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Functional Role of the Homeobox Transcription Factor Six1 in Neoplastic Transformation of Human Keratinocytes

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FUNCTIONAL ROLE OF THE HOMEOBOX TRANSCRIPTION FACTOR SIX1 IN NEOPLASTIC TRANSFORMATION OF HUMAN KERATINOCYTES

By

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DEDICATION

To my Mom and Dad:

Yesterday I was clever, so I wanted to change the world. Today I am wise, so I am changing myself… I ran from what was comfortable, and forgot safety. I lived where I feared to live, and destroyed my reputation. In the end, I became notorious.

Thank you.

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stopping in the hallways and talking to me. I enjoyed each and every conversation and will deeply treasure the memories and laughter.

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To all my lab members who have come and gone, thank you. Elena, Susannah and Carolyn- I will miss our lunches. Susannah- thank you for teaching me a lot of the skill sets that I have now developed. Dr. Phillip Buckhaults and Dr. Carolyn Banister- thank you for all the fun moments, allowing me to use the laboratory and training me whenever possible. Fadi, Justin, Geraldine, Erica and Rupa- thank you for all the help that you have provided me with over the years. Kiona- thank you for believing in me and making sure my head was always held up high. Diego- you provided me with guidance on our microarray studies and my western blot technique. Thank you for being kind and patient with me. I always felt comfortable in your presence and you were the tough, but caring, critic that I needed during my PhD studies. Swati- you taught me everything I know as a doctoral student in Lucia's lab. Hanwen- you taught me everything I know about SIX1. Yvon- Thank you for the deep conversations and the always insightful advice. I will never forget what you told me when we were both down in our luck as PhD students, "Never forget that we are truly blessed to be in Lucia's lab, because no other mentor could have possibly been more understanding, caring or encouraging as her. We both have gone through a lot and I cannot see any other professor giving us all the time we

need to complete our projects, grow as scientists and nurture us as if we were her own children and not just students in her lab."

Last but never the least, I would like to thank my family and close friends. Without their unconditional love and support, I would not have been able to achieve this great moment in my life. To my mom, dad, sister, Aunt Helen and grandmother Shahin- without them I would not be chasing my dreams and striving to excel on a daily basis. I feel very blessed to have them all in my life.

To the people I cherish and look up to. I dedicate this piece of work to them. To my role models whose eternal spirit is in a far better place than here on Earth- Dr. Raja Fayad and Dr. Maryam Mirzakhani.

ABSTRACT

The homeobox transcription factor SIX1 contributes to both tumor development and progression. Numerous studies have determined that the inappropriate expression of embryonic genes, in particular transcription factors, contributes to carcinogenesis. SIX1 is essential for the development of numerous organs including the auditory and olfactory system as well as the kidney, by promoting proliferation, survival and migration of progenitor cells during embryogenesis. SIX1 has also been shown to increase cancer cell proliferation, survival and invasion. The aberrant expression of SIX1 occurs in numerous adult and pediatric cancers. We have previously determined that our *in-vitro* model system for HPV16-mediated tumorigenesis shares many important features with cervical cancer and enables us to study the molecular mechanisms of transformation and immortalization in our cell human keratinocyte (HKc) lines. SIX1 mRNA and protein levels are overexpressed in our HPV16-transformed HKc lines at the differentiation-resistant stage (HKc/DR) compared with early passage, HPV16-immortalized HKc (HKc/HPV16) and in HKc/HPV16 compared to normal HKc. Furthermore, we have recently determined that SIX1 overexpression in HKc/HPV16 induces the differentiation-resistant phenotype characteristic of HKc/DR, and that SIX1 overexpression in HKc/DR induces tumorigenicity. In this study, we explored the role of SIX1 as a regulator of growth and transformation in normal HKc, and its role in the maintenance of growth and

a transformed phenotype in HKc/HPV16 and HKc/DR. We determined that loss of SIX1 is not tolerated by HKc/DR, which appear to be "addicted" to this oncogene. Decreased SIX1 expression results in slower proliferation and decreased HPV16 E6/E7 mRNA levels. We utilized Affymetrix GeneChip Arrays to explore the gene expression changes associated with decreased SIX1 expression in HKc/DR. Ingenuity Pathway Analysis, real-time PCR and functional cell-based assays determined that SIX1 is vital for cell survival; the decline in SIX1 causes a transition from the mesenchymal phenotype characteristic of HKc/DR towards the standard epithelial phenotype (mesenchymal-epithelial transition, MET). MET is accompanied by a switch in TGF-β signaling from an EMT-inducing tumor promoter to a tumor suppressor in HKc/DR cells. Additionally, we observed that SIX1 overexpression in normal HKc extends their lifespan and induces epithelial-mesenchymal transition, EMT. In summary, our studies suggest that SIX1 is necessary for cell survival in HPV-16 –transformed cells and may potentially become a suitable therapeutic target for HPV-driven cancers.

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LIST OF SYMBOLS

*	p values ≤ 0.05
**	p values ≤ 0.01
***	p values ≤ 0.001

LIST OF ABBREVIATIONS

BME ..... Basement Membrane Extract

BOR ..... Branchio-oto-renal Syndrome

BPE ..... Bovine Pituitary Extract

CCK8 ..... Cell Counting Kit-8

CCN2 ..... Connective Tissue Growth Factor

CIN ..... Cervical Intraepithelial Neoplasia

COX-2 ..... Cyclooxygenase-2

CRC ..... Colorectal Cancer

CSC ..... Cancer Stem Cell

CTGF ..... Connective Tissue Growth Factor

CTNNβ1 ..... β-catenin

DACH ..... Drosophila Dachshund (dac) Gene

ddPCR ..... Droplet Digital PCR

DMEM ..... Dulbecco's Modified Eagle's Medium

DR ..... Differentiation Resistant

EGF ..... Epidermal Growth Factor

EGFR ..... Epidermal Growth Factor Receptor

ELISA ..... Enzyme-linked Immunosorbent Assay

EMT ..... Epithelial-Mesenchymal Transition

ETS1 ..... ETS Proto-Oncogene 1, Transcription Factor

EYA ..... Eyes Absent Protein

FBS .....Fetal Bovine Serum

GAPDH.....Glyceraldehyde 3-Phosphate Dehydrogenase

gDNA..... Genomic DNA

GFI..... Growth Factor Independent

GFP .....Green Fluorescent Protein

GUSB..... Glucuronidase-beta

HFb .....Human Fibroblast

HFb/ALL3.....HFb transfected with human *SIX1* gene, Ras-V12 plasmid, p53i shRNA

HFb/SIX1.....HFb transfected with human SIX1 gene

HKc..... Human Keratinocyte

HKc/ALL3 ..... HKc transfected with human *SIX1* gene, Ras-V12 plasmid, p53i shRNA

HKc/SIX1 ..... HKc transfected with human SIX1 gene

HPV.....Human Papilloma Virus

HPV16..... Human Papilloma Virus Type 16

HRas.....Harvey Rat Sarcoma Viral Oncogene Homolog

HRas-V12 ..... HRas mutation replacing glycine with valine at position 12

HSV2.....Herpes Simplex Virus 2

IPA ..... Ingenuity Pathway Analysis

K14.....Keratin 14

K15.....Keratin 15

K19.....Keratin 19

KSFM..... Keratinocyte Serum-free Medium

MET .....Mesenchymal-Epithelial Transition

MMP1 ..... Matrix Metallopeptidase 1

MMP9 ..... Matrix Metallopeptidase 9

OCLN.....Occludin

p53i ..... p53 shRNA-based vector

PANC-1.....human Pancreatic Adenocarcinoma Cell Line 1

Pap.....Papanicolaou smear test

PPARγ.....Peroxisome Proliferator Activated Receptor Gamma

PTGS2.....Prostaglandin-endoperoxide Synthase 2

RDGN ..... Retinal Determination Gene Network

RPL26 ..... Ribosomal Protein L26

shRNA..... short-hairpin RNA

siRNA .....small (or short) interfering RNA

SIX .....Sine Oculis Homeobox Homolog Family Proteins

SIX1 ..... Sine Oculis Homeobox Homolog 1

STD .....Sexually Transmitted Disease

TAC..... Transcriptome Analysis Console

TβRI ..... Transforming Growth Factor-beta Receptor Type I

TβRII.....Transforming Growth Factor-beta Receptor Type II

TβRIII ..... Transforming Growth Factor-beta Receptor Type III

TGF-α..... Transforming Growth Factor-alpha

TGF-β..... Transforming Growth Factor-beta

Vim ..... Vimentin

CHAPTER 1

INTRODUCTION

1.1 SIX1 IN NORMAL AND HPV-16 TRANSFORMED HUMAN KERATINOCYTES

The retinal determination gene network (RDGN) was first discovered in *Drosophilia* eye formation and, over the years, has become a model system for analyzing the genetic and molecular mechanisms of cell fate determination. RDGN is composed of dachshund (*dac*/ Dach), tyrosine phosphatase eyes absent (*eya*/ Eya) and the SIX family sine oculis (*so/* SIX) (Liu *et. al.*, 2016). Genes from this network are highly conserved during evolution and encode nuclear transcription factors and cofactors that regulate morphogenesis in mammals. This network is aberrantly expressed in several pediatric and adult cancers: Eya and SIX genes are known to be upregulated and Dach genes are shown to be downregulated (Liu *et. al.*, 2016).

SIX1 belongs to the SIX superfamily of homeobox genes, which encode transcription factors. These proteins have an essential role in organogenesis and tumorigenesis through the activation and inhibition of numerous downstream targets. The SIX superfamily are homologous to Drosophilia *sine oculis (so), optix* and *DSix4* family of genes. The SIX family members in flies have homologs in many organisms within the animal kingdom, including mammals. Analysis of the SIX gene set within vertebrates has

shown that each of the SIX1 gene has two members of each subclass: Six 1/2 *(sine oculis, so), Six 3/6 (optix)* and *Six 4/5 (DSix4)* (Kumar *et. al.*, 2009).

The SIX family members are DNA-specific transcription factors that consist of two evolutionarily conserved domains. DNA binding is elicited through the homeobox nucleic acid recognition domain (HD), which are 60 amino acids in length. The SIX domain (SD) is directly adjacent to HD, 146 amino acids in length and contributes to protein-protein interactions (Kumar *et. al.*, 2009). SIX1 is ubiquitously expressed in humans and plays a critical role in the development of major organ systems including the muscle, kidney, brain, auditory and olfactory. SIX1 utilizes cofactors, coactivators and corepressors to regulate the transcription of downstream targets. In the absence of Eya, Dach regulates SIX1 to suppress gene expression. On the other hand, Eya phosphatase recruits coactivators to initiate transcription of target genes (Kumar *et. al.*, 2009).

Numerous studies have highlighted the importance of SIX1/EYA in development and disease, including cancer. For example, mutation of the SIX1 gene causes the human disease brancchio-oto-renal (BOR) syndrome. Classified as a developmental disorder, BOR syndrome is clinically characterized with hearing loss and malformations of the kidney and/-or urinary tract. Specifically, researchers have observed one SD mutation as well as two HD mutations that affect SIX1-EYA interaction and SIX1- DNA binding (Ruf *et. al.*, 2004) Overall, targeting this transcriptional complex will benefit patients in the clinical setting.

SIX1 is a master regulator of development where it plays a role in normal organogenesis. Numerous studies have identified that the inappropriate expression of

embryonic genes in cancer, in particular transcription factors, contribute to carcinogenesis (Abate-Shen *et. al.*, 2002). Elevated SIX1 levels in malignant cells is sufficient to increase cellular proliferation, survival and invasion, similar to what is observed during embryonic development (Christensen *et. al.*, 2008). In breast cancer cell lines, gene amplification is a type of mechanism shown to increase SIX1 expression (Reichenberger *et. al.*, 2005). The aberrant expression of SIX1 occurs in numerous adult human cancers, including breast, ovarian, cervical and hepatocellular carcinomas, and pediatric malignancies including rhabdomyosarcoma and Wilms' tumor (Coletta *et. al.*, 2010).

SIX1 overexpression has been shown to correlate with increased malignancy, lymph node metastasis and poor survival in cancer patients (Coletta *et. al.*, 2008; Micalizzi *et. al.*, 2009). For example, studies have established that the overexpression of SIX1 in immortalized, non-tumorigenic mammary epithelial cells induces malignant transformation, leading to highly aggressive and invasive tumors in nude mice. Studies have demonstrated that SIX1 is overexpressed in half of primary mammary carcinomas and most metastatic lesions (Coletta *et. al.*, 2008). Along the same lines, SIX1 overexpression in human breast cancer cell lines induces epithelial-mesenchymal transition (EMT), enhancing metastasis *in-vitro* and *in-vivo* (Coletta *et. al.*, 2008; Micalizzi *et. al.*, 2009). SIX1 has also been shown to be overexpressed in cervical cancer cell lines and tissues, which are correlated with increased malignancy and metastasis (Tan *et. al.*, 2011; Zheng *et. al.*, 2010). SIX1-induced EMT is associated with Smad-dependent transforming growth factor-beta (TGF-β) signaling and increased expression

of TGF-β receptor type I (TβRI), allowing SIX1 to induce lymphangiogenesis and metastasis by up-regulating VEGF-C, a lymphangiogenic factor (Wang *et. al.*, 2012).

1.2 EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

SIX1 overexpression is associated with tumor initiation, progression and metastasis. Whole genome sequencing of tumor tissues implicate SIX1 in its ability to decrease epithelial-related genes and increase mesenchymal-related genes and that SIX1-induced tumors undergo EMT, primarily depending on the activation of TGF-β and p38-MAPK (Xu H *et. al.*, 2014; Xu H *et. al.*, 2015). Through the interaction of SIX1 with Wnt and mitogen-activated protein kinase (MAPK)/ERK signaling, tumors are able to attain EMT and stem cell-like features (Xu H *et. al.*, 2014).

Epithelial and mesenchymal cell phenotypes are established early in normal development. However, these two cell phenotypes are highly dynamic and constantly in motion, depending on the cell type and environment. The conversion between these cell phenotypes, mechanistically labelled as Epithelial-Mesenchymal Transition (EMT) and the reverse Mesenchymal-Epithelial Transition (MET), provides elasticity during embryogenesis, wound healing and regeneration of differentiated tissues (Micalizzi *et. al.*, 2010). During developmental EMT, there are highly organized and well-defined multi-step events that allow the transition of immotile, polarized and cuboidal epithelial cells to gain a motile, apolar and fibroblastic phenotype. During carcinogenesis, epithelial cells undergoing EMT acquire resistance to suspension-induced cell death (anoikis) with the subsequent increase in cellular migration and invasion. This allows them to efficiently

respond to extracellular signals, involving extracellular matrix (ECM) components and soluble growth factors including transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF) (Lee *et. al.*, 2012; Micalizzi *et. al.*, 2010). The reverse holds true for mesenchymal cells where they are able to begin MET (Figure 1.1) (Micalizzi *et. al.*, 2010).

Cancer-associated EMT has been observed in several malignancies including breast, cervical and colon. EMT becomes exaggerated in malignant cells and is associated with the downregulation of epithelial markers, such as E-cadherin, and the upregulation of mesenchymal markers, such as N-cadherin and Vimentin- the major hallmarks in the EMT cascade (Lee *et. al.*, 2012; Micalizzi *et. al.*, 2010). The metastatic spread of malignant cells occurs in a multi-stepwise fashion, following EMT. Tumorigenic cells leave their primary site, invade the basement membrane, intravasate into the local lymphovascular, disseminate through the circulation, and extravasate into a secondary site, thereby colonizing distant organs, depending on the cancer type, setting the stage for metastasis (Figure 1.2) (Micalizzi *et. al.*, 2010). Additionally, numerous regulators of EMT have been misexpressed in cancers such as TGF-β, Wnt, Snail/Slug, Twist and SIX1. Potentially, these and various other transcription factors appear to have a clinical significance and have been recently identified to be prospective novel drug targets (Lee *et. al.*, 2012; Micalizzi *et. al.*, 2010).

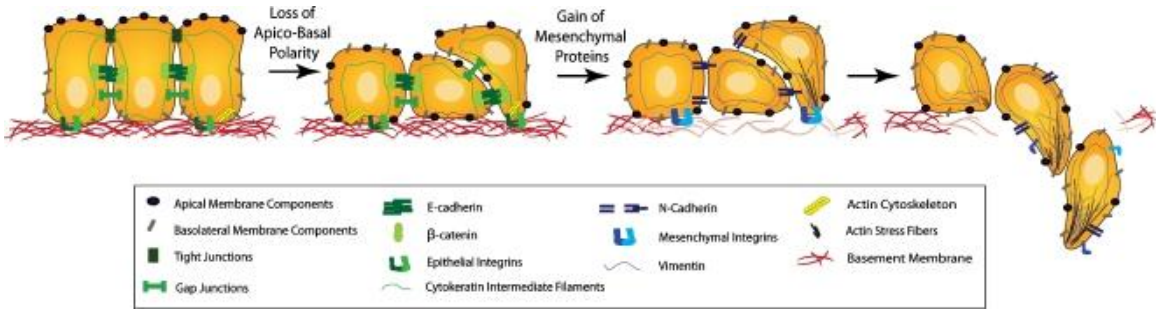


Figure 1.1 Transition between epithelial and mesenchymal state of cells.

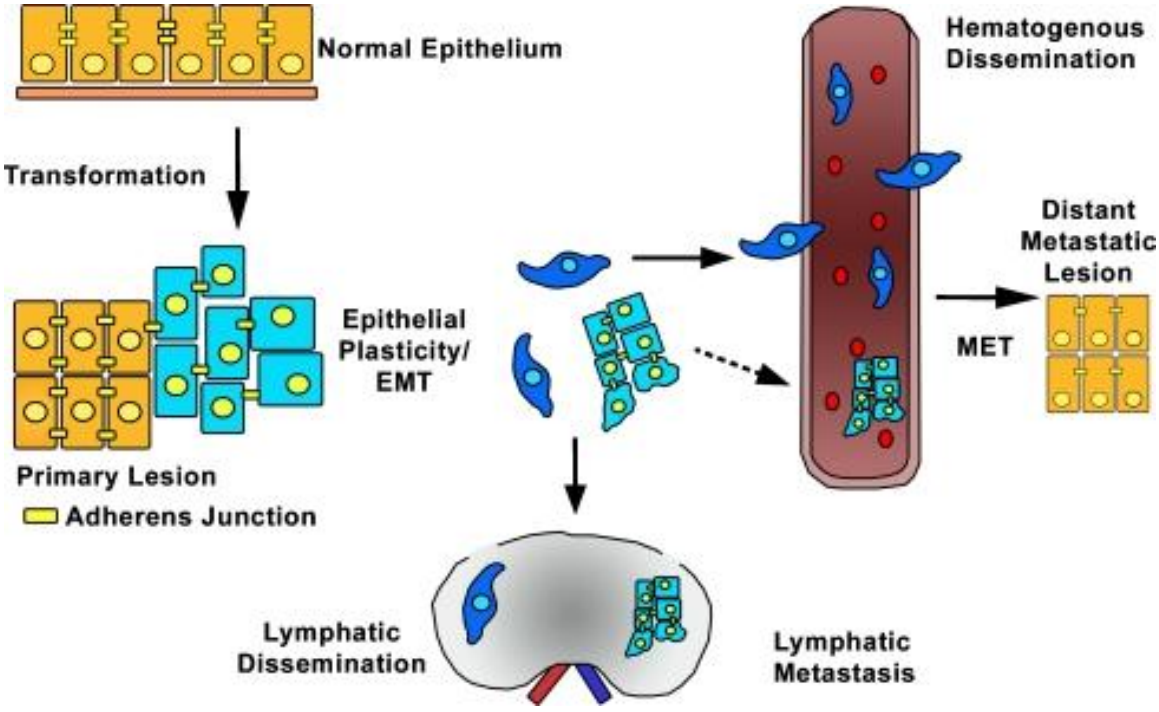


Figure 1.2 The metastatic spread of malignant cells facilitated through EMT. Tumorigenic cells leave their primary site, invade the basement membrane, intravasate into the local lymphovascular, disseminate through the circulation, and extravasate into a secondary site, leading to metastasis. At the distant colonized organ, cells can revert to an epithelial phenotype through MET.

1.3 TRANSFORMING GROWTH FACTOR-BETA (TGF-β)

Similar to many developmental signaling pathways misused in cancer, TGF-β signaling is known to be connected to numerous pathologic processes and its function during organogenesis parallels its role in neoplasia (Micalizzi *et. al.*, 2009). TGF-β is an ubiquitously expressed cytokine that contributes a dual role in tumor formation-suppressing tumor growth in normal cells and early neoplastic lesions, while promoting invasive migration and metastasis in the later stages of carcinogenesis (Micalizzi *et. al.*, 2009; Xu H *et. al.*, 2014; Xu H *et. al.*, 2015). This soluble growth factor is heavily involved in EMT and is a regulator of tumorigenesis. SIX1 has been shown to increase TGF-β signaling; this signaling pathway is a critical contributor to cancer progression and metastasis by inducing oncogenic EMT. SIX1 expression selectively promotes the pro-tumorigenic activity of TGF-β while halting its growth-inhibitory functions (Micalizzi *et. al.*, 2009). TGF-β contains three isoforms: TGFβ1, TGFβ2 and TGFβ3; and mediates signaling by binding to three receptors: TGF-β receptor type I (TβRI), type II (TβRII) and type III (TβRIII) (Meulmeester *et. al.*, 2011; Xu H *et. al.*, 2014; Xu H *et. al.*, 2015).

1.4 HUMAN PAPILLOMAVIRUS (HPV) AND CERVICAL CANCER

Cervical cancer is the leading cause of cancer morbidity and second leading cause of cancer mortality in women worldwide. There are about 500,000 new cases and 300,000 deaths annually due to cervical cancer (Peralta-Zaragoza *et. al.*, 2012). Virtually all cervical cancer cases are due to persistent infection with a high-risk human papilloma virus (HPV). Infection with HPV is the most common sexually transmitted viral disease

(STD). Currently, 79 million people in the USA have acquired an HPV infection. By the age of 50, about 80% of women will have acquired an HPV infection (Society AC., 2013; Peralta-Zaragoza *et. al.*, 2012; Saslow *et. al.*, 2012).

HPVs are small, non-enveloped viruses containing circular, double-stranded DNA. HPV infects the epithelial cells of skin and mucosae. The HPV genome encodes six to eight early and two late proteins. There are approximately 200 HPV types, 40 of which can infect the genital mucosa (Faridi *et. al*., 2011). HPVs can be further classified based on whether or not they lead to cervical cancer or benign genital warts: hence, we distinguish high-risk (oncogenic) types- that cause virtually 99% of cervical cancer cases- from low-risk (non-oncogenic) types that cause anogenital warts. Among the oncogenic HPV types, HPV16 and 18 are the most common, causing about 70% of cervical cancers. The non-oncogenic HPV types include HPV6 and 11, responsible for about 95% of genital warts (Crow JM, 2012; Grainge et. al., 2005).

