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Expression of cell cycle regulator *cdk2ap1* suppresses tumor cell phenotype by non-cell autonomous mechanisms

Olga Zolochovska and Marxa L. Figueiredo *

Department of Comparative Biomedical Sciences. School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA, 70803

Abstract

We evaluated the effect of expressing the cell cycle regulator *cdk2ap1* in epithelial or stromal cell compartments to reduce SCC growth in vitro and in vivo. Cell autonomous and/or non-cell autonomous expression of *cdk2ap1* reduced tumor growth and invasion and altered cell cycle, adhesion, invasion, angiogenesis, and apoptotic gene expression, as assessed by several in vitro phenotype assays, quantitative real time PCR, and in vivo molecular imaging using a novel three-way xenograft animal model. Our findings suggest that the interactions between cancer cells and fibroblasts that promote abnormal growth can be minimized by expressing *cdk2ap1*, supporting a novel concept by which tumor/growth suppressor genes can impact tumorigenesis phenotypes from non-cell autonomous interactions within the tumor microenvironment.

Keywords

Squamous cell carcinoma; oral cancer; *cdk2ap1*; stromal-epithelial interactions; non cell-autonomous; tumor suppression

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. Many types of stromal-epithelial interactions mediate or increase tumor invasion, including altered angiogenesis and adhesion/invasion profiles, contributing to MMP expression and activation in epithelial and/or stromal cells [1]. In many human cancers, the stromal microenvironment adjacent to carcinoma cells has an activated phenotype, with modified ECM composition and gene expression profiles [2-4], and exhibits markers similar to those found during wound repair [5]. Understanding of how HNSCC cells interact with their microenvironment is critical for achieving better prognostics and therapeutics to help prevent invasion and tumor spread.

Co culture models have been instrumental in elucidating the role of cell-specific regulation of tumor growth and invasion in vitro. Also, recent studies have addressed the role for stroma in

*Corresponding Author: Marxa L Figueiredo, PhD. Department of Comparative Biomedical Sciences. School of Veterinary Medicine, Louisiana State University, 1909 Skip Bertman Dr., Baton Rouge, LA, 70803. Ph: (225)578-9721; Fax: (225)578-9894; mfigueiredo@vetmed.lsu.edu.

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promoting tumorigenesis using in vivo models. Combination of transformed mammary epithelia with normal fibroblasts elevated the tumor incidence to twice that of epithelium only [6]. Other studies have shown that co-inoculation of essentially non-tumorigenic prostate carcinoma cells with either NIH3T3, human prostate stromal cell lines, or carcinoma-associated fibroblasts, dramatically increased the tumor incidence and growth by 2.4-fold [7]. In order to develop an epithelial-stromal HNSCC model of tumorigenesis, we chose human cell lines derived from tongue SCC, implanted in immune compromised SCID mice in the presence or absence of NIH3T3 fibroblasts. This model enables us to examine the effectiveness of therapeutic strategies on more complex tumors with a stromal-epithelial composition and the ability to manipulate epithelial or stromal cells by lentiviral transduction in order to examine the role of defined genetic alterations in regulating tumor growth in a cell autonomous and/or non-cell autonomous manner.

Targeting expression of tumor suppressor genes to the epithelia and/or stroma is one strategy for regulating tumor cell growth. For example, p53 and p21 are known to impact tumor suppression through cell-autonomous mechanisms; however, genetic manipulation of tumor/growth suppressors in stroma can dramatically reduce tumor progression. Downregulation of cdk-inhibitor p21^{WAF1/CIP1} in stroma correlates with tumor progression, while upregulation restores breast tumor cells to a more benign phenotype [8]. We have assessed the effect of expressing cdk2ap1 (p12^{CDK2AP1}) in the stroma and the impact of this strategy on HNSCC growth. Cdk2ap1 is an S-phase growth suppressor through association with DNA polymerase α /primase and/or CDK2 [9,10]. Additionally, cdk2ap1 can induce apoptosis and reduce cell proliferation [11-13], although its antitumor effects are unknown in the context of the epithelial-stromal tumor microenvironment. Our present study supports a novel role for cdk2ap1 as both a cell-autonomous and non cell-autonomous tumor suppressor, comprising a novel mechanism for tumor growth regulation.

