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Novel tumor growth inhibition mechanism by cell cycle regulator cdk2ap1 involves anti-angiogenesis modulation

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Abstract

We evaluated the effect of expressing the cell cycle regulator protein cdk2-associating protein1 (*cdk2ap1*) in inhibiting growth of squamous cell carcinoma (SCC). Expression of *cdk2ap1* correlated with reduction in several SCC malignant cell phenotypes, including reduced angiogenesis. We observed several alterations in gene expression consistent with classical functions of *cdk2ap1*, including upregulation of cell cycle inhibitory genes, and an upregulation in expression of genes belonging to both intrinsic and extrinsic apoptotic cascades. Interestingly, we also uncovered a profile of gene expression and activation of signaling pathways that may suggest new tumor-suppressive functions for *cdk2ap1* through downregulation of invasion/metastasis and modulation of antiangiogenesis by upregulation of the TGF β signaling pathway. Blocking of the TGF β 1 pathway resulted in inhibition of the *cdk2ap1* antiangiogenesis phenotype. In combination, these data support the role of *cdk2ap1* as a tumor suppressor gene that can regulate SCC tumor growth in a cell autonomous manner through decreases in invasiveness and a non cell-autonomous manner through decreases in angiogenesis phenotypes, and these are novel phenotypes induced by *cdk2ap1*.

Keywords

Squamous cell carcinoma; oral cancer; *cdk2ap1*; cell cycle; invasion; angiogenesis; TGF-beta/activin pathway

INTRODUCTION

Squamous cell carcinomas of the head and neck (HNSCC) are characterized by local tumor aggressiveness and a predisposition to metastasize to regional lymph nodes. Tumor development and progression of HNSCC includes the generation of variant cells with alterations in expression of adhesion receptors and associated signaling pathways, and accumulation of additional survival gene expression signals allows development of a highly invasive and metastatic phenotype. During tumor progression, many types of gene expression networks become altered that are responsible for mediating or increasing tumor progression, including altered angiogenesis and adhesion/invasion profiles. Understanding the mechanisms

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of how HNSCC cells alter the gene expression profile to acquire malignant cell phenotypes or escape growth regulation is critical to developing new preventative and therapeutic strategies. In special, it is important to understand how to regulate the growth and invasiveness of HNSCC in order to help prevent metastatic spread.

Current strategies for cancer treatment have begun to focus not only on the tumor cell population itself, but also on the complex interaction of tumor cells with other cells present in the tumor microenvironment. One critical microenvironment alteration favoring malignancy is the stimulation of expression of proangiogenic genes (Hanahan and Weinberg 2000). Inhibition of tumor-induced angiogenesis has now been recognized as a valuable strategy with several important advantages over conventional therapeutics. Tumor suppressor genes (TSGs) can impact not only the cell cycle and apoptosis pathways, but also have been implicated in suppressing angiogenesis and invasion pathways, functions that may greatly influence how tumor cells respond to their microenvironment. TSGs von Hippel-Lindau (VHL) or neurofibromatosis type I (Nf1), for example, can alter the response of endothelial cells in an indirect manner to proangiogenic factors produced by tumors harboring an inherited inactive form of the gene (Wu et al. 2005; Kurban et al. 2006). TSGs may also be direct regulators of angiogenesis, as it has been shown for the phosphatase and tensin homologue of chromosome 10 (PTEN), by reducing HIF1 α and VEGF expression (Hamada et al. 2005; Jiang and Liu 2009).

Consistent with the finding that TSGs can modulate tumor suppression by mechanisms beyond cell cycle and apoptosis regulation, we found that the TSG cdk2ap1 shows antitumor activity correlating with alterations in gene expression in several pathways including invasion, metastasis, and angiogenesis. Cdk2ap1 is an S-phase growth suppressor through association with DNA polymerase α /primase and/or CDK2 (Matsuo et al. 2000; Shintani et al. 2000), known to induce apoptosis and reduce cell proliferation *in vitro* and *in vivo* (Cwikla et al. 2000; Kohno et al. 2002; Figueiredo 2005). Our present study expands the characterization of cdk2ap1 growth inhibitory effects at the phenotypic and the gene expression levels to achieve its tumor suppression role through inhibition of angiogenesis via modulation of the TGF β signaling pathway. The sensitivity of both HNSCC tumor and endothelial cells to cdk2ap1 activation suggests that cdk2ap1-activating stimuli could potentially be exploited in developing therapies that can target both the tumor and its microvasculature.

