RhoC Expression and Head and Neck Cancer Metastasis

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Abstract
RhoC protein, a known marker of metastases in aggressive breast cancers and melanoma, has also been found to be overexpressed in certain head and neck cancers, thus we investigated the correlation between RhoC expression and the metastatic behavior of head and neck squamous cell carcinoma. Selective inhibition of RhoC expression was achieved using lentiviral small hairpin RNA (shRNA) transduced and tracked with green fluorescent protein to achieve 70% to 80% RhoC inhibition. Fluorescence microscopy of the RhoC knockdown stable clones showed strong green fluorescence in the majority of cells, signifying a high efficiency of transduction. Importantly, quantitative real-time PCR showed no significant decrease in the mRNA expression levels of other members of the Ras superfamily. Cell motility and invasion were markedly diminished in RhoC-depleted cell lines as compared with control transduced lines. H&E staining of lung tissue obtained from severe combined immunodeficiency mice, which had been implanted with RhoC knockdown cells, showed a marked decrease in lung metastasis and inflammation of the blood vessels. The cultured lung tissue showed a significant decrease in cell growth in mice implanted with RhoC-depleted cell lines as compared with shRNA-scrambled sequence control lines. Microscopic studies of CD31 expression revealed substantial quantitative and qualitative differences in the primary tumor microvessel density as compared with parental and shRNA-scrambled controls. This study is the first of its kind to establish the involvement of RhoC specifically in head and neck metastasis. These findings suggest that RhoC warrants further investigation to delineate its robustness as a novel potentially therapeutic target. (Mol Cancer Res 2009;7(11):OF1–10)

Introduction
Head and neck cancer is the sixth most common cancer worldwide (1). According to the statistical report of the American Cancer Society, ~70,000 new head and neck squamous cell carcinomas (HNSCC) will be diagnosed this year in the United States (2). In contrast to other epithelial cancers for which effective screening exists, most of the patients with head and neck cancer are diagnosed at a very late stage (stage III and IV). Despite advancements in surgical procedures, chemotherapy, and radiation therapy, survival rates have not improved in the last several decades (3). Furthermore, it has been shown that the high rate of morbidity is due to both locoregional recurrence and distant metastases.

In the past decade, numerous studies have shown that the Rho family of GTPases (RhoA, RhoB, RhoC, Rac1, Rac2, Rac3, and CDC42) is involved in instilling a metastatic phenotype into localized cancerous cells that are localized to the organ of origin. RhoA and RhoC are overexpressed in a number of tumor types (4, 5) suggesting an oncogenic role. Among the members of the Ras homology protein family, RhoC (molecular mass of 21 kDa) has been implicated in a wide range of cellular activities, including downstream expression of inflammatory genes and chemokines, cell proliferation, intracellular signaling, and cytoskeletal organization (6). More significantly, RhoC plays a central role in assembling focal adhesion by modulating the orientation of cytoskeletal fibers, resulting in cell polarity, increased cell motility, and consequently, increased invasiveness (7-9). In addition, signaling mediated by Rho proteins through Rho-activating kinase regulate proteins that in turn regulate actin polymerization such as cofilin, profilin, and formin homology proteins (10). Interestingly, high levels of RhoC and Rho-activating kinase are also associated with membrane blebbing, a phenomenon that is observed in motile or invasive cells (10, 11).

RhoC overexpression is now well documented in a wide range of malignant cancers, suggesting an important role in changing noninvasive carcinomas into invasive forms. Interestingly, overexpression of RhoC has been reported in inflammatory breast cancer and exclusively in invasive breast carcinoma (12-15). Other tumor types in which overexpression of RhoC has been reported are ovarian carcinoma (16), esophageal squamous cell carcinoma (17), pancreatic cancer (18), gastric cancer (17, 19), and human melanoma (11, 20). In addition, functional studies have shown that RhoC can act
as a transforming oncogene when it is overexpressed in human mammary epithelia converting these normally immobile cells into highly motile and invasive malignant cells (12, 21). Thus, a wide range of current studies reveal the important role of RhoC in cancer metastasis.