Most HPV infections are asymptomatic and spontaneously clear within 9-18 months, or (in some cases) the HPV remains latent at undetectable levels in the cervical epithelium. However, approximately 10% of infected individuals cannot naturally clear a high-risk HPV infection and are at risk for developing cervical cancer, which is known to be a slow but progressive disease. Progression to cervical carcinogenesis first starts with an infection to the genital mucosae of a high-risk HPV type. As the infection persists due to a multitude of factors, this can progress through early and then late stages of dysplasia- Cervical Intraepithelial Neoplasia I, II and III (CIN I, CIN II, CIN III) before developing into invasive cervical carcinoma (Saslow *et. al.,* 2012). Although new infections decrease with age, risk of persistence is known to increase with age. Currently, there are two main

methods known to prevent or detect persistent, high-risk HPV infection. Primary prevention with a prophylactic Gardasil-9 vaccine can prevent infections by nine HPV types: 6, 11, 16, 18, 31, 33, 45, 52 and 58 (Harper DM *et. al.*, 2017). Secondary prevention with the Papanicolaou (Pap) smear test can detect abnormal cytology of infected cervical cells (Figure 1. 3) (Crow JM, 2012).

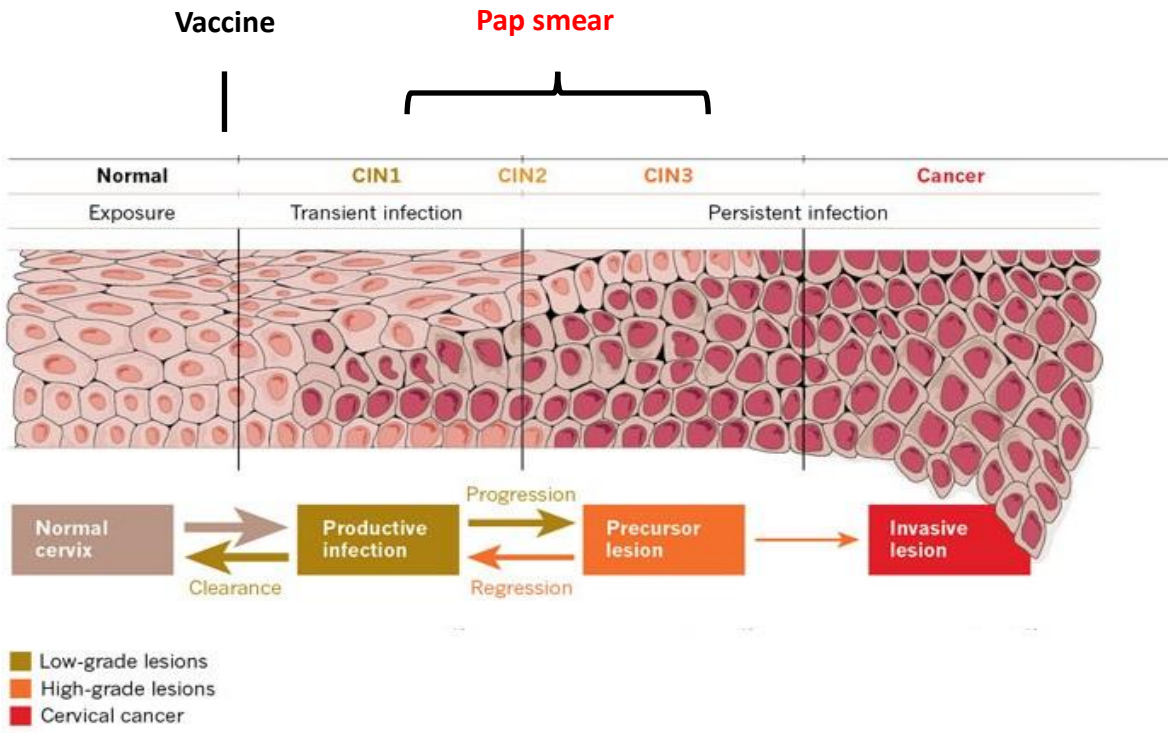


Figure 1.3 High-risk HPV infection and progression to invasive cervical carcinoma. Cervical cells can clear an infection through the host immune system or potentially progress. Persistors can progress to CIN I, which can potentially regress or progress to CIN II/III. At the later stages of pre-malignancy, clinical regression is possible but progression is probable. The main methods to prevent and/or detect an HPV infection include a prophylactic Gardasil-9 vaccine or the Pap test.

1.5 *IN-VITRO* MODEL SYSTEM OF HPV16-MEDIATED TRANSFORMATION OF NORMAL HUMAN KERATINOCYTES (HKc)

Our laboratory has developed an *in-vitro* model system of HPV16-mediated transformation of normal cells to explore the cellular and molecular events associated with HPV infection. Primary or secondary cultures of human foreskin keratinocytes go through a series of defined premalignant steps that represent the early and late stages of cervical carcinogenesis. Normal human keratinocytes (HKc) were isolated from neonatal foreskin tissue and transfected with pMHPV16d, which contains two copies of the HPV16 DNA in a head-to-tail configuration, and selected with G418. This resulted in immortalization of the transfected cells (HKc/HPV16), which represents the first transformed stage in the *in-vitro* model. With continuous passaging of HKc/HPV16, HPV16 sequences are amplified and integration into host chromosomes take place (Pirisi *et. al.*, 1987). Similar to normal HKc, these immortalized cells were cultured in keratinocyte-serum free medium (KSFM), requiring both epidermal growth factor (EGF) and bovine pituitary extract (BPE) for proliferation. In addition, normal HKc and HKc/HPV16 cells are sensitive to differentiation triggered by serum and high calcium levels. Nonetheless, HKc/HPV16 cells can be cultured in the absence of EGF and BPE. This gives rise to growth factor independent HKc (HKc/GFI) cells. An additional selection step was performed, where HKc/GFI were exposed to 5% fetal bovine serum (FBS) and 1mM calcium chloride (Figure 1.4). In this step, cells that do not differentiate in response to these two reagents are obtained and are consequently called differentiation resistant cells (HKc/DR) (Pirisi *et. al.*, 1987, 1988). This is the last stage of premalignant transformation in our model system and these cells are not tumorigenic when injected

into nude mice. In order to undergo malignant conversion, HKc/DR need to be transfected with other transforming genes such as viral *ras* or herpes simplex virus type 2 (HSV2) DNA (DiPaolo *et. al.,* 1989; DiPaolo *et. al.,* 1990). We have repeatedly shown that HKc/DR are completely resistant to the anti-proliferative effects of TGF-β, whereas HKc/HPV16 and normal HKc are sensitive to the tumor inhibiting effects of TGF-β (Creek *et. al.,* 1995).

We have established this *in-vitro* system in order to investigate various stages of malignant transformation. We utilized microarrays to identify genes whose altered expression may lead to transformation in this multi-step, pre-malignant process of *in-vitro* progression. Among the gene expression profiles that we had identified and that needed to be further studied was SIX1 (Wan F *et. al.,* 2008). This particular gene was shown to be altered during the early and later stages of our model system in comparison to normal cells.

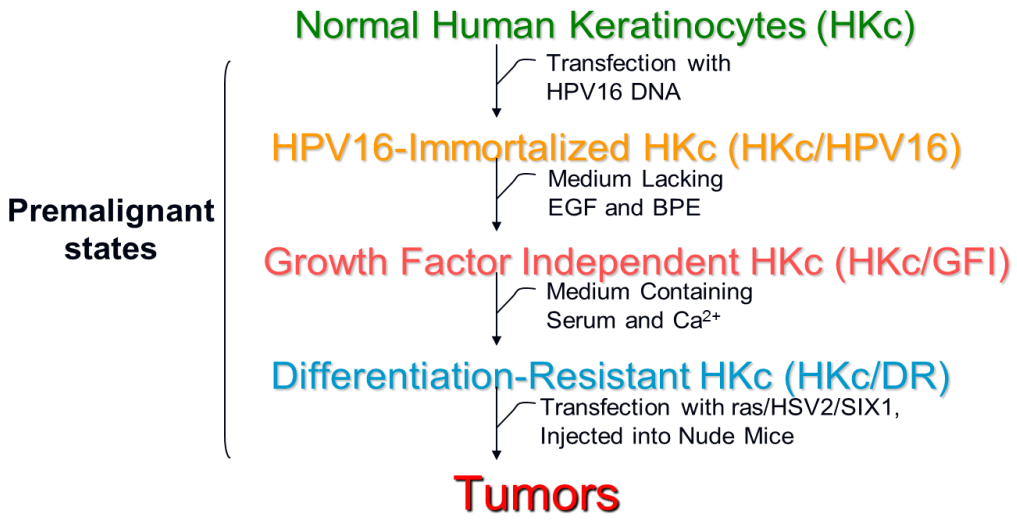


Figure 1.4 *in-vitro* model system of HPV16-mediated transformation of normal HKc.

1.6 SUMMARY AND GOAL OF RESEARCH

SIX1 overexpression has been shown to increase cancer cell proliferation, survival and invasion. The overexpression of SIX1 is associated with tumorigenesis and metastasis with poorer overall survival (Christensen *et. al.,* 2008). We have previously determined that SIX1 mRNA and protein levels are overexpressed in our HPV16-transformed HKc lines at the differentiation-resistant stage (HKc/DR) compared with early passage, HPV16-immortalized HKc (HKc/HPV16) and in HKc/HPV16 compared to normal HKc. Furthermore, we have recently determined that SIX1 overexpression in HKc/HPV16 induces the differentiation-resistant phenotype characteristic of HKc/DR, and that SIX1 overexpression in HKc/DR induces tumorigenicity. These findings strongly suggest that SIX1 has a role in regulating the growth and transformation of normal HKc, and that it plays an important role in the maintenance of growth and a transformed phenotype in HKc/HPV16 and HKc/DR. In summary, our studies suggest that SIX1 is necessary for cell survival in HPV-16 –transformed cells and may potentially become a suitable therapeutic target for HPV-driven cancers.

The main goal of this study is to explore the role of SIX1 in HPV16-transformed human keratinocytes by following the cellular and molecular changes associated with the inhibition of SIX1 expression.

CHAPTER 2

INHIBITION OF SIX1 EXPRESSION PRODUCES MESENCHYMAL-EPITHELIAL TRANSITION AND DECREASES E6/E7 EXPRESSION IN HPV16-TRANSFORMED HUMAN KERATINOCTYES

2.1 INTRODUCTION

Cervical cancer is the leading cause of cancer morbidity and second leading cause of cancer mortality in women worldwide. There are about 500,000 new cases and 300,000 deaths annually due to cervical cancer. Virtually all cervical cancer cases are due to persistent infection with a high-risk human papilloma virus (HPV). Infection with HPV is the most common sexually transmitted viral disease (STD). Currently, 79 million people in the USA have an HPV infection. By the age of 50, about 80% of women will have acquired an HPV infection (American Cancer Society; Peralta-Zaragoza *et. al.*, 2012; Saslow *et. al.*, 2012).

HPVs are small, non-enveloped viruses containing circular, double-stranded DNA. HPV infects the epithelial cells of skin and mucosae. The HPV genome encodes six to eight early and two late proteins. There are approximately 200 HPV types, 40 of which can infect the genital mucosa. HPVs can be further classified based on whether or not they lead to cervical cancer or benign genital warts: hence, we distinguish high-risk (oncogenic) types- that cause virtually 99% of cervical cancer cases- from low-risk (non-

oncogenic) types that cause anogenital warts. Among the oncogenic HPV types, HPV16 and 18 are the most common, causing about 70% of cervical cancers. The non-oncogenic HPV types include HPV6 and 11, responsible for about 95% of genital warts. Most HPV infections are asymptomatic and are spontaneously cleared within 9-18 months, or (in some cases) the HPV remains latent at undetectable levels in the cervical epithelium. However, approximately 10% of infected individuals cannot naturally clear a high-risk HPV infection and are at risk for developing cervical cancer. The Gardasil-9 vaccine can prevent infections by nine HPV types: 6, 11, 16, 18, 31, 33, 45, 52 and 58 (Harper DM *et. al.,* 2017).

Numerous studies have determined that the inappropriate expression of embryonic genes in cancer, in particular transcription factors, contribute to carcinogenesis (Abate-Shen C *et. al.,* 2002). Among the embryonic genes that also play a role in cancer development, the *sine oculis homeobox homolog 1* (*SIX1*) is essential for the development of numerous organs including the auditory and olfactory system as well as the kidney by promoting proliferation, survival and migration of progenitor cells during embryogenesis. SIX1 has been shown to increase cancer cell proliferation, survival and invasion, similar to what is observed during embryonic development (Christensen *et. al.,* 2008). In fact, many parallels exist between normal development and tumorigenesis. The aberrant expression of *SIX1* occurs in numerous adult human cancers, including breast, ovarian, cervical and hepatocellular carcinomas as well as pediatric malignancies including rhabdomyosarcoma and Wilms' tumor (Coletta *et. al.*, 2010).

SIX1 overexpression has been shown to correlate with increased malignancy, lymph node metastasis and poor survival in patients afflicted with cancer (Coletta *et. al.*, 2008; Micalizzi *et. al.*, 2009). For example, SIX1 is overexpressed in half of primary mammary carcinomas and most metastatic lesions (Coletta *et. al.*, 2008). SIX1 has also been shown to be overexpressed in cervical cancer cell lines and tissues, which are correlated with increased malignancy and metastasis (Tan *et. al.*, 2011; Zheng *et. al.*, 2010). Studies have established that the overexpression of SIX1 in immortalized and non-tumorigenic mammary epithelial cells induces malignant transformation, leading to highly aggressive and invasive tumors in nude mice. Moreover, SIX1 overexpression in human breast cancer cell lines induces epithelial-mesenchymal transition (EMT), enhancing metastasis *in-vitro* and *in-vivo* (Coletta *et. al.*, 2008; Micalizzi *et. al.*, 2009). Nonetheless, very few studies have elicited the effects of the loss of SIX1, especially in cervical carcinogenesis. Determining the role of SIX1 in the tumorigenesis and progression of cervical cancer could facilitate diagnosis and treatment. This work would contribute in our understanding of the deteriorative functions of SIX1 in cancers as a global regulator of malignant transformation.

In order to study the cellular and molecular mechanisms of HPV-mediated transformation, Pirisi *et. al.* established an *in-vitro* model system to study keratinocytes progression toward the premalignant stages of cervical cancer. In brief, primary human keratinocytes (HKc) were immortalized by transfection with pMHPV16d, which contains a head-to-tail HPV16 DNA dimer, and selection with G418. These immortalized human keratinocytes (HKc/HPV16) were selected in growth factor (epidermal growth factor and bovine pituitary extract) free medium and became growth factor independent (HKc/GFI).

A differentiation-resistant (HKc/DR) phenotype was then obtained by selection in serum and calcium -supplemented medium. HKc/DR cells are not tumorigenic but can form tumors in nude mice when these cells are transfected with activated *Ras*, Herpes Simplex Virus 2 (HSV2) or the *SIX1* oncogene (DiPaolo *et. al.*, 1989; DiPaolo *et. al.*, 1990).

Microarray studies conducted in our laboratory indicated that the *in-vitro* model system reflects many cellular and molecular alterations characteristic of cervical cancer. Therefore, our system for HPV16-mediated multi-step carcinogenesis shares important features with cervical cancer and enables us to study the molecular mechanisms of transformation and immortalization in our cell lines. It has been shown that SIX1 mRNA and protein levels are overexpressed in all 4 cell lines (HKc/DR vs. HKc/HPV16), and SIX1 mRNA and protein levels are consistently up-regulated during progression from HKc/HPV16 to HKc/DR. SIX1 overexpression was also confirmed in about 25% of cervical cancer tissue samples in a tissue array. Microarray results identified the increased expression of SIX1 as a potential biomarker for cervical cancer (Wan F *et. al.*, 2008; Xu H *et. al.*, 2014; Xu H *et. al.*, 2015).

HPV16 is the most common among the high-risk oncogenic papillomaviruses that contributes to cervical carcinogenesis (Zheng XH *et. al.*, 2010; Zheng ZM *et. al.*, 2011). HPV16-mediated transformation requires the continuous expression of the viral oncoproteins E6 and E7. These two oncogenes are necessary for malignant conversion where E6 binds to and promotes the degradation of the p53 tumor suppressor protein; and E7 binds to the Rb tumor suppressor protein thereby releasing the E2F transcription factor. Consequently, this leads to tumor initiation, proliferation and genomic instability (Ford HL *et. al.*, 2000; Ganguly *et. al.*, 2009; Li *et. al.*, 2011).

SIX1 overexpression in HKc/DR is associated to the increasing expression of HPV16 E7 that marks premalignant progression of these cells. SIX1 is known to be primarily under the control of E2F (Young AP *et. al.,* 2003). When E7 binds to unphosphorylated Rb, this tumor suppressor protein becomes inactivated and releases E2F. The displacement of E2F from Rb allows E2F to activate the cell cycle and promotes the aberrant expression of the embryonic gene SIX1 (Young AP *et. al.,* 2003).

We have determined that SIX1 overexpression in early-stage HPV16-immortalized human keratinocytes (HKc/HPV16) induces a differentiation-resistant phenotype, and that SIX1 overexpression in differentiation-resistant HPV16-transformed human keratinocytes (HKc/DR) induces tumorigenicity (Xu H *et. al.*, 2014; Xu H *et. al.*, 2015). Furthermore, we have also shown that SIX1 modulates TGF-beta signaling by decreasing canonical (Smad-mediated) responses and increasing non-canonical TGF-beta signaling pathways in both HKc/HPV16 and HKc/DR (Xu H *et. al.*, 2014; Xu H *et. al.*, 2015). We have also demonstrated that TGF-beta signaling through the Smads decreases E6 and E7 expression, and that in turn, E7 decreases cells' sensitivity to growth inhibition by TGF-beta (Baldwin *et. al*., 2004). Therefore, it is reasonable to expect that a decrease in SIX1 levels may be accompanied by a decrease in E6 and E7 expression, which would in turn be reflected in the increase of p53 and Rb levels.

With this knowledge in mind, we studied the role of SIX1 as a regulator of growth and transformation in HPV16-transformed human keratinocytes. Specifically, we studied the functional consequences of suppressing SIX1 at late stages of HPV16-mediated transformation of human keratinocytes by a variety of approaches. We could only obtain partial (up to 80%) and transient knockdown of SIX1, as treatment with different anti-

SIX1 sh- or siRNAs resulted in widespread cell death. The knockdown of SIX1 resulted in slower proliferation rates and was associated with, decreased E6 and E7 mRNA and increased p53 and Rb protein levels. We conclude that SIX1 expression is vital for cell survival, particularly in our HKc/DR cell lines. The results of these studies will give us valuable insight into the complex pathways affected through SIX1 and the functional significance of its inappropriate expression and activity in cancers, particularly those due to HPV.

2.2 MATERIALS AND METHODS

Cell culture and treatment

The cell lines discussed in this work, HKc/HPV16-d1, HKc/DRd-1 and HKc/DRd-2, have been described previously (Pirisi *et. al*., 1987, 1988) In short, normal HKc are isolated from neonatal foreskins and maintained in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) (KSFM, Invitrogen, Carlsbad, CA). This medium will be referred as complete medium. Normal HKc were transfected with a recombinant plasmid containing two copies of the HPV16 DNA in a head to tail configuration. This gave rise to HPV16-immortalized human keratinocytes, which are also cultured in KSFM. The immortalized cells were then selected in medium devoid of EGF and BPE, giving rise to Growth Factor Independent HKc (HKc/GFI). These cells were then further selected in basal KSFM supplemented with 5% fetal bovine serum (FBS) and 1 mM calcium chloride to give rise to differentiation resistant cell lines (HKc/DR). This medium will be referred as

differentiation-resistant (DR) medium. HKc/DR were transfected with SIX1, giving rise to HKc/DR-SIX1. These cells were also maintained in DR medium. All cells were maintained in a humidified atmosphere of 5% CO2 at 37° C.

Plasmid constructs and stable transfection

Independently-derived HKc/DRd-1 and HKc/DRd-2 cell lines at 70% confluency were transfected with a pSuper vector containing either a specific shRNA against human SIX1, HuSH-29 shRNA based vector (Oligoengine, Seattle, WA) or a scrambled shRNA. Additional plasmids utilized in this work include a pLXSN.neo vector expressing the HPV16- E7 viral oncogene and the Harvey rat sarcoma virus (Hras)-V12 oncogene in the pBABE.puro vector (Addgene, Cambridge, MA). Up to 5ug of the pLXSN.neo- E7 and pBABe.puro- Hras-V12 vector were used for transfection. Moreover, three different shRNA constructs against E7- that target three different positions within the HPV16 –E7 mRNA sequence, cloned in the backbone of the pSUPER.retro plasmid were used. 4ug or 5ug of anti-SIX1 shRNA construct and anti- E7 plasmid were used. Transfection efficiency was monitored with the co-transfection of the pSUPER/puro plasmid (3:1 w/v), expressing GFP. As a vector control, the pSUPER/puro plasmid was used. Cells were transfected with Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. Stable transfectants were selected with 3 ug/ml Puromycin (Toku-E, Bellingham, WA).

RNA interference (RNAi)

HKc and HKc/DRd-1 cells were seeded in 6-well plates, grown to 70% confluency and transfected with anti-SIX1 or anti-E7 siRNA or anti-GFP siRNA

(control) duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. The following sequences were utilized in this study for transfection with siRNAs targeting SIX1 and E7. SIX1-siR1: Forward 5'-CCAGCUCAGAAGAGGAAUU, Reverse 5'-AAUUCCUCUUCUGAGCUGG; SIX1-siR2: Forward 5'-CACGCCAGGAGCUCAAACU, Reverse 5'-AGUUUGAGCUCCUGGCGUG; E7- siR1 (position 141): Forward 5'-GGACAGAGCCCAUUACAAU, Reverse 5'-AUUGUAAUGGGCUCUGUCC; E7- siR2 (position 653): Forward 5'-GCUCAGAGGAGGAGGAUGA, Reverse 5'-UCAUCCUCCUCCUCUGAGC. 90 pmol siRNA was used per well and cells were incubated for 24, 48 and 72 hours before being harvested for RNA and protein extraction, for real-time PCR and Enzyme-linked immunosorbent assay analysis (ELISA), respectively.

Retroviral Infection

PA317 cells were transfected with four different shRNA constructs (Origene, Rockville, MD) against SIX1 that target four different positions within the human SIX1 mRNA sequence. A pSuper vector containing a scrambled shRNA was used as a control. Stable transfectants were selected with 3 ug/ml puromycin. After approximately 24 hours, virus-producing PA317 were cultured until sub confluent, fed fresh complete medium and the medium was collected after 24 h and filter-sterilized (0.22 u pore size).

Virus stocks were aliquoted and stored at -80 °C. High passage HKc/Drd-1 cells at 70% confluency were infected with control virus stock or anti-SIX1 shRNA virus in KSFM containing polybrene (6 ug/ml). HKc/DRd-1 cells were maintained in DR medium containing polybrene during the duration of the infection, approximately 24 hours. After

removal of virus-containing medium, the infected cells were re-plated and selected with 3 ug/ml puromycin.

Real-time PCR

Total RNA was isolated from cells using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out with 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad). All procedures followed the manufacturer's instructions. The sequences of the primers used for real-time PCR are listed in Table 2.1. GAPDH was used as an internal control. All samples were assayed in duplicates.