MATERIALS AND METHODS

Cell culture, viral transductions, and transfections

Human squamous cell carcinoma (SCC) cell lines UMSCC1 and 38 were obtained from Dr. T. Carey (Hoffman et al. 1990), and HUVEC2 cells were obtained from BD Biosciences (San Jose, CA). SCC were maintained in Ham's:F12 1:1 media (Mediatech, Manassas, VA) with 10% FBS/1% PS, and 400ng/mL hydrocortisone. HUVEC2 were maintained in Medium 200 supplemented with low serum growth supplement (Invitrogen, Carlsbad, CA). Lentiviral vectors used were either pNL.EF1 α -rtTA^{2SM2} (Zhang et al. 2002) or pNL.TRE.ires.RLuc cut with *Bam*HI and *Nhe*I to insert *cdk2ap1* upstream of the IRES sequence. Lentiviruses (Lv) were produced by triple transfections into 293T cells using calcium phosphate transfection (Soneoka et al. 1995) and supernatants harvested at 48, 74, and 96h, filtered (.45 μ m membrane), and titered by quantitative real-time PCR using genomic DNA from 293T cells infected with a serial dilution of each Lv and quantified using a positive control standard curve (Zolochovska and Figueiredo 2009). Lv were used at a multiplicity of infection of two (moi=2) using a transduction-enhancing reagent, Viraductin (CellBiolabs, Inc., San Diego, CA). For culture of cells with recombinant PEDF, 200ng/mL rPEDF were used (Leinco Technologies, St. Louis, MO). Transfections of signaling pathway reporter Luc constructs were performed with Lipofectamine 2000 using SCC cells stably transduced with lentiviruses encoding

doxycycline (dox)-inducible EF1 α -rtTA^{2SM2} activator and TRE-cdk2ap1.ires.Rluc responsive transgenes. 24h following transfection, target transgene expression was activated by adding 2 μ g/mL dox. The signaling pathway-responsive reporter (luc) constructs used were obtained from Addgene (Cambridge, MA): pG13-luc (p53 sites, #16442), FBE/SBE-Luc (Smad4 sites, TGF β 1 pathway, #16525), k3(long)-luc (TNF α promoter, #11110), 10-4 cycE-luc (E2F1 sites, #8458), 16xTCF/LEF-luc (TCF/LEF sites, #17165), and NF κ B-luc and AP1-luc were obtained from Clontech (Mountain View, CA).

Cell and tissue assays

For cell cycle analyses, $\sim 10^5$ cells were collected at 24 or 72 hours post gene expression activation by 2 μ g/mL doxycycline, resuspended in 70% Ethanol and placed at -20°C for at least 24 hours. Cells were then pelleted and resuspended in 0.5mL hypotonic propidium iodide buffer. Cell cycle analyses were performed with a FACScan (BD Biosciences, Franklin Lakes, NJ) flow cytometer and Modfit LT 3.0 software. at the LSU Flow Cytometry Core Facility. For conditioned culture media (CCM), we incubated SCC cells in DMEM:F12 containing 10% FBS for 48h, then collected the CCM, followed by centrifugation at 1600 rpm for 5 min at room temperature to exclude any cell debris (Pan et al. 2006). For examining angiogenesis potential of SCC conditioned media, an Endothelial Tube Formation Assay (CellBiolabs) was used, according to manufacturer's protocols. Following staining of the tubes formed with the provided fluorescence dye, the extent of tube formation (average tube length, branching points, or tube width) was quantified using NIH ImageJ software. For the TGF β 1 blocking assay, anti-TGF β 1 (A101NA) or control IgY (A101) antibodies (R&D Systems, Minneapolis, MN) were used at 20 μ g/mL in HUVEC tube formation assays, as described. For TGF β 1 ELISA, a human quantikine kit (DB100B, R&D systems) was used, and for Activin A ELISA, a human/mouse/rat quantikine kit was used (DAC00B, R&D systems) using CCM collected as described above, after culturing of cells for either 24h or 48h.