Materials and Methods

Cell culture and viral transductions

Human squamous cell carcinoma (SCC) line 9 and murine NIH3T3 fibroblasts were obtained from ATCC; human SCC38 [14] were obtained from Dr. S.Li. SCC were maintained in Ham's:F12 1:1 media (Mediatech, Manassas, VA) with 10% FBS/1% PS, and 400ng/mL hydrocortisone, and 3T3 in DMEM with 10% FBS/1% PS. Lentiviruses were constructed using the vector pCCL-CMV.ires.GFP [15] digested with BamHI and NheI to insert *cdk2ap1* upstream of the IRES, while the empty vector served as the GFP control. Lentiviruses (Lv) were produced by triple transfections into 293T cells using calcium phosphate transfection [16] and supernatants harvested at 48, 74, and 96h, filtered (.45 μ m membrane), and titered by p24 detection and GFP infection. Lv were used at a multiplicity of infection (moi)=2 for SCC and moi=3 for 3T3 using Viraductin reagent (CellBiolabs, Inc., San Diego, CA).

Flow Cytometry and Cell Growth Assays

Cells for flow cytometry detection of GFP expression were assayed on a FACScan (BD Biosciences, Franklin Lakes, NJ) at the LSU Flow Cytometry Core Facility. Growth assays were performed in 24-well plates for co-culture and matrigel invasion assays. For assaying cell number increase over time, 10⁴ SCC cells were plated at the bottom of a 24-well plate, and 10³ 3T3 plated at the top of a transwell 0.4 μ m insert (BD Biosciences). SCC were fixed in 10% buffered formalin, stained with 0.5% Crystal violet, washed 3 $\times$, the dye solubilized in 33% glacial acetic acid and 1/30 diluted sample read at absorbance 550. For invasion assay, 24-well 8 μ m inserts (BD Biosciences) contained 20 μ l 1:6 matrigel:serum free media dilution (BD Biosciences), and 10⁵ SCC cells were plated in the top chamber in 0.5mL 0.5%FBS Iscove's DMEM. The bottom chamber received 10⁴ 3T3 cells in the same media. At 48h, inserts were

fixed using 10% buffered formalin, matrigel removed with a cotton swab, membranes mounted on glass slides using DAPI mounting media, images acquired for 6-10 independent fields at 100 $\times$, and the total area fraction covered by cells determined using NIH ImageJ software.

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 2 $\times$ 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 $^{\circ}$ C/15sec; 56 $^{\circ}$ C/30sec, 72 $^{\circ}$ C/30sec, and analyzed using ABI7300 software. All samples were normalized to the internal β -actin control and expressed as change in Ct relative to β -actin. For experiments utilizing pathway-focused qPCR arrays, the Human Cancer PathwayFinder PCR array was used (PAHS-033A, SABiosciences, Frederick, MD), according to manufacturer's protocols.

In vivo studies and noninvasive imaging

Animal care and procedures were performed in accordance with the LSU Animal Research Committee guidelines. A 4:1 cell preparation (2 $\times 10^6$ SCC9: 5 $\times 10^5$ 3T3) was made in 30 μ l Matrigel (BD Biosciences) and implanted subcutaneously at the base of the mylohyoid muscle in SCID mice. The tumor growth was monitored over time using vernier calipers. For *in vivo* imaging studies, mice received SCC9 cells that were marked with a renilla luciferase-expressing lentivirus, and either Lv-CMV.i.res.GFP (control) or cdk2ap1-expressing lentivirus at moi=2. Mice were anesthetized, received a tail vein injection of coelenterazine (1mg/kg), images collected using a cooled IVIS CCD camera (Xenogen, Alameda, CA), and analyzed using Igor-PRO Living Image Software.