However, very few studies to date have investigated the role of RhoC in head and neck cancer. Studies on gene expression profiling of stage III and IV regionally metastatic HNSCC showed that there are elevated levels of RhoC when compared with stage I and II localized malignancy (22). Furthermore, in our laboratory, we have shown that RhoC expression is elevated in the tumors of patients with HNSCC when compared with normal squamous cell epithelium (21). More importantly, our study showed that increased RhoC expression is strongly associated with lymph node metastasis and could also be used to predict metastasis even in small (T1 and T2) primary tumors (23). In the present study, we investigated the role of RhoC in head and neck metastasis by inhibiting its function using RNA interference. Our in vitro findings determined that inhibiting RhoC function strongly reduced cell motility and invasion. Furthermore, we observed a remarkable reduction in tumor metastasis and microvessel density in severe combined immunodeficiency (SCID) mice injected with RhoC knockdown cell lines. These findings suggest that inhibition of RhoC function in HNSCC can diminish a tumor’s aggressive behavior, thus opening new possibilities for future drug therapies targeting this pathway.

Results
RhoC mRNA Expression Is Greatly Reduced in Knockdown Clones from HNSCC Cell Lines

To understand the role of RhoC expression in head and neck metastasis, we constructed cellular reagents in which RhoC expression was downregulated by small hairpin RNA (shRNA) in squamous cell carcinoma cell lines from the University of Michigan (UM-SCC-11A and UM-SCC-1). These cells exhibit a strong invasive phenotype and have shown in our previous studies that RhoC is constitutively active in these lines (23).

The inhibition of RhoC expression was achieved using RNA interference and lentiviral transfection and transduction technology. After lentiviral infection, positive (stable) clones were selected using puromycin antibiotics. Fluorescence microscopy of the stable clones showed a strong green fluorescence in the majority of the cells, signifying a high efficiency of transfection (Fig. 1).

We then tested the effectiveness of shRNA in depleting RhoC mRNA expression in the lentivirally infected cell lines using quantitative real-time PCR (qRT-PCR). Because only a small number of specific gene sequences are capable of activating the RNA degradation pathway, we used two different RhoC knockdown clones (i.e., C1 and C2 along with a parental and shRNA-scrambled sequence infected control) to ensure the effectiveness of depleting levels of RhoC. The results show greatly reduced expression levels of RhoC gene in the C1 and C2 RhoC knockdown clones, whereas normal RhoC expression was observed in clones with a shRNA-scrambled sequence (Fig. 2). The relative RhoC mRNA expression in parental, shRNA-scrambled control and RhoC knockdown clones 1 and 2 were evaluated by qRT-PCR and the Ct values thus obtained were normalized using two housekeeping genes as described in Materials and Methods. As shown in Fig. 2A, RhoC mRNA expression decreased ~75% and 80% in RhoC knockdown clone 1 and clone 2 of UM-SCC-1, respectively. A similar decrease of 40% and 70% in RhoC mRNA levels was observed in RhoC knockdown clone 1 and clone 2 of UM-SCC-11A, respectively. However, the control shRNA-scrambled sequence in either of the cell lines did not show any significant reduction in RhoC mRNA expression level (Fig. 2A). To confirm that only RhoC expression was being inhibited, the mRNA levels of other Rho family members, Cdc42, Rac1, and Rac2 were also analyzed by qRT-PCR. As shown in Fig. 2B, C, and D, the expression levels of Cdc42, Rac1, and Rac2 are very similar to the parental lines, thus confirming that our shRNA process is highly specific to RhoC only. These studies provided a clear insight about the “switching off” of the RhoC machinery by decreasing total levels of RhoC mRNA expression, and therefore, further detailed studies on its functional roles are definable. One of the most basic clinical questions that arise at this point is how inhibition of the RhoC transcript affects metastasis in head and neck cancer. To address this question, we investigated two characteristic behaviors of metastatic cells, invasion and motility in the transduced cell lines.