Real-time quantitative RT-PCR (qPCR) analyses

Total RNA from 5×10^5 cells was extracted using a SurePrep kit (Fisher Scientific, Pittsburgh, PA). 1 μ g total RNA was reverse transcribed using TaqMan kit (Applied Biosystems, Foster City, CA). 1 μ l template cDNA was used in a qPCR reaction with 2x Sybr green master mix (Applied Biosystems), and 10 μ M each forward and reverse primers for experimental or control housekeeping genes. Reactions were run on an ABI7300 cycler (Applied Biosystems), using: 40 repeats of $95^{\circ}\text{C}/15\text{sec}$; $56^{\circ}\text{C}/30\text{sec}$, $72^{\circ}\text{C}/30\text{sec}$, and analyzed using ABI7300 software. Samples were normalized to β -actin control and expressed as change in Ct relative to β -actin. For experiments utilizing pathway-focused arrays, the Human Cancer PathwayFinder qPCR array was used (PAHS-033A-2; SABiosciences, Frederick, MD), according to manufacturer's protocols and on an ABI7300 cycler. PubGene was utilized to examine cancer-specific networks derived from the gene expression changes observed using pathway mapping (Jenssen et al. 2001).

Statistical analysis

Assays were performed in triplicate, and values provided as mean $\pm$ SD or 95% confidence interval. Comparisons were performed using an unpaired t-test, and $p < 0.05$ was considered to indicate a significant difference.

RESULTS

Cdk2ap1 expression by a lentiviral system in human squamous cell carcinoma lines reduces cancer cell growth and malignancy phenotypes *in vitro*

To induce cdk2ap1 expression, we utilized an expression system inducible by doxycycline. Briefly, the activator construct encodes the reverse tetracycline transactivator (rtTA^{2SM2}), a fusion protein containing the DNA binding domain from the *E. coli* protein *tetR* and the transactivation domain from the Herpes simplex virus protein VP16 (Koponen et al. 2003). The rtTA^{2SM2} protein thus has the ability to bind DNA of responsive constructs at a TRE sequence containing tetO binding sites upstream of a minimal promoter. Upon doxycycline administration (dox), rtTA^{2SM2} can activate transcription of target genes cdk2ap1 and Renilla luc (Rluc). Two lentiviruses were used to overexpress cdk2ap1 and a reporter gene in SCC cells, Lv-EF1a.rtTA^{2SM2} (activator construct) and Lv-TRE.cdk2ap1.i.res.Rluc (responsive construct). Typical multiplicities of infection were moi=2 total. Expression of exogenous (Lv) cdk2ap1 was confirmed by qPCR using gene-specific primers for cdk2ap1 or B-actin. Cdk2ap1 was upregulated by 45 to 65-fold upon dox treatment as compared to untreated control cells (Figure 1a).

We examined the effect of expressing cdk2ap1 on different SCC cell lines and observed that its expression greatly reduced malignant cell phenotypes in several assays, including colony formation assays, where cdk2ap1 reduced SCC colony number by ~30%, and matrigel invasion assays, where cdk2ap1 expression (either in SCC or in stromal cells) dramatically reduced the SCC invasive ability by ~75% (data not shown). Additionally, cdk2ap1 appeared to be able to reduce malignant cell characteristics both in a cell autonomous (SCC) and also a non-cell autonomous (stromal) manner. These interesting results led us to therefore hypothesize that cdk2ap1 also could act as a TSG capable of promoting anti-angiogenesis as a non-cell autonomous antitumor mechanism. In order to examine cdk2ap1 effects in antiangiogenesis, we first compared its effect with that of a G1-S cell cycle regulator with a potent antiangiogenesis mechanism of action, the pigment-derived epithelial factor (PEDF).

Cdk2ap1 expression following dox treatment in SCC cells correlated with a decreased cellular viability as compared to control (untreated) SCC cells, as assessed by a crystal violet viability assay on days 3 or 6 post-treatment ($p < 0.02$; Figure 1b). Interestingly, cdk2ap1 appeared to reduce cell viability more effectively than PEDF, which only significantly reduced viability for SCC1 (day 3; $p < 0.01$). Addition of PEDF to cdk2ap1 samples did not augment the effect of cdk2ap1 on cell viability (data not shown). Next, we sought to determine whether the mechanism of reduced cell viability was related to alterations in the cell cycle or apoptosis profile. No significant changes were observed in the apoptosis profile of SCC cells as assessed by Caspase 3/7 luminescence assay, with cdk2ap1 inducing modest apoptosis changes of 0.5% ($p = 0.2$, SCC1) or 6% ($p = 0.41$, SCC38), and PEDF inducing modest apoptosis changes of 0.2% ($p = 0.19$, SCC38) or 3.4% ($p = 0.22$, SCC1). The cell cycle profiles, however, were modified by cdk2ap1 or PEDF, with the effects varying depending on the cell line examined (Figure 1c). At 24h, cdk2ap1 expression induces either G1 + G2 or a G1/S arrest, while at 72h, G1-S arrests were more prominent. PEDF induces G1 + G2 arrest in SCC38, but profiles were otherwise unchanged for SCC1 at either 24h or 72h. Combining cdk2ap1 and PEDF did not enhance the cell cycle arrests promoted by either molecule alone (data not shown).

