model, 100% of tumors are evident within a month after cell transplantation into the submandibular gland of SCID mice. Furthermore, we can use cells from digested orthotopic salivary gland tumors to conduct flow cytometry analysis of different cell markers including those that enrich for MEC CSCs (Figure 1.1, e). We employed this new

orthotopic model in studies presented in this thesis project that attempt to understand the effects of p53 signaling on MEC tumor pathobiology.

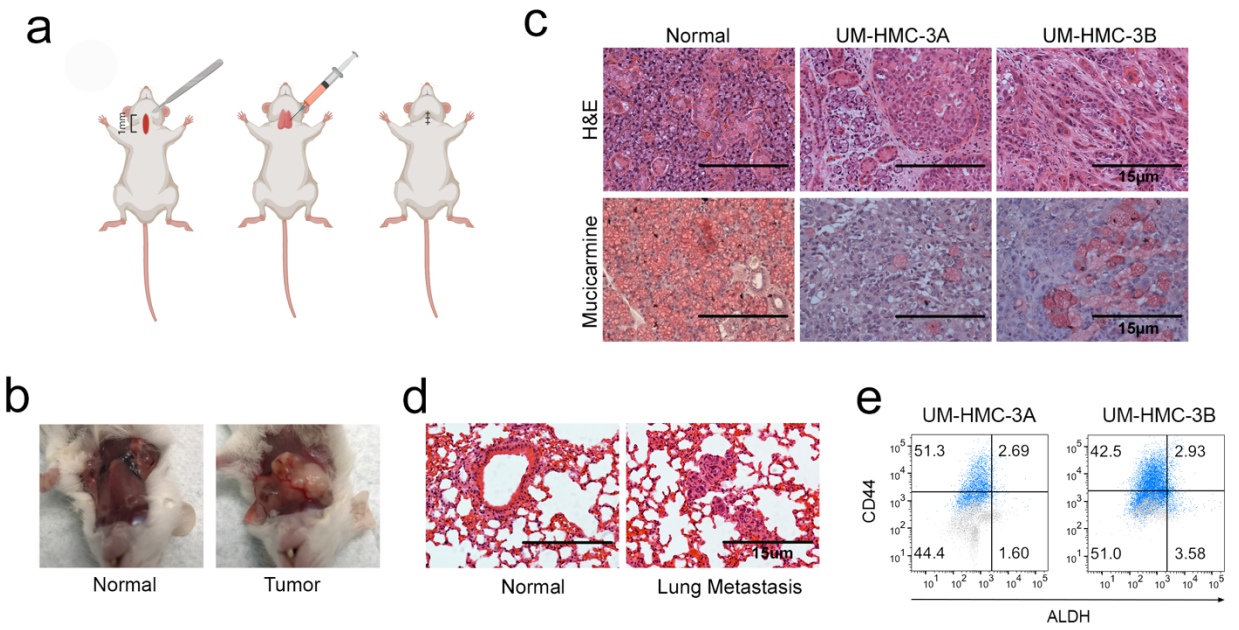


**Figure 1.1. Mucoepidermoid carcinoma orthotopic xenograft model. a** Demonstration of orthotopic injection of cells into the submandibular gland (SMG) of SCID mice. **b** Macroscopic view of salivary gland tumors and normal SMG glands. **c** Microscopic view of tumor composition (H&E) and mucin-production (Mucicarmine stain). **d** Micro-metastasis in lung tissues of tumor-bearing orthotopic xenograft mice. **e** Flow cytometry analysis of $ALDH^{high}CD44^{high}$ cells from digested orthotopic xenograft tumors. Dot plots include DEAB overlay (gray).

### 1.6 Specific Aims

The *long-term goal* of this work is to develop a therapeutic approach for ablation of cancer stem cells in MEC and for the sensitization of this cancer to systemic therapies. The *overall objective* is to understand the role of p53 signaling in the pathobiology of MEC cancer stem cells. The *central hypothesis* is that p53 signaling regulates MEC CSC maintenance. The *rationale* for this work is that understanding and targeting essential pathways involved in MEC stem cell maintenance will allow for the development of

effective therapeutic regimens that will increase survival rates and quality of life for MEC patients in the future. Here, we tested this hypothesis through the following specific aims:

**Specific Aim 1: Determine the role of p53 on mucoepidermoid carcinoma stem cell fate.** We observed that therapeutic inhibition of MDM2-p53 mediates a decrease in the fraction of MEC cancer stem cells. However, the mechanisms explaining this effect are unknown. Here, we hypothesized that inhibition of the MDM2-p53 interaction decreases the fraction of cancer stem cells by altering their cell fate, ultimately reducing their self-renewal and inducing their differentiation.

**Specific Aim 2: Investigate the therapeutic potential of inhibition of the MDM2-p53 protein interaction in *in vivo* models of MEC.** We observed that an inhibitor of the MDM2-p53 interaction reduces the fraction of cancer stem cells in MEC cell lines and xenograft tumors. Additionally, we previously showed that these inhibitors sensitize adenoid cystic carcinoma (ACC) patient-derived xenograft (PDX) tumors to cisplatin and eliminate tumor recurrence.[53] However, we do not know the clinical benefit of using these inhibitors in MEC. Here, we hypothesize that treatment with inhibitors of the MDM2-p53 interaction significantly decrease tumor growth and the recurrence of MEC xenograft tumors.

# CHAPTER 2. P53 and Cell Fate: Sensitizing Head and Neck Cancer Stem Cells to Chemotherapy*[2]

## 2.1 Summary

Head and neck cancers are deadly diseases that are diagnosed annually in approximately half a million individuals worldwide. Growing evidence supporting a role for cancer stem cells (CSCs) in the pathobiology of head and neck cancers has led to increasing interest in identifying therapeutics to target these cells. Platinum-based chemotherapies are the standard of care for most common head and neck cancers, yet they can induce stemness properties and increase the proportion of CSCs in tumors. Emerging research supports a significant role for p53 in physiological stem cell and CSC maintenance and reprogramming beyond its canonical tumor suppressor functions. Therefore, p53 has become a promising target to sensitize head and neck CSCs to chemotherapy. In this chapter, we highlight the role of p53 in stem cell maintenance and discuss potential implications of targeting p53 for the treatment of patients with head and neck cancers.

[2] This chapter was modified from work originally published in *Critical Reviews™ in Oncogenesis.* Christie Rodriguez-Ramirez & Jacques E. Nör. p53 and Cell Fate: Sensitizing Head and Neck Cancer Stem Cells to Chemotherapy. *Crit Rev Oncog* **23**, 173-187 (2018). © Begell House Inc.

## 2.2 Introduction

The tumor suppressor *TP53* is the most commonly mutated gene in cancer. Known as the "guardian of the genome", it can be considered a master regulator of cell fate. Although p53-null mice are viable, they have early onset of sporadic cancers.[54] In addition, people with Li-Fraumeni syndrome, which harbor p53 germline mutations, frequently develop cancers.[55] Given the major impact of p53 in cancer biology, most investigators have focused on its role as a tumor suppressor through its involvement in apoptosis, cell cycle arrest, and senescence. Nonetheless, p53 is important in normal cellular and developmental processes.[56,57] Notably, strong evidence shows involvement of p53 in stem cell self-renewal and differentiation.[58,59]

Two emerging roles for p53 include its ability to inhibit cancer stem cell (CSC) formation and regulate the CSC state. CSCs share many properties with adult stem cells. For example, they have the ability to self-renew and differentiate into committed progenitor cells with limited self-renewal potential.[60] CSCs are known to be resistant to standard chemotherapy and radiation therapies and have increased tumorigenic capacity, often playing a role in tumor recurrence and metastasis *in vivo*.[61] This chapter will highlight some of the functions of p53 relevant to CSC maintenance (*e.g.* self-renewal and differentiation) and the potential implications of targeting p53 to sensitize CSCs to conventional chemotherapy in head and neck malignancies.

### 2.3 p53: "The Guardian of the Genome"

Functioning primarily as a transcription factor, p53 regulates the expression of a large number of protein coding genes and microRNAs that mediate its downstream responses. Known as "the guardian of the genome", p53 is mutated in >50% of sporadic human cancers and is thought to be functionally inactivated in a significant portion of the remaining cancers.[62-64] p53 is involved in preserving genome integrity by eliminating cells with damaged or mutated DNA. It can transiently halt the cell cycle to allow for DNA damage repair or can irreversibly block proliferation through senescence or apoptosis (programmed cell death).

In normal conditions p53 is under tight regulation and its abundance is kept low in non-stressed cells. However, upon cytotoxic or genotoxic insults p53 is protected from degradation via several post-translational modifications.[65] The p53 protein is comprised of: an N-terminal transactivation domain, a proline-rich domain that mediates responses to DNA damage, a tetramerization domain, a sequence-specific DNA-binding domain, and a C-terminal regulatory domain with three nuclear localization signals (NLS) and a nuclear export signal (NES) (Figure 2.1). Post-translational modifications occur in different locations in these domains and have different roles in regulating p53 activity and protein stability.[65]

Mouse double minute 2 (MDM2) is the main negative regulator of p53. It functions as an E3 ubiquitin ligase that cooperates with MDMX to target p53 for proteasomal degradation.[66] Additionally, MDM2 can inhibit p53 transcriptional activity by binding to the N-terminal of p53.[67,68] MDM2 is also a transcriptional target of p53, resulting in a negative autoregulatory mechanism for p53 signaling (Figure 2.1).[69] The levels of p53, MDM2, and

MDMX are tightly controlled to sense and respond to stress.[70] This coordinated network is very important in normal homeostasis and development.[67,71] Some cancers bypass p53 function by overexpressing or amplifying MDM2.[72,73] Responses of p53 on a cell are stress and tissue specific. Below, we explore some of the evidence supporting the role of p53 in stem cells and CSCs.

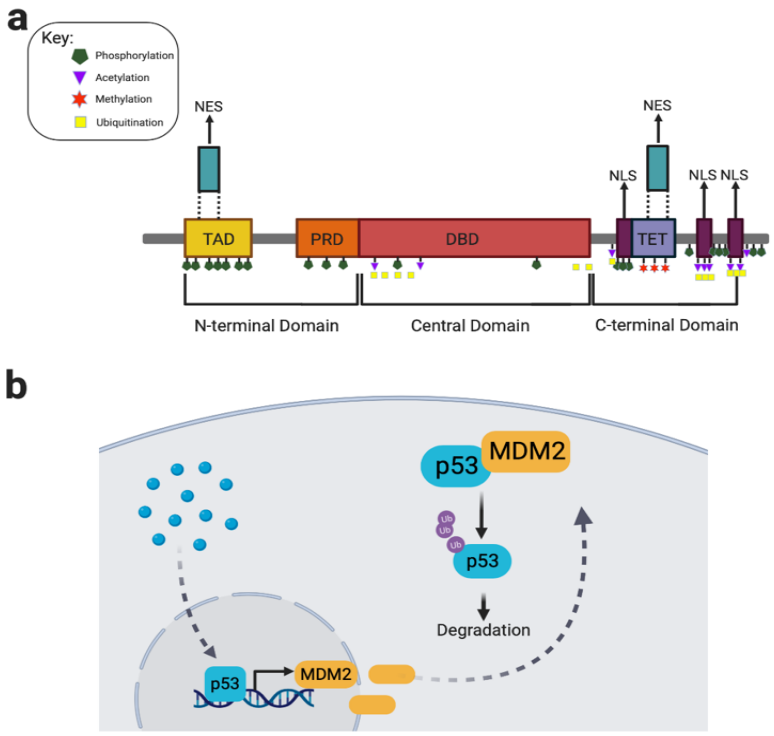


**Figure 2.1. The p53 structure and regulatory feedback loop.** **a** The p53 protein is comprised of an N-terminal transactivation domain (TAD), a proline-rich domain (PRD), a tetramerization domain (TET), a sequence-specific DNA-binding domain (DBD), and a C-terminal regulatory domain with three nuclear localization signals (NLS) and a nuclear export signal (NES). The p53 protein can get phosphorylated, acetylated, methylated or ubiquitinated throughout these different domains and these post-translational modifications regulate p53 stability and activity. **b** When p53 is activated it accumulates in the nucleus and act as a transcription factor regulating the expression of many genes and miRNAs. MDM2 is a transcriptional target of p53 and an E3 ubiquitin ligase that targets p53 for proteasomal degradation. This serves as an autoregulatory feedback for p53 protein levels. (Diagram in **(a)** was modified from Miller *et al.*[74]*).*

## 2.4 p53 in Stem Cells

There are several mechanisms by which p53 is thought to regulate both normal stem cells and CSCs.[75] In this chapter, we focus only on two of these mechanisms. The first involves the effects of p53 on stem cell self-renewal and the second on stem cell differentiation.

*2.4.1 p53 in self-renewal*

Stem cells are defined by their ability to self-renew and to produce progenitor cells that can ultimately generate multiple cell lineages (*i.e.* multipotency). Both of these tasks are centered on a single mitotic division event. Whereas, symmetric stem cell division yields two identical daughter cells, asymmetric division will produce one stem cell and one proliferative progenitor cell (Figure 2.2). When a stem cell undergoes symmetric division, it can yield two identical daughter stem cells or two proliferative progenitor cells. Both of these processes are essential during development and tissue homeostasis to maintain a stem cell pool and produce specialized cells. p53 plays an important role in the balance between self-renewal and differentiation in embryonic and adult stem cells, and this balance is important in cancer development and progression.[58,59]

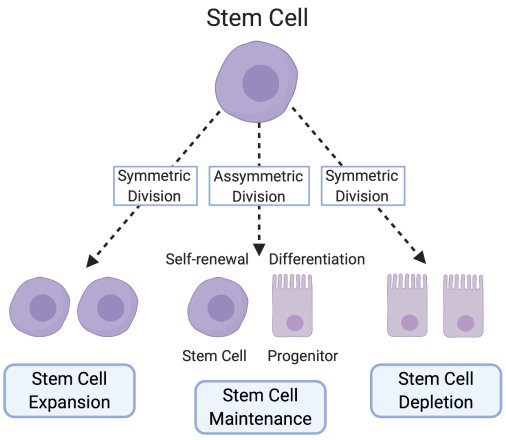


**Figure 2.2. Stem cell divisions.** Stem cells can undergo both symmetric and asymmetric division events, resulting in expansion, exhaustion, or maintenance of the stem cell pool. When a stem cell undergoes asymmetric division, it can produce an identical daughter cell (self-renewal) and committed progenitor (differentiation). If a stem cell divides symmetrically, two daughter stem cells or two daughter progenitor cells can result. These modes of division are regulated by pathways that govern self-renewal and differentiation. (This figure was modified from Rodriguez-Ramirez *et al*.[76])

In mammary stem cells, p53 has been found to regulate the polarity of cellular divisions.[77] The absence of p53 promotes self-renewal of these cells, allowing for the expansion of the stem cell pool and resulting in unlimited and symmetric self-renewing divisions. Confirming these findings, p53 knockout mice contain higher percentage of cells capable of forming mammospheres in culture and repopulating the mammary gland *in vivo,* corresponding to increased stem cell numbers.[78]

Furthermore, Numb can directly interact with p53 and MDM2 and is thought to regulate p53 signaling by preventing MDM2-mediated ubiquitination of p53.[79] Numb is a cell fate determinant that distributes asymmetrically in daughter cells during mitosis and inhibits Notch signaling. Asymmetric stem cell divisions can be measured by looking at its distribution in daughter cells.[80] Phosphorylation of Numb by NANOG destabilizes p53 and promotes cancer stem cell self-renewal.[81]

Several groups have shown that deregulation of pathways that control self-renewal of normal stem cells can lead to their transformation into cancer cells.[3,6,82] Indeed, p53 regulates the self-renewal of myeloid progenitor cells transforming them into leukemia-initiating cells that resemble CSCs.[3] Conversely, p53 loss promotes CSC-pool expansion.[83,84] Meanwhile, mutant p53 induces CSC marker expression in colorectal cancer.[85] Such findings suggest a strong role for p53 in regulating the self-renewal of CSCs, but the mechanism by which p53 regulates this process is not yet clear.