Table 2.1 Primer sequences used in Real-time PCR. Primer sequence 5'-3'.

Genes	Forward	Reverse
human β-catenin/ CTNNβ1	TTG AAA ATC CAG CGT GGA CA	TCG AGT CAT TGC ATA CTG TC
human CTGF	TTA CCA ATG ACA ACG CCT CCT	CTT TTT GCC CTT CTT AAT GTT CT
human E-cadherin	TGC TCT TGC TGT TTC TTC GG	TGC CCC ATT CGT TCA AGT AG
human EGFR	GGC CTA AGA TCC CGT CCA TC	GTA AGA GGC TCC ACA AGC TCC
human ETS1	GAT AGT TGT GAT CGC CTC ACC	GTC CTC TGA GTC GAA GCT GTC
human Fibronectin	ACC AAC CTA CGG ATG ACT CG	GCT CAT CAT CTG GCC ATT TT
human GAPDH	GGG AAG GTG AAG GTC GGA GTC AA	TCT CAG CCT TGA CGG TGC CAT
human GUSB	CTGTCAAGGGCAGTAACCTGT	AGATAGGCAGGGCGTTCGT
human keratin 14	TGA GCC GCA TTC TGA ACG AG	GAT GAC TGC GAT CCA GAG GA
human keratin 15	AATGTGGAGATGGACGCAG	TGGATCATTTCTGTGTTGGAGG
human keratin 19	CTT CCG AAC CAA GTT TGA GAC	AGC GTA CTG ATT TCC TCC TC

human ki-67	ACG CCT GGT TAC TAT CAA AAG G	CAG ACC CAT TTA CTT GTG TTG GA
human MMP1	AAA ATT ACA CGC CAG ATT TGC C	GGT GTG ACA TTA CTC CAG AGT TG
human MMP9	AGT CCA CCC TTG TGC TCT TCC C	TCT GCC ACC CGA GTG TAA CCA T
human N-cadherin	ACA GTG GCC ACC TAC AAA GG	CCG AGA TGG GGT TGA TAA TG
human Occludin	ATG ACA AGC GGT TTT ATC CA	CTC CAG CTC ATC ACA GGA CT
human p53	CGTGTGGAGTATTTGGATGAC	AGTCTTCCAGTGTGATGATGG
human PPARγ	GCC CTT TGG TGA CTT TAT GGA	GCA GCA GGT TGT CTT GGA TG
human PTGS2	GCC CAG CAC TTC ACG CAT CAG	AGA CCA GGC ACC AGA CCA AAG ACC
human Rb	CAA ACT TGG AGT TCG CTT GT	TTC AGA ATC CAT GGG AAA GA
human SIX1	ATT CTC ACC TCC CCA AAG TC	ACT TAG GAC CCC AAG TCC AC
human SNAIL	ACC CCA CAT CCT TCT CAC TG	TAC AAA AAC CCA CGC AGA CA
human TβRI	CTT AAT TCC TCG AGA TAG GC	GTG AGA TGC AGA CGA AGC
human TβRII	GGT TCC TGT GTG CCC TTA TT	TGC AAC CCA TGA AGG TAA AA
human TWIST	GTC CGC AGT CTT ACG AGG AG	GCT TGA GGG TCT GAA TCT TGC T
human Vimentin	GAG AAC TTT GCC GTT GAA GC	TCC AGC AGC TTC CTG TAG GT
HPV16- E6	CAC AGT TAT GCA CAG AGC TGC	CAT ATA TTC ATG CAA TGT AGG TGT A
HPV16- E7	CCG GAC AGA GCC CAT TAC AAT	ACG TGT GTG CTT TGT ACG CAC

Enzyme-linked immunosorbent assay (ELISA)

Protein was isolated from cells using the Cell Lysis Buffer from the PathScan Total p53 Sandwich ELISA kit (Cell Signaling, Danvers, MA). SigmaFAST Protease Inhibitor (Sigma, Saint Louis, MO) was added to the resulting supernatant. PathScan Total Rb Sandwich ELISA kit was then utilized according to the manufacturer's instructions. 50 ug of protein/sample was loaded into the p53 pre-coated wells. 150 ug of

protein/sample was loaded into the Rb pre-coated wells. All samples were assayed in replicates of two or three. Absorbance was read at 450 nm within 30 minutes using a microplate reader.

Cell proliferation assay

To compare the growth rates of either HKc/DRd-1- SIX1sh or HKc/Drd-2-SIX1sh and HKc/DRd-1-SCRAMsh or HKc/DRd-2-SCRAMsh, and HKc/HPV16d-1 the WST-8 dye reagent from the Cell Counting Kit-8 (CCK8) sensitive colorimetric assay was utilized (Dojindo, Rockville, MD). Proliferation was determined at 24-hour intervals. 5,000 cells were plated per well in 24-well plates in their respective media (1ml/well). Cells were plated in triplicate wells and at the appropriate times, 50 ul of WST-8 dye solution was added to each well and the plates were placed in a CO2 incubator for 2 hours. Absorbance was read at 450 nm with a microplate reader.

Gene-expression microarray analysis and validation by SYBR green real-time PCR

Total RNA from HKc/DR-SIX1-siRNA1, HKc/DR-SIX1-siRNA2 and HKc/DR-control-siRNA was isolated using Qiagen's RNeasy Plus Micro Kit according to the manufacturer's protocol. RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA Integrity Numbers ranged from 9.5 to 10.0. Microarray experiments were performed using the Affymetrix GeneChip Human Gene 2.0 ST Arrays as we have previously described (Xu H *et. al*., 2014).

Statistical Analysis

Results were expressed as the mean ± standard deviation (SD). Data were interpreted using student's t-test. $P \leq 0.05$, $P \leq 0.01$ or $P \leq 0.001$ were considered statistically significant and indicated in the figures by *, ** or ***, respectively.

2.3 RESULTS

2.3.1 THE KNOCKDOWN OF SIX1 AFFECTS CELL VIABILITY IN HKc/DR

We utilized shRNA constructs targeting the human SIX1 gene to explore the effects of suppressing this oncogene in HPV16-transformed human keratinocytes. Anti-SIX1-shRNA-encoding sequences and a control, scrambled shRNA were cloned in the pSuper-retro plasmid backbone (Oligoengine) and the resulting plasmids transfected into two HKc/DR cell lines, HKc/DRd-1 and HKc/DRd-2 (Pirisi *et. al*., 1987). Transfected cells were briefly selected with puromycin until vector controls died (approx. 72 h). Cells were then collected for RNA extraction and plated for a growth curve (Figure 2.1, A). The shRNA decreased SIX1 mRNA levels to about 20% of control (maximum) (Figure 2.1, B) and cells transfected with the anti-SIX1 shRNA exhibited much slower growth than their controls, although after 96h in culture, recovery could already be observed in one of the transfected lines (Figure 2.1, A). Further cultivation of these cells resulted in SIX1 mRNA levels returning to 100% of control and proliferation rates recovering completely (data not shown). Upon visual inspection, phase contrast photographs taken of cells co-transfected with pSuper-retro-scrambled or pSuper-retro-SIX1-shRNA and

GFP, at various times after transfection, demonstrated that loss of SIX1 leads to cell death in HKc/DR (Figure 2.1 D, E).

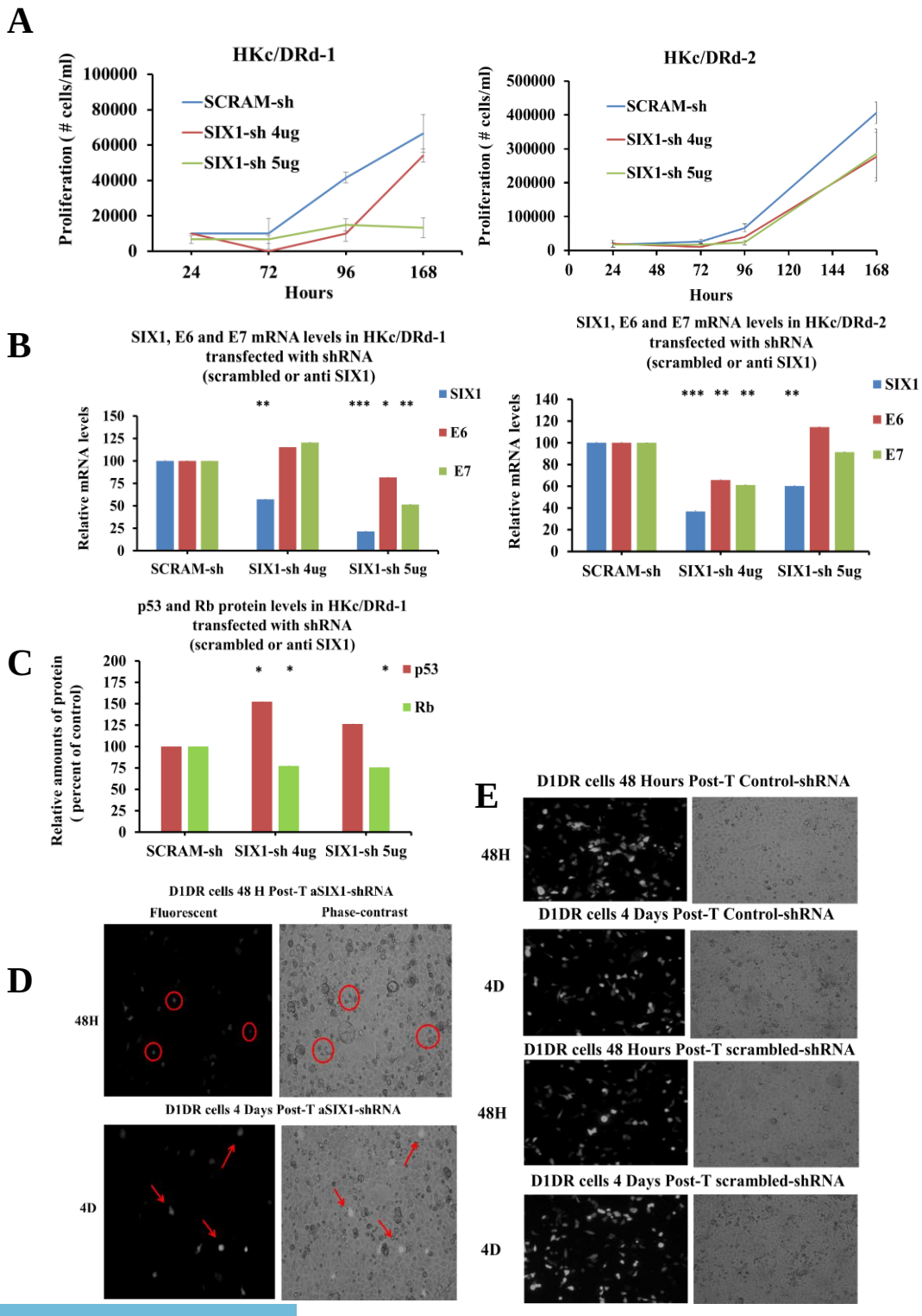


Figure 2.1 Loss of SIX1 in HKc/DR affects proliferation and HPV16- E6/E7 mRNA expression levels. (A) The rate of proliferation decreases in HKc/DRd-1 and HKc/DRd-2 transfected with SIX1-shRNA plasmid. Cell proliferation at 24-hour intervals was determined utilizing the WST-8 dye reagent from the Cell Counting Kit-8 (CCK8) sensitive colorimetric assay. (B) The mRNA expression levels of SIX1, E6 and E7 decrease in HKc/HPV16 and HKc/DR transfected with a plasmid encoding anti-SIX1-shRNA. Real-time PCR analysis for SIX1, E6 and E7 mRNA in HKc/DRd-1 and HKc/DRd-2 cell lines. Levels of expression were normalized to GAPDH. (C) The protein expression level of p53 increases while levels of Rb decrease in HKc/DRd-1 cell line transfected with SIX1-shRNA plasmid. ELISA for p53 and Rb in HKc/DRd-1 transfected with either control (anti-scram shRNA) or anti-SIX1 shRNA at indicated plasmid concentrations. (D, E) Loss of SIX1 affects cell viability in HKc/DRd-1. Fluorescent and phase-contrast images depicting transfection efficiency and morphology, respectively, of HKc/DRd-1 cells 48 hours (top) and 4 days (bottom) post-transfection with anti-SIX1 shRNA plasmid. (E) Fluorescent and phase-contrast images depicting transfection efficiency and morphology, respectively, of HKc/DRd-1 cells 48 hours (top) and 4 days (bottom) post-transfection with control-shRNA and scrambled-shRNA plasmids. Images are shown at 200X magnification.

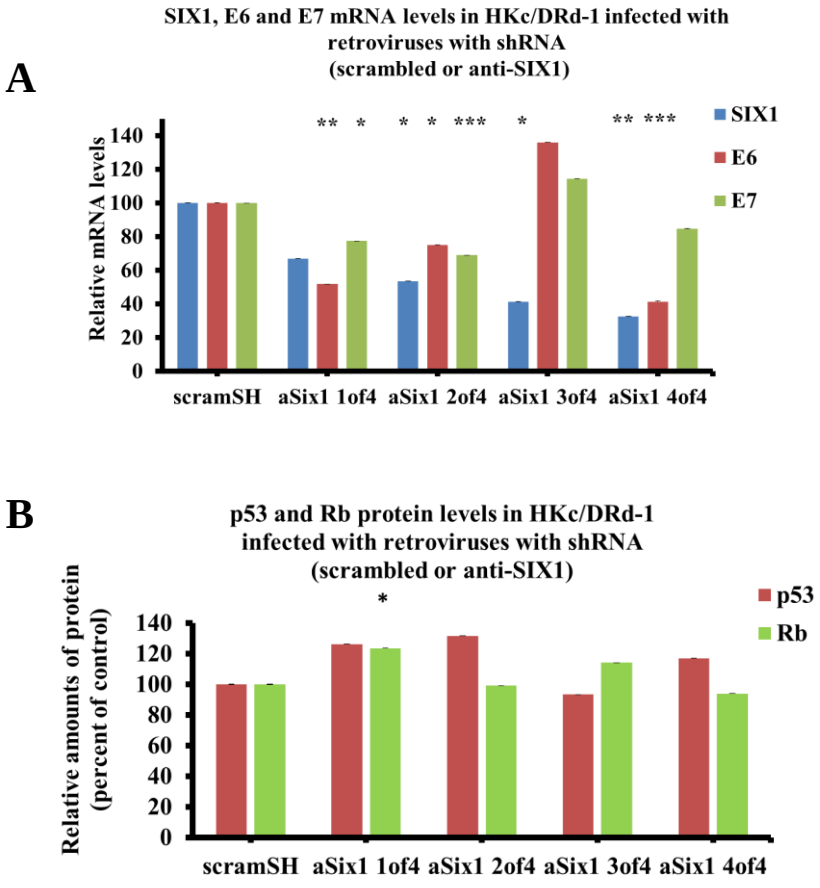


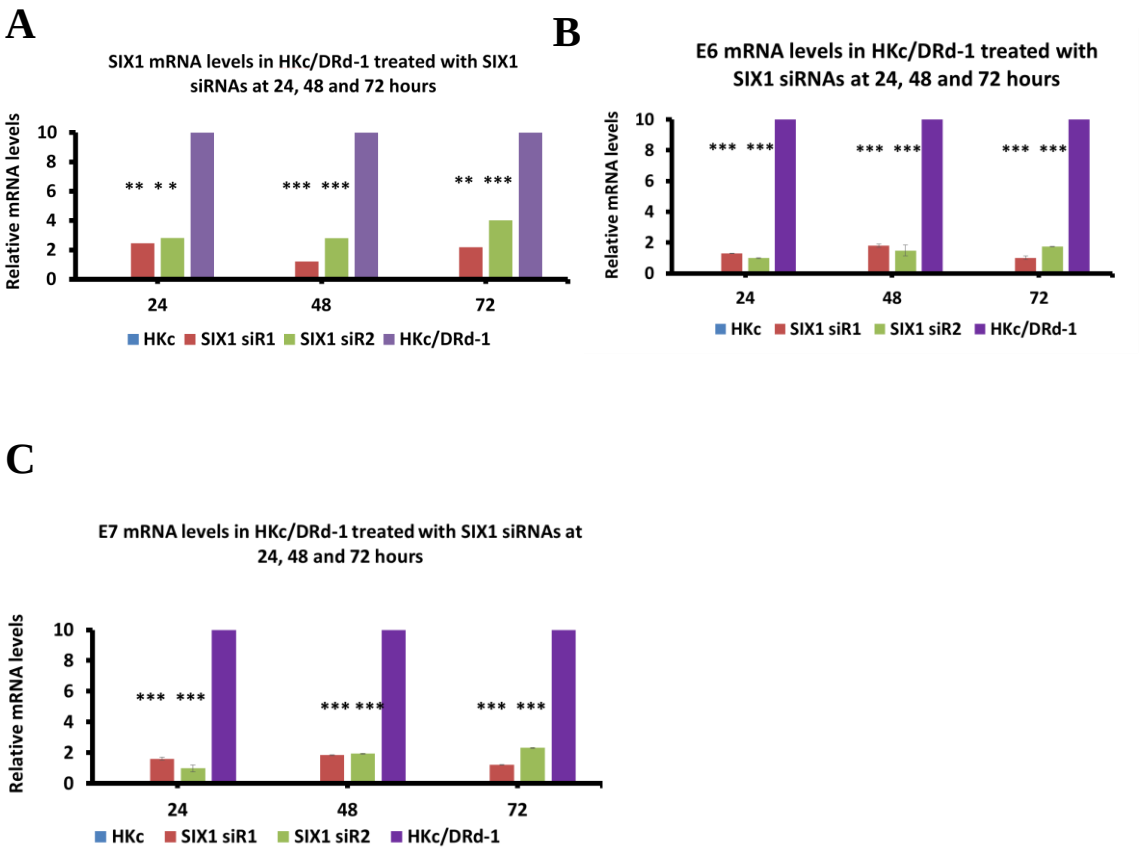
Figure 2.2 HKc/DR infected with four different shRNA constructs targeting human *SIX1* gene. mRNA and protein expression levels of SIX1, E6 and E7 in HKc/DRd-1 cells infected with four different shRNA constructs targeting four different positions within the human SIX1 mRNA sequence. (A) Real-time PCR analysis of SIX1, E6 and E7 mRNA in HKc/DRd-1 cell lines. Levels of expression were normalized to GAPDH. (B) ELISA for p53 and Rb in HKc/DRd-1 infected with either control (anti-scram shRNA) or the indicated anti-SIX1 shRNA constructs.

To fully understand the necessity of SIX1 in these differentiation resistant cell lines, HKc/DRd-1 cells were infected with anti-SIX1 shRNA PA317 conditioned medium. SIX1 loss was observed in cells treated with all four different constructs with concomitant E6 and E7 loss in three of the shRNA constructs (Figure 2.2, A). Similarly, in regards to p53 and Rb levels, we observed slight increases in protein levels (Figure 2.2, B). Overall, these results indicate that SIX1 expression is vital for cell survival in the late-stage HPV16 transformed human keratinocytes.

2.3.2 LOSS OF SIX1 CAUSES DECREASES OF HPV16 E6/E7 mRNA LEVELS IN HKc/DR

The experiments described above also provided a first hint that SIX1 expression may affect HPV16 E6/E7 mRNA expression. We have demonstrated that SIX1 knockdown in HKc/DRd-1 results in the decrease of HPV16- E6/E7 mRNA levels with a simultaneous increase in p53 and decrease in Rb protein expression levels (Figure 2.1 B, C). Therefore, we set out to further investigate this possibility through transient transfections with pre-formed anti-SIX1 siRNA. We utilized two different siRNA duplexes targeting different regions of the human SIX1 gene. In HKc/DRd-1 cells, SIX1

mRNA levels decreased significantly, under these transient transfection conditions, at 24, 48 and 72 hours (Figure 2.3, A). Decreased SIX1 expression was associated with decreases in HPV16-E6 and E7 mRNA levels (Figure 2.3 B, C). Consistent with a decrease of active E6 levels, p53 protein levels increased significantly, with the greatest rise observed 24 hours after SIX1 expression (Figure 2.3, D). Not surprisingly, p53 mRNA levels (which are high in HPV16-transformed cells) markedly decreased in HKc/DR transfected cells with either of the two anti-SIX1 siRNA constructs (Figure 2.3, E). Rb protein and mRNA levels followed similar trends, however to a lesser extent (Figure 2.3 F, G).



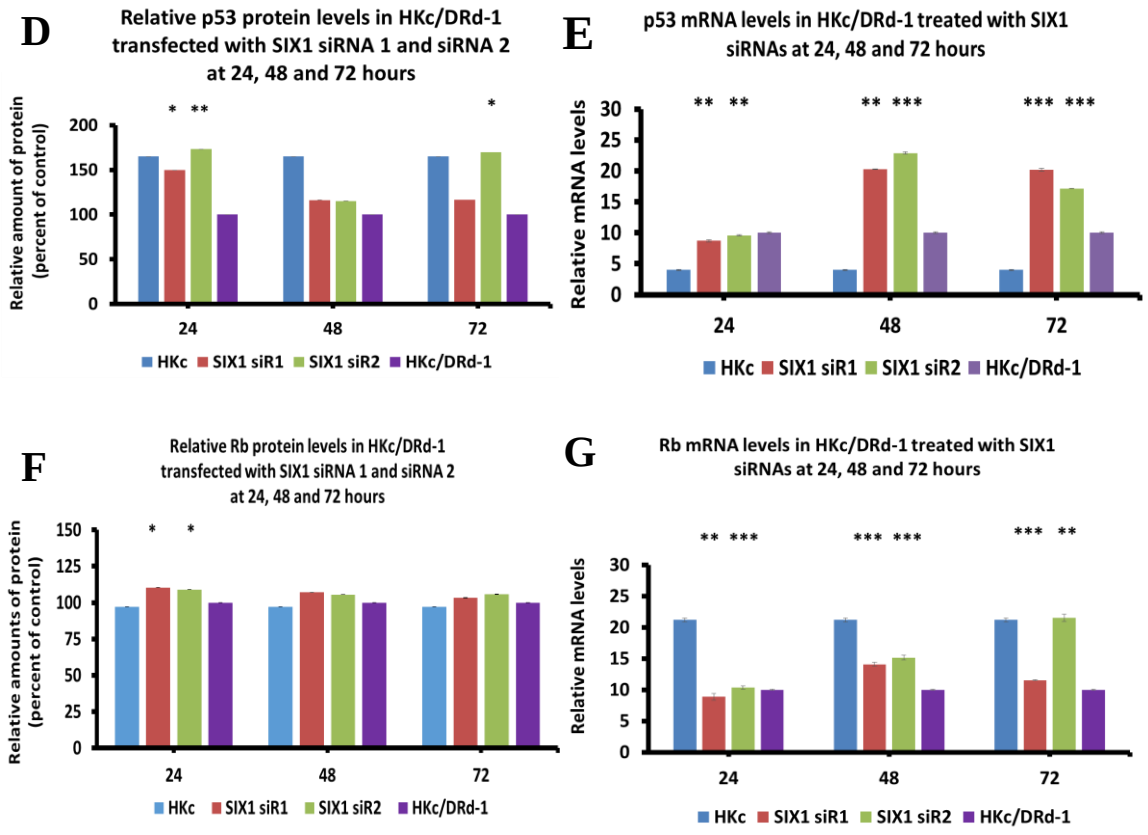


Figure 2.3 Loss of *SIX1* with pre-formed anti-SIX1 siRNAs causes decreases of HPV16- E6/E7 mRNA levels in HKc/DR. (A) (B) (C) Real-time PCR analysis for SIX1, E6 and E7 mRNA in HKc/DRd-1 under transient transfection conditions at the indicated time points. Levels of expression were normalized to GAPDH. (D) (F) Relative protein expression of p53 and Rb, respectively, after transfection with pre-formed anti-SIX1 siRNAs in HKc/DRd-1 cells. p53 and Rb protein expression was detected by ELISA in HKc/DRd-1 at indicated 24-hour interval time points. (E) (G) Relative mRNA expression of p53 and Rb, respectively, in the HKc/DRd-1 cell line transfected with pre-formed anti-SIX1 siRNAs. p53 and Rb mRNA were quantified by RT/PCR in HKc/DRd-1 at the indicated 24-hour interval time points. Levels of expression were normalized to GAPDH.