RhoC Knockdown Clones Show a Decrease in Cell Invasion and Motility

In the invasion assays, RhoC-depleted clones of UM-SCC-11A and UM-SCC-1 were remarkably less invasive and motile compared with the parental or shRNA-scrambled controls (Fig. 3). Notably, cell invasion was decreased by 50% and 75% in RhoC knockdown clones 1 and 2, respectively, in the transduced UM-SCC-11A cell line (Fig. 3I). A similar decrease of 60% and 80% in clones 1 and 2, respectively, was observed in UM-SCC-1 cell lines (Fig. 3J) when compared with their parental or shRNA-scrambled controls (n = 3; P < 0.003).

We hypothesized that RhoC plays an important role in cell motility in HNSCC. We therefore investigated the effect of RhoC on cell motility using the scratch model. A noticeable decrease in cell motility was observed in RhoC knockdown clones as compared with the parental or shRNA-scrambled sequence control lines (Fig. 4A and B; n = 3, P < 0.005). These in vitro assays provide evidence for the first time that RhoC plays an important role in cell invasion and motility and suggest that RhoC is important for metastasis in head and neck cancer.

RhoC Plays an Important Role in Lung Metastasis and Microvessel Density Formation

Besides localized tumor growth, lung metastasis is a common and frequent occurrence in patients with head and neck cancers (24). Keeping this aspect in view, we designed an in vivo study in which we could analyze the effect of RhoC inhibition on lung metastasis and primary tumor vascularity. This was achieved by injecting transduced cell lines through the tail veins of SCID mice and analyzing them for lung metastasis. Because both clones gave similar results in our
cell motility and invasion assays, we selected RhoC knockdown clone 2 for our subsequent in vivo studies and all results discussed hereafter are based on this clone. The xenograft mice were sacrificed 2 weeks after implantation and their lungs were analyzed for metastasis using H&E stain. As shown in Fig. 5A and B, in the mice injected with UM-SCC-11A parental or shRNA-scrambled control, a large metastatic focus and inflamed blood vessels were observed in the

**FIGURE 1.** Lentivirus-infected cells showing GFP expression levels. **A.** UM-SCC-11A cell line transfected with shRNA-scrambled sequence control (SR), RhoC knockdown clone 1 (C1), RhoC knockdown clone 2 (C2), and uninfected cells as controls (negative). Histograms obtained by flow cytometry (top), and GFP-labeled cells in fluorescent (middle) and bright lights (bottom). **B.** UM-SCC-1. All other notations are the same as described above. As shown by the GFP expression levels, a high number of cells (80-90%) were successfully infected with recombinant lentivirus.
lung region (marked by arrows). A similar set of results were obtained for the UM-SCC-1 parental and shRNA-scrambled sequence control (Fig. 5D and F). In contrast, mice injected with RhoC knockdown clone have very small metastatic tissue with barely visible patches of inflamed blood vessels in UM-SCC-11A and UM-SCC-1, respectively (Fig. 5C and F).

In addition, the remaining dissected lung tissues were cultured for observation of cell growth by the metastatic tumors. The bar graph shows the number of cancer cells grown in digested lung of mice which includes parental, shRNA-scrambled control and RhoC knockdown clones. Interestingly, there is a 67% and 58% decrease in cell number in RhoC knockdown clones of UM-SCC-11A and UM-SCC-1, respectively, when compared with their parental lines (Fig. 5G and H). These results strongly suggest that inhibition of RhoC expression greatly reduces metastasis in vivo.