Cdk2ap1 modifies HUVEC tube formation phenotypes *in vitro*

Stimulation of endothelial cells by tumor cell conditioned media establishes an endothelial phenotype consistent with the initial stages of angiogenesis. Conditioned media from SCC cells typically induces angiogenesis in a HUVEC cell tube formation assay *in vitro* (Shemirani and Crowe 2000). We therefore established the tube formation phenotype assay using SCC

cell line conditioned media (CM), and observed that CM from either SCC38 or SCC1 cell lines induced similar HUVEC tube branching, length, and width. We next used this assay to examine the effect of cdk2ap1 or PEDF on angiogenesis phenotypes. We collected conditioned culture media (CCM) from SCC cells where cdk2ap1 expression had been activated for 48 hours (cdk2ap1-CCM), or utilized recombinant PEDF treatment. HUVEC cells were assayed for their ability to form endothelial tubes following 18 hours in culture. cdk2ap1-CCM greatly reduced tube branching (Figure 2a), an inhibitory effect similar to that of PEDF in both cell lines. The phenotype of tube length was also modified, with both cdk2ap1-CCM and PEDF reducing the tube length in SCC1; in SCC38, both showed only a minimal increase over control (Figure 2b). For the phenotype of tube width, both PEDF and cdk2ap1-CCM reduced tube width, although in different cell lines. PEDF failed to reduce tube width in SCC1, and cdk2ap1-CCM failed to reduce tube width in SCC38. Nevertheless, both molecules showed an ability to reduce angiogenesis phenotypes in vitro. Representative images from the tube formation assays are shown in Figure 2d.

Cdk2ap1 expression correlates with changes in several transcriptional pathways in SCC lines

In order to examine potential pathways modulated upon cdk2ap1 expression in SCC cells that could be responsible for the antiangiogenic phenotype observed, we again took advantage of the inducible gene expression system to activate expression of the TRE-cdk2ap1.iRes.RLuc transgene. SCC cell lines transduced with lentiviruses harboring the expression cassette were treated with doxycycline (dox) and reporter gene activity assessed by Renilla Luc assay 48h later (Figure 3a). Levels of cassette expression induction by dox reached ~34-fold (SCC38) and ~46-fold (SCC1). Next, we examined the effect of expressing cdk2ap1 on several pathways related to angiogenesis using several luc-reporter constructs containing different transcriptional activation response elements (Figure 3b). The results differed for each SCC line following cdk2ap1 expression activation. SCC38 displayed moderately higher levels for TNF α , E2F1, and AP1 activity (~17%), high activation levels for NF κ B (39%) and TGF β 1 (57%), and a decrease in activation for p53 (-24%) and Wnt (-31%) pathways. For SCC1, AP1 activity was moderately increased (18%), while TNF α , E2F1, NF κ B, Wnt were all increased between 18–44%, with a dramatic increase in TGF β in particular (74% increase in activity). We proceeded to examine specific cancer-related and angiogenesis gene expression changes upon cdk2ap1 expression in these SCC cell lines to better understand the global mechanisms potentially involved in antiangiogenesis modulation.

Cdk2ap1 expression modifies anti-invasion and anti-angiogenic gene profiles

In order to better characterize the pathways modified by cdk2ap1 expression, we examined gene expression changes in SCC38 and SCC1 cells using cancer pathway-focused qPCR arrays. We observed 45 significant gene expression changes in SCC38 and 30 in SCC1 cells expressing cdk2ap1 as compared to control. The majority of these changes were consistent with anti-survival, anti-invasion, and anti-angiogenic phenotypes. Several alterations were associated with the more classical cell cycle regulation and proapoptotic functions of cdk2ap1 (Table 1). Several genes were altered for either cell cycle control/DNA repair or apoptosis/senescence pathways in both cell lines. Common alterations in both cell lines related to cell cycle arrest were upregulation of Cyclin E1, CDC25a, and E2F1. The only common change related to decreased cell survival was BAX upregulation. A common increase in BCL2L1 was also observed and could be associated with increased cell survival. Nevertheless, the overall profile of gene expression changes in each SCC cell line suggested a balance shift towards cell cycle arrest and reduced cell survival upon cdk2ap1 expression.