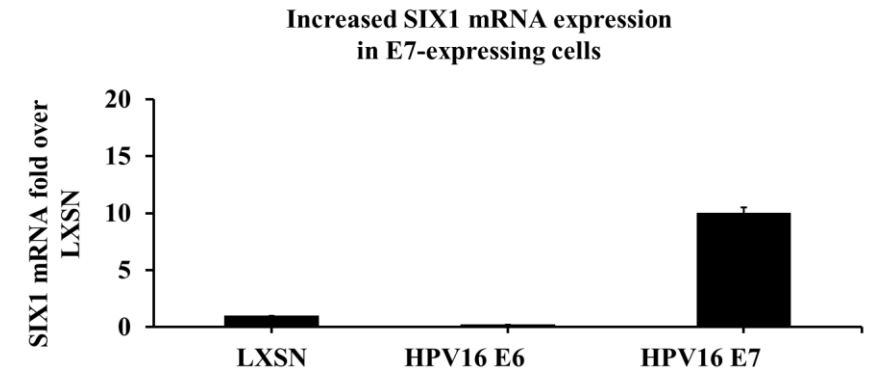
Taken together, our data indicate that a loss of SIX1 expression in HKc/DR cells is associated with decreased expression of the viral oncogenes E6 and E7. The mechanisms in which SIX1 directly, or perhaps indirectly, affect E6 and E7 still need to be elucidated.

2.3.3 KNOCKDOWN OF E7 RESULTS IN DECREASED mRNA LEVELS FOR SIX1

Previous unpublished work in our laboratory determined that SIX1 levels could be increased dramatically in normal HKc by the expression of HPV-16 E7, and by siRNA-mediated inhibition of Rb expression, provided that p53 levels were reduced as well by shRNA (Figure 2.4 A, B). We have also overexpressed HPV16 -E7 in HKc/DR cells. Increased E7 expression resulted in the subsequent increase of SIX1 and HPV16-E6 mRNA (Figure 2.5, A). Concomitantly, Rb and p53 protein levels, respectively, were downregulated (Figure 2.5 B-E).

To better pinpoint the functional consequence of E7 suppression in HKc/DR cells, the HKc/DRd-1 cell line was transiently transfected with two different siRNA duplexes targeting HPV16 -E7. Cells were then collected for RNA and protein extraction 48 hours after transfection. Both siRNAs produced a significant decrease in both E6 and E7 mRNA levels, up to 70% inhibition (Figure 2.6, A) accompanied by approximately 50% decrease in SIX1 mRNA levels (Figure 2.6, B). These changes in E6, E7 and SIX1 mRNA levels were also associated with the expected changes in p53 and Rb mRNA and protein levels (Figure 2.6 C-F).

A



B

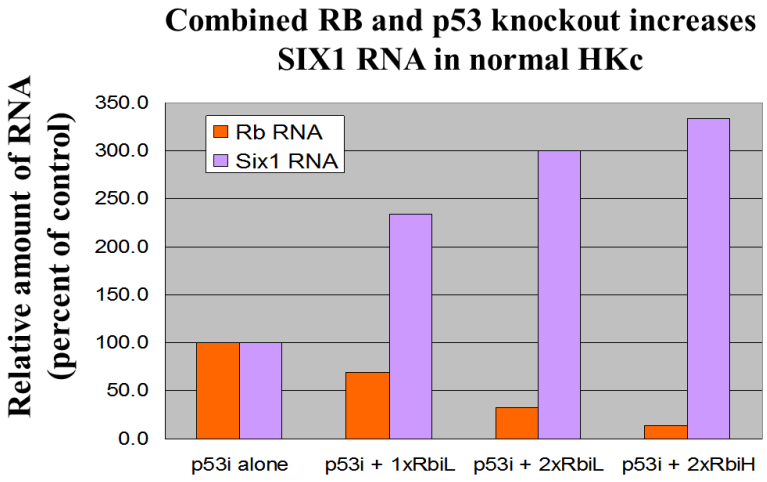


Figure 2.4 Increased SIX1 RNA expression in normal HKc and HKc/E7-expressing cells. (A) Real-time PCR analysis of SIX1 mRNA in normal HKc expressing HPV16E7. SIX1 mRNA expression was normalized to GAPDH; (B) Real-time PCR analysis for SIX1 and Rb mRNA in normal HKc co-transfected with a p53-shRNA plasmid and an anti-Rb siRNA duplex. Levels of expression were normalized to GUSB.

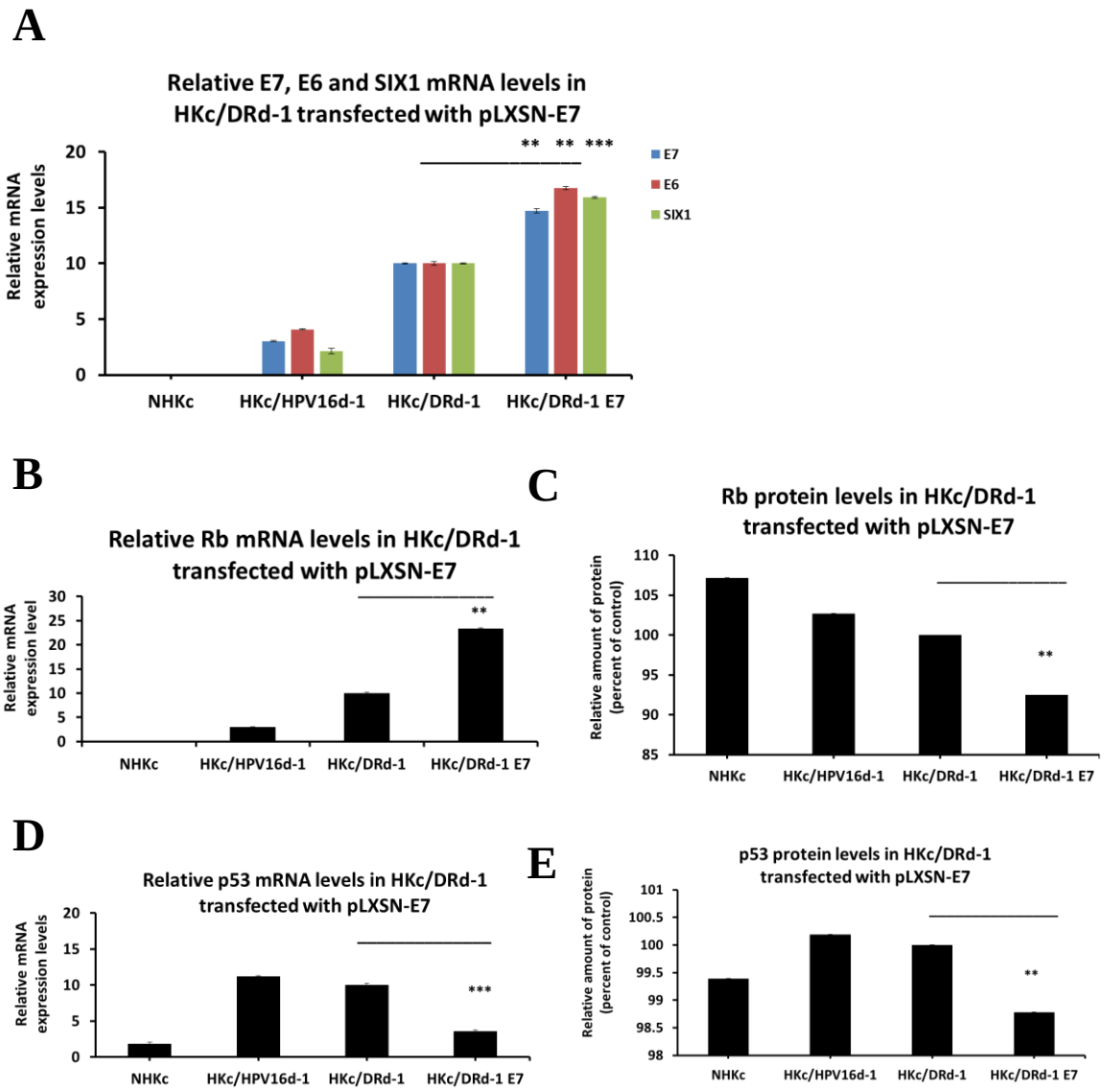


Figure 2.5 Increased SIX1 and HPV16- E6 mRNA expression in HKc/DR overexpressing HPV16 E7 from the pLXSN.neo- E7 vector. (A) Real-time PCR analysis for SIX1 and E6 mRNA in HKc/DR expressing HPV16 E7. Levels of expression were normalized to GAPDH. (B) (D) Real-time PCR analysis of Rb and p53 mRNA in HKc/DR overexpressing HPV16- E7. Levels of expression were normalized to GAPDH. (C) (E) ELISA for Rb and p53 protein expression in HKc/DR overexpressing HPV16- E7 with the use of pLXSN.neo- E7 vector.

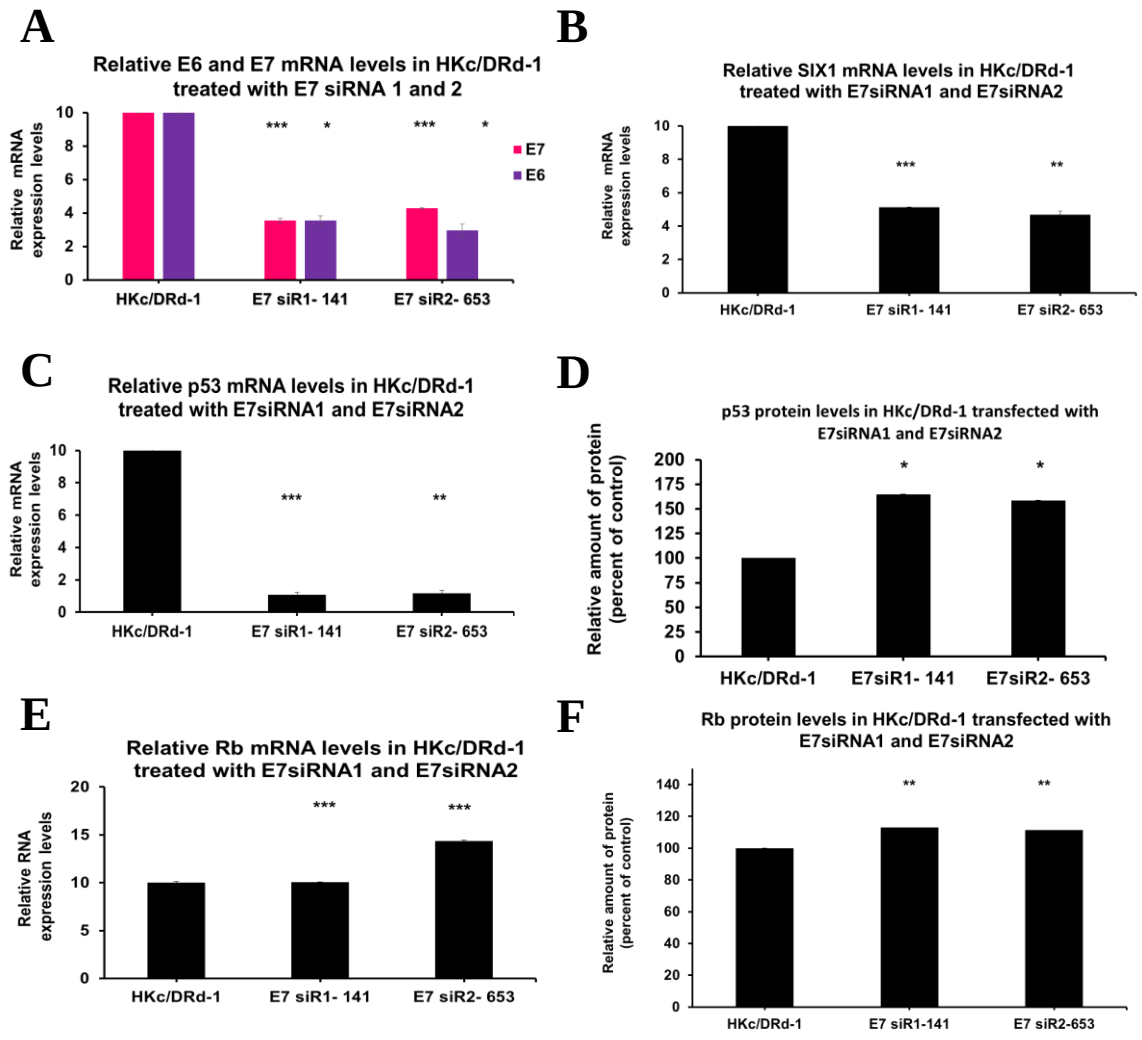


Figure 2.6 Knockdown of HPV16-E7 with pre-formed anti-E7 siRNAs results in decreased mRNA levels of HPV16-E6 and SIX1. (A) (B) Real-time PCR analysis for E6, E7 and SIX1 mRNA in HKc/DRd-1 under transient transfection conditions at 48 hours. Levels of expression were normalized to GAPDH. (C) Real-time PCR analysis for p53 mRNA in HKc/DRd-1 48 hours after transient transfection. p53 expression was normalized to GAPDH. (D) (F) ELISA for p53 and Rb protein expression in HKc/DRd-1 48 hours after transient transfection. (E) Real-time PCR analysis of p53 mRNA in HKc/DRd-1 at 48 hours. Rb expression was normalized to GAPDH.

Collectively, these data suggest that a loss of E6/E7 expression is associated with a decrease in the mRNA levels of SIX1. We tentatively explain these changes with the interrelated effects of E7 and SIX1 on TGF-β signaling, as detailed in the Discussion.

2.3.4 SIX1 KNOCKDOWN IN HKc/DR PROMOTES MESENCHYMAL-EPITHELIAL TRANSITION (MET) AND RESETS THE LEVELS OF TGF-B RECEPTORS 1 AND 2

We set out to investigate global gene expression during transient SIX1 knockdown in HKc/DRd-1 cells on Affymetrix GeneChip Human Gene 2.0 ST Arrays. Direct, paired comparisons of gene expression were made among four individual clones of HKc/DR-SIX1-siRNA1and HKc/DR-control-siRNA, and between HKc/DR-SIX1-siRNA2 and HKc/DR-control-siRNA. We used Transcriptome Analysis Console (TAC) software from Affymetrix to analyze the microarray results. To determine the differentially expressed genes in the pairwise comparisons, we used a fold change >1.4 (up and down) and a *P* value < 0.01 as cutoff values. In addition, Ingenuity Pathway Analysis (IPA) was performed on the differentially expressed genes. Pairwise comparisons identified 2447 differentially expressed genes after treatment with SIX1-siRNA1: 794 genes were upregulated and 1653 genes were downregulated. Correspondingly, 2693 differentially expressed genes were identified after treatment with SIX1-siRNA2: 1580 genes were upregulated and 1113 genes were downregulated. We then manually identified common genes between the two siRNA duplexes that were associated with a transition towards an epithelial phenotype. We were interested in nine genes that were downregulated and four genes that were upregulated through siRNA treatment in HKc/DRd-1 cells (Tables 2.2, 2.3).

Table 2.2 Expression of EMT-associated genes targeted by SIX1 and TGF-β in HKc/DR. Decrease in target gene expression during transient SIX1 knockdown in HKc/DRd-1 cell lines.

gene	Fold Change
CTGF	-1.72
CTNNβ1	Confounding results: -1.54 microarray; 185% (18.5) increase PCR
EGFR	predicted inhibition
ETS1	-1.75
MMP1	-6.38
MMP9	-3.46
PPARγ	-1.65
TβRII	-1.84
VIM	-2.05

Table 2.3 Expression of MET-associated genes targeted by SIX1 and TGF-β in HKc/DR. Increase in target gene expression during transient SIX1 knockdown in HKc/DRd-1 cell lines.

gene	Fold Change
K15	1.72
OCLN	2.11
PTGS2	1.64
TβRI	1.57

Unsupervised cluster analysis clearly differentiated the SIX1 knockdown cells from controls and allowed for the identification of a cluster of genes that best separate the two (Figure 2.7 A, B). IPA pathway analysis showed that the pathways most heavily affected by decreased SIX1 expression were consistent with MET (Figure 2.7, C).

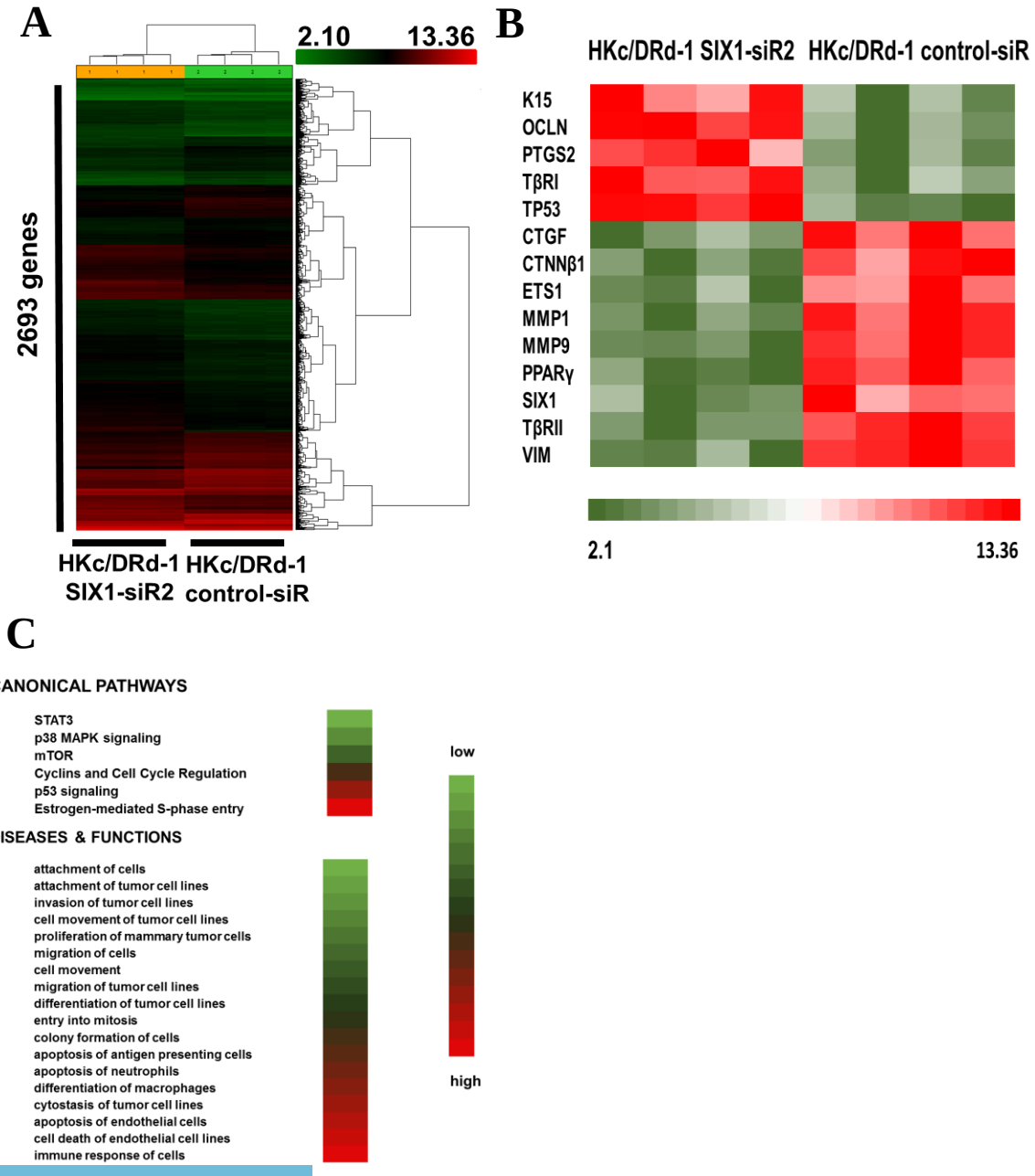


Figure 2.7 Gene expression profiles of SIX1 knockdown in HKc/DR involve TGF-β signaling, MET and apoptosis. (A) Unsupervised hierarchical clustering of differentially expressed genes between HKc/DR control siR (left) and HKc/DR SIX1-siR2 (right). The color represents the expression level of a gene above (red), below (green), or at neutral (black) the mean expression level of that gene across all samples. Pairwise comparisons identified 2693 differentially expressed genes after treatment with SIX1-siRNA2: 1580 genes were upregulated and 1113 genes were downregulated. (B) Heatmap depicting select genes relevant to MET. (C) Illustration of downstream functional effects of gene transcripts that are upregulated and downregulated. Canonical pathways and diseases & functions that are differentially expressed as a result of SIX1 loss in HKc/DR (1.4 fold change up and down; p-value < .01). Ingenuity Pathway Analysis (IPA) indicates deregulation of pathways related to invasion, proliferation, migration and apoptosis.

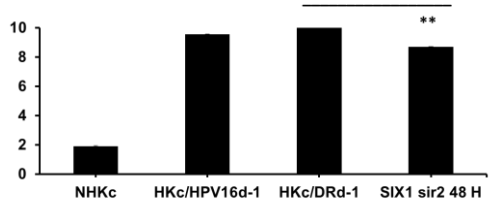
Among the many changes within these pathways, we observed a reduction in the expression of the mesenchymal-related gene Vimentin (VIM) and an increase in the expression of the epithelial-related genes Keratin 15 (K15) and Occludin (OCLN). Furthermore, there was an increase in expression of the inflammatory response gene prostaglandin-endoperoxide synthase 2, PTGS2 (cyclooxygenase-2, COX-2). There was a decrease in the expression of the cell-cell adhesion complex molecule, β-catenin (CTNNβ1) (Gumbiner *et. al.*, 2005). We noted a decrease in the pro-survival factor epidermal growth factor receptor (EGFR) and its associated ligand, transforming growth factor alpha (TGF-α). There was a decrease in the angiogenic matricellular marker connective tissue growth factor (CTGF/CCN2) and its downstream signaling effector ETS proto-oncogene 1, transcription factor (ETS1) (Geisinger *et. al.*, 2012; Ubink *et. al.*, 2016). mRNA levels for the markers for invasiveness, matrix metalloproteinase 1 and 9 (MMP1, MMP9), were also decreased. Lastly, there was a decrease in peroxisome proliferator activated receptor gamma (PPARγ), a ubiquitously expressed gene known to regulate differentiation and cell growth (Sarraf *et. al.*, 1999). These microarray data

suggest that mesenchymal to epithelial transition is a functional consequence of SIX1 loss in the late-stages of HPV16-transformed human keratinocytes.

