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HPV16-transformed human keratinocytes depend on SIX1 expression for proliferation and HPV E6/E7 gene expression

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ABSTRACT

The homeodomain transcription factor SIX1 plays a critical role in embryogenesis, is not expressed in normal adult tissue, but is expressed in many malignancies, including cervical cancer. SIX1 drives the progression of HPV16-immortalized human keratinocytes (HKc/HPV16) toward malignancy: HKc/HPV16 express high levels of SIX1 mRNA and protein; overexpression of SIX1 in HKc/HPV16 produces pre-malignant, differentiation-resistant lines (HKc/DR); SIX1 overexpression in HKc/DR induces tumorigenicity. In this paper, we explore the consequences of inhibition of SIX1 expression in premalignant HKc/DR. Only partial inhibition of SIX1 expression could be obtained in HKc/DR by RNA interference. Decreased SIX1 expression (up to 80%) in HKc/DR resulted in slower proliferation, decreased HPV16-E6/E7 mRNA levels, and increased p53 protein levels. Gene expression changes induced in HKc/DR by anti-SIX1 shRNA were indicative of mesenchymal-epithelial transition (MET) and changes in TGF-beta signaling. We conclude that HPV16-transformed cells depend on SIX1 for survival, HPV16 E6/E7 gene expression and epithelial-mesenchymal transition.

1. Introduction

According to the World Health Organization (WHO) there are about 500,000 new cases and 300,000 deaths annually due to cervical cancer (WHO/ICO, 2010; reviewed in Peralta-Zaragoza et al., 2012). Virtually all cervical cancer cases are due to persistent infection with a high-risk human papillomavirus (HPV). HPV infection is the most common sexually transmitted disease of viral origin (Saslow et al., 2012). Currently, 79 million people in the USA have an HPV infection. By age 50, about 80% of women have acquired an HPV infection (Saslow et al., 2012).

HPVs are small, non-enveloped viruses containing circular, double-stranded DNA encoding six to eight early and two late proteins. HPV infects the epithelial cells of skin and mucosae. There are approximately 200 HPV types, 40 of which infect the genital mucosa (Faridi et al., 2011). HPVs can be further classified into high-risk (oncogenic) types that cause virtually all cervical cancers, and low-risk (non-oncogenic) types that cause warts, including genital warts (condyloma acuminatum). 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, which are responsible for about 95% of genital warts (Crow, 2012). Most HPV infections are asymptomatic and clear spontaneously within 9–18 months, although in some cases HPV remains latent at undetectable levels in the cervical epithelium. However, approximately 10% of infected women cannot clear a high-risk HPV infection and are at risk for developing cervical cancer (Grainge et al., 2005). The Gardasil-9 vaccine prevents infections by nine HPV types: 6, 11, 16, 18, 31, 33, 45, 52 and 58 (Harper and Demars, 2017).

Numerous studies have determined that the inappropriate expression of embryonic genes in cancer, in particular transcription factors, contributes to carcinogenesis (Abate-Shen, 2002). Among the embryonic genes that play a role in cancer development, the homeobox gene *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 (Christensen et al., 2008). When aberrantly expressed in cancer cells, SIX1 has also been shown to increase 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

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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 (Tan et al., 2011; Zheng et al., 2010). For example, SIX1 is overexpressed in half of primary mammary carcinomas and most metastatic lesions (Reichenberger et al., 2005). SIX1 is also overexpressed in cervical cancer cell lines and tissues (Wan et al., 2008) correlating with increased malignancy and metastasis (Tan et al., 2011; Zheng et al., 2010). 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).

In order to study the cellular and molecular mechanisms of HPV-mediated transformation, we established an *in-vitro* model system to study the progression of HPV16-immortalized human keratinocytes toward premalignant and malignant phenotypes. In this model, primary human foreskin keratinocytes (HKc) isolated and cultured in low-calcium Keratinocyte Serum-Free Media (KSFM) were immortalized by transfection with the plasmid pMHPV16d, which contains two copies of the complete HPV16 genome in a head-to-tail configuration (Pirisi et al., 1987, 1988). The resulting HPV16-immortalized human keratinocytes (HKc/HPV16) were selected in KSFM devoid of Epidermal Growth Factor (EGF) and Bovine Pituitary Extract (BPE) giving rise to growth factor independent lines (HKc/GFI). Neither HKc/HPV16 or HKc/GFI were tumorigenic in nude mice (Pirisi et al., 1987, 1988). HKc/GFI were further selected in KSFM supplemented with 5% fetal bovine serum and 1.0 mM calcium chloride, to produce differentiation-resistant lines (HKc/DR). HKc/DR were not tumorigenic even at high passages (Pirisi et al., 1988; Xu et al., 2014) however they produced tumors in nude mice when transfected with plasmids expressing activated *Ras*, Herpes Simplex Virus 2 (DiPaolo et al., 1989, 1990) or SIX1 (Xu et al., 2014).

Microarray studies conducted in our laboratory indicated that this *in-vitro* model system reflects many cellular and molecular alterations characteristic of cervical cancer (Wan et al., 2008). Therefore, our system for HPV16-mediated multi-step carcinogenesis shares important features with cervical cancer and enables us to study the molecular mechanisms of HPV-mediated carcinogenesis *in vitro* with relative confidence that the molecular mechanisms we identify in this model system are likely to be active *in vivo*, as well. These gene expression profiling studies clearly pointed to *SIX1* as one of the genes consistently overexpressed during progression of HKc/HPV16, and further studies in our laboratory identified SIX1 as a key inducer of progression, EMT, resistance to growth inhibition by TGF-beta, and tumorigenesis in this model (Wan et al., 2008; Xu et al., 2014, 2015). Specifically, and importantly for the interpretation of the results we present in this paper, we have shown that: 1) SIX1 mRNA and protein levels increase during HPV16-mediated immortalization in all HPV16-immortalized cell lines established in our laboratory; 2) SIX1 mRNA and protein levels are further up-regulated during progression from HKc/HPV16 to HKc/DR (Xu et al., 2015); 3) SIX1 overexpression in HKc/DR leads to tumorigenicity (Xu et al., 2014); and 4) SIX1 overexpression occurs in about 26% of cervical cancer tissue samples in a tissue array (Wan et al., 2008). In addition, we investigated the mechanisms by which SIX1 acts as a master regulator of EMT and tumorigenicity in HPV16-immortalized cells, and have clearly 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 et al., 2014, 2015). We have previously 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 (Batova et al., 1992; Mi et al., 2000; Borger et al., 2000; Baldwin et al., 2004; Kowli et al., 2013). Therefore, it is reasonable to expect that a decrease in SIX1 levels may be accompanied by a decrease in E6 and E7 expression.

HPV16 is the most common among the high-risk oncogenic papillomaviruses that contribute to cervical carcinogenesis (Zheng and Wang, 2011). HPV16-mediated transformation usually requires the continuous expression of the viral oncoproteins E6 and E7. These two oncogenes are necessary for transformation: E6 binds to and promotes the degradation of the p53 tumor suppressor protein (Scheffner et al., 1993) and E7 binds to the Rb tumor suppressor protein thereby releasing the E2F transcription factor and promoting proliferation (Münger et al., 1989).

Our previous studies were performed by overexpressing SIX1 in HPV-transformed cells. Here, we show the functional and gene expression consequences of suppressing SIX1 expression at late stages of HPV16-mediated transformation of HKc 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. SIX1 knockdown in HKc/DR resulted in slower proliferation rates and was associated with decreased E6/E7 mRNA levels. We also investigated the gene expression profiles of HKc/DR with SIX1 knockdown, and determined that decreased SIX1 expression in these cells is associated with gene expression profiles that, based on our previous data obtained by overexpressing SIX1 in HPV16-transformed cells, are consistent with mesenchymal-epithelial transition (MET) and re-activation of canonical (Smad-mediated) TGF-beta signaling. We conclude that SIX1 expression is vital for cell survival in HKc/DR and, potentially, in advanced HPV-mediated cancers overexpressing SIX1. Hence, SIX1 constitutes an attractive target for treatment of HPV-positive cancers.