Changes in invasion/adhesion/metastasis genes were also observed, and the majority of these changes were consistent with an anti-invasive phenotype (Table 2). Downregulation of

integrins and MMPs were observed for SCC38, while changes in cell adhesion genes and increased expression of MMP inhibitor TIMP3 were observed for SCC1. The only common gene expression change between SCC38 and SCC1 was upregulation of integrin alpha 3 (ITGA3). Several gene expression changes were observed consistent with a reduction in angiogenesis, including downregulation of angiopoietins, interferons, IGF1, PDGF β , TEK (Tie-2), and TNF for SCC38, and reductions in proangiogenic and proinflammatory IL-8 for SCC1. The only common gene expression change related to angiogenesis in both lines was upregulation of TGF β 1. In SCC38, a decrease in TGF β R1 was also observed. Interestingly, several changes following cdk2ap1 expression were associated with genes important to signal transduction and transcription. These gene expression changes are complex and could favor apoptosis or survival, depending on the context, such as ETS2, MYC, FOS, JUN, and SNCG alterations. Other alterations are consistent with survival, such as AKT1 and ERBB2. Common alterations in both cell lines were upregulated FOS, PI3KR1, and SNCG.

Interestingly, we examined the expression of other members of the TGF β /activin signaling pathway in SCC1 cells. We found that TGF β 3 expression was not significantly altered by cdk2ap1 expression (-1.0 fold, $p=0.21$), while TGF β 2 expression was actually downregulated (-1.4 fold; $p<0.03$). We found that two members of the family were upregulated upon cdk2ap1 expression, TGF β 1 (2.0 fold, $p<0.04$), and Activin A (1.5 fold, $p<0.03$). Therefore, we pursued assaying of secreted levels of TGF β 1 and Activin A in SCC1 CCM using ELISA. We have determined that Activin A levels were unchanged in CCM following cdk2ap1 expression at either 24h (1.1 -fold change, $p=0.08$) or 48h (1.0 -fold change, $p=0.33$). In comparison, TGF β 1 level changes in CCM were yet undetectable at 24h (0.98 -fold change, $p=0.86$) upon cdk2ap1 expression, but later were upregulated at least by 48h (1.4 -fold change, $p<0.01$). This suggests that TGF β 1, not Activin A, is present in SCC1 CCM following cdk2ap1 expression.

In combination, the majority of gene expression alterations were consistent with reductions in invasion and angiogenesis pathways, and induction of cell cycle and apoptotic regulatory pathways. Interestingly, we analyzed the 3000bp region upstream of the regulated genes for identifying any common and enriched transcription factor binding sites by using CONFAC analyses (Karanam and Moreno 2004). For the genes upregulated by cdk2ap1 expression, we identified enriched binding sites for AP4 and AP1 (1), and Crebp1/c-Jun (0.75). For the downregulated genes, enriched binding sites were identified for Sry (6.1), Nkx25 (2.1), Oct1 (1.8), AP4 (1.7), MyoD (1.6), Cebp and S8 (1), and Pbx1 (0.8). Interestingly, the majority of these transcription factors actually are involved in TGF β /activin-related signaling pathways, and in particular, AP-1 is involved in modulating the effects of the TGF β pathway on transcription.

The antiangiogenic effect of cdk2ap1 can be inhibited by blocking TGF β signalling—Based on the potential TGF β -related mechanisms downstream of cdk2ap1 expression, we hypothesized that TGF β could be modulating cdk2ap1's antiangiogenic effect. We tested this possibility by examining whether the antiangiogenesis phenotype could be blocked by using a neutralizing antibody (anti-TGF β 1) in SCC1 cells, since they were more sensitive to the effects of cdk2ap1 expression on angiogenesis (Figure 2). SCC1 cells produce TGF β 1, and expression of cdk2ap1 enhanced the production of TGF β 1 by 48h of culture (Figure 4a). Blocking of TGF β signaling resulted in enhanced angiogenesis, reflected by a $\sim 20\%$ increase ($p<0.04$) in HUVEC tube branching and length compared either with control (conditioned culture media (CCM) containing control IgY antibody), or to HUVEC incubated with cdk2ap1-CCM (Figure 4b–c). This result suggested that TGF β could be involved in mediating the antiangiogenic effects of cdk2ap1. The mechanism of TGF β blockage appeared to involve enhanced HUVEC proliferation (by $\sim 40\%$ in a CCK8 assay). This effect was observed even in the presence of cdk2ap1-CCM (Figure 4d). This data suggests that TGF β mediates the antiangiogenesis effect of cdk2ap1 through inhibition of HUVEC cell

proliferation. An assay measuring Caspase 3/7 activity (CaspGLO) showed that blocking TGF β did not alter HUVEC apoptosis in the presence of either control or cdk2ap1-CCM. These results suggested that cdk2ap1 might utilize TGF β pathways to inhibit cell proliferation in order to reduce endothelial cell viability in the tumor microenvironment, effectively reducing angiogenesis.