Furthermore, to test the angiogenic role of RhoC, parental and RhoC knockdown cells were implanted in the flank region of the SCID mice. Microvessel density of the localized solid primary tumor, which grows into a sizable volume after 12 weeks of implantation in the flank region, was analyzed using CD31 antibody. Microscopic analysis of the CD31-stained tumor revealed a remarkable difference in microvessel formation in mice implanted with RhoC knockdown clones when compared with the corresponding either parental or shRNA-scrambled control. In the control groups, well developed microvessels were observed in the tumors, which was in strong contrast with the poorly developed microvessels in mice implanted with RhoC knockdown clone (Fig. 6). Our results are in coherence with the published work about the essential role of RhoC in angiogenesis (25, 26). A similar pattern of microvessel development was observed in UM-SCC-11A parental, shRNA-scrambled, and RhoC knockdown clone (data not shown). These results suggest that RhoC is required for proper formation of the vascular network in a developing tumor.

**Discussion**

Tumor metastasis is well correlated with the overexpression of certain oncogenes. The overexpression of the Rho gene family has been reported in many malignant forms of cancer (27), including pancreatic cancer (18), gastric cancer (17, 19), and human melanoma (11, 20). However, there have been very few studies on whether overexpression of RhoC is involved in head and neck tumor metastasis. Previous studies in our laboratories have shown that RhoC is actively expressed in several well established UM-SCC cell lines. Among the cell lines tested, the UM-SCC-11A and UM-SCC-1 lines exhibited considerably high levels of RhoC-protein (23). In particular, the active form of RhoC (RhoC GTPase) was observed to be constitutively expressed in the UM-SCC lines. Therefore, for our current study, we selected

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**FIGURE 2.** qRT-PCR of cell lines UM-SCC-1 and UM-SCC-11A showing the relative mRNA expression levels of RhoC (A), Cdc42 (B), Rac1 (C), and Rac2 (D) in parental (control), shRNA-scrambled sequence control, and RhoC knockdown clones 1 and 2 after selection and establishment of positive clones. Results were analyzed using 2−ΔΔCT methods. A significant decrease in mRNA levels of RhoC knockdown clones were obtained whereas the expression of Cdc42, Rac1, and Rac2 remained unchanged (P < 0.05).
FIGURE 3. Cell invasion assay of UM-SCC-11A and UM-SCC-1 lines transfected with RhoC shRNA. A and E, Parental cell lines; B and F, shRNA-scrambled controls; C and G, RhoC knockdown clone 1; D and H, RhoC knockdown clone 2 of UM-SCC-11A and UM-SCC-1, respectively (magnification, ×40 and ×100). I and J, Columns, rates of invasion; bars, 95% CI (P < 0.05).
two UM-SCC lines (UM-SCC-11A and UM-SCC-1) to evaluate the role of RhoC in HNSCC metastasis. Our first and foremost aim was to inhibit RhoC expression in the two selected cell lines and analyze its function in vitro. Our expectation was that the motility and invasion would be greatly reduced in RhoC-depleted cell lines as compared with parental lines. In this study, we have shown a successful inhibition of RhoC gene expression and, subsequently, its function using shRNA techniques (Fig. 2). Furthermore, our data show that cell invasiveness and motility which are characteristics of aggressive head and neck cancer cell lines were diminished when RhoC expression was inhibited (Figs. 3 and 4). Therefore, these results suggest that RhoC overexpression drives cell invasion and motility in HNSCC. It is reported that one of the major functions of the Rho family of proteins is to control cytoskeletal organization (28). Cytoskeletal proteins are involved predominantly in cell motility. Therefore, RhoC may control metastasis by modulating cell motility (29).
facilitate the movement of cells, they need to turn over both cell-extracellular matrices and cell to cell adhesions, which includes both adherence junctions and tight junctions (30, 31). It has also been reported that RhoC plays a predominant role over RhoA in the weakening of adherence junctions, which is an important step towards transforming cells into an invasive phenotype (6). These studies therefore, raise the question as to what effect RhoC inhibition would create in vivo. Our in vivo results showed that both inflamed blood vessels of lungs and a large volume of lung metastases were present in animals which were administered by tail vein injection of either parental or shRNA-scrambled sequence (control) cell lines. In contrast, the lungs of mice implanted or injected with RhoC knockdown lines were free from any pathologic findings, specifically very minimal lung metastases and very low level of inflammation in lung tissues and blood vessels (Fig. 5). Furthermore, the level of angiogenesis in the localized tumors was assessed using CD31 antibody and these results showed a remarkable difference both in quality as well quantity of the microvessels in the tumors. The mice implanted with RhoC knockdown lines showed markedly fewer and less poorly developed microvessels as compared with the far greater in number and clearly defined vessels in parental or shRNA-control cell lines (Fig. 6).