To validate data from the array analysis we performed real-time PCR on HKc/DRd-1 cells treated with SIX1-siRNA2 48 hours after transfection. The mRNA levels of K15 and OCLN both increased approximately 2.2-fold while VIM mRNA levels decreased approximately 20%. Additionally, there was a significant increase, up to 40%, in PTGS2/COX-2. These cells might be experiencing cell stress as a result of SIX1 loss; and therefore, they react by increasing levels of inflammatory-response genes. While the array data showed a 1.54-fold decrease in β-catenin expression, real-time PCR results revealed a 3-fold increase in mRNA levels. The shift away from a mesenchymal phenotype can be associated with changes that follow a gain in cell-cell adherens junctions typically seen in normal epithelial cells and β-catenin is a well-known epithelial marker. There was a 40% decrease in EGFR mRNA levels following transfection. Additionally, there was a 55% and 40% decrease in CTGF/CCN2 and ETS1 mRNA levels, respectively. Tumorigenic cells are known to metastasize via blood or lymphatic vessels through an increase in the mesenchymal-associated gene CTGF/CCN2 (Ubink *et. al*., 2016). In addition, there was a 70% and 40% decrease in MMP1 and MMP9 mRNA levels, respectively. Malignant cells can damage the basement membrane thru the increase of proteolytic enzymes such as MMP1 and MMP9. Lastly, there was a significant decrease in the proliferation and differentiation marker PPARγ. Therefore, a significant decrease of these EMT-inducing proteins is an indication of the functional consequences brought on by a deficit in SIX1 (Figure 2.8).

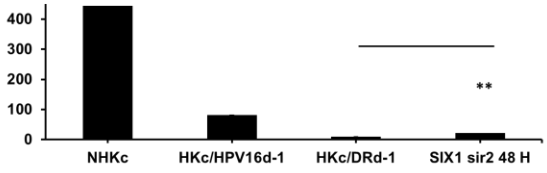
Mesenchymal-related gene

VIM mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours

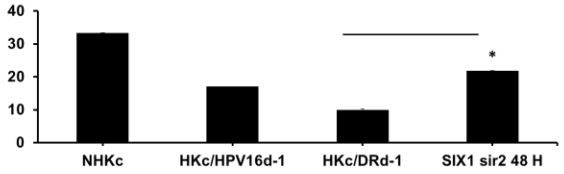


Epithelial-related genes

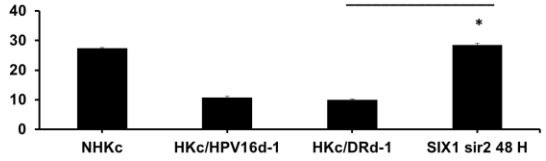
K15 mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours



OCLN mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours

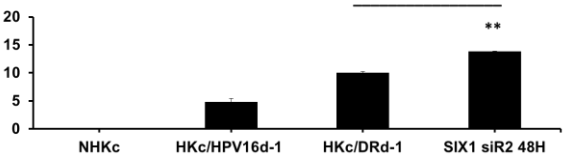


CTNNβ1 mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours



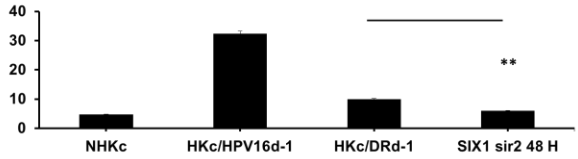
Inflammatory response

PTGS2 (COX-2) mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours

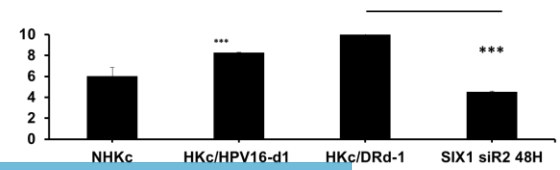


Metastasis-related genes

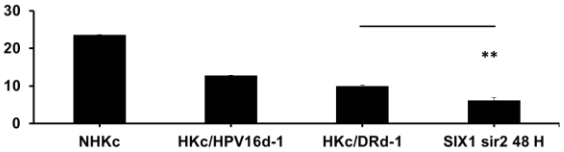
EGFR mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours



CTGF mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours



ETS1 mRNA levels in HKc/DRd-1 transfected with SIX1 siRNA 2 after 48 hours



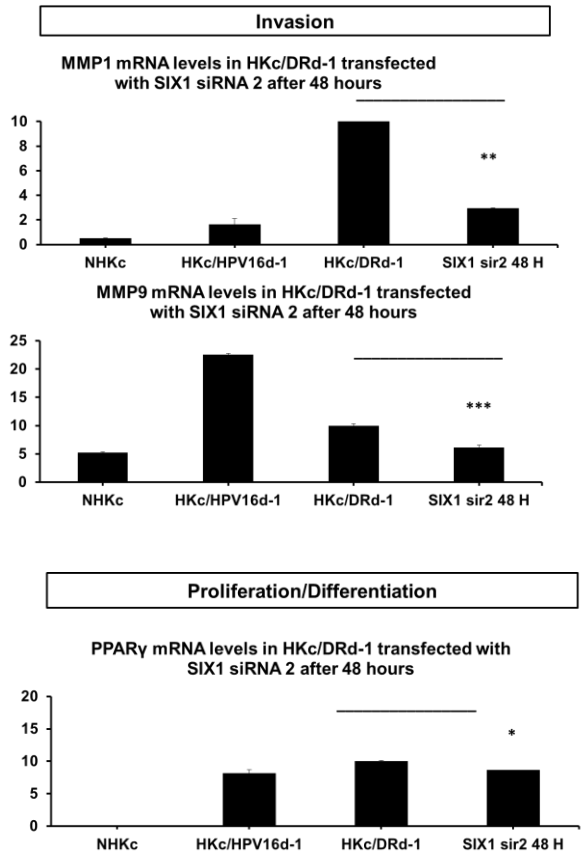


Figure 2.8 SIX1 knockdown induces markers of MET in HKc/DR. Levels of mRNA expression of CTGF, CTNNβ1, EGFR, ETS1, K15, MMP1 and 9, OCLN, PPARγ, PTGS2 and VIM were determined by real-time PCR analysis in HKc/DR control siR and HKc/DR SIX1-siR2. Data were normalized to GAPDH expression.

Last, but not least, microarray data revealed a 1.57-fold increase in TβRI and a 1.84-fold decrease in TβRII. Real-time PCR confirmed these changes. We analyzed our array data to locate gene expression changes that are regulated by TGF-β signaling (Figure 2.9). We also identified increases in K15 (1.72 fold) and OCLN (2.11 fold) and decreases in Hic1 (1.4), VIM (2.05), CTGF (1.72) and PLAT (3) in response to loss of SIX1 and the differential expression of TGF-β receptors.

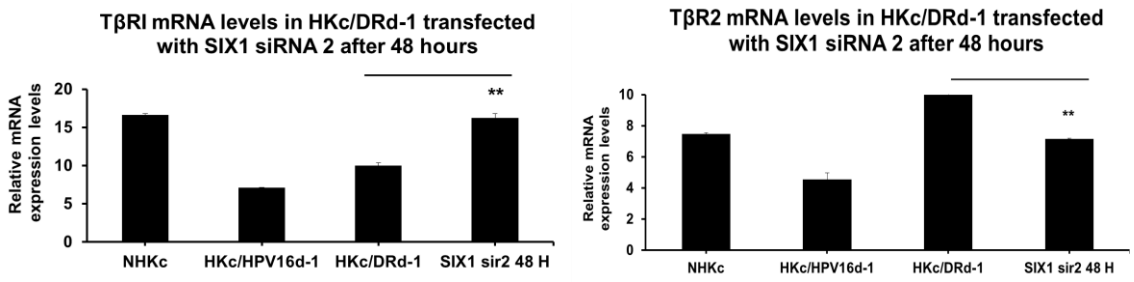


Figure 2.9 SIX1 knockdown in HKc/DR resets the levels of TGF-β receptors 1 and 2. Levels of mRNA expression of TGFβRI and TGFβRII were determined by Real-time PCR analysis in HKc/DR control siR and HKc/DR SIX1-siR2. Data were normalized to GAPDH expression.

2.4 DISCUSSION

The results shown above, taken together with our prior observation on the role of SIX1 in HPV16-mediated transformation, point to the fact that SIX1 expression is necessary for growth and survival of differentiation-resistant, HPV16-transformed human keratinocytes (HKc/DR) and that these cells do not tolerate a decrease in SIX1 levels. Given the fact that SIX1 is virtually not expressed in normal adult cells, this gene may become a suitable therapeutic target for HPV-driven cancers.

Deregulation of homeobox genes in cancer results in an out of context activation of their development-restricted functions. There have been countless studies identifying the link between normal organogenesis and neoplasia. Indeed, they both share many pathways and basic molecular processes between them, demonstrating undoubtedly that tumorigenesis and progression is an aberrant form of morphogenesis. The oncoprotein SIX1 has an important role in the expansion of progenitor cell populations during embryogenesis and is essential for the development of numerous organs. This transcription factor has been shown to be aberrantly misexpressed in various pediatric

and adult cancers. SIX1-induced EMT through TGF-β signaling enhances tumor progression, invasion and metastasis in several malignancies.

Our previous work demonstrated that SIX1 mRNA and protein levels are overexpressed in the *in-vitro* model system, and SIX1 mRNA and protein levels are consistently up-regulated during progression from HKc/HPV16 to HKc/DR. We further show that SIX1 overexpression in HKc/HPV16 induces a differentiation-resistant phenotype, and that SIX1 overexpression in HKc/DR induces tumorigenicity. In the current study, we focused on the role of SIX1 as a regulator of growth and transformation in HPV16-transformed human keratinocytes. We studied the functional consequences of suppressing SIX1 in the late stages of HPV16-transformed human keratinocytes.

In our studies, we utilized shRNA technology to suppress SIX1 expression levels in HKc/DR cells, resulting in decreased proliferation and widespread cell death. Interestingly, in select transfections, we also detected significant increase in SIX1 expression levels in comparison to the HKc/DR scrambled shRNA control population.

However, stable transfections resulted in only transient decreases in SIX1 expression and inconsistent results, which we attributed to the impression that cell populations were rapidly taken over by cells in which SIX1 was still being expressed. Therefore, we decided to explore the effects of SIX1 inhibition in transient transfection conditions, with pre-formed siRNA.

Previous studies have demonstrated that the transient inhibition of SIX1 in the pancreatic cancer cell line, PANC-1, efficiently results in an 86% decrease of SIX1

mRNA (Lerbs *et. al*., 2017). We utilized two siRNA duplexes targeting the human SIX1 gene and in doing so, we significantly reduced the mRNA expressions of SIX1, E6 and E7 at all three time points tested. There was an initial significant decrease in p53 mRNA levels; p53 protein levels increased as a result of SIX1 and E6 loss. Moreover, Rb mRNA levels were considerably low at the various time points; however, there were increases in Rb protein levels after the suppression of SIX1 and E7 mRNA expression. Therefore, with the brief reduction of SIX1 expression, we were able to drastically reduce E6 and E7 mRNA levels with the corresponding increases in p53 and Rb protein levels. Thus, HPV oncoprotein expression in our cell lines could be modulated by SIX1. These results also implied that SIX1 is able to promote malignant transformation and growth of infected cervical cells together with the concomitant expression of HPV16-E6 and E7 (Liu D. *et. al*., 2014).

It has been reported that HPV-E7 plays a role in inducing the expression of SIX1. HPV-negative C33a cells have very low SIX1 expression levels but the overexpression of E7, not E6, has been shown to induce the expression of SIX1 (Liu D. *et. al*., 2014). The data described in this paper on the reciprocal regulation of SIX1 and E6/E7 levels suggest that SIX1 expression in our cell lines is modulated by HPV oncoproteins, specifically HPV16 -E7. These results also imply that E7 relies on SIX1 to continuously promote malignant transformation and tumor progression by upregulating SIX1 expression.

Similar to many developmental signaling pathways dysregulated in cancer, TGF-β signaling is known to be connected to numerous pathologic processes and its function during ontogenesis parallels its role in neoplasia (Micalizzi *et. al*., 2009). TGF-β plays a

dual role in tumor formation and is known to be associated with tumorigenesis, including the progression of premalignant cervical lesions to invasive cervical cancer (Baritaki *et. al*., 2007). TGF-β is a potent suppressor of tumor growth in normal cells and early neoplastic lesions; however, this cytokine promotes invasive migration and metastasis in the later stages of tumorigenesis (Micalizzi *et. al*., 2009; Xu H *et. al*., 2014; Xu H *et. al*., 2015). We have previously shown that SIX1 modulates TGF-beta signaling by decreasing canonical (Smad-mediated) responses and increasing non-canonical TGF-beta signaling pathways in HKc/HPV16 and HKc/DR cell lines (Xu H *et. al*., 2014; Xu H *et. al*., 2015). Therefore, we analyzed signaling pathways in response to SIX1 knockdown in HKc/DR and observed alterations in the TGF-β pathway, including diminished expression of EGFR and its associated ligand TGF-α, CTGF and its downstream signaling effector ETS1, as well as MMP1 and MMP9. Further, the loss of SIX1 in HKc/DR results in enhanced expression of TβRI and a reduction in TβRII. Taken all together, these findings further confirm that SIX1 plays a very important role during progression of HPV16-transformed human keratinocytes- toward a malignant phenotype.

The effects of SIX1 knockdown, taken all together, corroborated and confirmed our prior observations made on the same cells, HKc/DR (and HKc/HPV16) in which we overexpressed SIX1, with major effects noted on pathways of EMT/MET, mainly through TGF-β signaling (Xu H *et. al*., 2014; Xu H *et. al*., 2015). The central EMT modulator, TGF-β, has a dual role in tumor development as well as in our cell lines where it switches from growth suppressive to EMT-inducing in the progression of HPV-

mediated transformation of human keratinocytes (Kowli *et. al*., 2013; Xu H *et. al*., 2014; Xu H *et. al*., 2015).

TGF-β signaling is a critical pathway in which SIX1 overexpression promotes EMT in HKc/DR cells. TGF-β receptors type I (TβRI), II (TβRII), and III (TβRIII) are part of the TGF-β pathway cascade. Microarray data revealed a 1.57-fold increase in TβRI and a 1.84-fold decrease in TβRII in HKc/DR in which SIX1 expression was downregulated by our siRNA approach. Real-time PCR demonstrated similar results with an approximate 70% increase in TβRI mRNA levels and a 30% decrease in TβRII mRNA levels. This may indicate that a decline in SIX1 expression permits HKc/DR cells transition from a differentiation resistant phenotype, with mesenchymal features, towards a phenotype associated more closely with epithelial cells such as the representation of TGF-β as a growth suppressive cytokine. The changes in the expression of genes targeted by TGF-β are consistent with our interpretation that a shift in TGF-β signaling from non-canonical to canonical, the opposite of what we detected with SIX1 overexpression, occurs as a consequence of SIX1 loss.

As we have previously demonstrated, this shift is pivotal to produce the DR phenotype and to further promote tumorigenicity in HPV-transformed cells (Xu H *et. al*., 2014; Xu H *et. al*., 2015). Based upon our previous body of work elucidating the mechanisms of TGF-β control of transcription from the HPV16 URR, we postulate that the "shift" in TGF-β signaling from non-canonical to canonical, which we observed as a consequence of inhibition of SIX1 expression, may be at the basis of the decrease in E6/E7 expression. However, the specific mechanisms by which SIX1 and E6/E7 regulate each other remain to be fully elucidated.

The decrease in EGFR expression is also of considerable interest. While we can explain it as a consequence of decreased E6 levels and increasing p53 levels, this observation not only corroborates our overall interpretation of an interplay between E6/E7 and SIX1 and a pivotal role of SIX1 in HPV16-mediated transformation and tumor progression (Bheda *et. al.*, 2008). EGFR loss has important functional consequences, as malignant cells are known to escape suspension-induced cell death (anoikis) through the increase of EGFR (Woappi *et. al*., 2017).

CHAPTER 3

SIX1 OVEREXPRESSION EXTENDS THE LIFESPAN OF NORMAL HUMAN KERATINOCYTES AND PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION

3.1 INTRODUCTION

Sine oculis homeobox homolog 1 (*SIX1*) is a homeodomain-containing transcription factor that has a critical role in the expansion of progenitor cells during embryogenesis (Coletta *et. al.*, 2008). SIX1 promotes tumor progression by induction of epithelial-mesenchymal transition (EMT). SIX1 overexpression has been found in various human cancers, including those of the breast, ovary, cervix and liver (Abate-Shen, 2002; Kumar JP, 2009). In HPV16-transformed human keratinocytes (HKc), SIX1 overexpression produces EMT and a differentiation-resistant phenotype at early stages of *in-vitro* progression, and transforms cells to malignancy at late stages (Xu H *et. al*., 2014; Xu H *et. al*., 2015).

Many studies have shown that inappropriate expression of embryonic genes, particularly homeodomain transcription factors, contributes to tumorigenesis and/-or tumor progression (Abate-Shen, 2002). In fact, genes critical for normal development are often exploited by neoplastic lesions. The homeobox gene *SIX1* encodes an embryonic transcription factor broadly expressed during development in different tissues and organs, including muscle, the auditory system, olfactory system and kidney, where it plays a

critical role (Coletta *et. al*., 2010). The overexpression of SIX1 in cancer cells is associated with tumor progression and metastasis, and decreased survival, mirroring its developmental roles (Behbakht *et. al*., 2007).

SIX1 overexpression in immortalized and nontumorigenic mammary epithelial cells and in premalignant HPV16-transformed cells in our model system induces malignant transformation, leading to tumor formation in nude mice (McCoy *et. al*., 2009). SIX1 is overexpressed in 50% of primary mammary carcinomas and 90% of metastatic lesions (Reichenberger *et. al*., 2005). Additionally, SIX1 overexpression in human breast cancer cell lines induces EMT and metastasis (Kumar JP, 2009; McCoy *et. al.*, 2009). SIX1 overexpression correlates with increased tumor malignancy and lymph node metastasis (Zheng *et. al*., 2010). Moreover, SIX1 overexpression is significantly associated with poorer overall survival in advanced-stage colorectal cancer (CRC) (Ono *et. al*., 2012).

The overexpression of SIX1 has been extensively studied in cancer and cancer-derived cells. In fact, SIX1 overexpression has been shown in immortalized mammary epithelial cells to induce malignant transformation, leading to aggressive and invasive tumors when injected into nude mice (Coletta *et. al*, 208). Furthermore, McCoy E. L. *et. al.*, 2009 have generated a transgenic mouse model that expresses the human *SIX1* gene in the mouse mammary epithelium. SIX1 expression in these non-transformed mammary glands of mice induces tumors and increases the mammary stem cell population. However, the ability of SIX1 to initiate malignant transformation in normal primary human cells have yet to be investigate. Hence, we set out to investigate the biological consequences of SIX1 overexpression, *in-vitro*, in primary normal HKc.

The focus of the present study was to explore the functional consequences of SIX1 overexpression, alone or in combination with the overexpression of HRas-V12 and an anti-p53 shRNA, in normal HKc. HKc/SIX1 and HKc/ALL3 grew continuously past the end of the normal HKc lifespan and exhibited a spindle-shape and fibroblastic appearance, rather than the cuboidal morphology of epithelial cells. Moreover, the growth rates of HKc/SIX1 and HKc/ALL3 were comparable to those of early-stage HPV16-immortalized HKc (HKc/HPV16). The invasiveness of HKc/SIX1 was comparable to that of HKc/HPV16 and the invasiveness of HKc/ALL3 is greater than HKc/DR. Finally, using microarrays, we demonstrated that SIX1 overexpression induces EMT in normal keratinocytes. Overall, our data demonstrate that SIX1 facilitates the establishment of primary human keratinocytes into cell lines with an extended lifespan, or perhaps into stem-like cells, and induces epithelial-mesenchymal transition.

3.2 MATERIALS AND METHODS

Cell culture and treatment

The cell lines, HKc/HPV16-d1 and HKc/DRd-1, discussed in this work have been previously described (Pirisi *et. al*., 1987; Pirisi *et. al*., 1988). Normal HKc were maintained in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract, BPE (KSFM, Invitrogen, Carlsbad, CA). HFb were cultured in Dulbecco's modified eagle's medium containing 4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate (DMEM, Invitrogen). HKc/HPV16-

d1, HKc/DRd-1 and normal HKc cells were maintained in a humidified atmosphere of 5% CO_2 at 37 ^oC. HFb was maintained in a humidified atmosphere of 10% CO_2 at 37 ^oC.

Plasmid constructs and stable transfection

First or second passage normal HKc and HFb at 70% confluency were transfected with either a pcDNA3.1/Zeo plasmid encoding human-SIX1 or a combination of three plasmids: pcDNA3.1/Zeo-SIX1, a pBABE/puro plasmid encoding HRas-V12 and a pSUPER/puro plasmid encoding a p53 shRNA. For transfection efficiency, the pSUPER/puro plasmid encoding the GFP reporter gene was used. As a vector control, the pSUPER/puro plasmid was used. Normal HKc and HFb were transfected with Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. Stable transfectants, HKc/SIX1, HKc/ALL3, HFb/SIX1 and HFb/ALL3, were selected in 50 ug/mL Zeocin (Invitrogen) and/or 3 ug/mL Puromycin (Toku-E).

RNA interference (RNAi)

HKc were transfected with two different anti-SIX1 siRNA or anti-GFP siRNA (control) duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. The protocol for transient transfections have been previously described (Chapter 1).

Real-time PCR

Total RNA was isolated from cells using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out with 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed

using iQ SYBR Green Supermix (Bio-Rad). All procedures were followed according to the manufacturer's instructions. The sequences of the primers used for real-time PCR have been previously listed (Chapter 1, Table 2.1). GAPDH was used as an internal control. All samples were assayed in duplicates.

Droplet digital PCR (ddPCR) for HRas-V12 mutation detection

Genomic DNA (gDNA) was isolated from cells using the AllPrep DNA/RNA mini kit (Qiagen). Detection, amplification and quantitation of HRas-V12 was performed by ddPCR, using the QX200 system (Bio-Rad) and probe-based assays (HRas p. G12V and HRas WT for p. G12V PrimePCR ddPCR Mutation Detection Assays; Bio-Rad and custom designed). All procedures were performed according to the manufacturer's instructions. The PCR mixture contained 12.5 µl of ddPCR droplet supermix for probes (no dUTPs), 1.25 µl of mutation primer/probe mix (FAM), 1.25 µl of wild type primer/probe mix (HEX) and 5 µl of gDNA (digested with MseI and diluted 1:10) in a final volume of 25 µl. Twenty-five microliters of this mixture and 70 µl of droplet generation oil were transferred to different wells of a droplet generation cartridge. After formation of droplets using the droplet generator, samples were transferred to a 96-well PCR plate. Plates were sealed at 180 °C using pierceable foil heat seal and a PX1 PCR plate sealer (Bio-Rad). Samples were subjected to PCR amplification for 40 cycles at 94°C for 30 secs and 55°C for 60 secs. Droplets were analyzed on the droplet reader, and Quantasoft software (version 1.4.0.99) was used for analyzing DNA concentrations. Threshold lines for each primer/probe mix, which divides the positive and negative droplets unequivocally, were determined and applied equally to all the samples. Experiments contained control samples, such as wild type gDNA, mutant HRas-V12

plasmid and no template. All samples were assayed in duplicates with at least three biological replicates.