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

Endogenous and Exogenous cdk2ap1 expression in squamous cell carcinoma lines

Endogenous cdk2ap1 expression was examined using real-time quantitative PCR with gene-specific primers to cdk2ap1 and actin. Two squamous cell carcinoma (SCC) lines, SCC9 and SCC38, NIH3T3 fibroblasts, and a positive control, normal HEK293 line, previously shown to have normal cdk2ap1 levels [17] were examined (Figure 1a). Cdk2ap1 was downregulated by 13-fold in SCC9, 2-fold in SCC38, and 800-fold in 3T3 cells compared to HEK293. Two lentiviruses were used to overexpress cdk2ap1 and a reporter gene in SCC or 3T3 cells, Lv-CMV.i.res.GFP (control) or Lv-CMV.cdk2ap1.i.res.GFP, and percentage of transduced cells examined by flow cytometry for GFP+%. Illustrated are the results from an experiment where SCC38 cells were transduced at moi=2 with Lvs (Figure 1b). The transduction efficiency typically ranged from 50-65% in all cell lines used. The expression of exogenous (Lv) cdk2ap1 was also confirmed by qPCR, and reached ~60-fold upregulation (SCC), and ~3000-fold (3T3) (Figure 1c).

Co culture with fibroblasts enhances SCC tumor cell growth *in vitro* and *in vivo*

We examined the effect of co culture with fibroblasts on SCC cell growth over time. SCC9 or SCC38 cells were grown in the presence or absence of 3T3 fibroblasts in Iscove's DMEM

media. By day 4 post-seeding, 3T3 effected a $1.2\times$ increase in absorbance of SCC9, and $1.6\times$ increase in SCC38 absorbance by day 6 (Figure 2a), indicating that 3T3 cells enhance the growth rate of SCC cells in co culture. The effect of 3T3 fibroblasts in enhancing SCC growth was examined *in vivo* using a 1:4 co culture with SCC9 and matrigel (three-way xenograft), conditions previously reported for enhancing epithelial cell growth in a prostate cancer model [7]. SCC9 alone were virtually nontumorigenic at the concentration used (2×10^6). When 5×10^5 3T3 cells were coinjected with SCC9, the growth was dramatically altered and tumors grew very rapidly at an orthotopic floor of mouth site in SCID mice (Figure 2b, $p<0.02$ at days 14 through 30). The tumor weight at sacrifice (as a % of body weight) was also greatly enhanced compared to controls (plot on the right, $p<0.001$).

Inhibitory effect of non-cell autonomous cdk2ap1 on SCC tumor phenotypes

The effect of expressing cdk2ap1 on either epithelial (SCC9 or 38) or stromal (3T3) compartments was assessed in the co culture model described (Figure 3). Cells were transduced with either control or cdk2ap1-expressing lentiviruses at moi=2 (SCC) or =3 (3T3). Co culture of SCC cells with 3T3 resulted in a $\sim 1.7\times$ increase in growth by days 4 or 6 post-seeding (Figure 3a, $p<0.03$), while expression of cdk2ap1 in the stromal compartment (3T3-cdk2ap1) resulted in a growth reduction of $\sim 1.3\times$ ($p<0.02$). The effect of cdk2ap1 in the epithelial behavior was assessed further in a colony formation assay, where cdk2ap1 reduced SCC9 colony number by $\sim 30\%$ (data not shown). The invasive potential of SCC cells grown in the presence or absence of co culture with control or cdk2ap1-expressing 3T3 was examined *in vitro* using a matrigel invasion assay. Cells were transduced with either control or cdk2ap1 Lvs (for SCC, moi=2; for 3T3, moi=3). Co culture of SCC9 with 3T3 significantly enhanced the invasiveness of cells compared to SCC9 alone ($p=0.006$) (Figure 3b). When cdk2ap1 was expressed from the stroma, SCC invasiveness was reduced by 68%, and virtually restored to regular SCC levels (Figure 3b). For SCC38, the changes were also similar, with a 75% reduction in invasion when co-cultured with cdk2ap1-expressing stroma. In conclusion, non-cell autonomous expression of cdk2ap1 appears to be effective in reducing SCC proliferation, colony formation, and invasive phenotypes in co-culture.