2. Materials and Methods

2.1. Cell culture and treatment

The cell lines used in this study, HKc/HPV16d-1, HKc/DRd-1 and HKc/DRd-2, were established in 1985–86 and have been described previously (Pirisi et al., 1987, 1988). Briefly, these cells were obtained from normal human keratinocytes (HKc) isolated from neonatal foreskin tissue (one cell line per individual donor) and maintained in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) (KSFM, Invitrogen, Carlsbad, CA). In those original experiments, normal HKc were transfected with a recombinant plasmid containing two copies of the HPV16 DNA in a head to tail configuration (pMHPV16d, Pirisi et al., 1987, 1988). This gave rise to HPV16-immortalized human keratinocytes (HKc/HPV16) also cultured in KSFM. HKc/HPV16 were then selected in medium devoid of EGF and BPE, giving rise to Growth Factor Independent lines (HKc/GFI), which were further selected in basal KSFM supplemented with 5% fetal bovine serum and 1.0 mM calcium chloride (DR medium) to give rise to differentiation resistant cell lines (HKc/DR). Over the years, we have utilized primarily four of the original cell lines, the HKc/HPV16d-1, d-2, d-4 and d-5 and their corresponding HKc/DR lines which have been expanded and stored in liquid nitrogen at specific passage levels to allow us to return to original low-passage cell populations when needed. In our previous studies of the role of SIX1 in HPV-mediated transformation and tumor progression, HKc/HPV16d-1 and HKc/DRd-1 were transfected with a SIX1 expression plasmid, giving rise to SIX1-overexpressing cell lines (Xu et al., 2014, 2015). For this reason, in order to be able to best compare results between the work of Xu et al. and the experiments described here, we utilized primarily HKc/DRd-1, after having ascertained that the effects of SIX1 on growth and HPV oncogene expression were the same in the independently-derived HKc/DRd-2. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Plasmid constructs and stable transfection

Two independently-derived HKc/DR lines (HKc/DRd-1 and HKc/DRd-2) at 70% confluency were transfected (in triplicate) with a pSuper vector containing either a specific shRNA against human SIX1 (Oligoengine, Seattle, WA) or a scrambled shRNA. A pLXSN vector expressing the HPV16-E7 oncogene (pLXSN-16E7) was a gift of Dr. Denise Galloway. Up to 5 μg of pLXSN-16E7 or its vector control, pLXSN (Promega) were used for transfection. Moreover, three different shRNA constructs against E7-that target three different regions within the HPV16 –E7 mRNA sequence, cloned in the backbone of the pSUPER.retro plasmid were used, at 4 μg or 5 μg per transfection (Bheda et al., 2008). Transfection efficiency was monitored with the co-transfection of the pSUPER/puro plasmid (3:1 w/v) expressing GFP. The empty pSUPER/puro plasmid was used as vector control. Cells were transfected with Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. Stable transfectants were selected with 3 μg/ml puromycin (Toku-E, Bellingham,WA).

2.3. Transient RNA interference

HKc/DRd-1 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 for transfection with siRNAs targeting SIX1 and E7.

SIX1-siR1: Forward 5′-CCAGCUCAGAAGAGGAAUU, Reverse 5′-AAUUCCUCUUCUGAGCUGG; SIX1-siR2: Forward 5′-CACGCCAGGA GCUCAAACU, Reverse 5′-AGUUUGAGCUCCUGGCGUG; E7-siR1 (position 141): Forward 5′-GGACAGAGCCCAUUACAAU, Reverse 5′-AUUG UAAUGGGCUCUGUCC;

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 h before being harvested for RNA and protein extraction, for real-time PCR and Enzyme-linked immunosorbent assay analysis (ELISA), respectively. Each transfection was performed in (at least) triplicate wells.

2.4. Retroviral infection

PA317 cells were transfected with four different pSuper-shRNA constructs (Origene, Rockville, MD) against SIX1, targeting 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 puromycin (3 μg/ml). After 24 h, virus-producing PA317 were cultured until sub confluent, fed fresh complete medium and the virus-containing medium was collected after 24 h and filter-sterilized (0.22 u pore size).

Virus stocks were aliquoted and stored at −80 °C. HKc/DRd-1 at 70% confluency were infected with control virus stock or anti-SIX1 shRNA virus in KSFM containing polybrene (6 μg/ml). HKc/DRd-1 were maintained in DR medium containing polybrene during the duration of the infection, approximately 24 h. After removal of virus-containing medium, the infected cells were trypsinized, re-plated and selected with puromycin (3 μg/ml).

2.5. Real-time PCR

Total RNA was isolated from cells using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with the iScript™ cDNA synthesis Kit (BioRad, Hercules, CA). To achieve uniform template concentration for RT-qPCR assays, c-DNA was quantified by the Quant-iT™ RiboGreen® RNA Assay (Invitrogen, Grand Island, NY). The sequences of the primers used for real-time are shown in Supplementary Table 1. We detected HPV16 E6 and E7 by separate primer pairs targeting the coding sequence of each ORF which, however, are contained in the same nascent mRNA. The E7 primers do not distinguish between the native early mRNA and spliced products that contain the intact E7 ORF. The E6 primers detect only products that contain the intact E6 ORF (for a recent review of the splicing patterns of the HPV16 early region, see Wu et al., 2017). GAPDH was used as an internal control. RT-qPCR assays were performed on the iCycler IQ detection system (BioRad) using iQ SYBR Green Supermix kit (BioRad). Reactions were performed in duplicate with 200 nm primer mix, 1X SYBR® Green Supermix, 560 pg template c-DNA and nuclease-free water to a final volume of 25 μl. The cycling conditions for the RT-qPCR assays were as follows: initial denaturation of template at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and primer annealing and elongation at the respective temperatures for 1 min. All samples were assayed in duplicate.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Protein was isolated from cells using the Cell Lysis Buffer from the PathScan Total p53 and PathScan Total Rb Sandwich ELISA kits (Cell Signaling, Danvers, MA). SigmaFAST Protease Inhibitor (Sigma, Saint Louis, MO) was added to the resulting supernatant. PathScan Sandwich ELISA kits were utilized according to the manufacturer's instructions. Protein (50 μg) was loaded into the p53 pre-coated wells. Protein (150 μg) was loaded into the Rb pre-coated wells. All samples were assayed in triplicate. Absorbance was read at 450 nm within 30 min using a microplate reader. A standard curve was constructed utilizing a manufacturer-provided standard.

2.7. Cell proliferation assay

To study cell proliferation, HKc/DRd-1 and HKc/DRd-2 (5000 cells/well) were plated in 24-well plates in their respective media (1.0 ml/well). Cells in triplicate wells were counted every 24 h, for each cell line investigated, using a hemocytometer.