DISCUSSION

Cdk2ap1 levels are commonly reduced in head and neck squamous cell carcinoma (HNSCC) (Todd et al. 1995; Shintani et al. 2001) and this gene has been implicated as a tumor/growth suppressor (Shintani et al. 2000). We have previously observed that expressing cdk2ap1 in SCCVII/SF could reduce cell growth in a xenograft model (Figueiredo 2005), and although only a fraction of the tumor cells was transfected with the plasmid gene therapy, the tumor inhibition was as high as 70–80% in some treatment groups. These results suggested that cdk2ap1 could be affecting tumor growth through some level of bystander activity that would help reduce tumor growth. The present investigation hypothesized that cdk2ap1 also might have a tumor suppressive effect impacting angiogenesis phenotypes, suggesting antiangiogenesis as a novel non-cell autonomous mechanism for cdk2ap1-mediated growth inhibition.

We observed a range of effects on several malignant cell phenotypes that included reduced colony formation and invasiveness when cdk2ap1 was expressed in SCC cells. However, induction of cdk2ap1 expression also significantly altered the activity of several cancer-related signal transduction pathways in SCC cells, resulting in a reduced ability of SCC to induce angiogenesis phenotypes. Upon activation of cdk2ap1 expression, conditioned culture media (CCM) from SCC cells displayed an antiangiogenic activity in HUVEC tube formation assays, and these effects were similar to that of a potent antiangiogenic and G1 cell cycle regulator, PEDF. Interestingly, both gene expression data and pathway reporter construct activation suggested that cdk2ap1's antiangiogenic effect could be related to upregulation of the TGF β signaling pathway. We assessed this hypothesis by incubating HUVEC with cdk2ap1-CCM in the presence or absence of a TGF β -neutralizing antibody (or isotype control), and the result was an inhibition of the effects of cdk2ap1 on HUVEC tube formation. This result suggested that cdk2ap1 expression in SCC cells might inhibit angiogenesis in the vascular microenvironment through paracrine stimulation of TGF β signaling.

The gene expression data in two separate SCC lines supports the observation that the TGF β pathway signaling may be involved in mediating the effects of cdk2ap1 on invasion and angiogenesis. For cell cycle control/DNA repair, the changes in general were consistent with increased cell cycle arrest. In particular, increases in Cyclin E1 and CDC25a in both cell lines suggest a common mechanism for enhanced cell cycle arrest. The increase in E2F1 observed can lead to apoptosis under certain conditions, and also the upregulation of BAX observed can induce apoptosis strongly. And although other genes were modulated differently in each cell line, it appears that both intrinsic and extrinsic apoptosis pathways could be activated by cdk2ap1 expression in SCC cells. Changes in invasion/adhesion/metastasis genes were also observed, and the majority of these changes were consistent with an anti-invasive phenotype. Downregulation of integrins and reduced MTSS1, MMPs, and PAI-1 in SCC38 potentially comprise strong anti-invasive and anti-metastatic signals. Upregulation of integrin A3 was observed for both cell lines, and may to some extent counteract the anti-invasion signals, but overall most of the gene expression changes appear to favor a reduction in tumor cell growth. Several gene expression changes were observed consistent with a reduction in angiogenesis, including downregulation of angiopoietins, interferons, IGF1, PDGF β , Tie-2, and TNF in SCC38, and downregulation of IL-8, a potent proinflammatory and angiogenesis modulator in SCC1. The TGF β signaling pathway was upregulated, with a TGF β 1 increase in both

lines, and a decrease in TGF β R1 in SCC38. Interestingly, TGF β 1 and/or other pathway molecules can mediate growth inhibition depending on the tissue or cellular context (Gohongi et al. 1999; Fenig et al. 2001; Ito et al. 2009). Thus, the balance between the ligand and receptor levels may help further determine if the final effect of this pathway converges onto growth or apoptosis.