Enzyme-linked immunosorbent assay (ELISA)

Protein was isolated from cells using 10x Cell Lysis Buffer (Cell Signaling, Danvers, MA). The extracted supernatant included a protease inhibitor (Sigma, Saint Louis, MO). PathScan Total p53 Sandwich kit and PathScan Total Rb Sandwich kit were used. Test procedure utilizing total protein was performed according to the manufacturer's instructions. 50 ug/ml of protein was loaded into the p53 pre-coated wells. 150 ug/ml of protein was loaded into the Rb pre-coated wells. All samples were assayed in replicates of two or three. Absorbance was read at 450 nm within 30 minutes using a microplate reader.

Cell proliferation assay

The growth rates of HKc, HKc/SIX1, HKc/ALL3, HKc/HPV16-d1 and HKc/DRd-1 were compared. Proliferation, at 24-hour intervals, was determined at different passage numbers through the Cell Counting Kit-8 (CCK8) sensitive colorimetric assay utilizing the WST-8 dye reagent (Dojindo, Rockville, MD). 5,000 cells in 500 ul of their respective media were plated per well in 24-well plates. Cells were plated in triplicate wells and 50 ul of WST-8 dye solution was added to each well and placed in a CO2 incubator for 2 hours for reaction to take place. Absorbance was read at 450 nm with a microplate reader.

Cell invasion assay

The invasive ability of HKc/SIX1 or HKc/ALL3 in comparison with HKc, HKc/HPV16-d1 and HKc/DRd-1 was measured using CultreCoat Basement Membrane Extract (BME)-Coated Cell Invasion assay (Trevigen, Gaithersburg, MD). Boyden chambers (24-well inserts) with an 8.0 μm polycarbonate (PC) membrane were used. The top side of the membranes was coated with laminin I, collagen IV, entactin and heparin sulfate proteoglycan. The upper surface of the matrix was challenged with 100,000 cells in complete medium. The lower chamber contained DR medium. After 24 hours, a mixture of Cell Dissociation Solution/Calcein AM was added to the bottom chamber of each well and incubated for 1 hour. The number of invading cells was quantified at 485 nm excitation, 520 nm emission with a microplate reader. Each experiment was performed in triplicate wells.

Wound-healing assay

To compare the migration of HKc, HKc/SIX1, HKc/ALL3, HKc/HPV16-d1 and HKc/DRd-1, cells were seeded into 60-mm plates and cultured until near confluency. Then, the cell monolayer was scraped with a 200-uL pipette tip through the central axis of the plate to generate artificial wounds. The wound distances were measured at 0, 4, 8, 12 and 24 hours under the microscope at 40X magnification. Images were captured in at least five different fields. The experiments were performed in triplicates.

Clonogenic assay

To compare the ability of a single cell to grow into a colony 50,000 cells of HKc, HKc/SIX1 and HKc/ALL3 were seeded onto 100-mm^2 plates and cultured for 10 days.

They were then washed twice with PBS, fixed in methyl acetate for 10 minutes and stained with 10% Giemsa (Thermo Fisher Scientific, Waltham, MA) to evaluate colony size. Experiments were performed in triplicate, and colonies with at least 50 cells (cells $\geq$ 1 mm in diameter) were enumerated by ImageJ software (NIH, Washington, D.C.) and compared between the different groups.

Population doubling (PD) and total cell output

To compare the replicative capacity between HKc and HKc/SIX1 or HKc/ALL3, 50,000 cells were seeded onto 100-mm^2 plates and cultured for 10 days. Cells were passaged and re-plated every 10 days up to 40 days. Experiments were performed in triplicate and generation time was calculated with the following equation:

$$\frac{\text{duration} * \log(2)}{\log(\text{Final concentration}) - \log(\text{Initial concentration})}$$

The same dataset was utilized to calculate the total cellular output of cells between HKc and HKc/SIX1 or HKc/ALL3. In general, the average number of cells after each time point (every 10 days) was subtracted from the initial number of seeded cells ($5x10^4$). Experiments were performed in triplicate.

Sphere-formation assay

To determine whether HKc/SIX1 and HKc/ALL3 exhibit anchorage-independence, spheroid-forming ability was tested. 96-well plates were coated with 100 ul of soft polymerized 0.5% agar in complete medium. 50-100 ul of cells

(1×10^6 cells/ml) were suspended as a single-cell suspension. After 24 hours, spheroids were photographed on a Nikon TMS phase microscope using Infinity 1 Analyze software. Each experiment was repeated in replicates of two or three.

Gene-expression microarray analysis and validation by SYBR green real-time PCR

To correlate the genes and pathways that are affected in comparison with those that are affected when SIX1 is suppressed in HKc/DR cells, we utilized the gene expression array analysis from our previous study (Chapter 1).

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Data were interpreted using student's t-test. $P \leq 0.05$, $P \leq 0.01$ or $P \leq 0.001$ were considered statistically significant and indicated in the figures by *, ** or ***, respectively.

3.3 RESULTS

3.3.1 OVEREXPRESSION OF SIX1 IN NORMAL HKc

To investigate the behavior of SIX1 in normal HKc, we set out to explore the effects of stably overexpressing SIX1 alone or in combination with HRas-V12 and p53i. Normal HKc and normal HFb were transfected with either a pcDNA3.1/Zeo plasmid encoding human-SIX1 or a combination of three plasmids: pcDNA3.1/Zeo-SIX1, a pBABE/puro plasmid encoding HRas-V12 and a pSUPER/puro plasmid encoding a p53 shRNA that we had previously demonstrated to be specific and selective (Bheda *et. al*.,

2008). Transfection efficiency was monitored with the co-transfection of the pSUPER/puro plasmid (3:1 w/v), expressing GFP (Figure 3.1, A). Real-time PCR confirmed that the mRNA levels of SIX1 were overexpressed in normal HKc (Figure 3.1, B). SIX1 mRNA levels in HKc/SIX1 were comparable to HKc/DR cells while the HKc/ALL3 cells were approximately 2.3-fold greater than the HKc/DR line. To compare mutant-to-wild type allele ratio of *ras*, primer/probe sets specific for each allele were utilized in ddPCR. The specificity of these primer/probe sets was shown with pBABE/puro. HRas-V12 plasmid as the positive control (Figure 3.2, A). Utilizing ddPCR, Ras-V12 to wild type *ras* ratios from equimolar amounts of genomic DNA of HKc, HKc/ALL3, HKc/HPV16 and HKc/DR were detected. Ras-V12 was observed in HKc/ALL3 and HKc/DR compared to HKc/HPV16 and HKc, which served as the negative control (Figure 3.2, B).

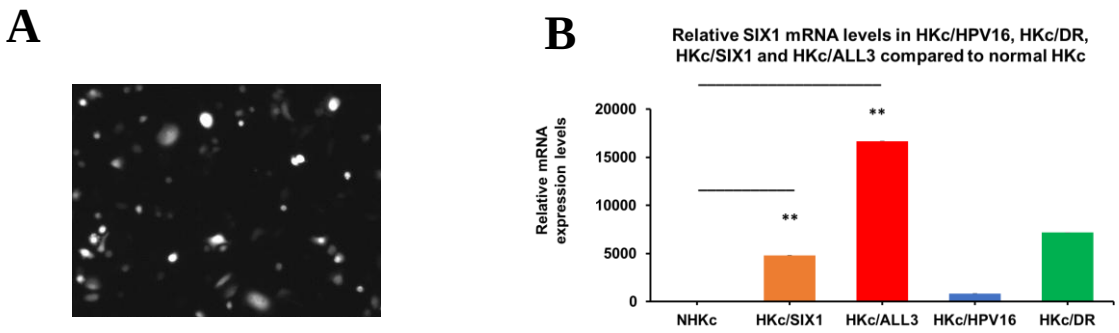


Figure 3.1 The expression of SIX1 significantly increases in normal HKc transfected with a SIX1 expression vector. (A) Representative image of successfully transfected cells. Normal HKc transfected with pcDNA3.1/Zeo-SIX1; a combination of pcDNA3.1/Zeo-SIX1pBABE/puro HRas-V12 and pSUPER/puro-p53 shRNA; or pSUPER/puro vector control plasmid, and co-transfected with GFP (3:1 w/w) to monitor transfection efficiency. GFP expression was analyzed 24-48 hours post-transfection at 200X magnification. (B) Real-time PCR analysis for SIX1 mRNA in normal HKc transfected with the SIX1 expression vector. SIX1 expression was normalized to GAPDH.

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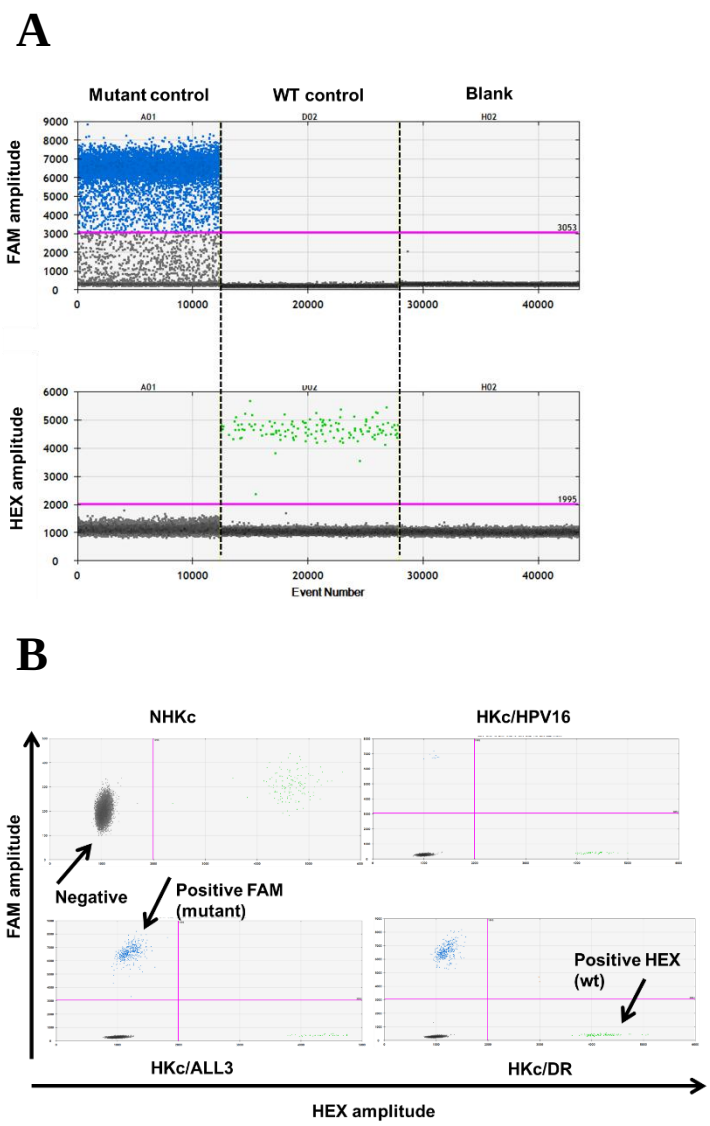


Figure 3.2 The expression levels of HRas-V12 in HKc/SIX1 and HKc/ALL3.
(A) Experimental validation.1D-Dot plot. The blue histogram indicates the number of droplets considered as positive for mutant Hras-V12 according to the fluorescence threshold. The green histogram corresponds to the number of wild-type droplets. Mutant control, DNA containing a 1:1 mix of mutant and wild type Hras-V12. WT control, DNA containing only KRAS wild-type. Blank, water, no template control. (B) Detection threshold of Hras-V12 from gDNA using ddPCR. 2D-Dot plot. Fluorescence results are plotted as two-dimensional dot plots (similar to the depiction of flow cytometry data). The region of these plots can be sequentially separated based on the fluorescence intensity of each droplet. Grey dots correspond to empty droplets. Green dots correspond to droplets containing wild-type copies of Hras. Blue dots correspond to droplets containing Hras-V12 mutation. Each analysis was performed in duplicate. Experiments were repeated at least three times. Nhkc contains no trace of mutant Hras-V12. HKc/ALL3 cells were transfected with the mutant Hras-V12 DNA.

Additionally, HKc and HFb cells transfected with the plasmids encoding SIX1, HRas-V12 and p53i were tested for contamination with the HPV16-E6 and E7 viral oncogenes. HKc/SIX1, HKc/ALL3, HFb/SIX1 and HFb/ALL3 were shown to not be contaminated with HPV DNA (results not shown).

3.3.2 SIX1 DOWNREGULATES p53 IN HKc/SIX1

Once we confirmed the robust SIX1 expression in SIX1-transfected HKc, we next explored the levels of the tumor suppressor genes p53 and Rb. p53 mRNA expression levels were decreased by more than 70% in both HKc/SIX1 and HKc/ALL3 cells (Figure 3.3, A). The levels of p53 were significantly smaller than those in the HKc/HPV16 and HKc/DR cell lines. p53 protein expression levels in HKc/SIX1 and HKc/ALL3 were decreased approximately 60% in comparison to HKc and their protein levels were smaller in comparison to the HKc/HPV16 and HKc/DR cell lines (Figure 3.3, B).

We next examined Rb mRNA levels in our transfected cell lines. Rb mRNA expression was significantly increased in HKc/SIX1 and HKc/ALL3 (Figure 3.3, C). There was a slight decrease in Rb protein levels in HKc/SIX1 with a slight increase in HKc/ALL3 (Figure 3.3, D). Similarly, we have previously demonstrated that the simultaneous knockdown of p53 and Rb in HKc results in the increase of SIX1 mRNA expression levels, as well as in our HKc/HPV16 cell lines (Chapter 1, Figure 2.4).

Given that SIX1 modulates p53 and Rb mRNA expression levels, we set out to assess levels of p53 and Rb after the transient knockdown of SIX1 in HKc. We demonstrated a significant reduction in p53 and Rb mRNA levels with a concomitant reduction in respective protein levels, in cells treated with anti-SIX1 siRNA (Figure 3.4). Collectively, we conclude that SIX1 regulates the expression of the p53 and Rb tumor suppressor genes. Pathways that are affected in response to the expression of the SIX1 oncoprotein warrant further investigation.

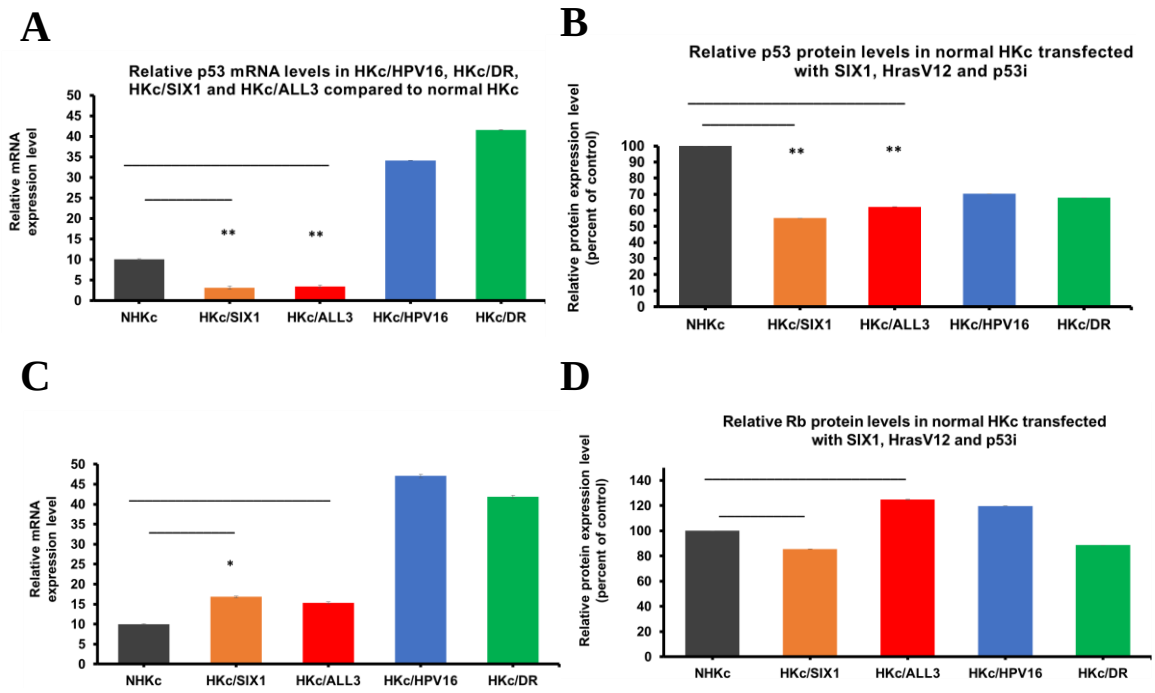


Figure 3.3 The expression levels of p53 and Rb in HKc/SIX1 and HKc/ALL3. (A) Real-time PCR analysis of p53 mRNA in normal HKc. p53 expression was normalized to GAPDH. (B) ELISA for p53 protein expression in normal HKc. (C) Real-time PCR analysis of Rb mRNA in normal HKc. Rb expression was normalized to GAPDH. (D) The relative protein expression of Rb in normal HKc. ELISA for Rb protein expression in normal HKc.

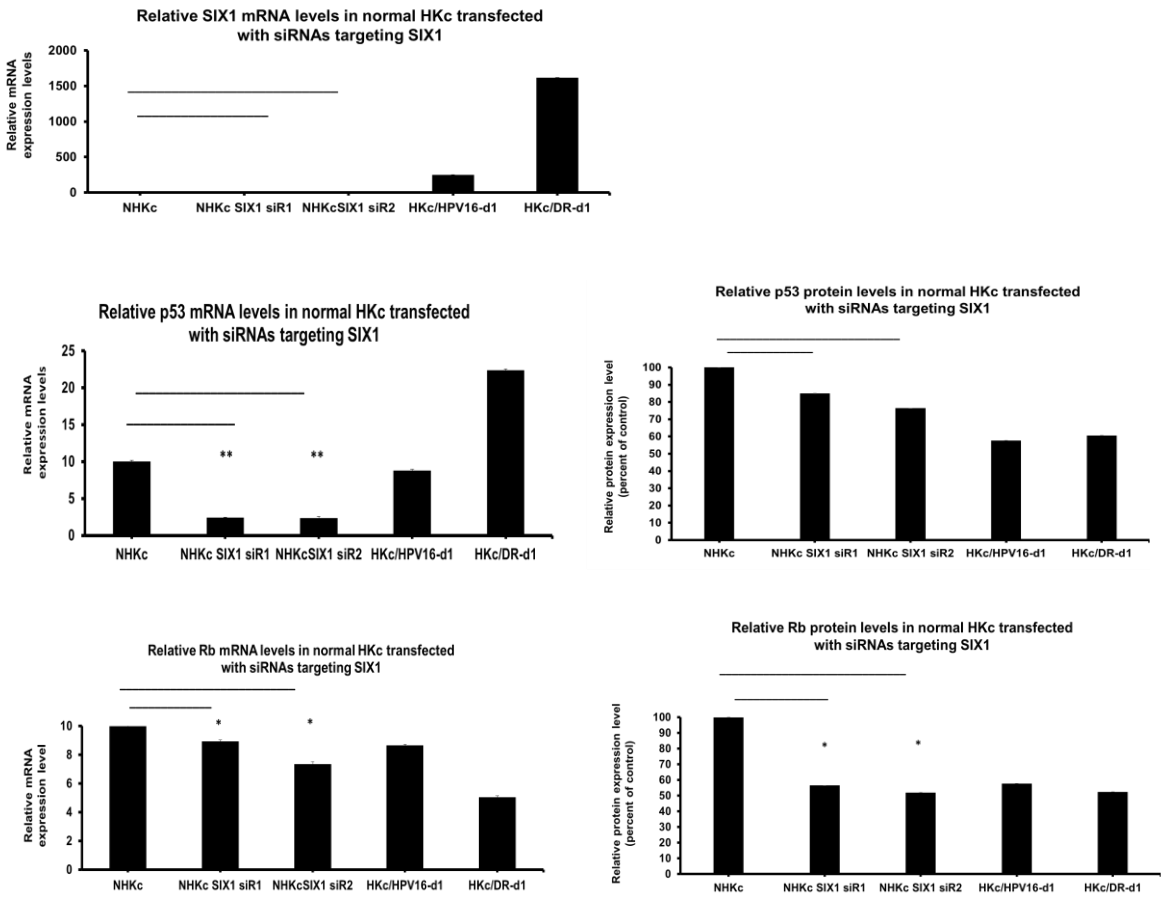


Figure 3.4 The expression of p53 and Rb decrease in normal HKc transfected with pre-formed anti-SIX1 siRNAs. Real-time PCR analysis for SIX1, p53 and Rb mRNA in SIX1-expressing HKc. Levels of expression were normalized to GAPDH; ELISA for p53 and Rb protein expression in SIX1-expressing HKc.

3.3.3 HKc/SIX1 AND HKc/ALL3 DISPLAY AN EXTENDED LIFESPAN AND RESISTANCE TO SERUM AND CALCIUM-INDUCED DIFFERENTIATION

A distinct feature of SIX1 overexpression in normal HKc was the continuous growth of HKc/SIX1 and HKc/ALL3. pSUPER vector-transfected controls senesced. Continuous growth past the end of the normal lifespan was also observed in HFb/SIX1 and HFb/ALL3. We have previously demonstrated that normal HKc and HKc/HPV16 can be cultured in conditions containing serum-free media and low Ca^{2+}, whereas media containing high-calcium or serum elicits terminal differentiation (Pirisi *et. al.*, 1988; Xu H *et. al.*, 2015). One of the features of HKc/DR is resistance to serum and calcium-induced differentiation (Xu H *et. al*., 2015). We investigated whether the expression of exogenous SIX1 in HKc/SIX1 and HKc/ALL3 induces differentiation resistance. Both HKc/SIX1 and HKc/ALL3 grew well in media supplemented with either 5% or 10% fetal bovine serum (FBS) and 1mM $CaCl_2$ while HKc/DR grow best in similar media containing 5% FBS. The results indicate that SIX1 generates differentiation resistance in cells. In addition, HKc/SIX1 and HKc/ALL3 exhibited a spindle-shaped and fibroblastic appearance (Figure 3.5, A). However, these epithelial cells remain morphologically distinct from SIX1-overexpressing normal HFb (Figure 3.5, B). The extended lifespan of HKc/SIX1 and HKc/ALL3 ultimately ended with senescence by passage 30, with or without the supplementation of serum and calcium. These data suggest that SIX1 prolongs the lifespan of epithelial cells but is not, by itself, capable of inducing immortalization.