*2.4.2 p53 in differentiation*

A correlation between the grade of differentiation and the presence of p53 mutations has been observed in several malignancies.[86,87] Cancers with p53 mutations or functional p53 inactivation by downstream regulators (*e.g.* overexpression of *MDM2*) correlate with poor-grade and undifferentiated tumors.[88] In many cancers, histological grade remains one of the best prognostic factors for patient survival.[89,90] This has certainly been the case for most squamous cell carcinomas and salivary gland cancers (SGC).[91,92]

Endogenous p53 was shown to induce the differentiation of mouse embryonic stem cells by suppressing *Nanog* expression after DNA damage.[93] *Nanog* is an important gene for embryonic stem cell self-renewal.[94] Not only does *Nanog* plays a role in embryonic stem cells, but it also regulates dedifferentiation of primary p53-deficient mouse astrocytes into CSC-like cells.[95] p53 can regulate the differentiation of several cell types,[96] and its loss can also induce reprogramming of pluripotent stem cells, further supporting a role for p53 in differentiation. Furthermore, it has been shown that p53 transcriptionally activates miRNAs responsible for downregulating stem cell transcription factors.[97] We find that the role of p53 in regulating cell fate is a balance between stem cell differentiation and self-renewal (Figure 2.3). This balance is "hijacked" in cancer to expand the CSC pool. Deregulation of these differentiation and self-renewal pathways can be responsible for the formation of cancer stem-like cells.

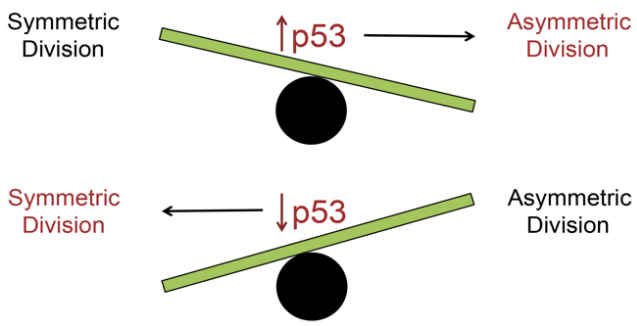


**Figure 2.3. The p53 protein is a key regulator of stem cell fate, balancing self-renewal and differentiation.** When p53 is activated in a stem cell, it tilts this balance towards asymmetric division. Cancer stem cells frequently downregulate p53 activity. This leads to changes in cell polarity increasing symmetric stem cell division, thus expanding the stem cell population. Activating p53 in cancer stem will differentiate these cells leading to a decreased stem cell pool and sensitizing these otherwise resistant cells to conventional chemotherapies. (This figure was modified from Rodriguez-Ramirez *et al*.[76])

## 2.5 Sensitizing Cancer Stem Cells to Chemotherapy

Two main approaches can be used to target CSCs. The first, and most studied, is to find agents that can specifically kill these cells.[98] CSCs are resistant to standard chemotherapy agents and radiotherapy used in the clinic.[99] For example, breast CSCs (*i.e.,* $CD44^{high}CD24^{low}$ cells) appear to be intrinsically resistant to conventional

chemotherapy and radiation therapy.[100-103] Their unique biology allows them to bypass many of the mechanisms used to target cancer cells. For this reason, a CSC-specific treatment must be developed to kill these cells. Several clinical trials targeting CSC-specific pathways are currently underway globally.[97]

The second approach is to find agents that can differentiate CSCs to sensitize them to chemotherapy and radiation therapy. CSCs exploit physiological stem cell pathways to maintain a more undifferentiated state in such a way that they can self-renew and give rise to the different cells comprising a tumor.[60] In theory, differentiating the CSCs will make them loose their potential to self-renew, therefore becoming short-lived. This approach is gaining increasing interest because the idea of sensitizing otherwise resistant cells to standard chemotherapy regimens is an attractive approach for the clinic. Nevertheless, more about the potential to differentiate CSCs must be understood because CSCs in many different tumor types (different from adult stem cells) have not been fully characterized and may present undistinguished differentiation fates.

One prominent challenge in studying CSCs is that the percentage and markers used to identify these cells in tumors varies by cancer and cancer subtype.[104] Compelling evidence for the importance of CSCs in tumorigenesis and correlations with patient outcomes has been observed in different tumors.[105-107] Apart from surgery, chemotherapy and ionizing radiation are the most common therapies used to treat cancer, but a good number of cancers do not respond to therapy and some develop resistance over time.[108] CSCs can be resistant to conventional chemotherapy through several mechanisms.[99] It is thought that these cells are intrinsically less susceptible to chemotherapy because they are slow cycling. Because chemotherapy agents are DNA damaging, they are most

effective on rapidly proliferating cancer cells. Apart from this, CSCs have ABC transporters that can effectively efflux chemotherapy drugs and are resistant to DNA damaged-induced death.[109,110]

Furthermore, intravenously injected chemotherapy drugs are capable of penetrating just a few layers of cells in tumor tissues.[111,112] This implies that deeper cell layers in the tumor receive lower doses of these agents. Given the intrinsic resistance of CSCs to chemotherapeutic drugs, sub-cytotoxic doses received by cells could explain the increase in the fraction of CSCs observed in tumors treated with chemotherapy.[41,113-115] This increase could also be due to the intrinsic nature of these cells to be more resistant to chemotherapeutic agents, thus surviving while the rest of the tumor cells succumb to treatment. Residual CSCs that remain after treatment are thought to be responsible for tumor recurrence and metastasis due to their increased cell invasiveness, survival, and tumorigenic potential.[61]

Chemotherapy is also capable of inducing transdifferentiation of cancer cells into cancer stem cells, creating a population of chemotherapy-resistant cells that rely on similar mechanisms to the generation and maintenance of induced pluripotent stem cells (iPSCs).[116,117] A study by Auffinger and colleagues showed that temozolomide induced an increase in the glioma CSC pool and that this increase is a result of a phenotypic shift of the nonCSC pool to a CSC-like state.[117] These authors showed that the newly transdifferentiated cells are more tumorigenic, invasive, and chemoresistant than the original tumor source.

Clinical studies monitoring the prevalence of CSCs before and after chemotherapy treatment have shown that CSCs are resistant to therapy, confirming results observed in

*in vitro* and in preclinical studies.[118,119] As an example, breast cancer patients undergoing neoadjuvant chemotherapy who underwent biopsies before and after treatment showed that cells with CSC markers persisted even as tumor mass regressed.[100] CSCs resistant to radiotherapy have also been observed in patients who have received high doses of irradiation.[120]

Using *Nanog* as a reporter of cancer stem cell formation, Saydaminova and colleagues identified GDM-1515, a regulator of histone demethylases, is capable of sensitizing CSCs to cisplatin-induced apoptosis.[99] They showed that this compound was able to inhibit EMT and the induction of CSCs by cisplatin. On the other hand, an unbiased screen for small-molecules that are cytotoxic to CSCs revealed salinomycin, a natural compound that can reduce the expression of CSC genes, mammary tumor growth, and can increase epithelial differentiation of mammary gland tumors.[99] Further studies have proven the effectiveness of this compound in targeting CSCs in other cancers.[121-124] However, severe toxicity of this compound in normal cells impedes its clinical use.[125,126] Meanwhile, other researchers have shown that targeting pathways that regulate CSC self-renewal can lead to sensitization of CSCs to chemotherapy.[127] *Bmi-1* (B cell-specific Moloney murine leukemia virus integration site 1) is a component of the polycomb-repressor complex I and a known regulator of self-renewal. Targeting *Bmi-1* results in sensitization of platinum-based chemotherapies in CSCs.[127]

### 2.6 p53 in Head and Neck Cancer

Head and neck cancers constitute ~4% of all cancers worldwide. They arise in mucosal surfaces of the oral cavity, nasopharynx, oropharynx, hypo-pharynx, larynx, paranasal sinuses, nasal cavity, and salivary glands. Among those, more than 90% are head and

neck squamous cell carcinomas (HNSCCs). HNSCC is the sixth most common cancer worldwide, occurring in more than 550,000 individuals and resulting in greater than 380,000 deaths each year.[128] Despite advancement in treatment modalities, there has been limited improvement in patient survival over the last three decades.

HNSCC can be stratified based on their human papilloma virus (HPV) infection status; HPV-positive HNSCC have a better survival rate than HPV-negative cancers.[129-133] Accumulating epidemiological, molecular, and clinical evidence supports HPV-positive cancer to be a distinct subtype of head and neck cancers.[134-136] Although the overall incidence of head and neck cancers has declined slightly in industrialized countries, there has been an increase in the incidence of HPV-positive cancers of the oropharynx.[137-140]

Mutations in *TP53* are the most frequent genomic alterations in HNSCC and have been correlated with poor patient survival.[130,141-143] An analysis of 279 HNSCC patients from The Cancer Genome Atlas (TCGA) showed *TP53* mutations in 70.4% of tumors.[130] *TP53* mutations are thought to be an early event in HNSCC carcinogenesis because they have been found in pre-malignant lesions, and their incidence is associated with cancer progression.[144] Although *TP53* is frequently mutated in HPV-negative tumors, it is typically wild-type in HPV-positive tumors.[130,145,146] Nevertheless, it has been shown that the HPV16 E6 protein in HPV-positive tumors can bind and target the p53 protein for proteasomal degradation, resulting in functional inactivation of p53 signaling.[147] These results indicate p53 signaling is key in HNSCC carcinogenesis of both HPV-negative and HPV-positive HNSCC.

Although *TP53* is commonly mutated in HNSCC, it is rarely mutated in SGC when compared to other neoplasms.[148] SGCs are rare malignant tumors that account for ~6%

of all head and neck cancers. There are 22 subtypes of malignant salivary gland tumors according to the most recent classification of the World Health Organization.[149] Their histological and clinical diversity results in diagnostic and management challenges for clinicians.[150] Standard of care commonly involves radical surgery and/or radiation therapy, with conventional chemotherapy employed as a palliative treatment for recurrent or metastatic disease. There is limited clinical trial data on systemic therapeutic approaches for SGCs, a problem that has largely been a result of challenges in recruiting enough patients (due to the rarity of these tumors), or lack of patient stratification by tumor type. SGCs can originate in any of the major or minor salivary glands. SGC-subtype and gland location are prognostic factors of patient survival.

Mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (ACC) are the two most common malignant SGCs.[149] They are characterized by recurrent chromosomal translocations which are thought to play an important role in tumorigenesis.[151] Despite these findings, the role of these translocations in tumor progression is not fully understood. Limited genomic studies of SGCs have hindered the understanding of the pathobiology of these cancers and of the identification of molecular candidates for targeted therapies.

Varying reports on the p53 mutational status in MEC samples have been reported.[152,153] These studies have been limited by small sample sizes and by unbiased detection of p53 mutations. Whole-exome sequencing of a cohort of 18 patients found p53 to be mutated in 30% of all MEC cases.[46] In this study, p53 mutations were only found in intermediate and high-grade tumors. A comprehensive genomic profiling of 48 MEC patient samples further confirmed p53 as a common genetic alteration (GA) in MEC,

found in about 40% of the cases analyzed and prevalent in higher disease grade samples.[154] Similar results were observed in a previous study using immunohistochemistry, where aberrant expression of p53 was detected in higher histological graded tumors.[153] p53 mutational status and its implications in MEC pathobiology need to be further evaluated to determine the frequency of these GAs and understand their implications in disease progression.

## 2.7 Head and Neck CSCs

### *2.7.1 CSCs in HNSCC*

Increasing evidence supports a role for CSCs in the pathogenesis and progression of HNSCC. CSCs in HNSCC were first described as $CD44^{high}$ cells by Prince and colleagues.[155] CD44, a type I transmembrane glycoprotein involved in cell-cell interactions, adhesion and migration, can act as a receptor for hyaluronic acid and other extracellular matrix proteins. In these experiments, cells expressing high levels of CD44 had increased tumorigenic potential over cells that expressed low levels.[128] Moreover, tumors generated from the $CD44^{high}$ cells were capable of reproducing the original tumor heterogeneity, observed by the presence of both $CD44^{high}$ and $CD44^{low}$ cells. Gene expression analysis showed that the $CD44^{high}$ cells differentially expressed the *Bmi-1* gene, a self-renewal protein. Their findings suggested self-renewal and differentiation potential of the $CD44^{high}$ cells, supporting the CSC hypothesis in HNSCC.

Similar experiments using ALDH showed that ALDH-positive cells had higher tumorigenic potential than ALDH-negative cells.[156,157] ALDH is an enzyme involved in the oxidation of aldehydes, cellular detoxification, retinoic acid metabolism, protection from reactive oxygen species, amongst other important cellular pathways.[158] Clay and

colleagues found that as few as 500 $ALDH^{high}$ cells were sufficient to form tumors in mice and that these tumors replicated the original tumor cell heterogeneity.[156] Notably, ALDH-positive cells isolated from patient samples were radioresistant and could initiate tumors in mice.[159] Krishnamurthy and colleagues went on to show that combined $ALDH^{high}$ and $CD44^{high}$ expression further enhanced the ability of enriching for CSCs and that tumors generated with $ALDH^{high}CD44^{high}$ cells resembled original patient histology.[160] Moreover, using serial transplantations *in vivo,* they showed these cells had increased Bmi-1 expression and self-renewal abilities. Further studies using these markers continue to prove the existence and role of these CSCs in the pathogenesis of HNSCC.

CD44 expression has been correlated with worse disease grade and prognosis in pharyngeal and laryngeal cancers.[161,162] Moreover, CD44 concentration in peripheral blood has potential to serve as a prognostic and diagnostic tool for HNSCC patients.[162] CD44-positive cells in HNSCC were found to have an epithelial-to-mesenchymal (EMT) phenotype, to overexpress PD-L1, and to be less immunogenic than CD44-negative cells.[163] ALDH1 isoenzyme expression has also been correlated with decreased overall patient survival.[164] Clinical studies have shown that ALDH1A1 expression correlates with poor tumor differentiation and worse patient prognosis.[165] A recent study showed that NCT-501, an ALDH1A1 inhibitor, is able to sensitize cisplatin-resistant HNSCC cells to cisplatin while decreasing CSC markers, self-renewal, and tumorigenic potential.[166] Furthermore, ALDH1A1 is immunogenic in HNSCC and an effective target for CD8(+) T-cell-mediated immune response.[167,168] A recent transcriptome analysis of TCGA data for five different cancers including 520 HNSCC tumors *versus* 40 normal samples found that differential expression of 19 ALDH isoforms correlated with cancer prognosis.[169]

*2.7.2 Salivary gland CSCs*

Recent studies have also identified $ALDH^{high}CD44^{high}$ as markers for CSCs in salivary MEC and ACC.[170,171] Similar to what was shown with HNSCC, serial transplantation and *in vitro* sphere assays demonstrated self-renewal and differentiation potential of these cells in both MEC and ACC. A recent study found that ALDH and CD44 markers enriched for CSCs in ACC and MEC patient-derived xenografts, validating the use of these markers in salivary gland CSC research.[172] Three patient-derived xenografts of a high grade MEC were generated from successive relapse surgeries (~9 months) in the same patient. Further genomic characterization of these tumors showed increased mutational burden and stem cell marker expression with decreased expression of tumor suppressors such as *TP53* during disease progression. Additionally, increased sphere-forming abilities and a larger fraction of $ALDH^{high}CD44^{high}$ cells in the later relapses.

## 2.8 Conclusion

Given the increasing evidence supporting a significant role for head and neck CSCs in tumor progression, we postulate that targeting pathways involved in CSC maintenance can potentially sensitize resistant cancers to conventional therapies and result in better eradication of tumor cells. In this chapter, we highlight some of the evidence showing the importance of p53 signaling in CSC maintenance. We know that p53 has a prominent role in self-renewal and differentiation of normal and tumorigenic stem cells.[75] Aberrant p53 signaling or inactivation in either HPV-positive or -negative tumors correlates with tumor progression in head and neck cancers. Moreover, p53 mutational status in head and neck cancers has been associated with poor patient prognosis.[144] Furthermore, p53 mutations correlate with higher disease grade in salivary gland cancers.

MDM2 inhibitors can activate endogenous wildtype p53[173] and reduce tumor volume, rate of recurrence, and CSC population in SGC.[53,174] They can also sensitize HNSCC to chemotherapy.[175] MDM2 inhibitors are in phase I and II clinical trials for the treatment of several malignancies.[176,177] Other methods for activating p53 include reactivation of wild-type p53 function in mutant p53 and the use of viral vectors to deliver wild-type p53 in p53-deficient cells.[152] A better understanding of the mechanisms by which p53 decreases self-renewal and induces differentiation of CSCs in head and neck tumors could inform a new therapeutic paradigm for cancer. Cancer patients might benefit from p53-induced differentiation of CSCs that can sensitize these cells to conventional chemotherapy.