Following a network analysis of these gene expression changes, we observed that central to all pathway links appeared to be the TGF β signaling pathway. Interestingly, cdk2ap1 itself has been implicated as a member of the TGF β pathway, and its overexpression may be able to tap into the growth-inhibitory function of this pathway, achieving a broad impact in the cell cycle, apoptosis, invasion, and angiogenesis. Also, several changes observed were associated with genes important to signal transduction and transcription. These gene expression changes are complex and could favor either apoptosis or survival, depending on the context, such as FOS or SNCG alterations. Interestingly, FOS and JUN form the AP1 transcription factor, which operates downstream of TGF β signaling pathway. Other alterations would be more consistent with cell survival, such as AKT1 and ERBB2. Also related to gene expression regulation was the finding of MTA1 upregulation in SCC38, an interesting finding since MTA1 is a NurD domain-containing protein potentially involved in transcription regulation and chromatin remodeling. Cdk2ap1 has recently been localized to a NurD complex (Le Guezennec et al. 2006) and this may be a mechanism by which it modifies malignant cell phenotypes through induction of transcription changes.

In summary, the overall balance of gene expression alterations following cdk2ap1 expression in HNSCC seems to favor a growth-inhibitory profile, with a novel antiangiogenesis and anti-invasion signature. In particular, the anti-angiogenesis phenotype appears to be in part mediated through the TGF β signaling pathway. The significance of these findings is that if malignant phenotypes and angiogenesis can be reduced by cdk2ap1 expression, this suggests that therapeutic applications could be envisioned for this molecule, especially if efficient delivery vectors such as stable or transient viral vectors can be harnessed to realize this concept. Based on this promising data, we are currently developing a promising adenoviral vector system for treatment of SCC and its microenvironment using expression of cdk2ap1 as a therapeutic gene and reporter genes for tracking gene therapy administration and efficacy noninvasively by molecular imaging in vivo.

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Figure 1. *Cdk2ap1* expression effect on reducing viability and cell cycle profiles

(A) An inducible *cdk2ap1* expression system was utilized whereby 2 μ g/mL doxycycline (dox) treatment can induce gene expression of a TRE.*cdk2ap1*.ires.Rluc cassette. *Cdk2ap1* upregulation in SCC cells transduced with a *cdk2ap1*-expressing lentivirus, as assessed by quantitative real-time qPCR using *cdk2ap1*- and actin-specific primers in either SCC38 or SCC1 cells following 2 μ g/mL dox treatment for 24h. Shown is the upregulation relative to control cells ($p < 0.001$). (B) A crystal violet cell viability assay was used in a 24-well format, and absorbance (A550) measured at days 1, 2, and 4 post-seeding. Shown is the percent change in cell viability compared to control, untreated cells. Treated SCC cells received either doxycycline (2 μ g/ml) to activate *cdk2ap1* or rPEDF (200ng/mL). (C) Flow cytometry cell cycle

analyses. A representative cell cycle analysis diagram by flow cytometry to assess profile of Propidium Iodide staining followed by Modfit analyses to determine the percentage in each phase of the cell cycle (shaded areas represent either G0–G1 or G2–M phases; the S phase is represented by crosshatched lines). Bar plots summarize the percentage of cells at each cell cycle phase for SCC1 or SCC38 at either 24h or 72h following the dox or rPEDF treatments, as in (B).

Figure 2. *Cdk2ap1* reduces angiogenesis phenotypes

A HUVEC tube formation assay was carried out using 6×10^4 HUVEC2 cells seeded with conditioned media from control cells or cells treated to activate cdk2ap1 expression (2 μ g/mL doxycycline) or treated with rPEDF (200ng/mL). Cells were incubated for 18h and images collected following staining and analyzed with ImageJ. Quantification is presented as percent change relative to control for (A) tube branching, (B) tube length, and (C) tube width. (D) Representative 100X fluorescence images of HUVEC tubes in the presence of conditioned media (SCC38 or SCC1), conditioned media from SCC cells expressing cdk2ap1, or conditioned media from SCC cells in the presence of rPEDF (200ng/mL).

Figure 3. *Cdk2ap1* modifies signal transduction pathways related to angiogenesis

(A) An inducible *cdk2ap1* expression system was utilized whereby 2 μ g/mL doxycycline (dox) treatment can induce gene expression of a TRE.*cdk2ap1*.ires.Rluc cassette. Shown is the cassette activation as assessed by Rluc reporter gene activity following 2 μ g/mL dox treatment for 48h. (B) SCC38 or SCC1 cells harboring the inducible expression system were transfected with firefly luc (Luc) responsive vectors to assess extent of signal transduction pathway activation, dox administered at 24h, and cells collected at 48h post-transfection for Luc assays. Presented is the percent up or downregulation of each construct relative to control (uninduced or 'no dox') cells. The luc-responsive constructs are described in Materials and Methods.