2.8. Gene-expression analysis by microarrays

Total RNA from HKc/DR-SIX1siRNA1 HKc/DR-SIX1siRNA2 and HKc/DR-control siRNA was isolated using the Qiagen's RNeasy Plus Micro Kit according to the manufacturer's protocol 48 h after transfection with the pre-formed siRNA. Transfections with each SIX1 siRNA were performed in four separate cell populations, each independently processed and hybridized to separate arrays; control siRNA was transfected into triplicate samples also independently transfected, processed and hybridized. 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 platform. Total RNA samples were amplified and biotinylated using GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA). Briefly, 100 ng of total RNA per sample was reverse transcribed into ds-cDNA using NNN random primers containing a T7 RNA polymerase promoter sequence. T7 RNA polymerase was then added to cDNA samples to amplify RNA, and then RNA was copied to ss-cDNA and degraded using RNase H. ss-cDNA molecules were then fragmented and labeled with biotin. Amplified and labeled samples were hybridized to GeneChip HuGene 2.0 ST Arrays (Affymetrix) for 16 h at 45 °C using a GeneChip Hybridization Oven 640 and a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Hybridized arrays were washed and stained using GeneChip Fluidics Stations 450 (Affymetrix). Arrays were scanned using a GeneChip Scanner 3000 7G system and computer workstation equipped with GeneChip Command Console 4.0 software (Affymetrix). Following completion of array scans, probe cell intensity (CEL) files were imported into Expression Console Software (Affymetrix) and processed at

the gene-level using Affymetrix's HuGene-2_0-st library file and Robust Multichip Analysis (RMA) algorithm to generate CHP files. After confirming data quality within Expression Console, CHP files containing log2 expression signals for each probe were then imported into Transcriptome Analysis Console (TAC) Software version 3.0.0.466 (Affymetrix) to analyze cell type specific transcriptional responses using one-way between-subject analysis of variance. 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. The selection of the > 1.4-fold change as a threshold was made based upon the consideration that our SIX1 knockdown populations were not homogenous, as they were produced by transient transfection of pre-formed siRNA and utilized without selection. Validation of microarray data was conducted on selected genes by real-time PCR.

3. Results

3.1. Knockdown of SIX1 causes cell death in HKc/DR

We first determined SIX1 mRNA levels in normal HKc, HKc/HPV16, HKc/DR, and several cervical cancer-derived cell lines, and demonstrated a progressive increase of SIX1 levels in HPV16-immortalized keratinocytes and in their corresponding HKc/DR lines; in addition, we determined that SIX1 is overexpressed to various levels in cervical cancer cell lines, including the HPV-negative C33a cells (Supplementary Fig. 1). At a first glance, the fact that C33a cells also overexpress SIX1 may seem unexpected, however normal RB function is disrupted in these cells by the lack of BRG-1 (Strobeck et al., 2000). As we will see later on, loss of RB function may explain the rise of SIX1 levels in these cells. Hence, the increase in SIX1 mRNA levels occurs during HPV-mediated immortalization and progression in all four HPV16-transformed human keratinocyte lines, and SIX1 is also overexpressed in cervical cancer cells. Therefore, we set out to explore the effects of suppressing SIX1 in HPV16-transformed human keratinocytes, utilizing a variety of approaches. At first, we designed shRNA constructs targeting the human SIX1 gene and a control, scrambled shRNA, cloned them in the pSuper-retro plasmid (Oligoengine) and transfected the resulting plasmids into two independently-derived HKc/DR cell lines, HKc/DRd-1 and HKc/DRd-2 (Pirisi et al., 1987, 1988). Transfected cells were selected with puromycin until non-transfected controls died (approximately 72 h). Cells were then collected for RNA extraction and plated for a growth curve. The shRNA decreased SIX1 mRNA levels to about 60% and 20% of controls in HKc/DRd-1 transfected with 4 μg and 5 μg of the anti-SIX1-shRNA, respectively (Fig. 1, A). HKc/DRd-1 transfected with the anti-SIX1 shRNA grew slowly, compared to their controls (Fig. 1, B). However, after 96 h in culture, growth recovery was observed in cells transfected with 4 μg of anti-SIX1-shRNA plasmid (Fig. 1, B). Similar results were obtained with HKc/HPV16d-2 cells (data not shown). Upon further culturing these cells, SIX1 mRNA levels returned to 100% of control and proliferation rates recovered completely in both cell lines (data not shown) indicating that cells expressing SIX1 have a strong growth advantage over those in which SIX1 expression is suppressed. We then co-transfected HKc/DRd-1 with pSuper-retro-scrambled shRNA, or pSuper-retro-SIX1-shRNA, or control empty pSuper vector together with a GFP expression vector, and observed and photographed the cells using a fluorescent microscope or under phase contrast, without passaging or puromycin selection, at 2 and 4 days after transfection. The resulting images showed profound morphological alterations in GFP-positive cells in the dishes transfected with the SIX1 shRNA (Fig. 2C and D,G,H), but not in the controls (Fig. 2A and B,E,F). We quantified the extent of cytopathic effects of the anti-SIX1 shRNA by counting the numbers of GFP positive cells and determining how many of those were morphologically abnormal (i.e.: had altered size, altered nuclear shape, condensed nuclei, fragmentation) and how many had normal morphology 2 days after transfection: over 90% of the GFP positive cells had normal morphology in the two

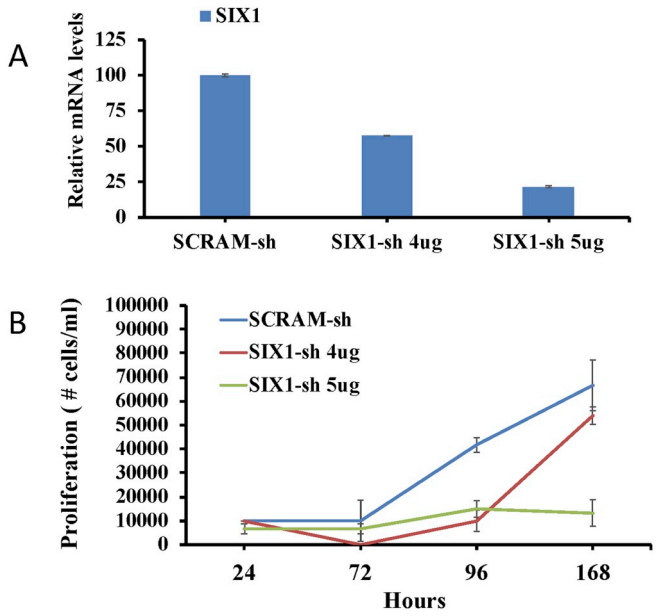

Fig. 1. Proliferation of HKc/DR-d1 transfected with SIX1 shRNA. The pre-malignant, HPV16-immortalized, differentiation-resistant HKcDR-d1 cell line at 70% confluency was transfected with pSuper vectors containing either a specific shRNA against human SIX1 (SIX1-sh, at 4 or 5 μg/well in six-well plates, three wells for each dose) or a scrambled, control shRNA (SCRAM-sh, 5 μg/well in six-well plates). **A**, RT PCR results for SIX1; **B**, proliferation curves for the cell lines resulting from the transfection: 5000 cells were plated per well in 24-well plates (1 ml medium/well). Cells in triplicate wells were trypsinized and counted at the times indicated over a 7-day period using a hemocytometer. Bars represent standard deviations.

controls, but only 37% appeared morphologically normal in the anti-SIX1 shRNA-transfected cells (Fig. 3). At 4 days after transfection, all residual GFP fluorescence originated from cell remnants in the shRNA-transfected cells (Fig. 2G and H), while cells transfected with control plasmids were still morphologically normal (Fig. 2E and F). Due to the need for sub-confluency at the beginning of the experiment, to ensure maximum transfection efficiency, we could not ascertain in these experiments whether cell density affected in any way the dynamics of cell death in the cultures transfected with anti-SIX1 siRNA. However, GFP-positive cells in the controls remained morphologically healthy at higher cell densities, indicating that the cell deaths observed were due to SIX1 loss and not to high cell density per se.