# CHAPTER 3. P53 Regulates Stemness in Mucoepidermoid Carcinoma

## 3.1 Summary

Mucoepidermoid carcinoma (MEC) is a poorly understood salivary gland malignancy with limited therapeutic options. Cancer stem cells (CSCs) are considered drivers of cancer progression by mediating tumor recurrence and metastasis. We have shown that clinically relevant small molecule inhibitors of the MDM2-p53 interaction activate p53 signaling and reduce the fraction of cancer stem cells (CSCs) in MEC. Here, we examined the functional role of p53 in regulating cell fate processes of MEC CSCs. We found, that although p53 activation does not induce apoptosis of MEC CSCs, it reduces stemness properties such as self-renewal by regulating Bmi-1 protein stability and driving CSCs towards differentiation. In contrast, downregulation of p53 causes an expansion of the CSC population while promoting tumor growth. Remarkably, therapeutic activation of p53 prevented tumor recurrence in a preclinical trial in mice. Collectively, these results demonstrate that p53 regulates stemness properties in MEC and suggest that therapeutic activation of p53 may have clinical utility in patients with mucoepidermoid carcinoma.

### 3.2 Introduction

Few recurrent genetic alterations have been found in MEC, with CRCT1/MAML2 fusions being the most common.[46,49] In this fusion, the NOTCH-binding domain of MAML2 is replaced by the CREB-binding domain of CRCT1 resulting in disruption of NOTCH signaling and activation of c-AMP responsive target genes.[178,179] Although this fusion is thought to play a role in tumorigenesis, there has been conflicting evidence as to how its presence affects patient outcomes.[180-183] Unlike other head and neck cancers, p53 is not frequently mutated in MEC suggesting that p53 activating therapeutics may have clinical utility for the treatment of this disease.[148,184,185] Such therapeutics include MDM2 inhibitors which activate p53 signaling by disrupting the binding between MDM2 (Mouse double minute 2) and p53. MDM2 is an E3 ubiquitin ligase that targets p53 for proteasomal degradation. MDM2 is also a transcriptional target of p53 resulting in a negative feedback loop that keeps p53 levels in check.[69] p53 is the most commonly mutated gene in cancer. Cancers that do not directly have mutated p53 inactivate p53 signaling by overexpressing MDM2 or deregulating downstream p53 effectors. Importantly, p53 is considered a master regulator of cell fate by controlling processes involved in cell survival, cell division and stem cell and cancer stem cell self-renewal and differentiation.[76] Studies have shown cancer stem cells are resistant to conventional therapeutics and are thought to be responsible for tumor recurrence and metastasis.[99,186] Furthermore, they can serve as predictive or prognostic markers for different cancers.[161,162]

MEC cancer stem cells (CSCs) are defined by high ALDH enzymatic activity and CD44 expression and are highly tumorigenic, have self-renewal capacity, and have the

ability to generate the different cell phenotypes that make up MEC tumors.[171,172] We have previously demonstrated that treatment of MEC cells with the MDM2 inhibitor MI-773 activates p53 signaling and decreases the MEC CSC population.[1,173] However, the mechanisms underlying this effect are not known. Here, we evaluated the functional role of p53 in regulating MEC cancer stem cell fate and unveiled the therapeutic potential of targeting this pathway in pre-clinical trials in murine models of MEC.

## 3.3 Results

### 3.3.1 p53 depletion leads to an expansion of the cancer stem cell population

We evaluated the baseline levels of several proteins involved in p53 signaling in a panel of human mucoepidermoid carcinoma cell lines, *i.e.* UM-HMC-1, UM-HMC-3A, and UM-HMC-3B (Figure 3.1, a). The UM-HMC-1 cell line was generated from a primary tumor; meanwhile, the UM-HMC-3A cell line was generated from a recurrent tumor and the UM-HMC-3B cell line was generated from a lymph node metastasis in the same patient as UM-HMC-3A.[52] Interestingly, the UM-HMC-3B cell line expresses higher p53 and lower MDM2 levels then UM-HMC-3A, suggesting p53 signaling might be regulated differently in this cell line (Figure 3.1, a). Whole-exome sequencing on these cells revealed no mutations in either *MDM2* or *TP53* that might explain this differential expression.

A small molecule inhibitor of the MDM2-p53 interaction (MI-773) was used as a tool to activate p53 signaling in MEC cells. Cytotoxicity assays confirmed our prior findings that UM-HMC-1 and UM-HMC-3A cells are more responsive than UM-HMC-3B cells to inhibition of the MDM2-p53 interaction (Figure 3.1, b).[1] Meanwhile, western blotting confirmed that p53 signaling is activated in a dose-dependent manner evidenced by accumulation of p53 transcriptional targets, p21 and MDM2 (Figure 3.1, c). Consistent

with the cytotoxicity assays, UM-HMC-3B cells are less responsive to MI-773 than UM-HMC-1 or UM-HMC-3A. Of note, MI-773 treatment decreases Bmi-1 expression in all three cell lines (Figure 3.1, c). This polycomb protein is an important regulator of stem cell self-renewal and is frequently used as a CSC marker.[187] Confirming this observation, low cytotoxic doses of MI-773 caused a significant decrease in the CSC fraction ($ALDH^{high}CD44^{high}$) in the sensitive MEC cells, while no significant difference was observed in the less responsive UM-HMC-3B cells (Figure 3.1, d).

A second generation inhibitor of MDM2-p53 interaction called APG-115 was used to activate p53 signaling and verify the effects on MEC CSCs[188]. As observed with MI-773, cytotoxicity assays confirmed UM-HMC-3B cells are more resistant than UM-HMC-1 and UM-HMC-3A to the MDM2 inhibitor (Figure 3.1, e). p53 pathway activation as well as decreased Bmi-1 expression was confirmed via Western blot (Figure 3.1, f), and a decrease in the CSC fraction was observed in the sensitive UM-HMC-1 cells (Figure 3.1, g). Of note, we found multiple MDM2 protein bands in the UM-HMC-3B cells that we do not observe in the other cell lines. These multiple band patterns are an artifact of using higher antibody concentrations and doing longer exposures times of the WB membranes in order to detect MDM2 proteins in the UM-HMC-3B cell lysates. The same band patterns are observed in whole cell lysates in the other cell lines when we use higher primary antibody concentrations.

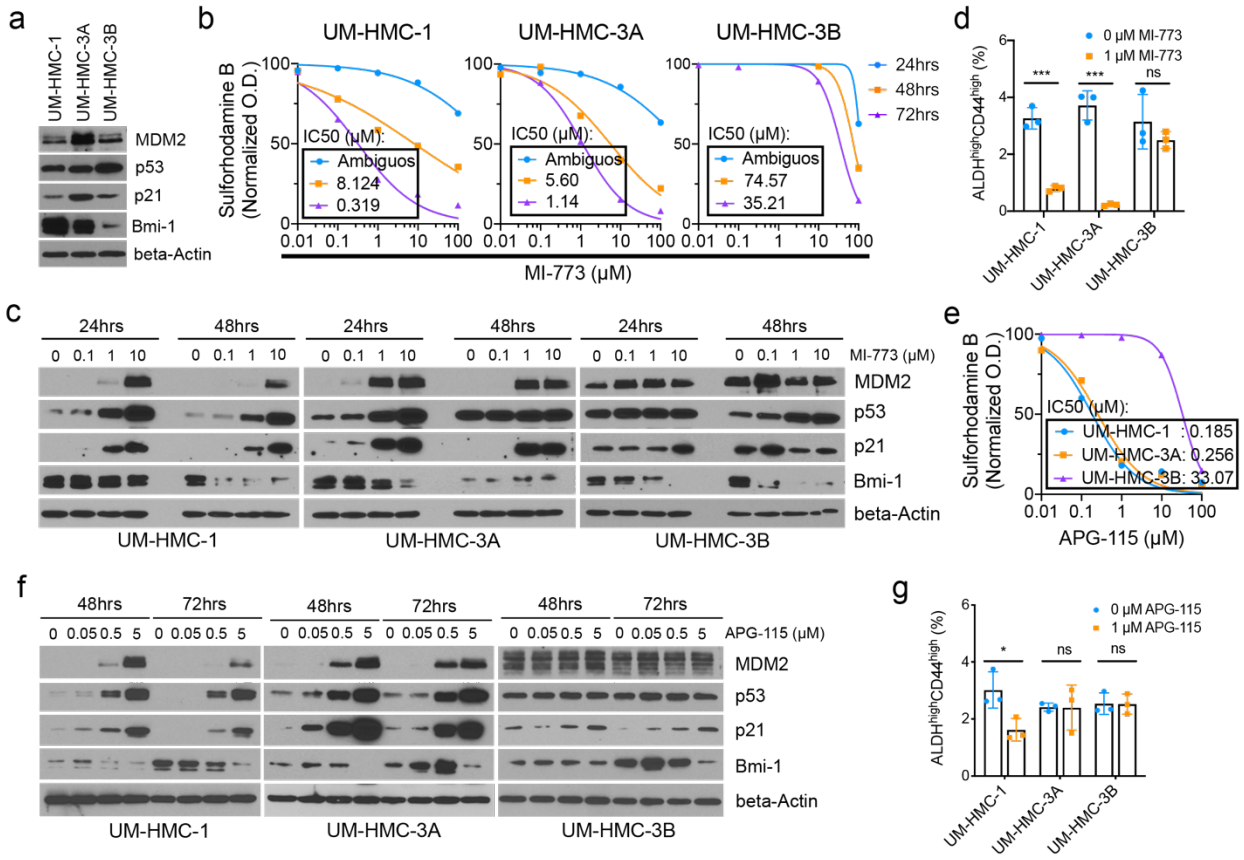


**Figure 3.1. MDM2 inhibitors decrease the fraction of mucoepidermoid carcinoma cancer stem cells *in vitro*. a** Western blot showing baseline protein levels for MDM2, p53, p21 and Bmi-1 in human mucoepidermoid carcinoma (MEC) cell lines (UM-HMC-1, UM-HMC-3A, UM-HMC-3B). **b** Graph showing the half-maximal inhibitory concentration ($IC_{50}$) of MI-773 in UM-HMC cells. **c** Accumulation of p53 and downstream effectors (MDM2 and p21) and decrease in Bmi-1 levels by MI-773, a 1st generation small molecule inhibitor of the MDM2-p53 interaction. **d** MEC cell lines were treated with MI-773 for 72 hours and the fraction of cancer stem cells ($ALDH^{high}CD44^{high}$) was measured using flow cytometry. **e** Sulforhodamine-B assay measuring the effect of APG-115 on cell viability after 72 hours. Data was normalized against vehicle controls. **f** Western blot verifying activation of p53 signaling with APG-115, a 2nd generation small molecule inhibitor of the MDM2-p53 interaction. **g** Fraction of cancer stem cells ($ALDH^{high}CD44^{high}$) measured using flow cytometry in MEC cell lines treated with APG-115 for 72 hours. All results are representative of at least two independent experiments. Data was analyzed by one-way ANOVA followed by post-hoc Tukey. ** $P$<0.01, *** $P$<0.001, ns=not significant.

To confirm that the decrease in MEC CSCs is due to activation of p53 signaling and not due to off target effects of the MDM2-p53 inhibitor, we used short hairpin RNAs (shRNA) to silence p53 expression in MEC cells. Successful p53 silencing was obtained

with sequences -1 and -2, while sequence -3 did not mediate adequate silencing and was used as a control (Figure 3.2, a). In p53 knockdown cells MI-773 failed to activate p53 downstream signaling and caused significantly less cytotoxicity compared to scrambled vector-transduced cells, confirming the specificity of the inhibitor (Figure 3.2, b, c). Importantly, no significant difference in cell proliferation was observed between the control and p53-silenced cells as determined from their growth curves and doubling times (DT) (Figure 3.2, d). Nonetheless, in the absence of accumulated p53 we no longer observed a decrease in the CSC population upon inhibition of the MDM2-p53 interaction (Figure 3.2, e, f), suggesting that increased p53 protein levels can regulate the maintenance of MEC CSCs.

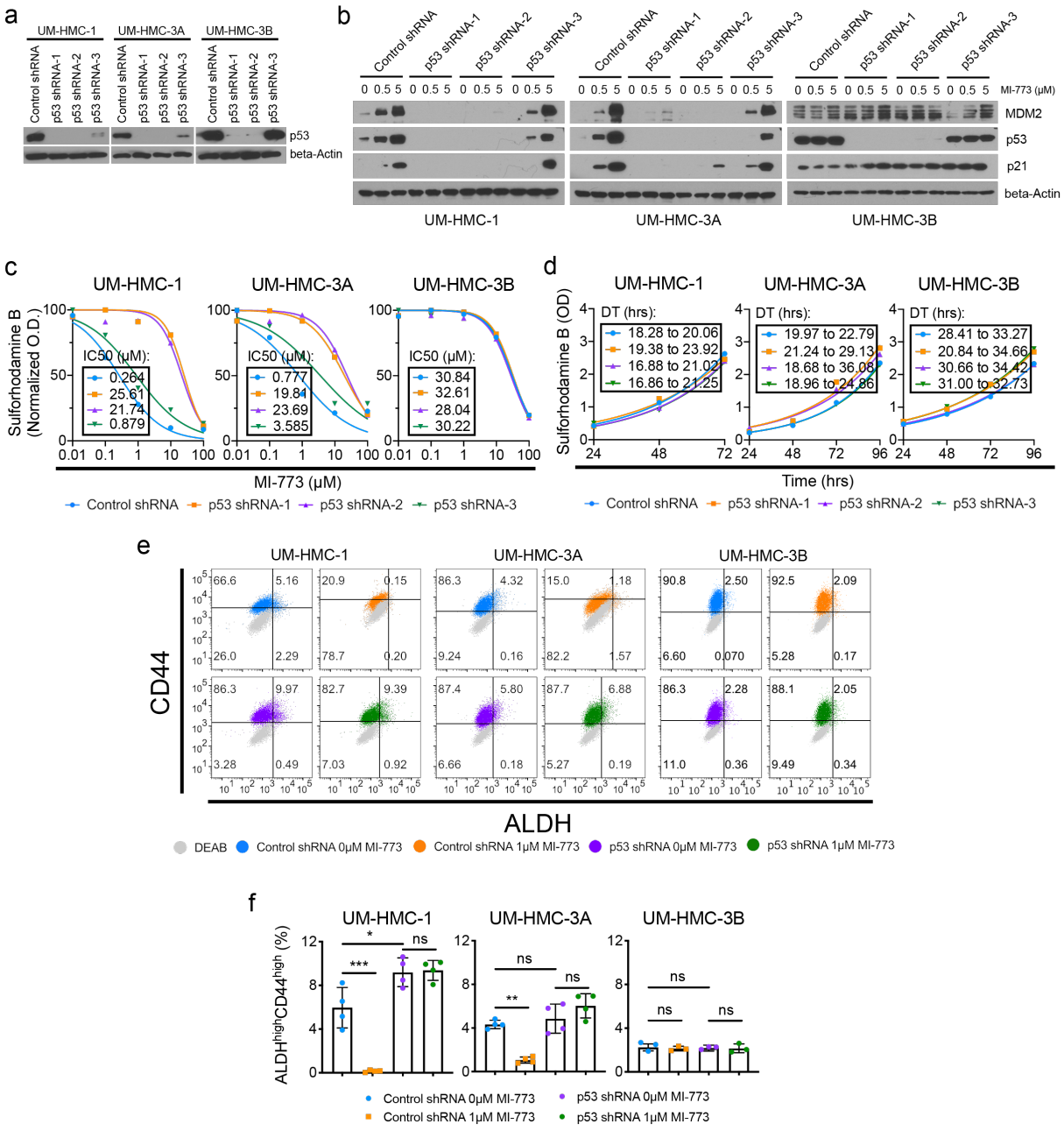


**Figure 3.2. p53 silencing abrogates the decrease on cancer stem cells by MDM2 inhibitors.** **a** Western blot showing knockdown levels of p53 with different shRNA constructs in the UM-HMC cell lines. **b** Western blots of p53-silenced and control cells treated with MI-773 for 48 hours. **c** Sulforhodamine-B assay measuring the effect of increasing doses of MI-773 for 72 hours on cell viability ($IC_{50}$). Data was normalized against vehicle controls. **d** Growth curves and doubling time (DT) calculations in control and p53-silenced cells. **e** Dot plots of flow cytometry analysis of the $ALDH^{high}CD44^{high}$ cell fraction in vector control and p53-silenced cells treated with MI-773 for 72 hours. **f** Quantification of (**e**). All results are representative of at least two independent

experiments. Data was analyzed by one-way ANOVA followed by post-hoc Tukey ($\alpha$=0.05). ** $P$<0.01, *** $P$<0.001, ns=not significant.