Non-cell autonomous cdk2ap1 modifies SCC gene expression profiles

In order to understand the pathways modified by non-cell autonomous cdk2ap1 expression, we examined gene expression changes in SCC38 cells co-cultured with control or 3T3-cdk2ap1 cells, using a cancer pathway-focused qPCR array (Table 1). The only alteration in cell cycle/DNA repair genes was p53 tumor suppressor gene upregulation. Four apoptosis genes were modulated, with upregulation of TNFRSF1A and downregulation of GZMA, TERT, and TNFRSF25. For angiogenesis, eight genes were downregulated, including ANGPT1 and 2, IFN α and β , IGF1, Tie-2, TGF β R1, and TNF. Another category with a high number of downregulated genes was adhesion/invasion/metastasis, including several integrin genes (ITGA1, A1, and B3), MTSS1, TIMP3, NME4, and MMPs 1 and 9. The genes up- and down-regulated in SCC upon exposure to non-cell autonomous cdk2ap1 conferred a particular signature, with specific alterations in apoptosis, angiogenesis, and perhaps most significantly, an altered adhesion/invasion/metastasis profile, supporting the concept that extrinsic cdk2ap1 expression may represent a novel mechanism for suppressing HNSCC growth and invasiveness.

Non-cell autonomous cdk2ap1 reduces SCC tumor growth *in vivo*

We next examined whether cdk2ap1 expression could be effective in reducing SCC tumor growth *in vivo*, using the three-way orthotopic floor of mouth model (Figure 2b). Tumors were established, and growth was tracked by noninvasive bioluminescence molecular imaging. SCC9 cells were transduced with an imageable Lv (Renilla luc-expressing) at moi=0.5 for

tumor growth tracking over time. Also, SCC cells were transduced with moi=2 of either control or cdk2ap1-expressing Lvs. At week 4, tumor volume measurements confirmed that both cell-autonomous (SCC-expressed) and non-cell autonomous (fibroblast-expressed) cdk2ap1 appeared to have a growth-suppressive effect on SCC tumors, although this difference was only significant for non-cell autonomous cdk2ap1 (Figure 4a, $p<0.01$). We have labeled either SCC9 or 3T3 cells using transduction by a lentivirus expressing renilla luc. Following implantation and growth with matrigel for 3-4 weeks, the SCC give a renilla luc signal following substrate administration, but not the 3T3 cells, indicating that they could not establish and form a visible or detectable tumor. Renilla luc imaging at weeks 1, 2, and 4 post-implantation allowed a quantitative measurement of tumor growth with time and also displayed a role for cdk2ap1 in reducing or suppressing SCC tumor growth *in vivo* (Figure 4b). Interestingly, either cell-autonomous or non-cell autonomous cdk2ap1 expression reduced tumor growth in the presence of 3T3 fibroblast stimuli. However, the tumors expressing non-cell autonomous cdk2ap1 displayed lower tumor signals than both SCC:3T3 tumors ($p=0.04$), and tumors expressing cdk2ap1 cell-autonomously (in SCC, $p=0.012$). In Figure 4c, representative animals are shown with the color bar quantification and scale used.

Discussion

Cdk2ap1 levels are commonly reduced in HNSCC [18,19] and this gene has been implicated as a cell-autonomous tumor/growth suppressor [9]. Although we had previously shown that cell-autonomous cdk2ap1 overexpression in SCC could reduce cell growth in a SCCVII/SF xenograft [12], the present investigation uncovers a novel role for cdk2ap1 in suppressing tumorigenesis in a non-cell autonomous manner. We have developed a three-way xenograft model, establishing a novel way to examine biology of epithelial-stromal cell interactions. In the absence of fibroblastic stimulus, SCC cells were virtually non-tumorigenic, however, upon commixing with 3T3 cells, their growth rate was enhanced by ~9-fold. Interestingly, it has been reported that inoculation of 3T3 cells in matrigel can render these cells tumorigenic in nu/nu mice [20]. Since we and others [7] have not observed this effect, we propose that several factors could modify the ability of 3T3 to grow *in vivo*. For example, the concentration and volume of matrigel, the site of inoculation, and the time of tumor follow-up (restricted by the choice of inoculation sites) all might affect the efficacy of tumor formation.