3.2. Loss of SIX1 is associated with decreased HPV16 E6/E7 mRNA levels in HKc/DR

The extent and duration of inhibition of SIX1 expression in the experiments described above were not optimal for us to conduct in depth studies of a possible relationship between SIX1 and E6/E7 expression. We explored the use of retroviral vectors expressing anti-SIX1 shRNA, but that approach did not allow us to isolate cell lines with stable knockdown of SIX1. Therefore, we set out to investigate the connections between SIX1 and HPV16 oncogene expression through transient transfections with pre-formed anti-SIX1 siRNA, in the hope of both knocking down SIX1 more effectively and being able to observe the effects of SIX1 knockdown before widespread death of the transfected cells. We utilized two different siRNA duplexes targeting different regions of the human SIX1 gene. In HKc/DRd-1, SIX1 mRNA levels decreased significantly, under these transient transfection conditions, at 24, 48 and 72 h (Fig. 4A). Decreased SIX1 expression was associated with marked decreases in HPV16 E6/E7 mRNA levels: a typical experiment utilizing two pre-formed siRNAs in transient transfection conditions, in triplicate wells per siRNA, is shown in Fig. 4A. Fig. 4B

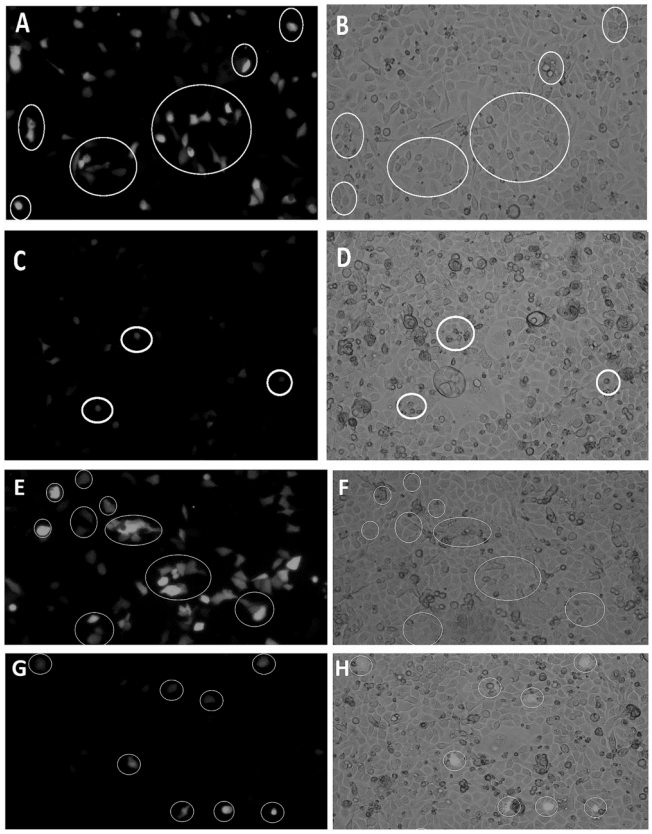

Fig. 2. Cytopathic effects of SIX1 shRNA. Representative fluorescent (**A,C,E,G**) and phase-contrast (**B,D,F,H**) images of HKc/DRd-1 transfected with either the pSuper-SCRAM-sh or the pSuper-SIX1sh RNA, co-transfected with pSuper-GFP as described in Materials and Methods. In these experiments, cells were transfected, incubated for the indicated times and photographed, without passaging or antibiotic selection. **A,B**: control pSuper-SCRAM-sh, 2 days after transfection; **C,D**: pSuper-SIX1-sh RNA, 2 days after transfection. **E,F**: control pSuper-SCRAM-sh, 4 days after transfection; **G,H**: pSuper-SIX1-sh RNA, 4 days after transfection.

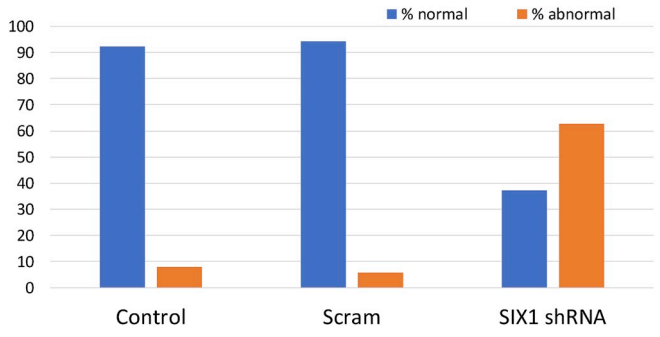

Fig. 3. Analysis of cytopathic effects of SIX1 shRNA. Images such as those shown in Fig. 2, obtained from cells transfected with empty vector (control), pSuper-SCRAM-sh (scram) or pSuperSIX1-sh RNA (SIX1shRNA) plasmids, together with pSuper-GFP, were utilized to manually count all the visible GFP-positive cells, locate them in the corresponding phase contrast images by electronically overlaying the two, and observing the morphology of each GFP-positive cell. Total counts of morphologically-abnormal cells were expressed as % of all GFP-positive cells in each set of images. All detectable GFP-positive cells, at least 100 per experimental condition across multiple fields, were counted in this analysis.

shows a more comprehensive picture of the correlation between SIX1 and E6/E7 mRNA levels measured by real-time RT/PCR, across a variety of experimental approaches, utilizing shRNA delivered by plasmid transfection or retroviral infection, or transfected, pre-formed siRNA (Fig. 4B). These results show that a loss of SIX1 expression in HKc/DR is associated with decreased expression of the viral oncogenes E6/E7. Consistent with a decrease of E6 expression, p53 protein levels increased 24 h after SIX1 knockdown (Fig. 5). However, by 48 h p53 levels had already returned to those found in HKc/DR, in anti-SIX1 siRNA transfected cells (Fig. 5). This experiment was repeated several times with essentially the same results: a detectable but transient increase in p53 protein levels was associated with SIX1 loss and a reduction of E6/E7 expression. The quick return to low p53 protein levels while E6/E7 mRNA levels are still low prompted us to speculate that compensation mechanisms (perhaps a stabilization of the E6 protein?) might come into place to cope with the reduction in HPV oncoproteins which are necessary for cell survival, in the cells that survive the loss of SIX1/E6/E7. Alternatively, or in addition, perhaps the decrease in p53 protein observed at 48 h is an indication of the widespread death of cells in which the anti-SIX1 siRNA has been successful, in these mixed populations. However, the specific mechanisms for this behavior cannot be ascertained without further investigation.

E7 overexpression is associated with a rise in SIX1 levels, and knockdown of E7 results in decreased mRNA levels for SIX1.

We determined that SIX1 mRNA levels increased about 10-fold in HKc expressing HPV16 E7 but not E6, from LXSN retroviral vectors (Supplementary Fig. 2A). SIX1 mRNA levels also increased in normal HKc expressing anti-p53 shRNA, upon transfection with anti-RB siRNA (Supplementary Fig. 2B) while transfection of normal HKc with anti-RB siRNA led to widespread cell death (not shown). We therefore asked whether E7 overexpression in HKc/DR would further increase SIX1 levels: HKc/DRd-1 were co-transfected with the pLXSN-E7 plasmid and the pSuper-puro vector, selected with puromycin and cultured to establish stable lines from which to extract RNA and perform real-time RT/PCR with primers specifically directed at E6, E7, and SIX1, in comparison with HKc/DRd-1 and HKc/HPV16d-1 (Fig. 6). As expected, endogenous E6/E7 levels were increased in HKc/DRd-1 (used here as the reference) compared to their parental line, HKc/HPV16d-1. Transfection with the E7 expression vector caused a modest (about 50%) increase in mRNA detected with E7 primers, and also increased the levels of mRNA detected by E6 primers, implying an increase in endogenous HPV16 early mRNA expression. Despite its modest size, the increase in E7 mRNA led to a further increase of SIX1 mRNA levels in HKc/DRd-1 (Fig. 6).