To verify these *in vitro* observations, we evaluated the effects of p53 silencing on MEC tumors using subcutaneous and orthotopic xenograft models. Cancer cells either transplanted into the subcutaneous space or in the submandibular glands (SMG) of immunodeficient mice were monitored for tumor growth and all mice were euthanized when the first tumors reached study endpoint (2mm$^3$). We found that p53 knockdown dramatically enhanced tumor growth in xenograft tumors generated with the sensitive UM-HMC-3A cell line (Figure 3.3, a-f). The p53-silenced tumors reached endpoint ~2 months after transplantation. Importantly, no proliferative advantage was observed in the p53-silenced cells that might explain the increased growth rate of these tumors (Figure 3.2, d). Given the limited tumor growth by the vector control cells at study endpoint, we had insufficient tissue to perform flow cytometry analysis of the ALDH$^{high}$CD44$^{high}$ CSC population. As an alternative, we performed immunohistochemistry for ALDH1 in cells seeded in the scaffold of the UM-HMC-3A xenograft tumors in order to understand the impact of p53 silencing on the fraction of the cancer stem cells *in vivo*. While all tumors in the p53 shRNA group contained CSCs as determined by high ALDH1 expression, very few ALDH1-positive cells were found in control specimens (Figure 3.3, g, h).

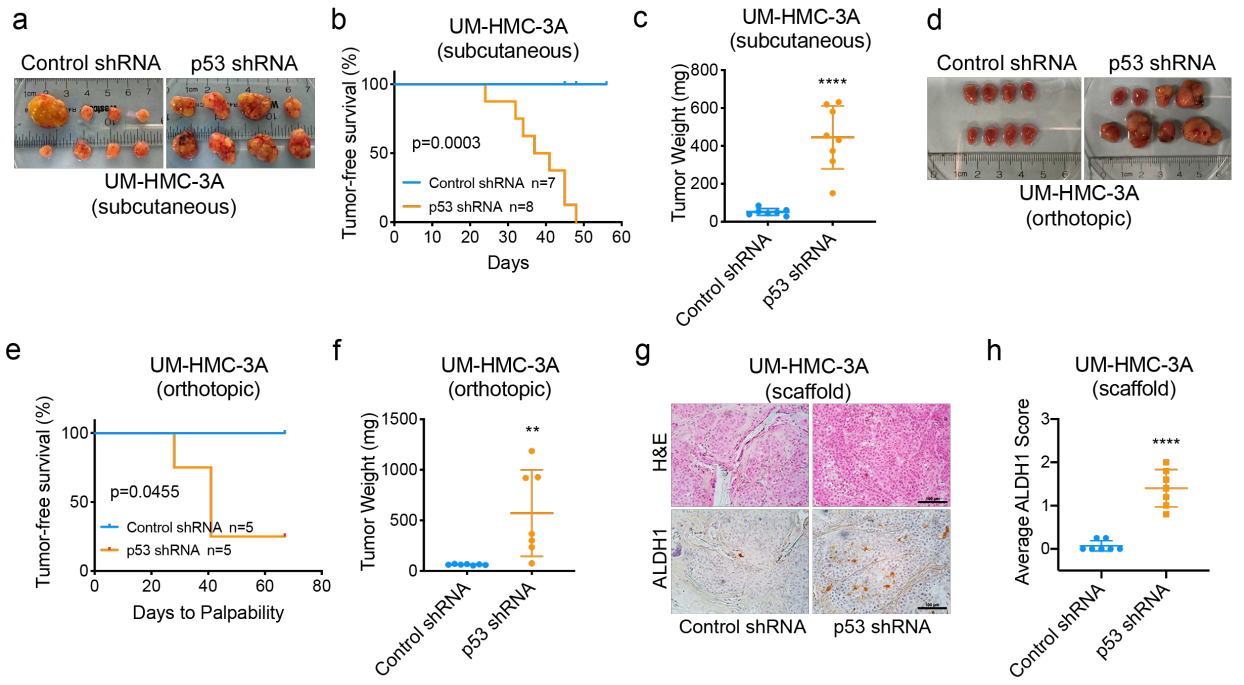


**Figure 3.3. p53 depletion increases tumor growth and leads to an expansion of ALDH-expressing cancer stem cells *in vivo*. a** Macroscopic image of subcutaneous xenograft tumors generated with UM-HMC-3A cells transduced with p53 shRNA or vector control. Outlier tumor in Control shRNA group was excluded from further analysis. **b** Kaplan-Meier curves depicting tumor-free survival. Failure was defined as tumors that reached a volume of 200 $mm^3$. **c** Tumor weights at study endpoint. **d** Macroscopic image of orthotopic xenograft tumors generated with UM-HMC-3A cells injected in the submandibular glands of mice. **e** Kaplan-Meier curves depicting tumor-free survival. Failure was defined as palpable salivary gland tumors. **f** Weight of the submandibular glands at study endpoint. **g** H&E and immunohistochemical analysis of ALDH1 in subcutaneous tumors from **(a)**, scale bar = 100 μM. **h** Average ALDH1 scores of 5 randomly selected microscopic fields per tumor **(g)**. Microscopic fields were scored as follows: 0 – no cells with ALDH1 staining; 1 – 1 to 10 cells stained for ALDH1; 2 – more than 10 cells with high ALDH1 expression. Kaplan-Meier graphs were analyzed using Gehan-Breslow-Wilcoxon test. All other data was analyzed by two-tailed student's *t*-test. ***P*<0.01, *****P*<0.0001.

These experiments were repeated with the less responsive UM-HMC-3B cells and no detectable difference in the establishment and growth of the xenograft tumors was found between the control and the p53 shRNA groups (Figure 3.4, a-c, e-g). Nevertheless, even in tumors generated with these less responsive cells we observed a trend of higher percentages of CSCs in the p53 shRNA tumors when compared to vector control tumors, as measured through flow cytometry (Figure 3.4, d, h).

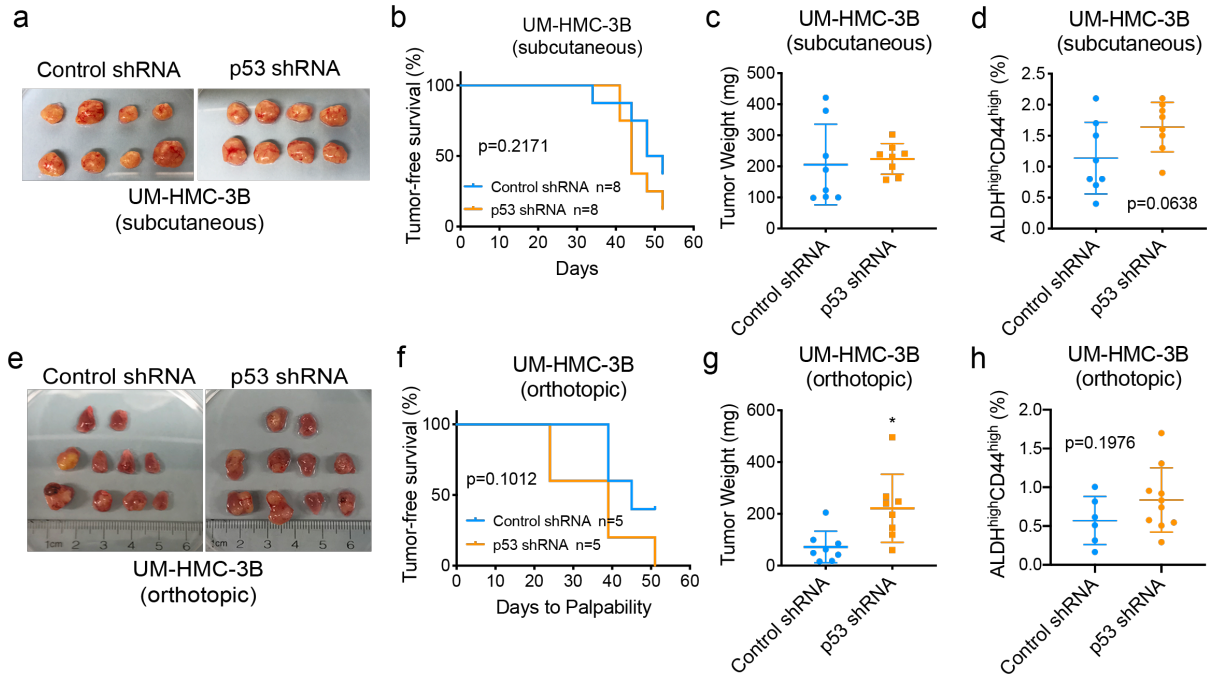


**Figure 3.4. p53 depletion does not increase tumor growth in xenograft tumors with the resistant UM-HMC-3B cells. a** Macroscopic image of subcutaneous xenograft tumors generated with UM-HMC-3B cells. **b** Kaplan-Meier curves depicting tumor-free survival. Failure was defined as tumors that reached a volume of 200 $mm^3$. **c** Tumor weights at study endpoint. **d** Fraction of cancer stem cells ($ALDH^{high}CD44^{high}$) in tumors generated with UM-HMC-3B cells transduced with p53 shRNA or vector control. **e** Macroscopic image of orthotopic xenograft tumors generated with UM-HMC-3B cells. **f** Kaplan-Meier curves depicting tumor-free survival. Failure was defined as palpable salivary gland tumors. **g** Weight of submandibular glands at study endpoint. **h** Orthotopic xenograft experiment with UM-HMC-3B cells was repeated (n=13) and the fraction of cancer stem cells was analyzed. Graph depicting the fraction of cancer stem cells ($ALDH^{high}CD44^{high}$) in tumors generated with UM-HMC-3B cells transduced with p53 shRNA or vector control. Samples with less than 3,000 tumor cells obtained from digested tissues were excluded from flow cytometry analysis. Kaplan-Meier graphs were analyzed using Gehan-Breslow-Wilcoxon test. All other data was analyzed by two-tailed student's *t*-test. **P*<0.05.

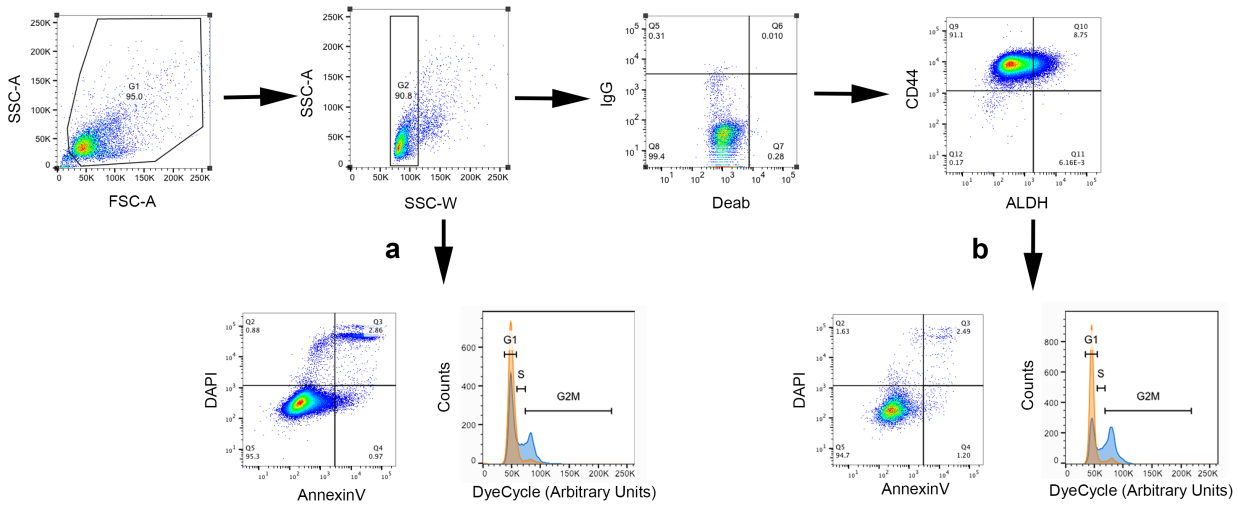


**Figure 3.5. Gating schematic for apoptosis and cell cycle profile analysis of bulk and cancer stem cells.** Representative gating shows how apoptosis and cell cycle profiles were measured in the bulk cell population **(a)** and in the cancer stem cells **(b)**, $ALDH^{high}CD44^{high}$. Cancer stem cells were analyzed by gating on high ALDH enzymatic activity using DEAB as a negative control for AldeRed substrate, followed by high CD44 expression. Afterwards, gates for Annexin V or DyeCycle-Orange based on the bulk cell population were applied to the cancer stem cells.

### 3.3.2 p53 activation does not preferentially induce apoptosis of cancer stem cells

To understand how induction of p53 decreases the population of MEC CSCs, we first assessed whether activating p53 signaling depletes this population by preferentially inducing its apoptosis. Annexin V staining is commonly used to detect early apoptotic events in viable cells and is compatible with ALDH enzymatic detection assays such as ALDEFLUOR™ and ALDERED™. This approach allowed for the examination of apoptotic events specifically in the CSCs (Figure 3.5). Cells were treated with a low dose of MI-773 (1 μM) to activate p53 signaling and apoptosis was measured at different time points. Under these conditions, apoptosis was observed only in the bulk cell population of the UM-HMC-3A cell line (Figure 3.6, a). Nevertheless, treatment was sufficient to cause a decrease in the CSCs fraction at 48 and 72 hours in both sensitive cell lines (UM-HMC-1, UM-HMC-3A) (Figure 3.6, b). Remarkably, no apoptosis was detected in the CSC

fraction that could account for this decrease (Figure 3.6, 3c) suggesting p53 activation does not preferentially induce apoptosis of MEC CSCs.

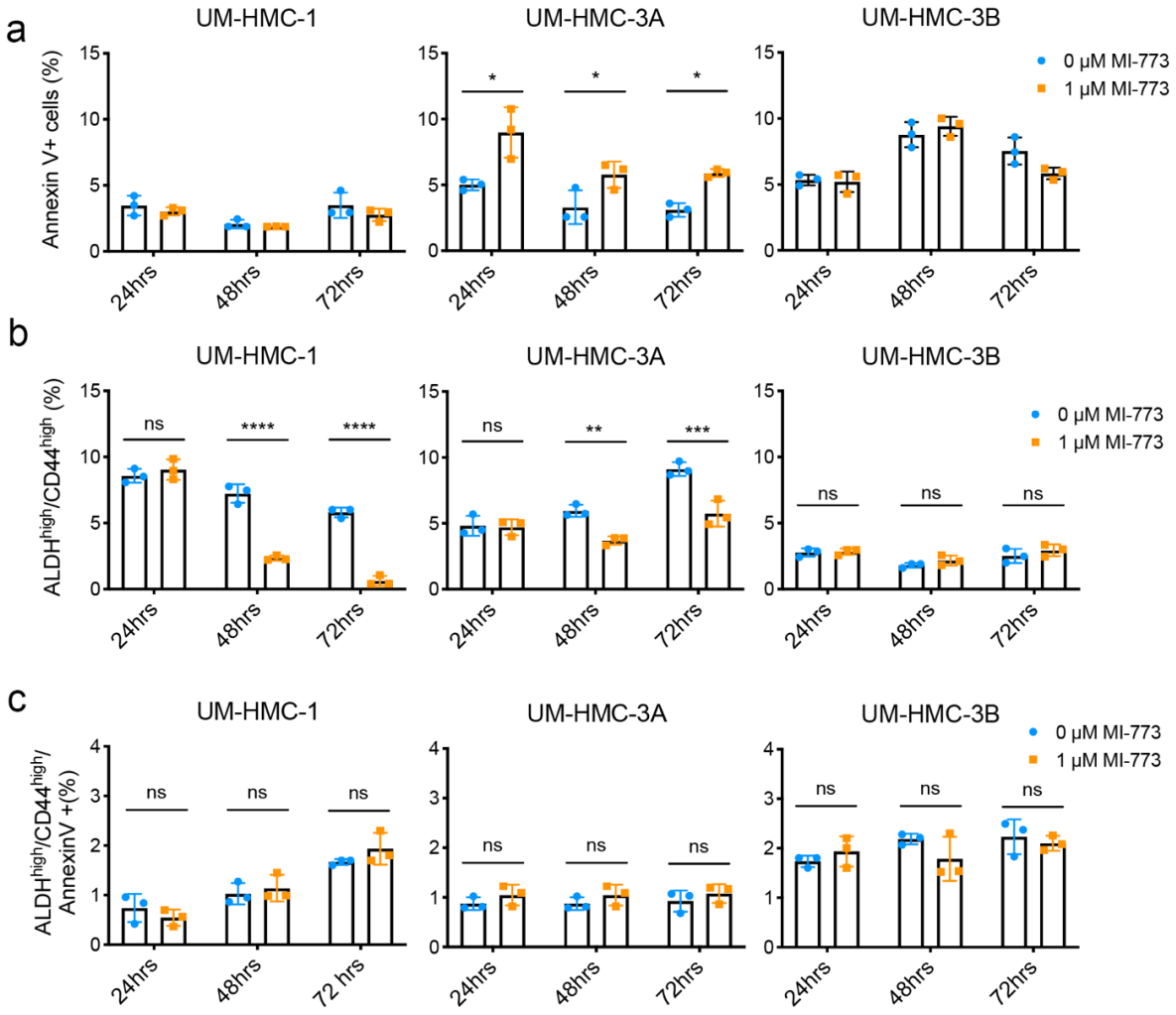


**Figure 3.6. p53 activation does not preferentially induce apoptosis of cancer stem cells.** UM-HMC cell lines were treated with either vehicle or MI-773 and subsequently analyzed for apoptotic cells using Annexin V staining. **a** Apoptosis measured in the bulk cell population after 24-72-hour treatment with 1 μM MI-773 or vehicle control. **b** Fraction of cancer stem cells ($ALDH^{high}CD44^{high}$) after 24-72-hour treatment with 1 μM MI-773 or vehicle control. **c** Graphs depicting the fraction of apoptotic cancer stem cells ($ALDH^{high}CD44^{high}$) after 24-72-hour treatment with MI-773 or vehicle control. All results are representative of at least two independent experiments. Data was analyzed by two-way ANOVA followed by post-hoc Bonferroni. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, ns=not significant.