Figure 4. The antiangiogenic effect of *cdk2ap1* can be inhibited by blocking TGF β pathway signaling (A) Activin A and TGF β 1 production by SCC1 cells as detected using either a human/rat/mouse Activin A or human TGF β 1 quantikine ELISA kits at either 24h or 48h following dox treatment. (B) Blocking of TGF β signaling by using a neutralizing antibody resulted in reversal of several *cdk2ap1* antiangiogenic phenotypes, as assessed by HUVEC tube formation. HUVEC2 cells were incubated either with conditioned media (CCM)-plus anti-TGF β 1 antibody or CCM containing isotype control antibody. (C) Representative images of HUVEC tube formation assay showing the effect of incubating HUVEC with anti-TGF β 1 antibody (+) or isotype control (-). The TGF β antibody reversed the antiangiogenesis phenotype of *cdk2ap1*. (D) HUVEC CCK8 cell viability assay. In a CCK8 HUVEC proliferation assay, blocking TGF β

reverted cdk2ap1 antigrowth effects on HUVEC cells on day 4 post-treatment ($p < 0.03$). (E) A CaspGlo luminescence assay for assessing Casp3/7 activity as a measure of HUVEC apoptosis was performed on day 4 post-treatment in a 96-well format. No effect on caspase activity was observed with TGF β blockage.

Table 1

Gene expression profiles modified in SCC related to classical cdk2ap1 functions related to regulation of cell cycle and apoptosis.

Function	Gene symbols	Fold up- or Down-regulation SCC38	Fold up- or Down- regulation SCC1
<i>Cell cycle control/ DNA repair</i>	ATM	-1.4	
	CCNE1 *	1.5	1.4
	CDC25a	1.2	1.2
	CDK2		1.2
	CHEK2	-1.3	
	E2F1	1.3	1.2
	MDM2	1.6	
	RB1		-1.4
	S100A4	-1.5	
<i>Apoptosis/ Senescence</i>	TP53	3.2	
	BAD	1.7	
	BAX	1.8	1.3
	BCL2L1	3.0	1.3
	GZMA	-1.3	
	TNFRSF1A	1.7	
	TNFRSF10B	1.2	
	TERT	-1.5	1.19
	BCL2		1.27
<i></i>	CASP8		1.21

* Bold type face indicates gene expression altered in a similar pattern in both SCC cell lines.

Table 2

Gene expression profiles modified in SCC related to novel cdk2ap1 tumor-suppressive functions related to invasion and angiogenesis processes

Function	Gene symbols	Fold up- or down-regulation in SCC38	Fold up- or down-regulation In SCC1
<i>Adhesion/ Invasion/ Metastasis</i>	EPDRB		1.3
	ITGA1	-1.2	1.3
	ITGA4	-1.4	
	ITGA3*	3.4	1.3
	ITGAV		-1.2
	ITGB1	1.2	
	ITGB3	-1.3	
	MCAM		1.2
	MTSS1	-1.5	
	MMP1	-1.3	1.3
	MMP2		1.3
	MMP9	-1.3	
	MTA1	2.0	
	MTA2	1.3	
	NME4		1.2
	PLAUR (PAI-1)	-1.3	
	PNN		-1.3
	TIMP3		1.2
	TWIST1		1.2
<i>Angiogenesis</i>	ANGPT1	-1.5	
	ANGPT2	-1.5	
	IFNa	-1.5	
	IFNb	-1.4	
	IGF1	-1.4	1.2
	IL8		-1.3
	HTATIP2	1.2	
	PDGFa		1.2
	PDGFb	-1.3	
	TEK (Tie-2)	-1.4	1.3
	TGFb1	2.0	1.5
	TGFbR1	-1.4	
<i>Signal Transduction/ Transcription Factors</i>	TNF	-1.4	
	AKT1	2.6	
	ERBB2	1.5	
	ETS2	-1.4	
	FOS	1.8	1.2
	JUN	2.7	

Function	Gene symbols	Fold up- or down-regulation in SCC38	Fold up- or down-regulation In SCC1
	MAP2K1		−1.3
	MYC	2	
	PI3KR1	1.5	1.3
	SNCG	1.6	1.3

* Bold type face indicates gene expression altered in a similar pattern in both SCC cell lines.