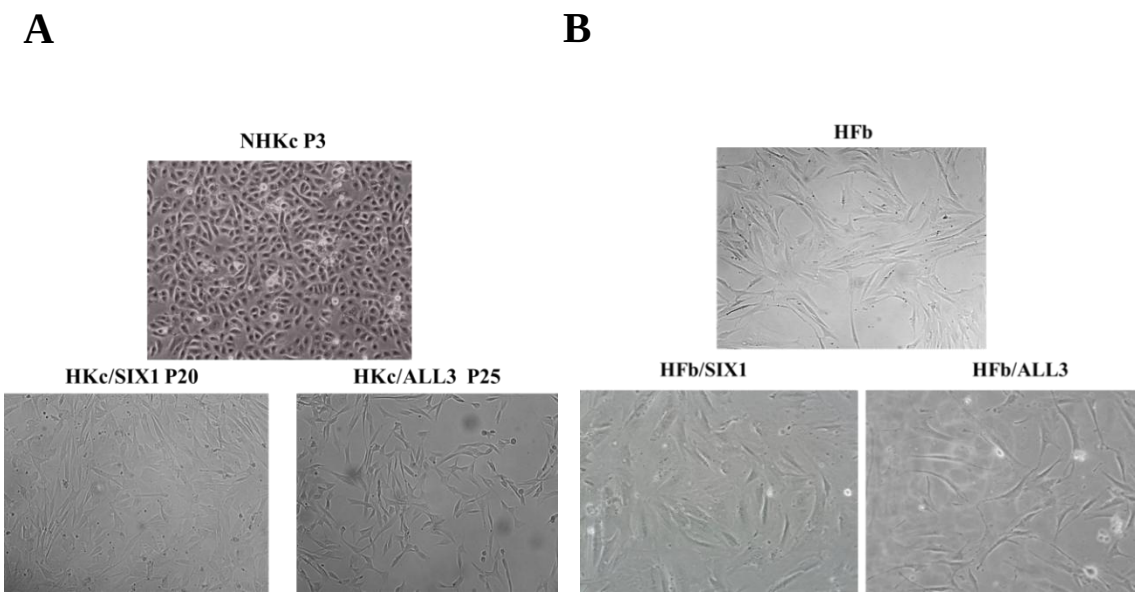
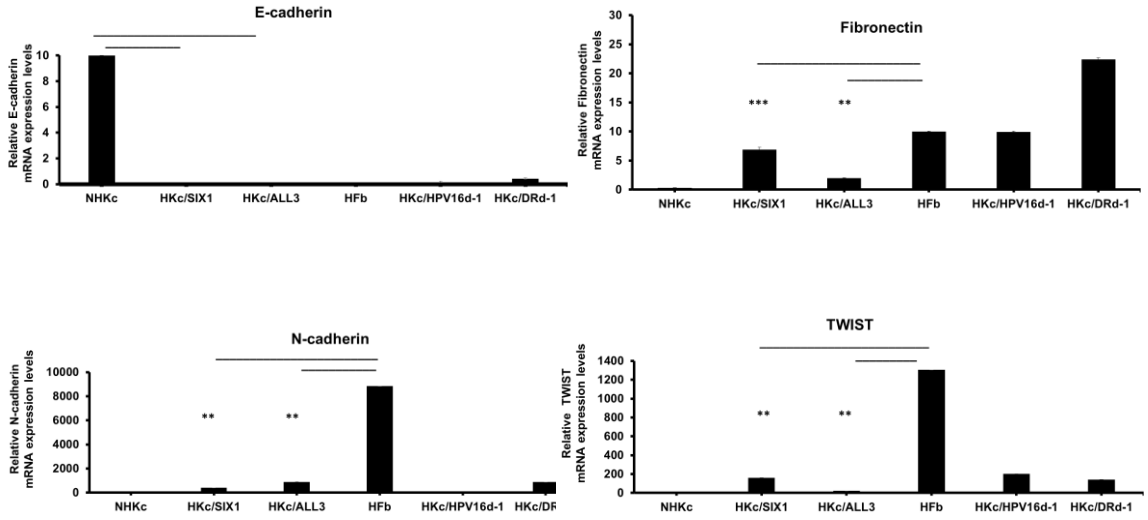


Figure 3.5 Morphological changes due to SIX1 overexpression in normal HKc and HFb. (A) Cell morphology of normal HKc, HKc/SIX1 and HKc/ALL3. Images are shown at 200X magnification. (B) SIX1 overexpression in normal HFb. Cell morphology of normal HFb, HFb/SIX1 and HFb/ALL3. Images are shown at 200X magnification.

3.3.4 SIX1-EXPRESSING CELLS ARE CULTURED FROM KERATINOCYTES AND EXHIBIT EPITHELIAL FEATURES, DESPITE THEIR FIBROBLASTIC MORPHOLOGY

During the beginning stages of foreskin processing, a necessary step is the separation of the dermis from the epidermis. To verify that the SIX1-expressing epithelial cells were of keratinocyte origin, we compared mRNA levels of E-cadherin, Fibronectin, N-cadherin, SNAIL, TWIST and Vimentin (VIM) with that of non-transfected HFb. We also analyzed expression levels of Keratin 14 (K14), an epidermal gene, as well as Keratin 19 (K19), a transformation marker. There were nominal levels of E-cadherin in HKc/SIX1 and HKc/ALL3, possibly due to their ongoing epithelial to mesenchymal

transition. There were significant expression of the mesenchymal markers Fibronectin and N-cadherin but their mRNA levels were smaller in comparison to HFb (Figure 3.6). HKc/SIX1 and HKc/ALL3 seem to demonstrate an intermediate "metastable" state of EMT. There was a significant increase in TWIST but not in SNAIL for the HKc/SIX1 and HKc/ALL3 cells. Moreover, there was a significant increase in the mesenchymal marker VIM in the HKc/SIX1 cells. HKc/SIX1 seems to demonstrate a more advanced metastable stage of EMT in comparison to HKc/ALL3. Lastly, mRNA expression levels of K14 were nominal in the SIX1-expressing cells, perhaps due to EMT, and K19 levels were depicted to be significant, possibly demonstrating that these cells are undergoing transformation.



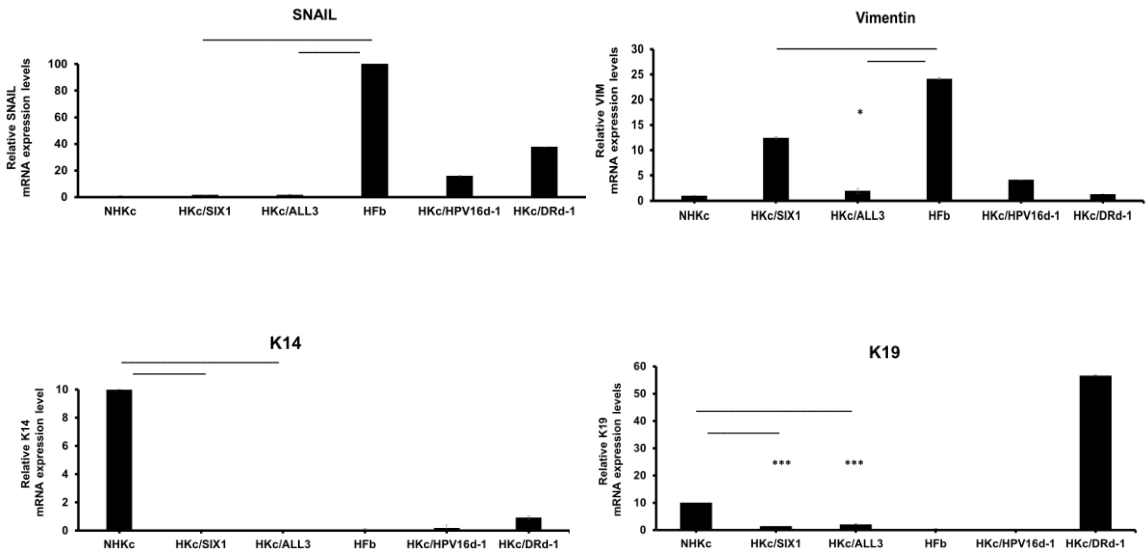


Figure 3.6 Expression of epithelial and mesenchymal-associated genes in SIX1-expressing normal cells. The levels of mRNA expression of E-cadherin, Fibronectin, N-cadherin, TWIST, SNAIL, VIM, K14 and K19 were determined by real-time PCR analysis in normal HKc, normal HFb, HKc/SIX1 and HKc/ALL3. Data were normalized to GAPDH expression.

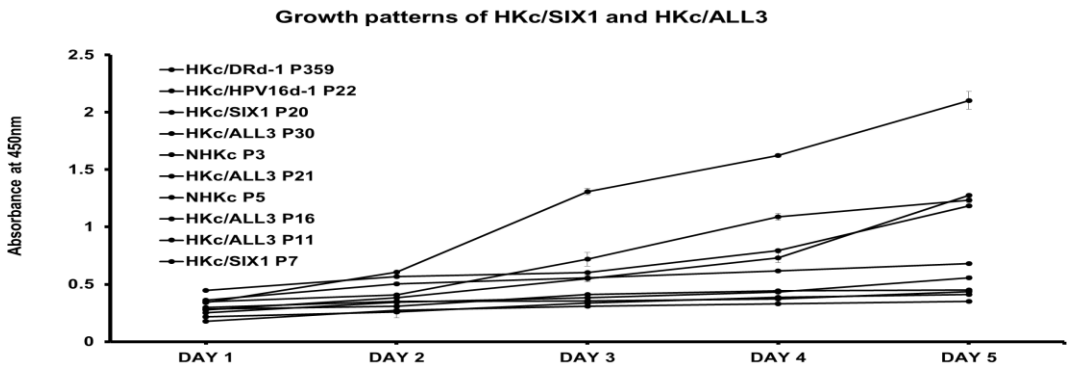
3.3.5 INCREASE IN SIX1 INDUCES ALTERATIONS IN GROWTH, INVASION AND MIGRATION IN HKc/SIX1 AND HKc/ALL3

To further elucidate the functional role of SIX1 in HKc, we proceeded to demonstrate their proliferative capacity and invasive ability. The growth rates of HKc/SIX1 and HKc/ALL3 were comparable to the HKc/HPV16 cells (Figure 3.7, A). We also demonstrated that as the passage number increased, the proliferative capacity of HKc/SIX1 and HKc/ALL3 increased. Moreover, invasion assays demonstrated that HKc/SIX1 is more invasive than normal HKc, and their invasiveness is comparable to that of HKc/HPV16. The invasiveness of HKc/ALL3 is greater than the HKc/DR cells,

which are about 2.5-fold more invasive than HKc/HPV16 (Figure 3.7, B). Additionally, we demonstrated that as the passage number increased, the invasive abilities of our SIX1-expressing cell lines increased as well.

Given that the SIX1-expressing cells presented an enhanced rate of proliferation and invasion, we set out to explore their migratory potential by utilizing a wound-scratch assay. Furthermore, HKc/SIX1 and HKc/ALL3 restored 77% and 79%, respectively, of the wounded area within 24 hours in culture (Figure 3.7 C, D). Their rate of wound closure fell in-between that of HKc/HPV16 and HKc/DR (Table 3.1). These results further validate that HKc/SIX1 and HKc/ALL3 exhibit a mesenchymal phenotype and demonstrate their increases in cell proliferation, invasion and migration in comparison to HKc.

A



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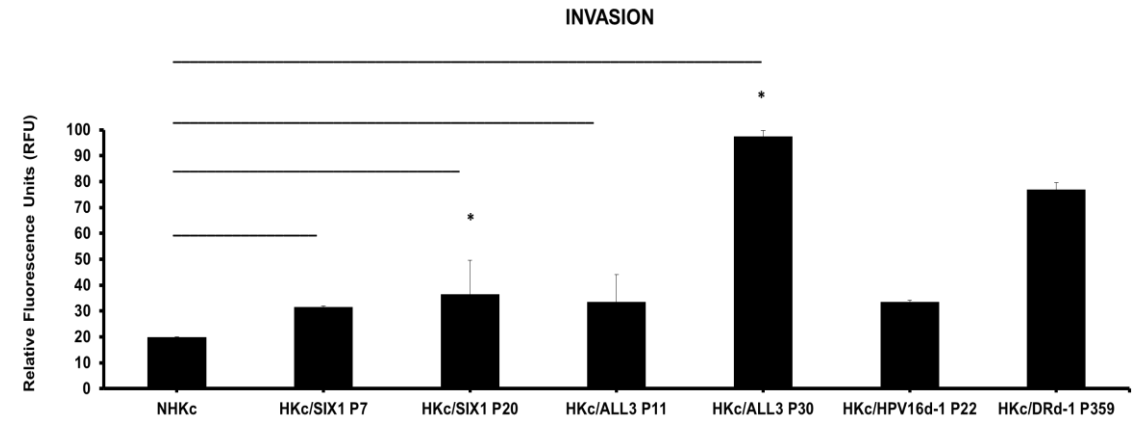

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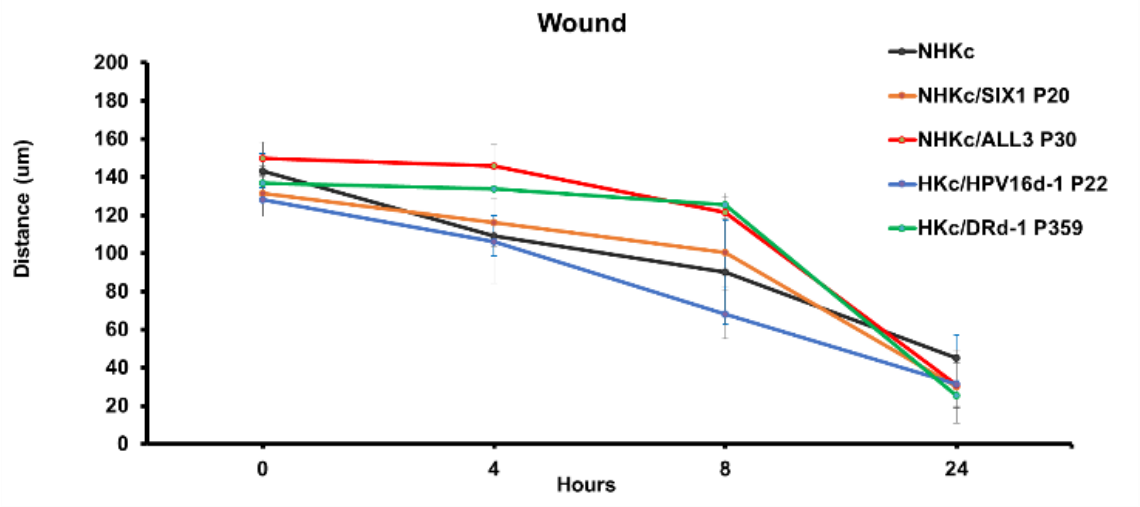

D

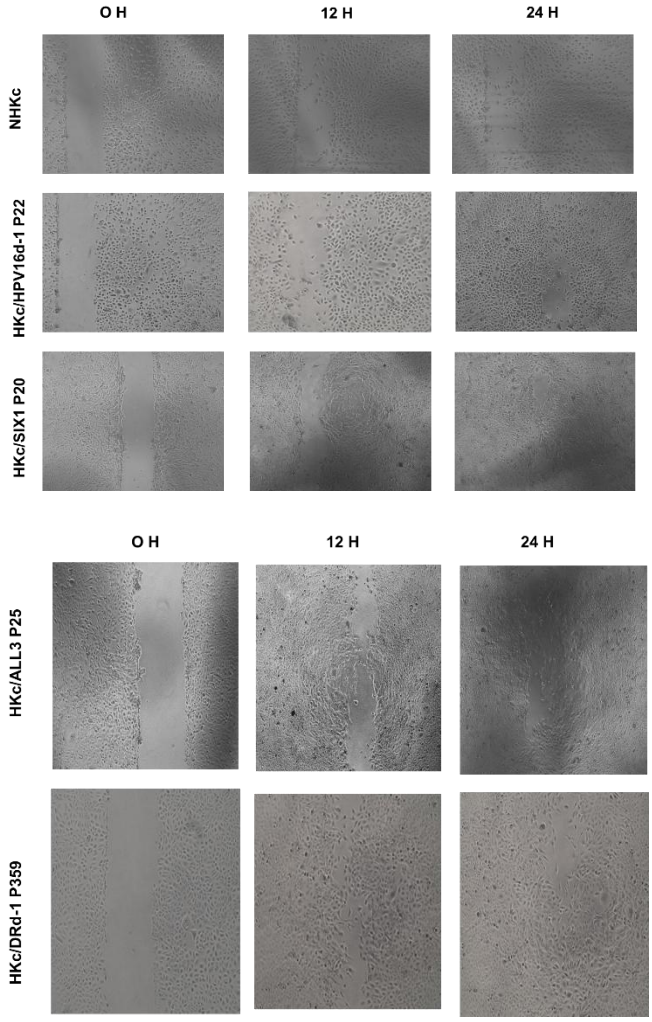

Figure 3.7 SIX1 overexpression induces alterations in growth, invasion and migration in normal HKc. (A) Cell proliferation was determined by the Cell Counting Kit-8 (CCK8) colorimetric assay utilizing the WST-8 dye reagent at the days indicated in normal HKc, HKc/SIX1, HKc/ALL3, HKc/HPV16 and HKc/DR. (B) Invasion assays for normal HKc, HKc/SIX1, HKc/ALL3, HKc/HPV16 and HKc/DR. Basement membrane extract (BME) cell invasion as determined by transwell migration. Quantification of invading cells was determined by the addition of calcein AM after 24 hours. (C) Percentage of wound closure after a scratch in normal HKc, HKc/SIX1, HKc/ALL3, HKc/HPV16 and HKc/DR at the time points indicated. (D) Scratch-wound assay depicting the migratory behaviors of normal HKc, HKc/SIX1, HKc/ALL3, HKc/HPV16 and HKc/DR at 12-hour intervals.

Table 3.1 Percentage of wound closure between NHKc, HKc/SIX1, HKc/ALL3, HKc/HPV16 and HKc/DR.

sample	% wound closure
HKc/DRd-1 P359	81
HKc/ALL3 P30	79
HKc/SIX1 P20	77
HKc/HPV16-d1 P22	76
NHKc	69

3.3.6 SIX1-EXPRESSING EPITHELIAL CELLS DISPLAY HIGH CLONAL GROWTH AND INCREASED REPLICATIVE CAPABILITY

To assess the ability of HKc to form colonies, $5x10^4$ cells were plated in 100-mm^2 dishes and sub-cultured for 10 days. Normal HKc were used as controls. HKc/SIX1 and HKc/ALL3 demonstrated more robust colony formation, more than 3-fold, in comparison to HKc (Figure 3.8, A; Table 3.2). Additionally, HKc/SIX1 and HKc/ALL3 both underwent nearly 24 population doublings (PD) within 40 days in culture, compared to the 9 PD acquired by HKc (Figure 3.8, B; Tables 3.3, 3.4). The approximate doubling time of HKc/SIX1 and HKc/ALL3 was between 48-60 hours in comparison to the doubling time of 72 hours in normal HKc. Lastly, the total concentration of cells in HKc/SIX1 and HKc/ALL3 were up to 14- and 16- fold greater, respectively, than normal HKc after 30 days in culture (Figure 3.8, C; Table 3.5). These findings suggest that SIX1-expressing epithelial cells have the increased capacity to replicate and produce a significant number of colonies within a shorter period.

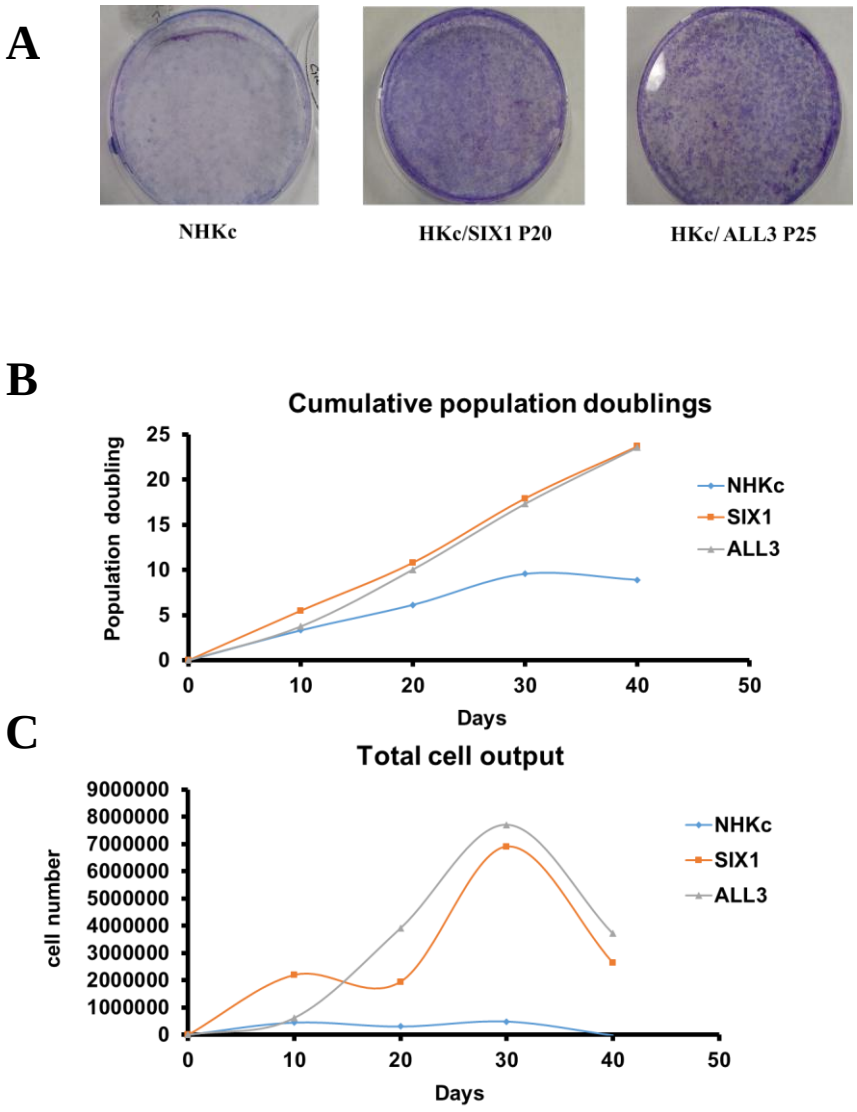

Figure 3.8 HKc/SIX1 and HKc/ALL3 display high colony density and increased replicative capability. (A) $5x10^4$ cells were plated in 100-mm^2 dishes, sub-cultured and colony patterns were examined by Giemsa staining after 10 days. Normal HKc, HKc/SIX1 and HKc/ALL3 after 10 days in culture. (B) Cumulative population doublings (PD) observed in HKc/SIX1 and HKc/ALL3 relative to normal HKc over the course of 40 days. (C) Total cell output in HKc/SIX1 and HKc/ALL3 relative to normal HKc over the course of 40 days.

Table 3.2 Clonal growth of NHKc, HKc/SIX1 and HKc/ALL3 10 days in culture. Significant increase in dish occupancy of HKc/SIX1 and HKc/ALL3 relative to normal HKc after 10 days in culture. Cells were manually counted and quantified by ImageJ software to determine colony density 10 days after being in culture.