### 3.3.3 p53 activation changes the cell cycle profile of the cancer stem cells through p21 signaling

A non-toxic cell permeable DNA dye (DyeCycle-Orange) compatible with the ALDH enzymatic assay was used to evaluate the effect of p53 activation on the cell cycle of MEC CSCs (Figure 3.5). Focusing on the sensitive cell lines, MEC cells were again treated with a low dose of MI-773 (1μM) to activate p53 signaling without appreciable cytotoxicity. The cell cycle profiles were analyzed 24 hours after treatment. MI-773 increased the proportion of bulk cells in G1 suggestive of a G1 cell cycle arrest (Figure 3.7, a, b), an effect that correlates with increased p21 expression.[1] When we focused our cell cycle analysis on the CSCs, we observed that a high percentage of CSCs were in G2/M at baseline, which is the opposite of the bulk cell population in which most untreated cells are in G1 (Figure 3.7, a, b). Nevertheless, activation of p53 with MI-773 increased the proportion of CSCs in G1 when compared to untreated cells, a response similar to that seen in the bulk cell population (Figure 3.7, a, b).

Since p53 affects the cell cycle by transcriptional activation of p21, we knocked down p21 using shRNA to determine its role in cell cycle regulation of the CSCs. Knockdown was confirmed with two different shRNA constructs, *i.e.* sequence -2 and -3 (Figure 3.7, c). Sequence -2 was used for the remaining experiments. As expected, p21 silencing did not affect p53 protein accumulation and activation of its downstream signaling by MI-773, as evidenced by the accumulation of MDM2 (Figure 3.7, d). Additionally, Bmi-1 protein levels decreased with MI-773 treatment, suggesting that the regulation of Bmi-1 is independent of the p21 signaling axis. Although p21 knockdown had little effect on the cell cycle of the bulk cell population, it attenuated the MI-773-

mediated G2/M to G1 shift in the CSCs (Figure 3.7, e, f). Collectively, these results demonstrate that MI-773-induced activation of p53 causes a shift towards G1 in the cell cycle of the CSCs that is dependent on downstream p21 signaling.

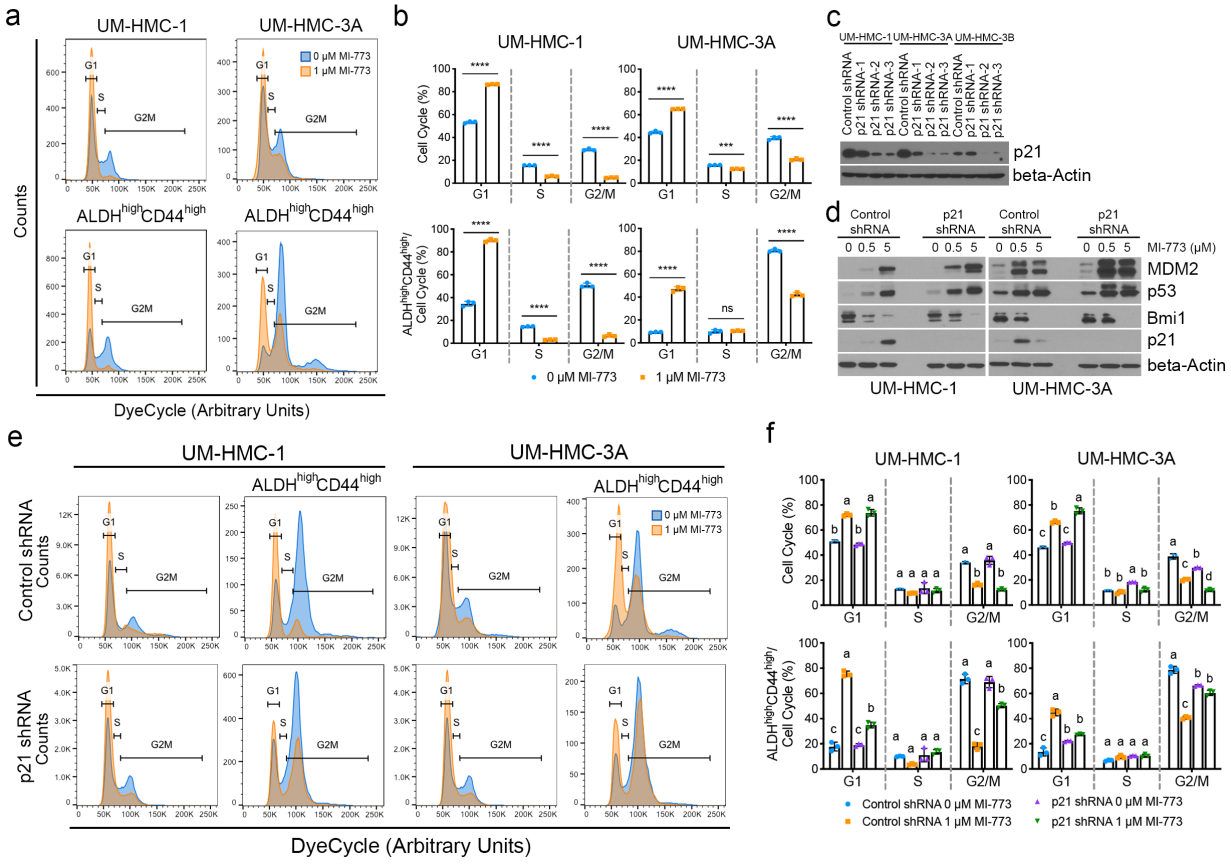


**Figure 3.7. Activation p53-p21 signaling shifts the cell cycle of mucoepidermoid carcinoma stem cells**. The cell cycle of UM-HMC cells was analyzed with DyeCycle-Orange after being treated with vehicle or MI-773 for 24 hours. **a** Representative cell cycle plots for bulk (top) and cancer stem cells (bottom), ALDH$^{high}$CD44$^{high}$, treated with vehicle or 1 μM MI-773 for 24 hours. **b** Quantification of bulk and cancer stem cells in each phase of the cell cycle from **(a)**. **c** Western blot showing knockdown levels of p21 with different shRNA constructs in UM-HMC cell lines. **d** Western blot depicting the impact of increasing concentrations of MI-773 for 48 hours on p53 pathway activation in UM-HMC cells transduced with p21 shRNA or scrambled-vector control. **e** Representative cell cycle plots of bulk cells or cancer stem cells (ALDH$^{high}$CD44$^{high}$) in UM-HMC cells transduced with p21 shRNA or vector control and treated with vehicle or MI-773 for 24 hours. **f** Graphs depicting the quantification of the cell cycle phases of UM-HMC cells in **(e)**. All results are representative of at least two independent experiments. Means not sharing any lower-case letters are significantly different by two-way ANOVA followed by post-hoc Tukey ($\alpha$=0.001). ***$P$<0.001, ****$P$<0.0001, ns=not significant.

### 3.3.4 p53 signaling blocks self-renewal and induces differentiation of cancer stem cells

To understand how p53 activation affects the stemness phenotype of MEC CSCs, protein expression of CSC and bulk cell fractions were analyzed after treatment with MI-773 for 72 hours (Figure 3.8, a). As expected, we observed higher baseline Bmi-1 expression in the CSC fraction when compared to nonCSCs. Although a modest decrease in Bmi-1 was observed in the resistant UM-HMC-3B cell line, we observed a significant reduction in Bmi-1 protein levels in the sensitive UM-HMC-3A CSCs treated with MI-773. These results indicate that p53 signaling decreases CSC self-renewal and/or increases differentiation. To further explore this finding, pan-Cytokeratin was used as a marker of differentiation. We observed significantly higher expression of pan-Cytokeratin in CSCs treated with MI-773, when compared to controls (Figure 3.8, b, c). CSCs were co-stained for Bmi-1 and pan-Cytokeratin to identify a possible inverse relationship between these markers (Figure 3.8, d-f). As expected, activation of p53 with MI-773 caused a progressive loss in Bmi-1 expression and a gain in pan-Cytokeratin over time (Figure 3.8, e, f). Remarkably, cells expressing high levels of pan-Cytokeratin rarely co-expressed high levels of Bmi-1 (Figure 3.8, d). These results suggest that activation of p53 signaling with MI-773 induces differentiation of MEC CSCs.

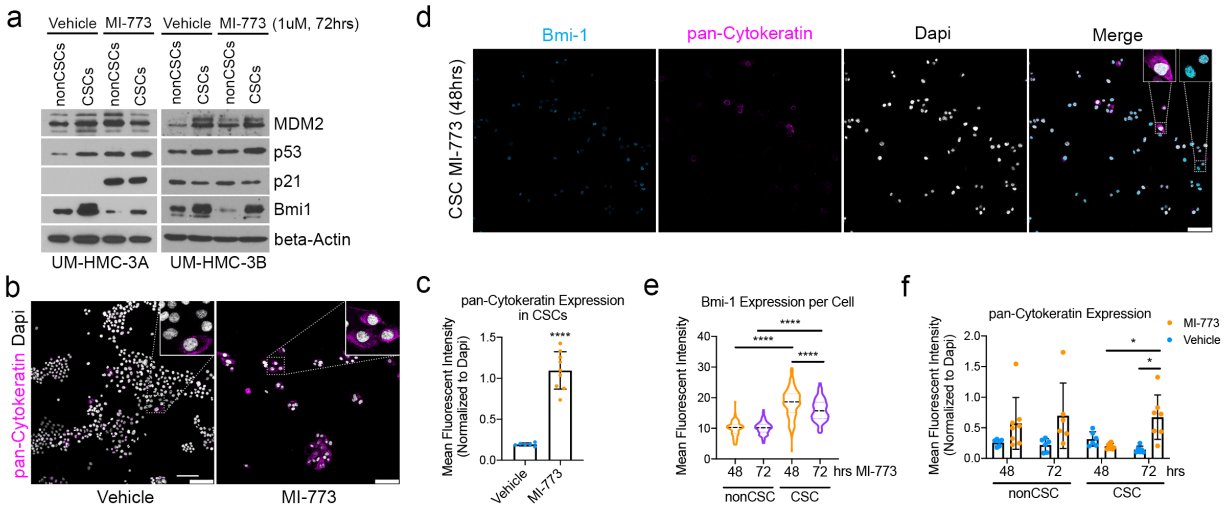


**Figure 3.8. p53 activation induces pan-Cytokeratin expression and decreases Bmi-1 in cancer stem cells. a-c** UM-HMC cell lines were sorted for cancer stem cells ($ALDH^{high}CD44^{high}$) and non-cancer stem cells ($ALDH^{low}CD44^{low}$) after being treated with MI-773 for 72 hours. Immediately after, **(a)** the collected cells were used to make whole-cell lysates for western blot analysis or **(b)** cultured in a 4-well chamber slide for two days prior to fixing and staining for pan-Cytokeratin and DAPI (scale bar=100μm). **c** Graph depicting quantification of pan-Cytokeratin expression in **(b)**. **d-f** Cells were sorted for cancer stem cells and immediately plated in a 4-well chamber slide and cultured for 24 hours prior to treatment with 1 μM MI-773. **d** Slides were fixed at 48- and 72-hours post-treatment and subsequently stained for pan-Cytokeratin and Bmi-1 (scale bar=100μm). **e** Graph depicting quantification of nuclear Bmi-1 expression in cancer stem cells or non-cancer stem cells. **f** Graph depicting quantification of pan-Cytokeratin expression in cells treated with 1 μM MI-773 or vehicle. Immunofluorescence was measured as the mean gray value normalized to DAPI. At least 5 arbitrary areas per chamber were selected for quantification. Two-tailed student's *t*-test was used for two group comparisons, two-way ANOVA with post-hoc Tukey was used in **(f)** and one-way ANOVA was used for all other comparisons. **P*<0.05, *****P*<0.0001.

Salispheres can be used to measure the stemness and self-renewal of salivary gland cancer stem cells[171,172]. Since UM-HMC-1 cells have limited sphere forming capacity, only the UM-HMC-3A and UM-HMC-3B cells were used for these studies. Although the resistant UM-HMC-3B cells do not show a significant difference in the fraction of CSCs after treatment with inhibitors of the MDM2-p53 interaction *in vitro*, treatment significantly decreased primary salisphere formation in both MEC cell lines (Figure 3.9 a-c). Nevertheless, UM-HMC-3B spheres were less sensitive to MI-773 than

UM-HMC-3A spheres. To confirm that MI-773 affects primary sphere development and is not just toxic to cells grown in these conditions, spheres were allowed to form for 5 days prior to being treated with MI-773 (1μM) and were monitored for 5 to 14 days post-treatment. No significant difference in salisphere formation was observed under these conditions (Figure 3.9 d, e).

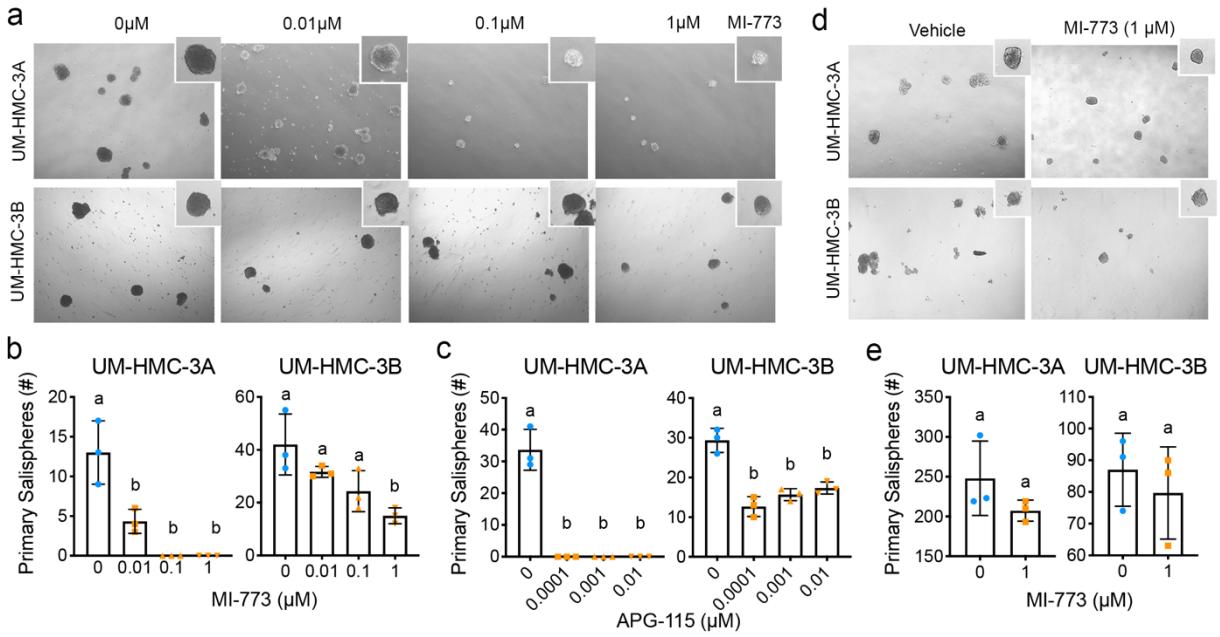


**Figure 3.9. Activation of p53 by MDM2 inhibitors decreases the sphere forming ability of mucoepidermoid carcinoma cells. a** Unsorted cells were plated in sphere conditions and treated the following day with increasing doses of MI-773. **b** Graph depicting the number of primary salispheres 7-9 days after MI-773 treatment. **c** Graph depicting the number of primary salispheres 7-9 days after APG-115 treatment. **d** Unsorted cells were plated in sphere conditions and spheres were treated 5 days after cells were plated. **e** Quantification of **(d)**. All results are representative of at least two independent experiments. Means not sharing any lower-case letters are significantly different by one-way ANOVA followed by post-hoc Tukey ($\alpha$=0.05).