Next, we transiently transfected HKc/DRd-1 with two different siRNA duplexes targeting HPV16 E7. We postulated that these siRNAs would cause the degradation of the HPV16 early mRNAs containing intact E6 coding sequences together with E7, as well as the spliced products encoding E7 (reviewed in Wu et al., 2017). Cells were then collected for RNA and protein extraction 48 h after transfection. Both siRNAs produced a significant decrease in HPV16 early mRNA levels, detected by real-time RT PCR with both E6-and E7-specific primers: up to 70% inhibition of E6/E7 mRNA levels was obtained, accompanied by approximately a 50% decrease in SIX1 mRNA levels (Fig. 7). These changes in E6/E7 and SIX1 mRNA were associated with an increase in p53 and (to a much lesser extent) Rb protein levels (Supplementary Fig. 3). Taken together, these data suggest that E7 and SIX1 increase each other's expression in differentiation-resistant, HPV16-transformed human keratinocytes.

SIX1 knockdown in HKc/DR activates gene expression markers of mesenchymal-epithelial transition (MET) and resets the levels of TGF-beta receptors type 1 and 2.

We next investigated global gene expression during transient SIX1 knockdown by transfection with pre-formed siRNA duplexes in HKc/DRd-1, using Affymetrix GeneChip Human Gene 2.0 ST Arrays. Direct, paired comparisons of gene expression were made among HKc/DR-SIX1-siRNA1 and HKc/DR-control-siRNA, and between HKc/DR-SIX1-siRNA2 and HKc/DR-control-siRNA, utilizing four independently-transfected cell populations for each SIX1 siRNA, and three for the

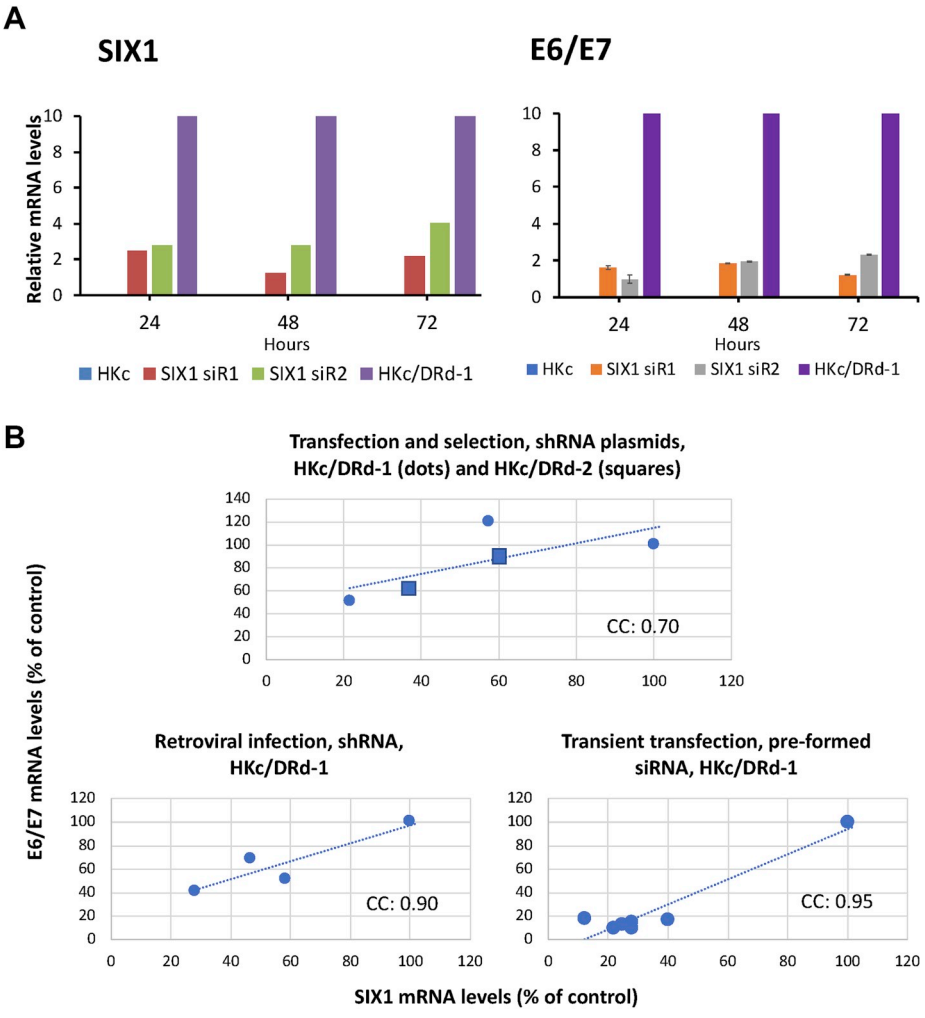

Fig. 4. SIX1 knockdown decreases SIX1 and E6/E7 mRNA levels. **A**: HKc/DRd-1 were cultured in six-well plates until 60% confluent and transfected in triplicate wells per each siRNA, with two different siRNA constructs against SIX1 as described in Materials and Methods. At the indicated times after transfection, RNA was extracted from the transfected cells, and from parallel cultures of normal human keratinocytes (HKc) and mock-transfected HKc/DRd-1, both utilized as controls. Relative SIX1 and E6/E7 mRNA levels were determined by real-time RT/PCR with primers specific for SIX1, HPV16 E6 and HPV16 E7. Results are averages ± standard deviation of triplicate determinations. There was barely detectable SIX1 expression, and no HPV16 E6/E7 expression in normal HKc, which were used here as negative controls. Data are normalized to HKc/DR. **B**: Correlation of E6/E7 mRNA levels with SIX1 levels (measured by real-time RT/PCR and expressed as % of control) across different experiments with the indicated experimental approaches and tools.

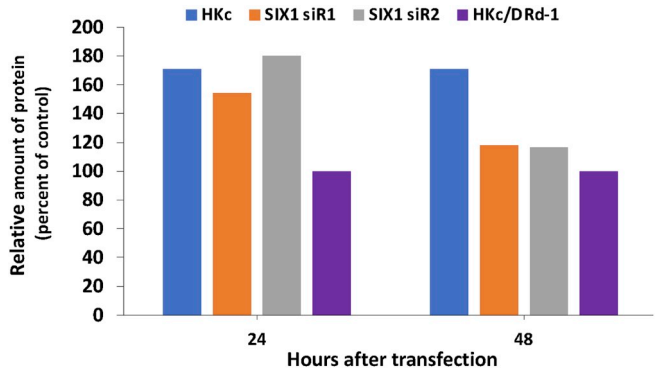

Fig. 5. p53 levels in HKc/DRd-1 transfected with SIX1 siRNA. Protein extracts from HKc/DRd-1 transfected as described in Fig. 4A were utilized to measure p53 levels by ELISA at 24 and 48 h after transfection. p53 expression in HKc/DR was set to 100%. This experiment was repeated multiple times with similar results.