Induction of cdk2ap1 expression in the stroma significantly reduced SCC growth and invasiveness in co culture and altered expression profiles of several cancer-related pathways. Upregulation of the classical tumor suppressor gene p53 was observed, and many other relevant gene alterations could be contributing to the reduced SCC growth/invasion phenotypes. These alterations included downregulation of TERT, TGFBR1, and IGF1, factors associated with tumor progression and survival, and changes in apoptotic pathways, with modifications in TNF α receptor and ligand expression levels. Several angiogenesis pathways also were modified including angiopoietin-Tie2, and Interferon. Most alterations occurred in adhesion/invasion/metastasis pathways, including reductions in expression of integrin genes, matrix metalloproteinase genes, and the invasion gene NME4. Matrix metalloproteinases (MMPs) have been implicated at many levels in carcinogenesis, and can impact ECM degradation, cell proliferation, and angiogenesis. MMP9 is associated with poor survival in HNSCC [21]. MMP1 and TIMP (MMP inhibitors) gene upregulation are characteristic of HNSCC progression [22]. Cells upregulating TIMP have been proposed to exhibit a lower metastatic potential and represent an earlier stage of tumor progression [23]. Interestingly, NME4 has been implicated among a predictive gene set for HNSCC locoregional response [24]. In summary, the majority of gene expression changes observed on HNSCC upon non cell-autonomous cdk2ap1 expression are consistent with strong anti-tumorigenic signals, and can modulate apoptosis and reduce tumor survival and invasion.

We further investigated the effects of non-cell autonomous cdk2ap1 *in vivo*, using a novel three-way xenograft model for examining SCC:stromal interactions. We found that non-cell autonomous cdk2ap1 could reduce tumor volume and growth rate to an extent greater than cell-autonomous cdk2ap1. However, there was no tumor eradication, suggesting that SCC proliferative/survival interactions may have to be manipulated in both epithelia and stroma in order to achieve complete tumor reduction. Cdk2ap1 or other cell cycle regulators could be utilized to disrupt paracrine activation loops and achieve “normalization” of the stroma. This concept has been tested using a basement membrane reconstitution model, where induction of cell growth arrest restored breast tumor cells to more normal and polarized alveolar structures [25]. If fibroblasts can be modulated to reduce tumor phenotypes by cdk2ap1 expression, therapeutic applications can be envisioned for this molecule. Viral vectors or cellular therapies may be utilized to realize this concept, with stromal therapy collaborating with therapies targeted at the epithelial tumor cells, to effectively treat the tumor and its supporting microenvironment.

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Abbreviations Used

Cdk2ap1	cyclin-dependent kinase 2 associating protein 1
SCC	squamous cell carcinoma
ECM	extracellular matrix
MMP	matrix metalloproteinase
CMV	cytomegalovirus promoter
GFP	green fluorescent protein
Lv	

	lentivirus
qPCR	real-time quantitative polymerase chain reaction
CCD	charge-coupled device
IGF	insulin-like growth factor
IL	interleukin
FGF	fibroblast growth factor
TGF	transforming growth factor
TNF	transforming nuclear factor
TNFR	TNF receptor
DR	death receptor

Figure 1. Endogenous and transgene-derived cdk2ap1 and reporter expression in SCC and 3T3 cells

(A) Quantitative real-time PCR results for cdk2ap1 levels relative to actin endogenous expression. (B) Assessment of transgene expression in lentivirally (Lv)-transduced SCC or NIH3T3 (3T3) cells. The percentage of cells expressing the reporter gene green fluorescent protein (GFP) is shown as assessed by FACS for cells transduced with either CMV-ires-GFP or CMV-cdk2ap1-ires-GFP Lvs. (C) Real-time qPCR analyses of cdk2ap1 levels relative to actin following transduction with control or cdk2ap1-expressing Lvs.