To confirm the data obtained with the MDM2 inhibitors, we performed the reverse experiment with p53-silenced MEC cells. We observed that p53-silenced cells formed more primary salispheres than cells transduced with scrambled vectors (Figure 3.10, a, b). To confirm these results, high-throughput sphere culture microfluidic devices were

used to monitor single-cell derived spheres with higher precision (Figure 3.10, c). Each microfluidic device contained 3,200 individual sphere culture chambers allowing for rapid assessment of sphere formation. Single cells were guided by hydrodynamic flow and captured at the trapping site in each sphere culture chamber. p53-silenced cells presented a higher proportion of cells capable of sphere formation as well as increased sphere size (Figure 3.10, d). Additionally, spheres formed from p53 shRNA cells had higher Bmi-1 protein levels than those formed from control cells, indicating a role for p53 in regulating CSC self-renewal (Figure 3.10, e). Self-renewal was measured by evaluating secondary salisphere formation from primary spheres treated with MI-773. Treatment of primary salispheres caused a significant decrease in secondary sphere formation while p53 knock-down abrogated that effect (Figure 3.10, f). Meanwhile, there was no difference in primary sphere formation in response to MI-773 or Bmi-1 levels in p21 shRNA-derived spheres (Figure 3.10, g-i). Collectively, these results demonstrate that p53 activation mediates blockade of CSC self-renewal and that this effect is independent of p21 signaling.

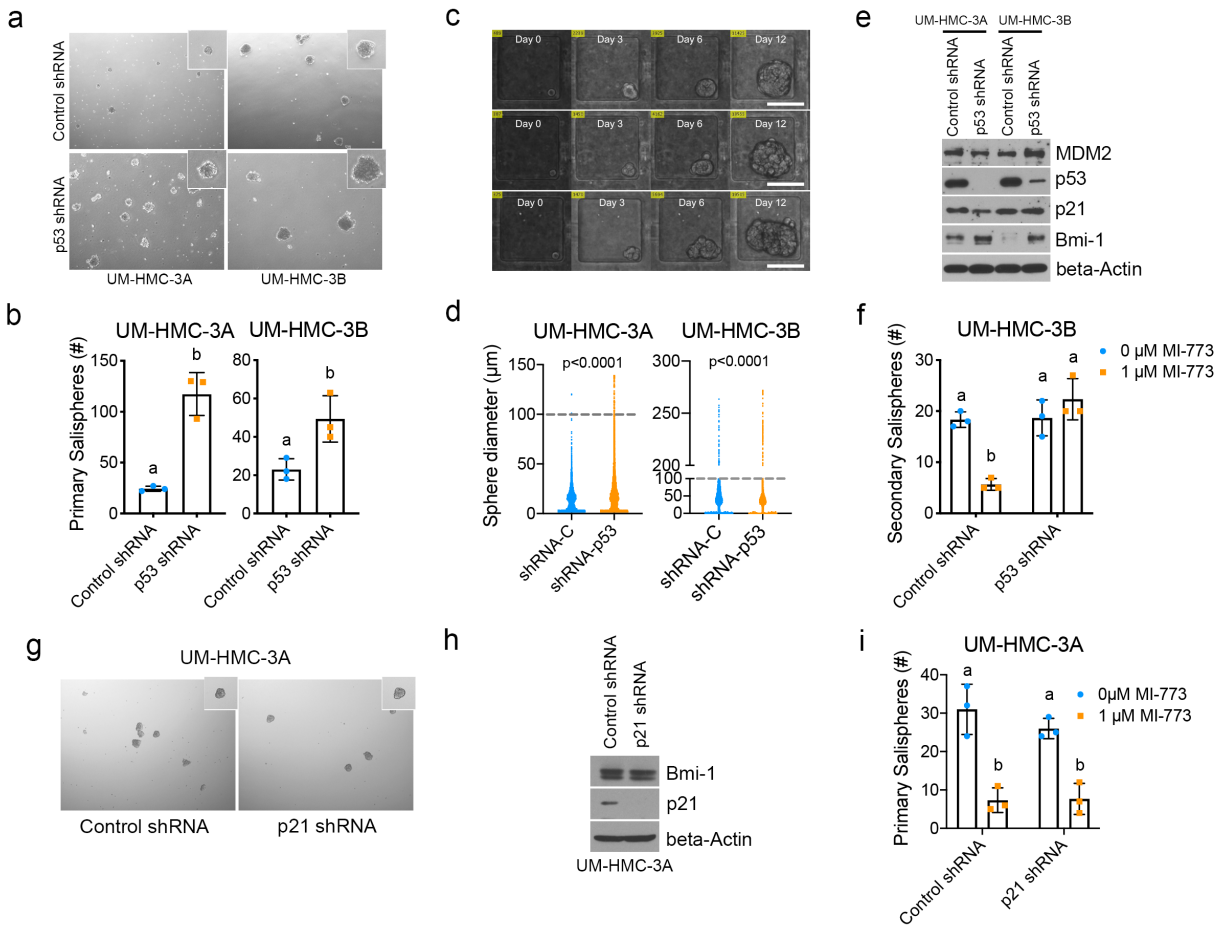


**Figure 3.10. p53 levels regulate primary salisphere formation and self-renewal independent of the p53-p21 signaling axis. a** Microscopic view of primary salispheres formed by UM-HMC cells transduced with p53 shRNA or vector control cells. **b** Quantification of spheres represented in **(a)**. **c** Microscopic view of single cell capture microfluidic device showing sphere growth over time (scale bar = 100 μm). **d** Graph depicting sphere diameters from single-cell salispheres generated in single-cell sphere microfluidic device. Dotted lines depict cut-off for minimum sphere size (100 μM). **e** Western blot analysis of primary salispheres generated by UM-HMC cells transduced with p53 shRNA or vector control cells. **f** Secondary salispheres generated from primary salispheres treated with MI-773 (1μM) or vehicle control. **g** Representative micrographs of primary salispheres generated with UM-HMC-3A cells transduced with p21 shRNA or scrambled vector control. **h** Western blot showing effects of p21 knockdown on Bmi-1 protein levels in primary salispheres. **i** Graph depicting the number of primary salispheres 7-9 days after MI-773 treatment. Means not sharing any lower-case letters are significantly different by two-tailed student's *t*-test ($\alpha$=0.05) in two group comparisons or one-way ANOVA with post-hoc Tukey ($\alpha$=0.05) in multiple group comparisons.

### 3.3.5 p53 regulates Bmi-1 protein stability

Although Bmi-1 protein levels decrease with MI-773 treatment, there was no difference in mRNA levels when cells were treated with varying doses and timepoints of MI-773 (Figure 3.11 a, b). Nevertheless, we observed increasing p21 mRNA levels confirming the transcriptional activity of p53 (Figure 3.11, a). This prompted us to look at Bmi-1 protein stability. It is known that Bmi-1 is phosphorylated upon cycloheximide treatment resulting in an increase in molecular weight.[189,190] The lower band of Bmi-1 was used to calculate protein degradation rates, as shown.[189] Under these conditions, Bmi-1 had a half-life of 42 minutes, similar to the 20-40 minute half-life reported by others (Figure 3.11, c, d).[189,190] When cells were treated with MI-773, Bmi-1 half-life decreased by almost 50% ($T_{1/2}$=22 minutes). Interestingly, even the phosphorylated Bmi-1 is degraded faster in the MI-773 treated cells when monitored for longer periods of time (Figure 3.11, e). As a control, we calculated the half-life of p53 ($T_{1/2}$=3 hours) and, as expected, found that MI-773 increases its half-life ($T_{1/2}$=12 hours) (Figure 3.11, f). These results suggest accumulation of p53 regulates Bmi-1 protein stability, indicating a potential mechanism by which p53 regulates CSC self-renewal.

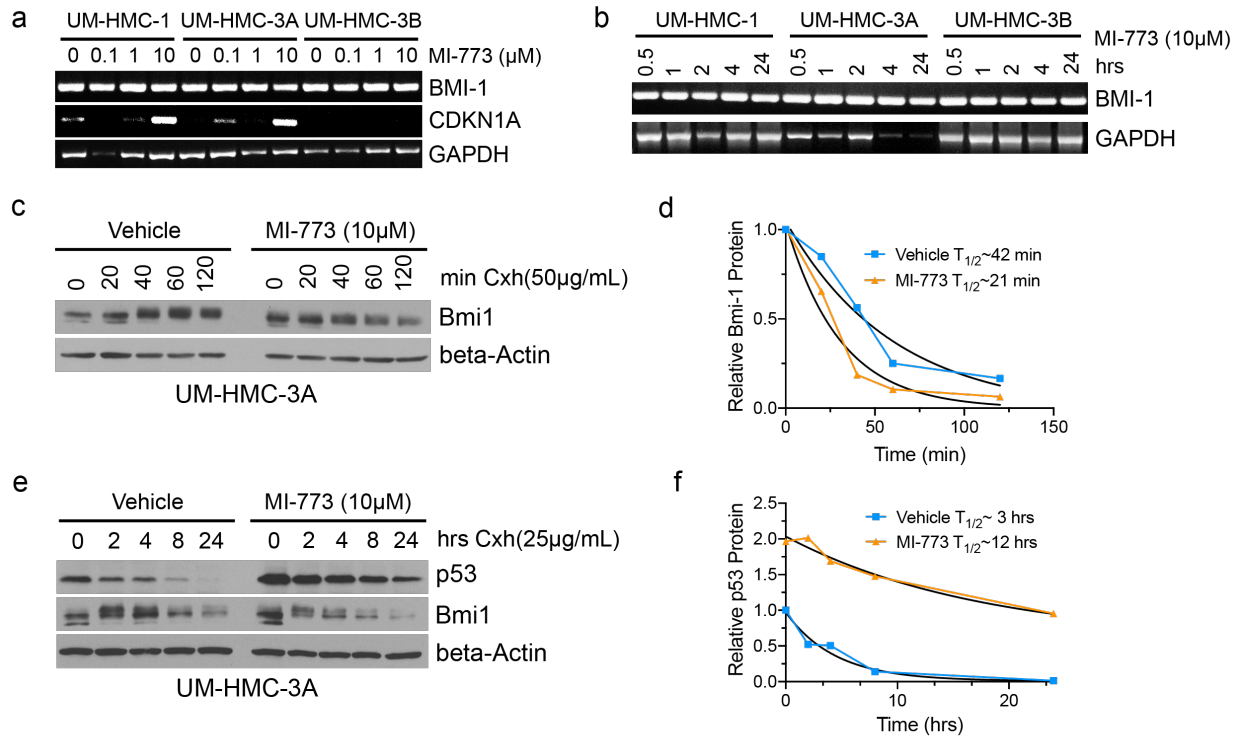


**Figure 3.11. p53 activation decreases Bmi-1 protein stability. a** Reverse transcription polymerase chain reaction (RT-PCR) for Bmi-1, p21 and GAPDH of cells treated for 24 hours with increasing concentrations of MI-773. **b** RT-PCR depicting time-course expression of Bmi-1 and GAPDH in cells treated with 10 μM MI-773. **c** Western blot of cells pre-treated for 3 hours with 10 μM MI-773 followed by treatment with 50 μg/mL cycloheximide (CXH) for up to 2 hours. **d** Graph depicting protein half-life ($T_{1/2}$) calculation based on unphosphorylated Bmi-1 (lower band). **e** Western blot of cells treated with 10 μM MI-773 for 3 hours prior to addition of 25 μg/mL cycloheximide for up to 24 hours. **f** Graph depicting protein half-life ($T_{1/2}$) calculation based on p53 from **(e).**

### 3.3.6 Therapeutic induction of p53 prevents MEC tumor recurrence in mice

A recurrence study was conducted to examine the therapeutic benefit of the small molecule inhibitor of the MDM2-p53 interaction in a clinically relevant neoadjuvant setting simulating treatment of patients with advanced MEC. We generated subcutaneous xenografts by transplanting MEC cells into the dorsal region of mice and waited until tumors reached an average of 800 $mm^3$ (Figure 3.12 a). At that stage, tumors were randomly assigned to receive either MI-773 or vehicle control (Figure 3.12, b). Treatment

with MI-773 started 3 days prior to tumor resection and continued as weekly maintenance doses for 1 month (Figure 3.12, a). Tumor recurrence was monitored until palpable tumors were detected. At that stage, mice were euthanized and the presence of recurrent tumors was verified macroscopically. While 7 out of 9 tumors (78%) recurred in the vehicle control group, only 3 out of 8 (37.5%) recurred in the MI-773-treated group (Figure 3.12, c). All tumor-free mice were monitored for 250 days after which the experiment was terminated and the absence of recurrent tumor lesions was verified upon autopsy. This pre-clinical trial demonstrated that adjuvant therapy with MI-773 prevents tumor recurrence in mice. Notably, post-surgical tumor progression involving local recurrence is the most common cause of treatment failure in human patients with salivary gland mucoepidermoid carcinoma.[191]

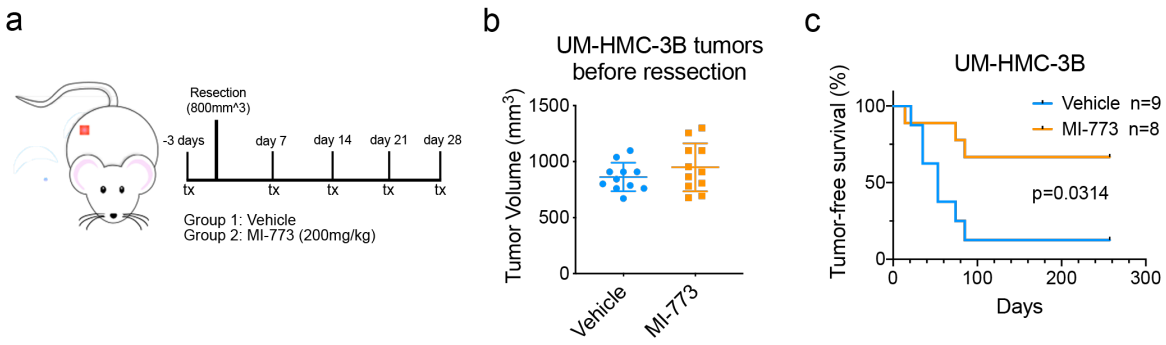


**Figure 3.12. Therapeutic induction of p53 signaling prevents MEC tumor recurrence in mice. a** Schematic showing experimental design of tumor recurrence study. UM-HMC-3B subcutaneous xenograft tumors were allowed to grow to an average volume of 800mm$^3$ then randomly assigned to a treatment group. Mice were treated via oral gavage with either vehicle or one dose of 200 mg/kg of MI-773 3 days prior to tumor resection. Weekly maintenance treatments of MI-773 (200 mg/kg) were given for four weeks after tumor resection. Mice were monitored weekly for tumor recurrence by palpability. **b** Graph depicting tumor volumes in both experimental groups at start of treatment. **c** Kaplan-Meier curves depicting tumor-free survival. Failure was defined as palpable subcutaneous tumors. Kaplan-Meier graphs were analyzed using the Gehan-Breslow-Wilcoxon test.

### 3.4 Discussion

Much of the focus in understanding the pathobiology of mucoepidermoid carcinomas has been centered on the CRCT/MAML fusion. *TP53* mutations have been described as rare events in these tumors, especially when compared to other head and neck cancers such as squamous cell carcinoma.[148,184,185] Nevertheless, as more interest develops in understanding the genetic landscape of this rare malignancy and potential drivers of this disease, we find that p53 regulation might play an important role in MEC tumor biology. The first whole-exome sequencing study in MEC patients revealed that *TP53* mutations are found in only about 30% of patients.[46] Interestingly, *TP53* mutations are present primarily in intermediate and high-grade disease and associated with higher number of overall mutations.[154] Additionally, a group who studied serial tumor relapses from one MEC patient observed loss of expression of *TP53* upon tumor evolution.[172] Importantly they found that tumor recurrence in this patient correlated with a time-dependent increase in the CSC fraction and higher tumorigenic potential. These findings reveal that a loss in p53 signaling might contribute to MEC progression. Consistent with this, we showed here that MEC xenograft tumors with silenced p53 expression grew faster than tumors with functional levels of p53. Furthermore, this increased growth rate was not attributed to a proliferative advantage in the silenced cells. Importantly, these tumors also contained a higher proportion of CSCs.