Cumulative population doublings			
Days	NHKc	SIX1	ALL3
0	0	0	0
10	3.321928	5.491853096	3.744161096
20	6.142958	10.81378119	10.05401636
30	9.575917	17.93617782	17.33324
40	8.916954	23.69106532	23.56845646

Table 3.3 Cumulative population doublings NHKc, HKc/SIX1 and HKc/ALL3.

NHKc: 72-80H doubling time
HKc/SIX1: 48-60H doubling time
HKc/ALL3: 48-60H doubling time

Table 3.4 Estimated doubling times of NHKc, HKc/SIX1 and HKc/ALL3.

Total cell output			
Days	NHKc	SIX1	ALL3
0	0	0	0
10	450,000	2,200,000	620,000
20	303,333	1,950,000	3,916,667
30	490,000	6,916,667	7,716,667
40	-18,333	2,650,000	3,716,667

Table 3.5 Total cell output between NHKc, HKc/SIX1 and HKc/ALL3 over the course of 40 days. Initial number of seeded cells was $5x10^4$.

	NHKc	HKc/SIX1	HKc/ALL3
manual colony count	165	450-500	350-400
ImageJ	120	460	353

3.3.7 HKc/SIX1 AND HKc/ALL3 SURVIVE IN SUSPENSION CULTURE

To understand whether HKc/SIX1 and HKc/ALL3 have the capability to evade programmed cell death upon loss of adhesion to a monolayer culture substrate, we utilized a spheroid culture model containing a mixture of agarose and KSFM to investigate their spheroid forming abilities. HKc/SIX1 and HKc/ALL3 had the capability to aggregate into multicellular spheroids (Figure 3.9 A, B). Furthermore, when the suspended spheroids were transferred from the spheroid culture back to a monolayer culture, they readily adhered and began proliferating (Figure 3.9 C-F). These findings not only demonstrate that SIX1-expressing keratinocytes can overcome suspension-induced cell death (anoikis), accumulate and assemble into spheroids in a suspension culture, they remain functionally viable (up to at least eight passages) once re-plated from the spheroid culture back into a monolayer culture (Woappi Y *et. al.*, 2017). In addition, spheroid-derived HKc/SIX1 and HKc/ALL3 returned to a cobblestone-type morphology when re-plated in monolayers.

3.3.8 SIX1 OVEREXPRESSION IN NORMAL HKc PROMOTES EMT

Due to the changes in cellular morphology and behavior, such as growth and invasion, in HKc/SIX1 and HKc/ALL3, we set out to investigate the expression of EMT related genes. This study explored the effects of SIX1 overexpression in normal HKc on global gene expression through the utilization of Affymetrix GeneChip Human Gene 2.0 ST Arrays. We used Transcriptome Analysis Console (TAC) software from Affymetrix to analyze the microarray results. We were interested in the same set of genes as our paper (Chapter 1) that discussed the role of SIX1 knockdown on global gene expression

in HKc/DR cell lines. To determine the differentially expressed genes from the microarray dataset, we used a fold change >1.4 (up and down) and a *P* value > 0.01 as cutoff values.

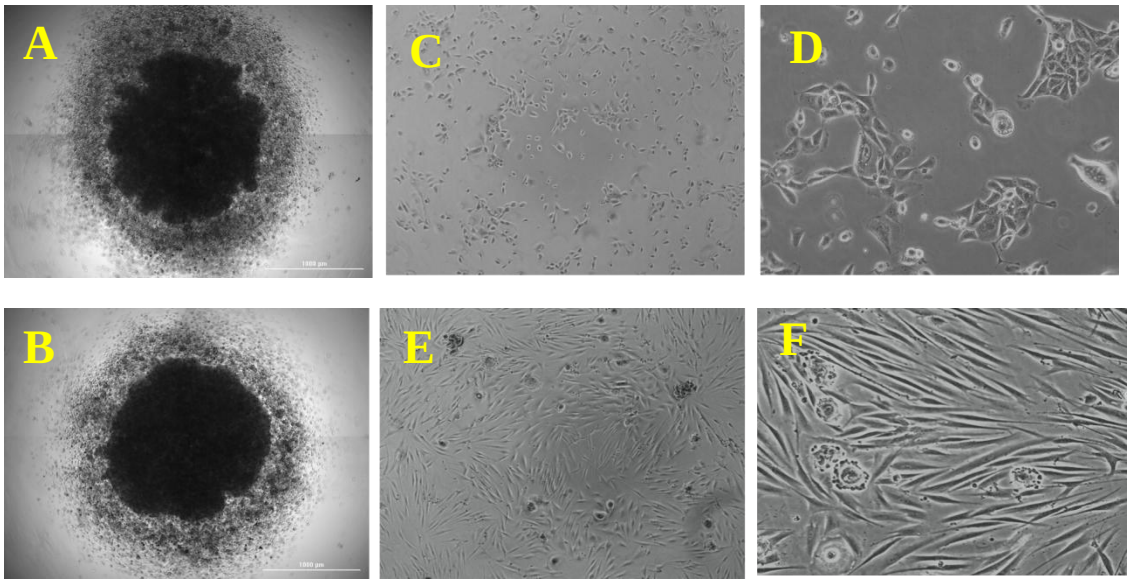

Figure 3.9 HKc/SIX1 and HKc/ALL3 survive in suspension culture. (A) HKc/SIX1 in spheroid (B) HKc/ALL3 in spheroid (C) (D) HKc/SIX1 at eight passages after suspension culture. 40X and 100X, respectively. (E) (F) HKc/ALL3 at 24 hours after suspension culture. 40X and 100X, respectively. Data not shown for eight passages after suspension culture.

The following images (Figure 3.10; Tables 3.6, 3.7) demonstrate the differentially expressed genes that were either upregulated or downregulated in HKc/SIX1 and HKc/ALL3. Based on our microarray data from our original paper (Chapter 1), we analyzed the following set of genes, which included EMT-associated genes regulated by TGF-β (Wendt *et. al.*, 2009): connective tissue growth factor (CTGF/CCN2), β-catenin (CTNNβ1), ETS proto-oncogene 1, transcription factor (ETS1), Keratin-15 (K15), Occludin (OCLN) and TGF-β receptors type I and II (TβRI, TβRII). We also investigated the epidermal proliferation gene, ki-67, and epidermal growth factor receptor, EGFR. To validate the array data, we performed real-time PCR. In the HKc/SIX1 and HKc/ALL3 cells, we observed an increase in the angiogenic matricellular marker CTGF and a reduction in its downstream signaling effector ETS1 (Ubink *et. al*., 2016; Geisinger *et. al*., 2012). HKc/SIX1 displayed significant increases in expression of the inflammatory response gene prostaglandin-endoperoxide synthase 2, PTGS2 (cyclooxygenase-2, COX-2). There were significant increases in peroxisome proliferator activated receptor gamma (PPARγ), a ubiquitously expressed gene known to regulate differentiation and cell growth (Sarraf *et. al*., 1999).

Additionally, there was a decrease in the expression of the cell-cell adhesion complex molecule CTNNβ1 (Gumbiner *et. al*., 2005). As expected, we observed a reduction in the expression of the epithelial-related genes K15 and OCLN. We noted an increase in the pro-survival factor EGFR. Lastly, we noted an increase in ki-67 mRNA level. These data suggest that a consequence of SIX1 is the activation of EMT in normal HKc.

Table 3.6 Expression of EMT and MET-associated genes targeted by SIX1 and TGF-β in normal HKc.

gene	HKc/SIX1 expression	HKc/ALL3 expression
Fibronectin	increase	increase
N-cadherin	increase	increase
TWIST	increase	increase
Vimentin	increase	Not significant
K19	increase	increase
ki67	increase	increase
CTGF	increase	Not significant
EGFR	increase	increase
PPARγ	increase	increase
PTGS2	increase	Not significant
TGFβRII	increase	increase

Table 3.7 Expression of EMT and MET-associated genes targeted by SIX1 and TGF-β in normal HKc.

gene	HKc/SIX1 expression	HKc/ALL3 expression
E-cadherin	Not significant	Not significant
OCLN	decrease	decrease
CTNNβ1	decrease	decrease
ETS1	decrease	decrease
TβRI	decrease	decrease

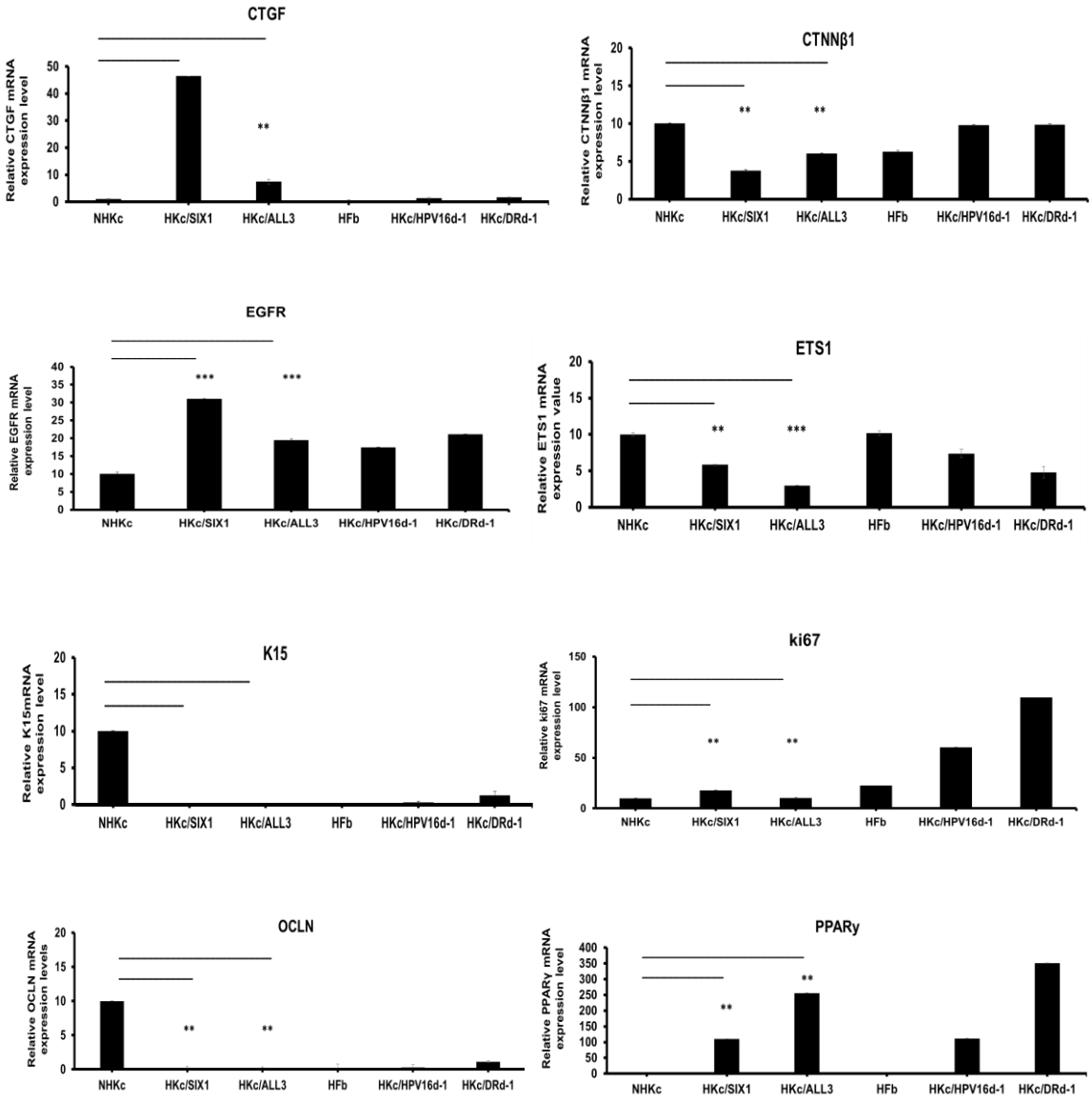

Figure 3.10 SIX1 overexpression in normal HKc promotes EMT. Levels of mRNA expression of CTGF, CTNNβ1, EGFR, ETS1, ki-67, K15, OCLN, PPARy and PTGS2 (COX-2) were determined by real-time PCR analysis in normal HKc, HKc/SIX1 and HKc/ALL3. Data were normalized to GAPDH expression.

3.3.9 INCREASE IN SIX1 EXPRESSION MODULATES TGF-β RECEPTOR mRNA LEVELS IN NORMAL HKc

We attempted to identify pathways known to dictate SIX1-induced EMT within the normal HKc population. TGF-β signaling is a critical pathway in which SIX1 overexpression promotes EMT in malignant cells. The central EMT modulator, TGF-β, is known to exhibit a dual role in tumor development as well as in our HKc/HPV16 and HKc/DR cell lines. TGF-β has been shown to switch from growth suppressive to EMT-inducing in the progression of HPV-mediated transformation of human keratinocytes (Wendt *et. al.*, 2009). The majority of genes described here are known to be regulated by TGF-β signaling. TGF-β receptors type I (TβRI), II (TβRII), and III (TβRIII) are part of the TGF-β pathway cascade. Previous microarray data revealed an increase in TβRI and a decrease in TβRII when SIX1 was decreased in the HKc/DR lines (Chapter 1). Real-time PCR demonstrated similar results. In contrast, when we tested TβRI and TβRII mRNA expression levels in HKc/SIX1 and HKc/ALL3 we observed a decrease in TβRI and an increase in TβRII (Figure 3.11), similar to what we have observed when we increase SIX1 levels in our HKc/HPV16 and HKc/DR cell lines (Xu H *et. al.*, 2014; Xu H *et. al.*, 2015).

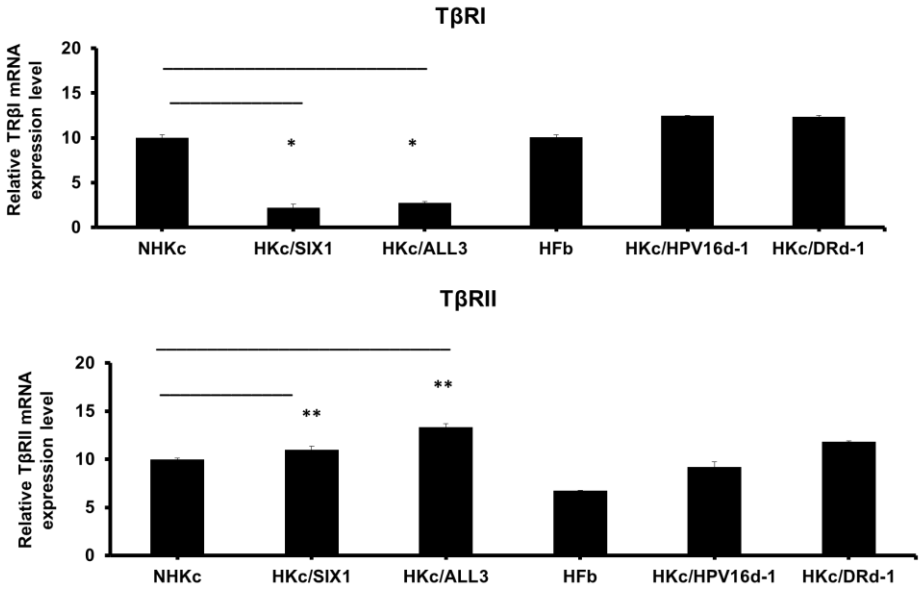

Figure 3.11 SIX1 modulates TGF-β receptor mRNA levels in normal HKc. Levels of mRNA expression of TGFβRI and TGFβRII were determined by real-time PCR analysis in normal HKc, HKc/SIX1 and HKc/ALL3. Data were normalized to GAPDH expression.

3.4 DISCUSSION

It is well documented that the signaling pathways involved in carcinogenesis often represent aberrations of molecular activities that occur during early development. Misexpression of embryonic transcription factors are known to lead to carcinogenesis (Abate-Shen *et. al*., 2002). These proteins reestablish developmental programs out of context. SIX1 is a developmentally restricted transcription factor that inappropriately reinstates numerous embryonic pathways, including growth, proliferation, survival and migration during oncogenesis (Kumar *et. al.*, 2009). Indeed, the SIX1 oncofetal protein

participates in the regulation of numerous pediatric and adult cancers (Abate-Shen *et. al.*, 2002; Coletta *et. al.*, 2010; Kumar *et. al.*, 2009).

Serrano M. *et. al*., 1997 demonstrated that transformation of primary cells by *ras* requires either an oncogene or the inactivation of tumor suppressors such as p53, p21 or p16. Oncogenic *ras* induces senescence through p53 and p16 in primary human or rodent cells; and therefore, inactivation of p53 and/-or p16 prevents *ras*-induced arrest in these cell types that are not transformed nor immortalized (Serrano *et. al*., 1997). With this in mind, we have shown that the overexpression of SIX1 extends the lifespan of normal HKc and pushes these cells to become resistant to serum and calcium-induced differentiation in culture. SIX1 expression in normal HKc induces a spindle-shaped and fibroblastic appearance rather than the known cuboidal morphology of epithelial cells. Moreover, HKc/SIX1 and HKc/ALL3 cells demonstrated increases in cell proliferation, invasion and migration compared to HKc.

As a result of SIX1 expression in normal HKc, p53 mRNA and protein expression levels were drastically decreased. SIX1 is not implicated to affect levels of p53 until Towers *et. al.*, 2015, discovered that SIX1 downregulates p53 through a mechanism that involves the direct upregulation of microRNA-27a and the simultaneous downregulation of the p53-translational regulator, ribosomal protein L26 (RPL26) (Towers *et. al*., 2015). Similarly, we have shown that the simultaneous knockdown of p53 and Rb in HKc results in the increase of SIX1 mRNA expression levels. Together, these findings demonstrate that SIX1 has a relationship with p53 that warrants further investigation.

The development of metastatic lesions has been known to contribute to the dysregulation of oncogenes and tumor suppressor genes. Li *et. al.,* 2013 have shown that silencing of SIX1 decreased phosphorylation levels of Rb in human pancreatic carcinoma cell lines (PANC-1). This may explain SIX1 induced hyperproliferation, cell growth and cell cycle progression (Li Z *et. al.,* 2013). Similarly, in normal HKc, we have shown that overexpressing SIX1 leads to an increased expression of Rb. This tumor suppressor gene is known to bind to E2F and inhibit transcriptional regulation by E2F and cell cycle progression (Young *et. al.,* 2003). SIX1 expression is known to be regulated by the E2F family member E2F1. The overexpression of SIX1 leads to the deregulation of cell cycle checkpoints including E2F (Kumar *et. al.,* 2009). The mechanism for this action is unknown in the HKc/SIX1 and HKc/ALL3 cell lines and needs further elucidation. We hypothesize that E2F detaches from Rb during the upregulation of SIX1 that leads to cell cycle progression and aberrant proliferation of SIX1-expressing cells. E2F can therefore activate cell cycle genes, while allowing for the aberrant expression of the embryonic gene SIX1. In turn, Rb mRNA levels rise to increase the production of Rb protein levels. However, the rate of Rb protein production cannot be significantly increased in order to attach to E2F and inhibit increased cell growth and cell cycle de-regulation (Kumar *et. al.,* 2009; Young *et. al.,* 2003). Thus, the misexpression of SIX1 in normal or tumorigenic cell lines leads to deregulation of cell-cycle checkpoints, promoting cellular proliferation.

It has been shown that SIX1-expressing malignant cells display larger colony size and higher colony formation rate in soft agar while evading anoikis. SIX1 has been shown to promote tumor cell proliferation *in-vitro* and growth *in-vivo* (Liu *et. al*., 2014).

In concordance with these findings, our experiments of colony formation, including in soft agar, demonstrated that HKc/SIX1 and HKc/ALL3 produce robust colony formation in monolayer, and survive in suspension, suggesting that SIX1-expressing epithelial cells have the increased capacity to replicate and produce a significant number of colonies within a shorter period.

The changes in cellular morphology and behavior, such as growth and invasion, which were exhibited in HKc/SIX1 and HKc/ALL3 as a result of SIX1 overexpression, lead us to investigate the expression of EMT related genes. Based on our published array data from our previous work (Chapter 1), we analyzed the differential expression of numerous genes. We validated, through real-time PCR, the increased expression of CTGF and decreased expression of ETS1. Tumorigenic cells are known to metastasize via blood or lymphatic vessels through an increase in CTGF/CCN2 (Ubink *et. al*., 2016). Moreover, there was a decrease in the expression of CTNNβ1, in contrast to what we observed in our HKc/DR cells that underwent a loss of SIX1. In this present context, this could be attributed to a shift away from an epithelial phenotype with changes that follow a loss in cell adherens junctions. We also observed a reduction in the expression of the epithelial-related genes K15 and OCLN. We noted an increase in the pro-survival factor EGFR. Malignant cells are known to escape anoikis through the increase of EGFR (Woappi *et. al.*, 2017). Lastly, we noted an increase in ki-67 mRNA level. These data suggest that EMT is due to SIX1 overexpression in normal HKc. Therefore, a significant increase in these markers parallels an EMT phenotype and is an indication of the functional consequences brought on by an increase in SIX1.

We have demonstrated that activation of TGF-β pathway is necessary to maintain most of the epithelial plasticity from forced SIX1 expression. We have also shown that SIX1 is a critical mediator of epithelial plasticity and TβRII induction through its ability to induce TGF-β-dependent EMT. We demonstrate that SIX1 expression in HKc/SIX1 and HKc/ALL3 induces properties of oncogenic EMT. Further, we observed a decrease in TβRI and an increase in TβRII in SIX1-expressing epithelial cells. This may indicate that an increase in SIX1 expression permits normal HKc to begin their transition away from an epithelial and towards a mesenchymal phenotype, as TGF-β switches from a growth suppressive to a growth promoter cytokine.

Stem cells, which are found in normal tissue, have the ability of self-renewal through cell division along with the generation of progenitor cells that will eventually differentiate into cells and tissues of various functions. Many studies have also determined that a small proportion of malignant cells and tissues have self-renewing, stem cell like properties (Mani *et. al.,* 2008). These cells have been termed cancer stem cells (CSCs). CSCs have the ability to produce new tumors within the heterogeneous malignant cell populations (Mani *et. al.,* 2008). Along similar lines, activation of EMT in immortalized cells produces many of the properties associated with stem cells, such as self-renewal (Mani *et. al*., 2008). The process of EMT is known to mediate tumor progression, metastasis and CSC properties (Christiansen *et. al*., 2006). Therefore, the transient induction of EMT in our HKc/SIX1 and HKc/ALL3 cells may allow for the possibility of generating stem cell like features.

In summary, SIX1 promotes hyperproliferation and invasiveness, indicative of cellular transformation, in the background of normal HKc. SIX1 facilitates the

establishment of primary human keratinocytes into cell lines with extended life span, or perhaps into stem-like cells with extended proliferation potential. The mechanisms underlying the pro-tumorigenic and pro-metastatic properties of SIX1 include the activation of multiple signaling pathways. We have shown that SIX1 has the ability to extend the lifespan of normal epithelial cells and induce an oncogenic EMT pathway through the activation of TGF-β and its downstream targets.

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