Figure 2. Stromal conditions regulate proliferation and tumor growth *in vitro* and *in vivo*
 (A) Fibroblasts (3T3) enhance SCC proliferation (SCC9 or 38) in culture as assessed by a crystal violet assay at A550. (B) A three-way SCC:3T3:matrigel xenograft model greatly accelerates the growth of SCC9 tumors (open triangles) at the orthotopic floor of mouth implantation site. The right panel shows the tumor weight as a percentage of the body weight at endpoint (day 30), (*, $p < 0.001$).

Figure 3. Effect of expressing cdk2ap1 (p12) in epithelial and/or stromal cellular compartments on SCC cell growth phenotypes

(A) Cell viability assay for SCC cells co-cultured with 3T3 fibroblasts transduced with moi=3 of either control (CMV-ires-GFP) or cdk2ap1-expressing (CMV-cdk2ap1-ires-GFP) Lvs. The growth of SCC cells in the presence or absence of co culture with 3T3 was assessed by crystal violet staining assay at A550 at either days 2 and 4 (SCC9) or days 3 and 6 (SCC38). (B) Invasion Assay. 10^5 SCC9 cells transduced with control Lv at moi=2 were seeded on the top chamber of an invasion assay membrane (8 μ m) in 0.5% FBS media. The bottom chamber received 0.5% FBS media plus or minus 3T3 cells, transduced with either control or cdk2ap1-Lv, at moi=3. At 48h, membranes were fixed, mounted in DAPI mounting media, images captured, and ImageJ used to obtain an area fraction (%) occupied by invasive cells. On the right are 100X images of representative fields showing blue DAPI-stained nuclei.

Figure 4. Effect of cdk2ap1 (p12)-expressing stromal cells on SCC tumor growth suppression *in vivo*

SCC9 or 3T3 cells were transduced with moi 2-3 control or cdk2ap1-expressing Lvs. 2×10^6 SCC9 and 5×10^5 3T3 cells were isolated, mixed in a three-way xenograft with matrigel and implanted into the floor of mouth in SCID mice. SCC9 cells also were infected with a Lv expressing renilla luciferase to follow growth over time using noninvasive bioluminescence imaging, using tail vein injection of coelenterazine and 1min image collection using a CCD cooled camera (IVIS). Light units are expressed as maximum bioluminescence photons signal ($\text{p}\cdot\text{s}^{-1}\cdot\text{cm}^{-2}\cdot\text{sr}^{-1}$) at weeks 1, 2, and 4 post-implantation. (C) Representative mouse images are shown for week 2 with color bar.

Table 1
Effect of non-cell autonomous cdk2ap1 on SCC38 gene expression using a cancer pathway-focused array

Function	Gene symbols	Fold up- or Down-regulation	P value
Cell cycle control/DNA repair	<i>Tp53 (p53)</i>	+1.4	0.03
	<i>TNFRSF1A</i>	+1.3	0.05
Apoptosis/Senescence	<i>GZMA</i>	-1.5	0.01
	<i>TERT</i>	-1.5	0.03
	<i>TNFRSF25 (DR3)</i>	-1.3	0.04
Angiogenesis	<i>ANGPT1</i>	-1.6	0.015
	<i>ANGPT2</i>	-1.6	0.02
	<i>IFNA1 (IFNα)</i>	-1.5	0.01
	<i>IFNB1 (IFNβ)</i>	-1.6	0.015
	<i>IGF1</i>	-1.4	0.03
	<i>TEK (Tie-2)</i>	-1.6	0.015
	<i>TGFBR1 (ALK-5)</i>	-1.5	0.015
	<i>TNF</i>	-1.4	0.04
	<i>ITGA1</i>	-1.5	0.02
Adhesion/Invasion/Metastasis	<i>ITGA4</i>	-1.4	0.03
	<i>ITGB3</i>	-1.4	0.04
	<i>MTSS1</i>	-1.5	0.02
	<i>MMP1</i>	-1.5	0.025
	<i>MMP9</i>	-1.5	0.02
	<i>NME4</i>	-1.4	0.04
	<i>TIMP3</i>	-2.3	0.001