Some of the more commonly studied cell fate processes regulated by p53 are apoptosis and cell cycle progression. However, emergent evidence is providing support for a role for p53 in regulating self-renewal and differentiation. These cell fate processes are centered in a single mitotic event and a balance is needed in order to maintain the

stem cell pool. For example, studies in mammary stem cells and breast cancer have shown that p53 can regulate the asymmetric division of stem cells, where the absence of p53 can lead to an expansion of the stem cell pool through an increase in symmetric self-renewal while overexpression leads to stem cell depletion.[77,78] Our observations that MDM2 inhibitors cause a decrease in the cancer stem cell population while p53 knockdown causes an increase, led us to postulate that p53 signaling also regulates this balance in MEC. While MEC CSCs were depleted upon activation of p53 via inhibition of its binding to MDM2, this decrease was not accounted for by apoptosis. Surprisingly, most CSCs were in a G2/M cell cycle state indicating these cells might have longer G2/M cell cycle phases or might be in a quiescent state in G2. Few studies have reported on G2-quiescent stem cells and on their biological role.[192,193] Stem cells in quiescence are considered to be in a "poised state" and serve as a reservoir that protects against stem cell depletion.[194] Whether CSCs in MEC are in a similar G2-quiescent state needs further exploration. Importantly, inhibitors of the MDM2-p53 interaction shifted the cell cycle state of the CSCs towards G1. This G1 shift could be associated with an exit from G2/M-quiescence. An exit from G2/M-quiescence coupled with a loss in self-renewal could account for the decreased CSC fraction that we observed with therapeutic inhibition of the MDM2-p53 interaction. Furthermore, this shift was mediated through the p53-p21 signaling axis. As p21 has been implicated in differentiation of stem cells[195], our data suggested that p53 activation might lead to decreased CSC fraction by promoting differentiation of the CSCs.

Bmi-1 is an important regulator of stem cell self-renewal and is frequently used as a marker of stemness in different malignancies.[196] We detected a progressive loss of Bmi-

1 protein expression coupled with increased expression of pan-Cytokeratin (marker of epithelial differentiation) when MEC CSCs are treated with inhibitors of the MDM2-p53 interaction. This suggested that p53 activation caused cells to lose their stem-like state while acquiring a more differentiated phenotype. We corroborated the loss of stemness in MEC CSCs through sphere assays and found that p53 levels are an important regulator of the sphere forming ability of MEC cells and their self-renewal. Importantly, while p53 did not affect Bmi-1 mRNA levels, it decreased protein stability. It was previously reported that Bmi-1 and p53 can directly interact and that Bmi-1 can regulate p53 protein stability, but not the other way around.[36] This is the first time p53 signaling has been found to regulate Bmi-1 protein degradation, although the mechanism by which this occurs still needs elucidation. Nevertheless, we propose here that p53 regulates CSC fate through parallel signaling mechanisms that result in induced expression of p53 targets (*e.g*. p21) and increased Bmi-1 degradation.

Since CSCs are thought to be responsible for recurrence and metastasis, having abrogated p53 signaling can give an important advantage to cancer cells and can partly explain why cancers with high p53 mutations have higher recurrence and metastatic rates.[197,198] Importantly, it also strengthens the rationale for using p53-activating therapies for the treatment of cancers that usually have functional p53 signaling, such as mucoepidermoid carcinomas. To date, small molecule inhibitors of MDM2-p53 such as MI-773 have passed phase I safety clinical trials for several solid malignancies.[177] In these trials, although no objective response was observed, disease stabilization occurred in 58% of patients. Additionally, preclinical studies have shown therapeutic benefit of MI-773 treatment in salivary gland adenoid cystic carcinoma.[53,174] These results have led to

the recent approval of a Phase I/II trial of APG-115 (small molecule inhibitor of MDM2-p53) for patients with salivary gland tumors (NCT03781986). In support of the rationale for using this class of drugs in salivary gland MEC, we observed here that tumor recurrence was prevented in mice treated with MI-773 in an adjuvant setting. Tumors in mice were allowed to grow to a big size (800mm$^3$) before surgical resection to ensure our study was reflective of patients with advanced disease. Furthermore, a low maintenance regimen was given to these mice with just one weekly oral treatment of MI-773. These mice were followed for ~9 months to identify any delays in tumor recurrence. Our findings have major translational implications for the treatment of this orphan disease, as the most common reason of escape from therapy leading to MEC tumor progression and death is the high incidence of local and regional recurrences.

This work represents the first report on the role of p53 signaling in the pathobiology of salivary gland tumors. Our results give insight into the potential therapeutic benefits of small molecule inhibitors of MDM2-p53 for patients with salivary gland mucoepidermoid carcinomas. It also provides a strong rationale for the exploration of combination therapies that target both the cancer stem cells (*e.g.* small molecule inhibitor of MDM2-p53) as well as bulk tumor cells (*e.g.* conventional chemotherapy, radiotherapy) to treat patients with unresectable or advanced salivary gland tumors.

## 3.5 Methods

### 3.5.1 Cell culture and reagents

University of Michigan Human Mucoepidermoid Carcinoma cell lines (UM-HMC-1, UM-HMC-3A, and UM-HMC-3B)[22] were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Atlanta

Biologicals), 1% L-Glutamine (Gibco), 1% Antibiotic-Antimycotic (Gibco,), 400 ng/mL hydrocortisone (StemCell Technologies), 20 ng/mL recombinant human epidermal growth factor (rhEGF; R&D Systems), and 5 µg/mL recombinant human insulin (Sigma-Aldrich). Low passage primary human microvascular endothelial cells (HDMEC; Lonza) were cultured in endothelial growth medium-2 for microvascular cells (EGM-2 MV; Lonza). Small molecule inhibitors of MDM2-p53 interaction (*i.e.* MI-773 and APG-115) were gifted from Dr. Shaomeng Wang (University of Michigan).[173,188]

### 3.5.2 Animals

Female 236-CB17 SCID mice obtained from Charles Rivers were used to generate mucoepidermoid carcinoma xenografts. Mouse sex matched the original cell line donor.[22] All studies were performed according to the experimental protocols approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC), and all procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### 3.5.3 Lentiviral knockdown

Lentiviral particles were produced in HEK293T cells using the calcium phosphate method by co-transfecting pMD2.G and psPAX2 packaging vectors with either shRNA-control (pGIPZ scrambled or pLKO.1 scramble), p53 shRNA constructs on a pGIPZ backbone (University of Michigan Vector Core; seq #1: TACACATGTAGTTGTAGTG, seq #2: TAACTGCAAGAACATTTCT, seq #3: TACACATGTAGTTGTAGTG), or p21 shRNA constructs on a pLKO backbone (Sigma, seq #1: GACAGATTTCTACCACTCCAACTCGAGTTGGAGTGGTAGAAATCTGTC, seq #2: CGCTCTACATCTTCTGCCTTACTCGAGTAAGGCAGAAGATGTAGAGCG, seq #3:

GACACCACTGGAGGGTGACTTCTCGAGAAGTCACCCTCCAGTGGTGTC). The UM-HMC-1,-3A,-3B cells were infected with supernatant containing the lentiviral particles and with 4 µg/mL polybrene (Sigma-Aldrich) overnight. Infected cells were selected with 1 µg/mL puromycin (InvivoGen) for at least one week. pGIPZ constructs were subsequently sorted via FACS for GFP positive cells. Immunoblotting was used to verify p53 and p21 knockdown of the selected cells.

### 3.5.4 Cytotoxicity assays

Sulforhodamine B assays were used to measure the cytotoxicity of small molecule inhibitors of MDM2-p53 interaction in UM-HMC cells. Here, 800-1,000 cells/well were plated in 96-well plates and were exposed the following day to either vehicle, MI-773, or APG-115 for 24 to 72 hours. Cells were fixed in 10% trichloroacetic acid for 1 hour at 4ºC. After drying, plates were stained with a 0.4% Sulforhodamine B solution (Sigma Aldrich) at room temperature for 30 minutes. Unbound dye was washed away with 1% acetic acid. The absorbed dye was resolubilized in 10 mM unbuffered Tris base and the plates were read in a microplate reader at 565 nm (GENios, TECAN). Results were normalized to vehicle control and $IC_{50}$ values were calculated using the nonlinear fit of variable slope function in GraphPad PRISM. All conditions were evaluated in triplicate and results are representative of at least two independent experiments.

### 3.5.5 Salisphere assays

UM-HMC cells were cultured in ultra-low attachment (ULA) plates or flasks (Corning) in DMEM/F12 (Gibco) supplemented with 1% N2 Supplement (Gibco), 1% Glutamax (Gibco), 1% antibiotic-antimycotic (Gibco), 20 ng/mL rhEGF (R&D Systems), 20 ng/mL recombinant human basic FGF (R&D Systems), 10 ng/mL recombinant human insulin

(Sigma-Aldrich), and 1 μM Dexamethasone (Sigma-Aldrich). 2-4,000 cells/well were plated in 6-well ultra-low attachment plates and treated the following day with either MI-773 or APG-115, unless otherwise stated. Secondary salispheres were generated by dissociating primary spheres into single cell suspensions with Accutase (StemCell Technologies) and plating 4,000 cells/well in 6-well ultra-low attachment plates. Salispheres were defined as non-adherent spheres containing ≥30 cells. All conditions were evaluated in triplicate and results are representative of at least two independent experiments.

### 3.5.6 Single-cell microfluidic spheres

The microfluidic sphere culture chip was fabricated with a patterned PDMS (polydimethylsiloxane, Sylgard 184, Dow Corning) bonded to another piece of blank PDMS. Standard soft-lithography process was used to pattern PDMS piece. A silicon wafer patterned with SU8 photoresist (MicroChem) was used as mold for soft lithography. The mold was created by a 3-layer photolithography process with a 5 μm-thick-layer for cell capture site, a 40 μm-thick-layer for meander escape channel, and a 100 μm-thick-layer for sphere chambers and flow channels, following the protocol described in the previous work.[199] 25 grams of PDMS reagent was poured on silicon mold. After curing at 100 °C for 1 day, the PDMS piece was peeled off. Then, an inlet and an outlet were created using a biopsy punch. Finally, the patterned PDMS pieces were activated by oxygen plasma treatment (80 Watts, 60 seconds) and bonded to another piece of blank PDMS. The microfluidic chips were sanitized using UV radiation and primed using a 5% (w/w) PEO-terminated triblock polymer (Pluronic® F108, BASF) 1 day before usage.

The sphere culture chambers were designed to be 150 μm × 150 μm × 150 μm to provide enough room for sphere growth. A 40 μm-high meander escape channel was designed at the end of main flow channels to release the residue flow and avoid multiple cell capture cases. Sphere culture media was exchanged every 24h. Microfluidic device images were taken right after cell loading, as well as on day 6 and day 12, to keep track of sphere growth. The cells were stained with CellTracker green (ThermoFisher, C2925) for automated sphere size analysis with a Matlab program.

### 3.5.7 Flow cytometry

All flow cytometry analysis was conducted in a BD LSRFortessa instrument. An iCyt Synergy SY3200 cell sorter was used for fluorescence-activated cell sorting (FACS) at the University of Michigan Flow Cytometry Core. Staining for ALDH enzymatic activity was carried out using the ALDEFLUOR™ Kit (StemCell Technologies) or the ALDERED™ ALDH Detection Assay (EMD Millipore). For analysis of the cancer stem cell fraction *in vitro*, $5x10^5$ cells were incubated with 2.5 μL of activated Aldefluor or Aldered substrate in 250 μL of buffer at 37°C for 40 minutes. DEAB controls for all treatment conditions were included. After incubation, cells were washed with PBS and subsequently stained for CD44 using one of the following antibodies: CD44-PE, CD44-APC, CD44-BV450 (R&D Systems), or CD44-APC-Cy7 (BioLegend). CD44 staining was done at 4°C for 15-20 minutes. Cells were washed with PBS and DAPI was added for live/dead discrimination. To look at the proportion of cancer stem cells undergoing apoptosis, cells stained for ALDH and CD44 were subsequently incubated with 10 μL of Annexin V-PE in 100 μl of 1X binding buffer (BD Biosciences) for 15 minutes at room temperature. Staining was immediately quenched with 200 μL of 1X binding buffer and DAPI was added. To

analyze cell cycle of the $ALDH^{high}CD44^{high}$ cells, $1.5x10^6$ cells/tube were co-incubated with 7.5 µL of activated Aldefluor substrate and 3 µL of Vibrant DyeCycle-Orange Stain (Invitrogen) in 1 mL of PBS (Gibco) for 35 minutes at 37°C. As previously described, DEAB was used as a negative control for Aldefluor. After incubation, cells were spun down at 800rpm for 5 min, the supernatant was removed, and cells were resuspended in 2.5 µl anti-human CD44-APC-Cy7 in 300 µL PBS for 15 minutes at 4°C. DAPI was added right after incubation and cells were taken immediately for flow cytometry analysis. All conditions were evaluated in triplicate and results are representative of at least two independent experiments.

### 3.5.8 Western blot

Whole-cell lysates from UM-HMC cells were prepared using a 1% Nonidet P-40 (NP-40) lysis buffer. Lysates were loaded onto 9-15% SDS-PAGE gels for protein separation. Proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences) and probed with the following primary antibodies: mouse anti-p53, mouse anti-MDM2, HRP-conjugated anti beta-Actin (Santa Cruz Biotechnology), rabbit anti-p21, and rabbit anti-Bmi-1 (Cell Signaling). Membranes were exposed to HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Immuno Research Laboratories) and proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

### 3.5.9 Bmi-1 protein stability

Cells were pre-treated with DMSO vehicle control or 10 µM MI-773 for 3 hours before being treated with 25-50 µM Cycloheximide to stop protein synthesis. Whole cell lysates were generated at different timepoints using an NP-40 lysis buffer and subsequently

analyzed through western blot. Protein half-life was calculated using one-phase decay function in GraphPad PRISM.

### 3.5.10 RT-PCR

RNA was isolated from cells with *Quick*-RNA Miniprep (Zymogen). 1 µg of RNA was used to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) and PCR was performed with Platinum Taq DNA Polymerase (Invitrogen). The following primers were used to generate the PCR products: Bmi-1 (Sense 5'- CAGCGGTAACCACCAATCTT -3', Antisense: 5'- AAAGTCTTGCCTGCTTTCCA -3'), p21 (Sense: 5'- AGTCAGTTCCTTGTGGAGCC -3', Antisense: 5'- GAAGGTAGAGCTTGGGCAGG -3'), and GAPDH (Sense: 5′- GACCCCTTCATTGACCTCAACT -3′, Antisense: 5′- CACCACCTTCTTGATGTCATC -3′). RT-PCR products were verified through gel electrophoresis.

### 3.5.11 Immunohistochemistry

Paraffin embedded tissue section slides were incubated with Trypsin (Sigma) at 37°C for antigen retrieval followed by 0.1% Triton-x100 (Sigma) at room temperature. Endogenous peroxidase activity was inhibited by incubating with 3% hydrogen peroxide (Fisher) and nonspecific background antibody binding was blocked using Background Sniper (Biocare Medical) at room temperature. Slides were incubated overnight at 4°C with mouse anti-ALDH (BD Transduction). Following incubation, sections were washed with 1X Immunohistochemistry (IHC) Wash Buffer (Dako) and afterwards incubated with MACHI 3 Probe (Biocare Medical). Sections were washed again with 1X IHC wash buffer followed by incubation with MACH 3 HRP (Biocare Medical) and washed again with IHC wash buffer. DAB peroxidase substrate (Biocare Medical, Concord, CA, USA) was used for

chromogenic development at room temperature. Finally, sections were incubated with hematoxylin, dehydrated, and permanent mounting solution (Vectamount, Vector) was used to fix the slide coverslip on the stained tissue section.

### 3.5.12 Immunocytochemistry

Cells were plated in 4-well chamber slides and incubated overnight before any treatment. Cells were fixed in formaldehyde/glutaraldehyde. After fixation, chambers were removed from the slides and slides were incubated first with 0.1% Triton-X 100 (Sigma), followed by 3% hydrogen peroxide, then background sniper (Biocare Medical), and finally overnight at 4ºC with one of the following primary antibodies: mouse anti-pan-cytokeratin (Santa Cruz) and rabbit anti-Bmi-1 (Cell Signaling). The following day, slides were washed with 1X IHC wash buffer and incubated with fluorophore-conjugated secondary antibodies (Life Technologies) for 1 hour at room temperature. Slides were washed again with 1X IHC wash buffer and coverslip was placed with DAPI-containing mounting solution (Vectashield, Vector). Imaging was achieved using an inverted Leica SP5 confocal microscope (Leica microsystems; Germany). To excite fluorescence in all channels, the 405 nm laser and the tunable white light laser were used. The detectors spectral range were set to: blue spectral range from 415 to 478 nm; green spectral range from 498 to 550 nm and red spectral range was from 571 to 727 nm bandpass. Channels were acquired sequentially between lines. Scanning speed was set to 50 Hz in bidirectional mode with line average set to 2. All Images were recorded in 1,024 x 1,024 format and pinhole size was set to 60 µm. Image analysis was conducted using Fiji[200]. The mean gray values of our channels of interest, reported as Mean Fluorescence Intensity, were measured using the built in “Measure” function in Fiji. For nuclear

quantification, the “Make Binary” function from Fiji was used in the Dapi channel and the resulting ROIs were used to quantify per cell nuclear expression of our channel of interest.