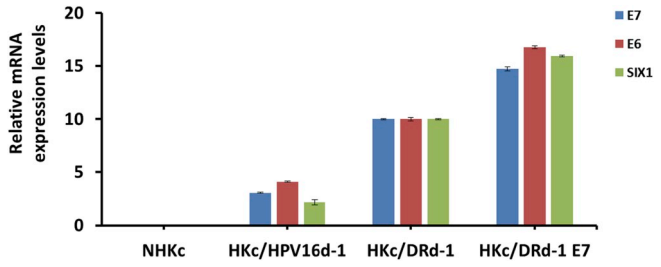

Fig. 6. E7 overexpression increases SIX1 expression in HKc/DR. HKc/DRd-1 were co-transfected with the pLXSN-E7 construct and the pSuper-puro vector, selected with puromycin, and the resulting cell line was utilized for RNA and protein extraction together with control HKc/DRd-1 and their parental line, HKc/HPV16d-1. Real-time RT/PCR was performed in triplicate samples, with primers targeting the HPV16 E6 and E7 sequences and SIX1, and GAPDH as a control for normalization. Data are expressed as % of the GAPDH-normalized levels detected in HKc/DRd-1, for each primer set. Error bars represent SD.

control. We used the 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 or down) and a *P* value > 0.01 as cutoff values. The choice of a slightly lower than usual fold change threshold was guided by the fact that we were examining transfected cell populations that had not been selected, and therefore contained mixtures of transfected and non-transfected cells, which thus limited our ability to detect effects on gene expression. Ingenuity Pathway Analysis (IPA, Qiagen) 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. After determining that the gene expression profiles generated by the two siRNAs were similar in terms of gene lists, we elected to continue the analysis with

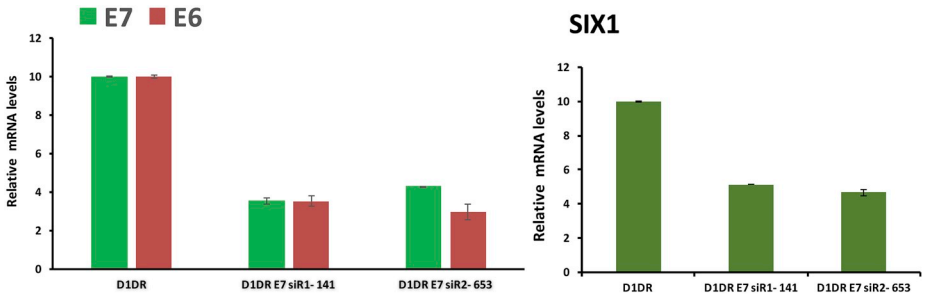

Fig. 7. E7 knockdown reduces SIX1 mRNA levels. Real-time RT/PCR with E6-and E7-specific primers, and SIX1 primers, on RNA isolated from mock-transfected HKc/DRd-1 (D1DR) and HKc/DRd-1 transiently-transfected, in triplicate, with two different siRNA's against HPV16 E7. RNA was isolated 48 h after transfection. Data are normalized against GAPDH and are expressed relative to the values obtained for mock-transfected HKc/DRd-1. Error bars represent SD.

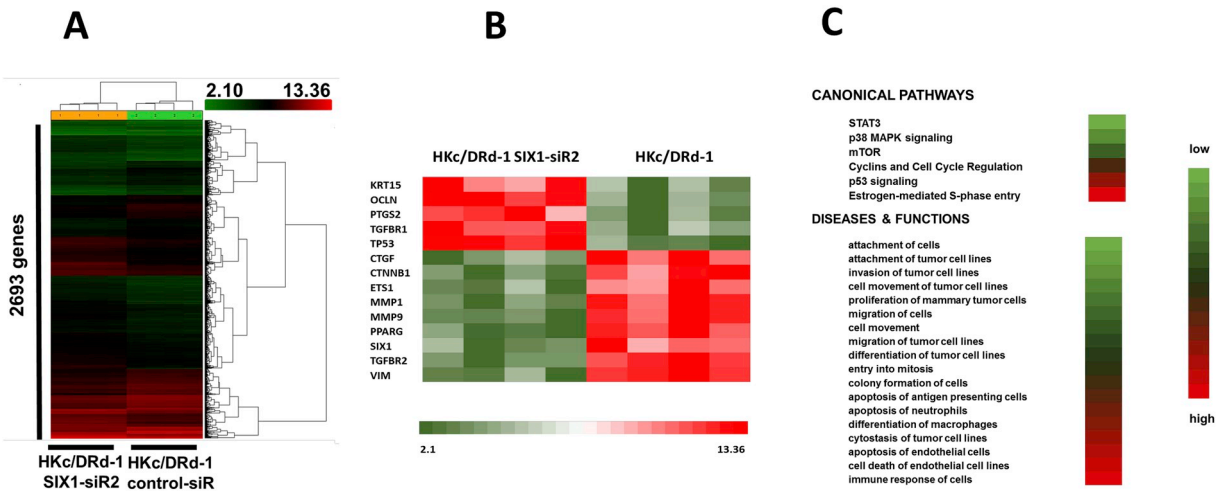

Fig. 8. Gene expression profiles of HKc/DRd-1 transfected with anti-SIX1 or control siRNA. **A**, unsupervised hierarchical clustering of differentially expressed genes between HKc/DR control siRNA, and HKc/DR SIX1-siR2. The color represents the expression level of a gene above (red), below (green), or at neutral (black) the mean expression level of that gene across all samples. **B,** heatmap of selected genes relevant to MET in the SIX1-siR2 dataset. **C**, canonical pathways and diseases & functions differentially affected by SIX1 loss in HKc/DRd-1 identified by Ingenuity Pathway Analysis (IPA) of the SIX1-siR2 dataset (1.4 fold change up and down; p-value < 0.01).

the dataset from siRNA2, which had produced a more marked decrease in SIX1 mRNA levels than siRNA1, as detected by real-time PCR.

Unsupervised cluster analysis clearly differentiated the SIX1 knockdown cells from controls and allowed for the identification of a cluster of genes that best separated the two (Fig. 8A and B). IPA pathway analysis showed that the pathways most heavily affected by decreased SIX1 expression were consistent with MET and cell death (Fig. 8, C).

As we show above, it proved impossible to produce cells stably transfected with anti-SIX1 siRNA that retained reduced levels of SIX1 expression long enough to allow us to reliably perform functional studies, in order to experimentally corroborate the notion that SIX1 suppression causes MET. However, extensive previous studies from our group show that the same markers of EMT/MET were modified in the opposite direction by SIX1 overexpression, which is very well-tolerated by HKc/HPV16 and HKc/DR, and has very important functional consequences, including the induction of resistance to differentiation stimuli and tumorigenic behavior (Xu et al., 2014, 2015).

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 cytokeratin 15 (KRT15) 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 (CTNNB1). We noted a decrease in the pro-survival factor epidermal growth factor receptor (EGFR) and its associated ligand, transforming growth factor alpha (TGF-alpha) (not shown). 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 markers of invasiveness, matrix metalloproteinase 1 and 9 (MMP1, MMP9), were also decreased. Lastly, there was a significant inhibition of peroxisome proliferator activated receptor gamma (PPARγ) a ubiquitously expressed gene known to regulate differentiation and cell growth (Sarraf et al., 1999).

To validate gene expression data from the array analysis we performed real time PCR on RNA isolated 48 h after transfection of HKc/DRd-1 treated with SIX1-siRNA2 (Fig. 9). The mRNA levels of cytokeratin 15 (K15) and occludin (OCLN), both of which are expressed at much lower levels in HKc/HPV16 and HKc/DR in comparison with normal HKc, increased approximately 2.2-fold. Vimentin (VIM) mRNA levels, which increase dramatically upon HPV-mediated immortalization, decreased approximately 20% in HKc/DR transfected with SIX1-siRNA2 (Fig. 9A). Surprisingly, real time PCR results also revealed a 3-fold increase in β-catenin (CTNNβ1) mRNA levels in HKc/DR transfected with SIX1-siRNA2, compared with HKc/DR (Fig. 9A) while CTNNβ1 mRNA levels decreased considerably in HKc/HPV16 and HKc/DR compared to normal HKc. This result contrasted with the microarray data, where a decrease of CTNNβ1 mRNA with loss of SIX1 was detected in both siRNA datasets. This gene has a large number of splice variants, therefore the discrepancy may be due to the exact specificity of the primers used for RT/PCR versus the probes present on the microarray; in any case, the direction of β-catenin responses to SIX1 loss will need to be resolved at the protein level. In previous work, we observed a decrease in β-catenin protein levels in HKc/DR overexpressing SIX1 from an exogenous plasmid, therefore our RT/PCR data that show an increase in β-catenin mRNA with SIX1 loss are in agreement with