### 3.5.13 Mucoepidermoid carcinoma xenografts (subcutaneous)

Mucoepidermoid carcinoma subcutaneous xenograft tumors were generated, as we described.[171] In brief, poly-L-lactic acid scaffolds were seeded with 600,000 UM-HMC-3A or UM-HMC-3B cells with or without 400,000 primary human HDMEC cells (Lonza) in a cell growth media and Matrigel (Corning) mix. The scaffolds were implanted subcutaneously in the dorsal region of CB17 SCID mice (Charles River). Tumor measurements were taken along the x and y axis using a caliper and volumes were calculated using the equation $V=(height*width^2)/2$. For tumor growth studies, mice were monitored twice a week until tumor volumes reached 2 $cm^3$, at which point all groups were euthanized. Outlier criteria for mouse tumors was defined as tumor volumes greater than two standard deviations away from the group mean.

### 3.5.14 Mucoepidermoid carcinoma xenografts (orthotopic)

Orthotopic xenograft tumors were generated by injecting 500,000 UM-HMC-3A or UM-HMC-3B cells into the submandibular glands of CB17 SCID mice. Mice were euthanized 80 days post-injection or upon significant adverse events such as weight loss. For flow cytometry analysis of the cancer stem cell population, tumor tissues were digested using collagenase (StemCell Technologies) and single cell suspensions were stained for ALDH and CD44 as described above.

### 3.5.15 Statistical analysis

All statistical analysis was done using GraphPad PRISM. Two-tailed student’s *t*-test was conducted on two group comparisons and analysis of variance (ANOVA) with multiple-

comparisons post-hoc Tukey or Bonferroni was conducted on comparisons between more than two groups. All *in vitro* results were done with a minimum of three biological replicates and represent at least two independent experiments. Kaplan-Meier graphs were analyzed using the Gehan-Breslow-Wilcoxon test. Significance level was set at $P<0.05$.

# CHAPTER 4. Conclusions

## 4.1 Summary of Research Findings

Despite being the most common type of salivary gland cancer in both adults and children, mucoepidermoid carcinoma (MEC) is a poorly understood malignancy. There have been no significant advances for its treatment in over two decades. Therapies have been limited to radical head and neck dissections and radiation that cause severe patient morbidities. Little is known about MEC etiology and pathobiology, but it is thought to arise from stem cells in the excretory duct of the salivary gland.[45] New genomic studies have found *TP*53 genomic alterations (GAs) to be the second most common GA in MEC tumors.[46,154] These GAs are found only in intermediate and high-grade disease. Nevertheless, p53 is wild-type in 60-70% of MEC patients making p53 activating therapeutics promising for the treatment of this disease.

CSCs are chemoresistant and are responsible for tumor recurrence and metastasis in head and neck cancers. Cancer stem cells (CSCs) in MEC are marked by high ALDH enzymatic activity and CD44 cell-surface expression ($ALDH^{high}CD44^{high}$). These cells are highly tumorigenic, have the ability to self-renew, and can recapitulate the cell heterogeneity in a tumor.[171] This thesis explored the functional roles of p53 in regulating CSC maintenance in MEC.

Our work stemmed from the observation that MI-773, an inhibitor of the MDM2-p53 interaction, decreases the CSC population in MEC cell lines and xenograft tumors.

Importantly, we found that this decrease occurred after 48 hours of treatment. Given apoptosis commonly occurs 12 - 24 hours after exposure to death inducing signals, we believed that the delayed decrease in the CSC population was not due to p53-dependent apoptosis. Furthermore, we found a decrease in Bmi-1 protein levels before a decrease in the CSC population was evident, indicating that inhibition of the MDM2-p53 interaction could be affecting stem cell self-renewal, thus leading to an exhaustion of the stem cell pool.

Using genetic silencing of p53, we confirmed that the accumulation of p53 protein and activation of subsequent downstream signaling was responsible for the decrease in the CSC population. Furthermore, we found that both orthotopic and subcutaneous xenograft tumors generated with p53 knockdown cells grew faster and had a higher fraction of CSCs than those generated with control cells. These results indicated that regulation of p53 protein levels is important in CSC maintenance.

The tumor suppressor p53 is a master regulator of cell survival and cell fate decisions. To understand the functional role of p53 in CSC biology, we first explored the effects of p53 activation on apoptosis and cell cycle. Importantly, we found no induction of apoptosis that could account for the decrease in CSCs seen by p53 activation. Meanwhile, we found MEC CSCs have a different cell cycle profile than bulk cancer cells and that this profile changes when p53 is activated. We found that while most bulk cells are in a G1 cell cycle state at a given time, the CSCs are predominantly in a G2/M state. Whether this G2/M state indicates that the CSCs have a longer G2/M cell cycle phase or whether they are quiescent in G2 needs further evaluation. Once p53 signaling is activated, there is a shift in the cell cycle profile of the CSCs towards G1, resembling the

cell cycle state of the bulk cell population. This suggests that the CSCs are differentiating as they cycle, thus losing their characteristic cell cycle profile and resembling the nonCSCs. In agreement with this, we found that while p53 activation decreases Bmi-1 protein levels in the CSCs, it increases the expression of pan-cytokeratin. Pan-cytokeratin is used as a marker of differentiation, as it is differentially expressed between the CSC and nonCSC populations. Moreover, we found that this cell-cycle shift was mediated through the p53-p21 signaling axis. However, we found that this signaling axis does not regulate Bmi-1 protein levels. This indicates that p53 may be regulating the CSC population through parallel signaling pathways of self-renewal and differentiation (*i.e.* p53-Bmi-1 and p53-p21).

We found that activation of p53 leads to decreased sphere forming ability and increased self-renewal of MEC cells. Additionally, we found that Bmi-1 protein levels are upregulated when p53 is knocked down. On the other hand, p53 upregulation leads to decreased Bmi-1 protein stability. This is the first time p53 signaling has been found to regulate Bmi-1 protein degradation, however the mechanism by which this occurs still needs elucidation.

Finally, we assessed the therapeutic potential of activating p53 using MDM2 inhibitors and found that mice treated with MI-773 had fewer tumor recurrences then vehicle controls. These findings provide a strong rationale for the use of MDM2 inhibitors for the treatment of MEC. Importantly, it leads us to further explore the therapeutic potential of MDM2 inhibitors for patients with advanced disease.

## 4.2 Future Works and Conclusions

Several unanswered questions remain when identifying the molecular mechanism by which p53 regulates cell fate decisions in MEC CSCs. Understanding the cell cycles of the CSCs vs nonCSCs remains an area of further exploration. We propose that by using the FUCCI cell cycle reporter system, we can track cell divisions in the CSCs using live-cell imaging. This will allow us to measure the cell cycle phases of the CSCs and distinguish between proliferating and quiescent cells. One limitation of studying MEC CSCs is having to rely on ALDH enzymatic assays for their detection. These assays are incompatible with live-cell imaging systems, making it impossible to identify CSCs and their progeny. For this reason, a reliable ALDH reporter system will be essential for identifying CSCs and being able to track the polarity of cell division events.

Although we looked at the potential of MDM2 inhibitors to prevent tumor recurrence in pre-clinical studies of MEC, we did not look at whether these inhibitors have other anti-tumor effects. Preliminary studies show that MDM2 inhibitors are capable of causing tumor regression as a first-line therapy when given in frequent dosing in mice (Figure 4.1, a, c). However, we find that tumors continue to grow once treatment is stopped (Figure 4.1, c). We know that there is plasticity in the CSC-state and that environmental cues can cause cells to acquire or loose these CSC-characteristics. Our results indicate that monotherapy is not sufficient to cause a durable response in MEC tumors.

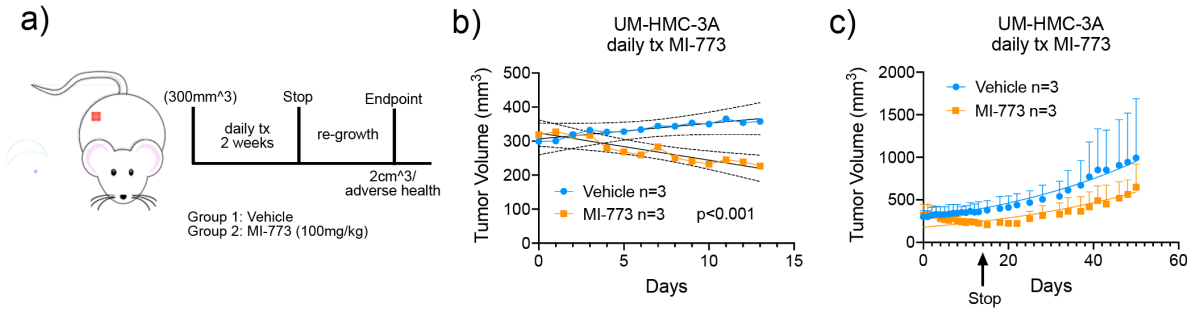


**Figure 4.1. Tumor regression with MI-773 treatment. a** UM-HMC-3A subcutaneous xenograft tumors were allowed to grow to ~300mm$^3$ before starting daily oral gavage treatments of vehicle or MI-773 (100mg/kg) for two weeks. Tumor growth was monitored until tumor endpoint of 2mm$^3$ or adverse health events. **b** Average tumor volumes throughout duration of treatment showed tumor regression with MI-773 treatment. Tumor volumes were fitted with a simple linear regression model including 95% confidence intervals. **c** Tumor volumes were monitored after cessation of treatment and fitted with an exponential growth rate curve.

In order to effectively eradicate all cancer cells, tumors must be treated with a dual-approach by using agents that target bulk cancer cells together with CSC-specific agents (Figure 4.2). This thesis discussed the potential of combining p53-activating therapies with standard chemotherapies for head and neck cancers. Previous studies conducted in our lab showed that short-term cisplatin treatment (< 60 days) does not cause significant tumor regression of MEC xenografts (Figure 4.2, b). We conducted a preliminary study comparing MI-773 monotherapy to combination therapy with cisplatin and found a significantly better response in combination treatment (Figure 4.2, c-f). A more complete study must be made to compare the effectiveness of mono- vs. combination therapies for the treatment of advanced MEC. Nevertheless, these results seem promising for patients with unresectable and advanced disease.

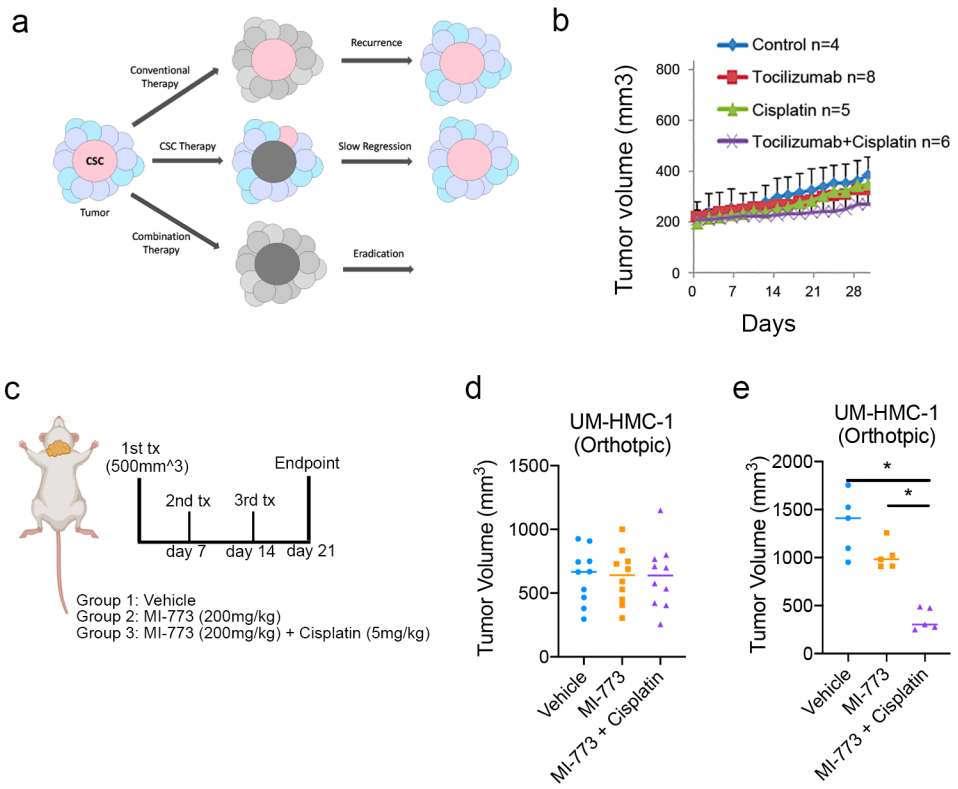


**Figure 4.2. Combination therapy. a** Cancer stem cells (CSCs) are resistant to conventional chemotherapy and radiation therapy leading to tumor recurrence. CSC-therapy alone will eliminate the CSCs, but because of the plasticity of the CSC-state the remaining bulk cells can become new CSCs. To completely eradicate a tumor, combination therapy targeting both the CSCs and the bulk tumor cells must be used. **b** Graph depicting tumor volume over time of tumors treated weekly with IgG Control (5mg/kg), Tocilizumab (5mg/kg), Cisplatin (15mg/kg), Tocilizumab + cisplatin. **c** Mice with orthotopic tumors were randomly distributed into groups and treated once weekly with vehicle, MI-773 (200mg/kg) alone, or combination MI-773 (200mg/kg) + cisplatin (5mg/kg). **d** Tumor volumes before treatment. **e** Tumor volumes at study endpoint (day 21). (Panel (**b)** was modified from Mochizuki *et al.*)[201]

In conclusion, this thesis identified p53 as an important regulator of cell fate decisions in MEC CSCs (Figure 4.3). Furthermore, it confirms that loss of p53 signaling contributes to disease progression in MEC tumors. Importantly, it demonstrates the clinical utility of using p53-activating therapies for the treatment of MEC. Finally, it provides a rationale for the use of combination therapy to target CSCs and bulk tumor cells as a form to effectively eradicate cancer cells and reduce tumor recurrence rates in patient with advanced MEC.

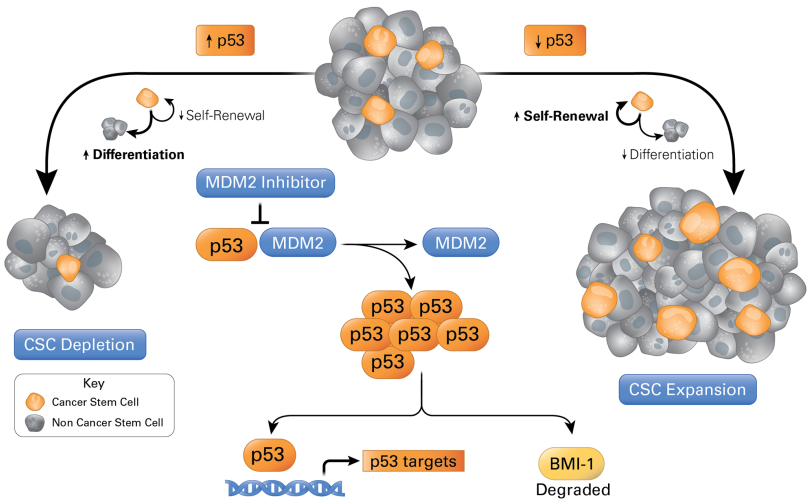


**Figure 4.3. Thesis Summary.** p53 activation can be achieved by using small molecule inhibitors that block the interaction between p53 and its negative regulator MDM2. This leads to accumulation of p53 protein and activation of downstream signaling such as transcriptional activation of p21 and regulation of BMI-1 protein stability. Downregulation of p53 results in increased cancer stem cell self-renewal leading to an expansion of the cancer stem cell population and increased tumor growth. Meanwhile, p53 activation results in decreased cancer stem cell self-renewal and increased differentiation resulting in depletion of the cancer stem pool.

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# APPENDICES

## Appendix 1: Author Contributions

CHAPTER 1

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