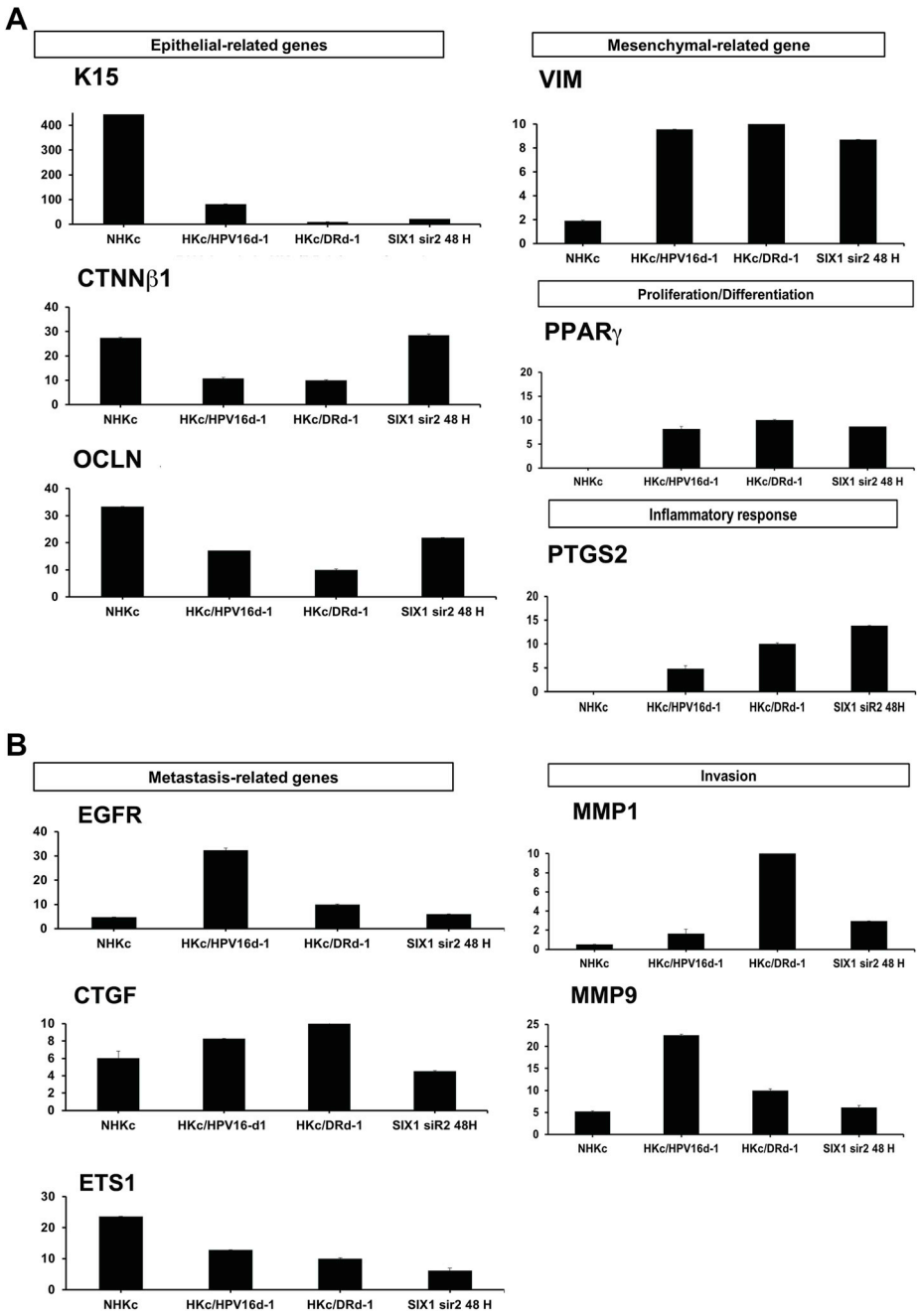

Fig. 9. Real-time RT/PCR validation of gene expression profiles of HKc/DRd-1 transfected with anti-SIX1 or control siRNA. mRNA levels of: **A,** KRT15 (K15), CTNNβ1, OCLN, VIM, PPARγ, PTGS2, and **B**, EGFR, CTGF, ETS1, MMP1/9 were determined by Real-time RT/PCR analysis in HKc/DR control siR and HKc/DR SIX1-siR2. Data were normalized to GAPDH expression. Bars indicate SD among multiple samples derived from independent transfections (4 for anti-SIX1 siRNA, 3 for control siRNA) as detailed in Materials and Methods.

that observation (Xu et al., 2014). We also detected changes in the expression of PPARγ and PTGS2, both of which are expressed in HKc/HPV16 and HKc/DR but not in normal HKc (Fig. 9A). As we had previously shown (Akerman et al., 2001; Behda et al., 2008; Zyzak et al., 1994) EGFR mRNA levels increased dramatically in HKc/HPV16 compared to normal HKc, and are still about twice the normal HKc levels, in HKc/DR (Fig. 9B). Transfection with anti SIX1 siRNA reduced EGFR mRNA levels by about 40% in HKc/DR. Additionally, there was a 55% and 40% decrease in CTGF/CCN2 and ETS1 mRNA levels, respectively, in HKc/DR transfected with the SIX1 siRNA (Fig. 9B). 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, 70% and 40% decrease in MMP1 and MMP9 mRNA levels, respectively, were observed in cells transfected with the SIX1 siRNA (Fig. 9B). Malignant cells can damage the basement membrane thru the increase of proteolytic enzymes such as MMP1 and MMP9. Therefore, a significant decrease of these EMT-inducing proteins is an indication of the functional consequences brought on by decreased SIX1 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 (Fig. 10).

4. Discussion

The current results, taken together with our prior observations on the role of SIX1 in HPV16-mediated transformation (Xu et al., 2014,

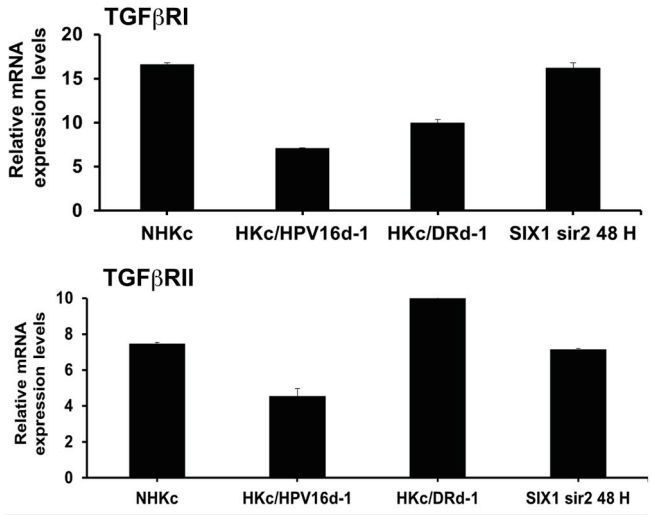

Fig. 10. mRNA levels for TGFβ receptors I and II in normal HKc, HKc/HPV16, HKc/DR and HKc/DR transfected with anti-SIX1 siRNA. mRNA levels of TGFβRI and TGFβRII were determined by Real-time RT PCR in HKc/DRd-1 control siR and HKc/DRd-1 SIX1-siR2, in comparison with normal HKc (NHKc) and HKc/HPV16. Data were normalized to GAPDH expression. Bars indicate SD among multiple samples derived from independent transfections (4 for anti-SIX1 siRNA, 3 for control siRNA) as detailed in Materials and Methods.

2015) lead to the conclusion 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 marked decrease in SIX1 levels. Since SIX1 is expressed at extremely low levels in normal adult cells, SIX1 may be a suitable therapeutic target for HPV-driven cancers, and possibly also for other cancers that overexpress SIX1.

Deregulation of homeobox genes in cancer results in an out of context activation of their development-restricted functions (Abate-Shen, 2002; Behbakht et al., 2007). There have been many studies identifying the link between normal organogenesis and neoplasia. Indeed, they both share many pathways and basic molecular processes, suggesting that tumor development and progression are an aberrant form of morphogenesis (Behbakht et al., 2007; Yu et al., 2006). 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 (Coletta et al., 2010; Kumar, 2009). This transcription factor has been shown to be aberrantly expressed in various pediatric and adult cancers (Christensen et al., 2008). SIX1-induced EMT through TGF-beta signaling enhances tumor progression, invasion and metastasis in several malignancies (Micalizzi et al., 2009).

We demonstrated that SIX1 mRNA and protein levels are overexpressed in all cell lines in our *in-vitro* model system for HPV16-mediated transformation (in which each cell line derives from a different donor) and SIX1 mRNA and protein levels are consistently upregulated during progression from HKc/HPV16 to HKc/DR (Xu et al., 2014, 2015). We have also shown previously that SIX1 overexpression in HKc/HPV16 induces a differentiation-resistant phenotype, and that SIX1 overexpression in HKc/DR induces tumorigenicity (Xu et al., 2014, 2015). In the current study, we focused on the functional consequences of suppressing SIX1 expression in the premalignant, differentiation-resistant HPV16-transformed human keratinocyte lines (HKc/DR).

We utilized shRNA technology to suppress SIX1 expression levels in HKc/DR and observed slower proliferation and widespread cell death. However, attempts at stable transfection resulted in only transient decreases in SIX1 expression and yielded inconsistent results, which we attributed to the fact that cell populations were rapidly taken over by cells in which SIX1 was still being expressed. Therefore, after testing retroviral infection for delivery of anti-SIX1 shRNA, again with mixed results, 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 markedly reduced the mRNA expressions of SIX1 and, consequently, E6/E7. We determined that p53 protein levels transiently increased as a result of SIX1 and E6 loss. Therefore, with the brief reduction of SIX1 expression, we were able to substantially (albeit transiently) reduce E6/E7 levels. In addition, we show that expression of HPV16 E7, or anti-RB siRNA (but not E6) increases SIX1 levels in human keratinocytes. Thus, HPV E6/E7 mRNA expression in HPV16-tranformed cells is supported by SIX1, and in turn, E7 increases SIX1 mRNA levels. These results are in agreement with published evidence that SIX1 promotes malignant transformation and growth of HPV-infected cervical cells, where it is induced by HPV E7 (Liu et al., 2014). In HPV-negative C33a cells, the overexpression of exogenous HPV E7, not E6, induces SIX1 expression (Liu et al., 2014). Our results point to a reciprocal regulation of SIX1 and E7 levels, one inducing expression of the other. These results, together with our previous extensive work on SIX1 overexpression in HPV16-transformed cells (Xu et al., 2014, 2015) also imply that E7 acts (at least in part) through SIX1 to promote malignant transformation and tumor progression.

Similar to many developmental signaling pathways dysregulated in cancer, TGF-beta 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-beta plays 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 tumorigenesis (Micalizzi et al., 2009; Xu et al., 2014, 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 (Xu et al., 2014, 2015). Therefore, we analyzed signaling pathways in response to SIX1 knockdown in HKc/DR and observed alterations in the expression of genes within the TGF-beta pathway, including diminished expression of EGFR and its associated ligand TGF-alpha, CTGF and its downstream signaling effector ETS1, as well as MMP1 and MMP9. Furthermore, decreased expression of SIX1 in HKc/DR results in enhanced expression of TGF-beta receptor type I (TβRI) and a reduction in TβRII. The gene expression changes we observed are relatively modest, due to the fact (we believe) that the cell populations we analyzed were derived through transient transfection of pre-formed siRNA and were not selected, and therefore not homogenous. Despite this limitation, the changes we observed affected the same patterns and genes we had previously determined to be changed in the opposite direction by SIX1 overexpression (Xu et al., 2014, 2015). Taken 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 corroborated and confirmed our prior observations made on the same cells, HKc/DR (and also HKc/HPV16) in which we overexpressed SIX1, with major effects noted on pathways of EMT/MET, mainly through a shift in TGF-beta signaling from canonical (Smad-mediated) to non-canonical (Xu et al., 2014, 2015). The central EMT modulator, TGF-beta, 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 et al., 2014, 2015).

TGF-beta signaling is a critical pathway by which SIX1 overexpression promotes EMT in HKc/DR. TβRI, TβRII, and 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 of RNA deriving from an independent transfection demonstrated similar results with an approximate 70% increase in TβRI mRNA levels and a 30% decrease in TβRII mRNA levels. The changes in the expression of genes targeted by TGF-beta are consistent with our previous in-depth studies of the effects of SIX1 on TGF-beta signaling, and point to the possibility that a shift in TGF-beta signaling from non-canonical to canonical, the opposite of what we have documented with SIX1 overexpression, occurs as a consequence of SIX1 loss. As we have previously demonstrated, this shift is pivotal to produce the HKc/DR phenotype and to further promote tumorigenicity in HPV-transformed cells (Xu et al., 2014, 2015). Based upon our previous body of work elucidating the mechanisms of TGF-beta control of transcription from the HPV16 URR (Baldwin et al., 2004; Kowli et al., 2013) and the role of TGF-beta signaling in mediating SIX1's role in premalignant progression of HKc/HPV16 and malignant conversion of HKc/DR (Xu et al., 2014, 2015), we postulate that a “shift” in TGF-beta signaling from non-canonical to canonical, which is suggested by the gene expression patterns we documented, may also be at the basis of the decrease in E6/E7 expression. However, a full demonstration that TGF-beta signaling indeed returns to canonical pathways upon SIX1 knockdown will require direct studies of Smad activation, using for example reporter constructs containing Smad responsive elements. Our overarching hypothesis is that TGF-beta signaling mediates the effects of SIX1 on E6/E7 expression: we have previously shown that E7 decreases TGF-beta signaling (Kowli et al., 2013) and have also shown that canonical, SMAD-mediated TGF-beta signaling inhibits E6/E7 expression by decreasing NF1-ski complexes, which activate E6/E7 transcription via the HPV16 URR (Baldwin et al., 2004). In addition, SIX1 expression depends on the activity of E2F1, which is activated when RB levels and activity fall due to E7 (Young et al., 2003). Taken all together, these observations shed some light onto the mechanisms by which E7 and SIX1 regulate each other's expression. Nevertheless, the specific molecular mechanisms by which SIX1 causes a switch in TGF-beta signaling from canonical to non-canonical pathways remain to be explored.

The decrease in EGFR expression is also of considerable interest. We have previously shown that EGFR levels increase dramatically in HPV16-immortalized human keratinocytes, and that this increase is due to p53 degradation by E6, which in turn causes an increase in YY1 protein levels: p53 and YY1 negatively regulate each other and YY1 induces EGFR transcription (Bheda et al., 2008). Therefore, decreased E6 levels and increasing p53 levels should cause a decrease in EGFR expression.

In conclusion, we show that loss of SIX1 causes decreased expression of the HPV oncogenes E6 and E7 and widespread cell death in the HPV16-immortalized, differentiation-resistant, pre-malignant HKc/DR. We conclude that these cells depend on SIX1 for survival. Our results corroborate our previous observations that SIX1 overexpression is a key factor in the progression of HPV16-transformed cells to malignancy and reinforce the notion of strong ties between SIX1 and TGF-beta signaling. Since SIX1 is either not expressed or expressed at extremely low levels in normal adult cells, this transcription factor may be a useful target for therapy of HPV-induced cancers, as well as other cancers overexpressing SIX1.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.009.

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