The implications of the findings in this study provide a fertile area of research in HNSCC. For instance, recent work has shown that matrix metalloproteinases (MMP), which are well-known mediators of invasive tumor behavior, have been identified as a specific and critical player for the formation of lung metastasis (32, 33). Li et al. reported that the oncogene, AF1Q, which is responsible for primary breast tumor growth and pulmonary metastasis are at least, in part, regulates other MMPs and RhoC expression (34). The remodeling of the actin cytoskeleton is a critical and important step in the formation of pulmonary metastasis due to changes in cell shape, polarity, cell interactions, and eventual migration of the cancer cells. Interestingly, studies by Nelson et al. (35) have shown that expression of MMP3 gene which induces epithelial-mesenchymal transition in mammary epithelial cells is brought about by change in cell shape through Rac1 (also a member of the Rho family) mediated changes in cytoskeletal structure. Clearly, future studies elucidating the specific interactions between MMP2, MMP3, and MMP9 (major MMP proteins in HNSCC) and RhoC are indicated, and may prove to be one of the signaling pathways for RhoC-mediated function.

In conclusion, the findings presented in this study illustrate that in both in vivo and in vitro conditions, RhoC plays an important role in head and neck cancer progression and metastasis. With additional investigations and ongoing development of RhoC specific inhibitors, this may prove to be an important therapeutic target in this patient population.

Materials and Methods

Cell Culture and Generation of Stable RhoC Knockdown Clones

UM-SCC-11A and UM-SCC-1 are well established cell lines derived, respectively, from a 65-y-old patient with a T2N2a of the epiglottis and from a 46-y-old patient with T2N0 of the false vocal cord (36, 37). These cell lines were grown at 37°C in a humidified atmosphere with 95% air-5% CO2. The cultures were maintained in DMEM (Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone) and supplemented with 50 μg/mL of penicillin G and 50 μg/mL of streptomycin sulfate.

RhoC knockdown and scrambled sequence constructs with green fluorescence protein (GFP) tag and puromycin resistance sites were synthesized by the vector core facility of...
the University of Michigan. The sequences used for RhoC constructs are available in Open Biosystems and include oligo ID V2LHS_69446 and V2LHS_69410, accession number NM_001042678. The sequences of the constructs are 69446 = 5'-ATACTGTCTTTGAGAACTATAT (sense; for RhoC knockdown clone 1) and 69410 = 5'-CACCAGCACTTTATACACTTC (sense; for RhoC knockdown clone 2). The sequence of shRNA miR nonsilencing (scrambled) control is ATCTCGCTTGGGCGAGAGTAAGTGCTGGACAGTAAGCTGGCAGAGTAAGTAGTGAAAGGCGCAAGATGCTACTTCGCCCAGCAGAGTGCCCTACTGCCTCGGA. This control sequence does not match any known mammalian genes (the sequence had at least three or more mismatches against any gene which was determined via nucleotide alignment/BLAST of target 22mer sequence). This is the nonsilencing shRNAAmir hairpin sequence found in the pSM2, pSMF, pGIPZ, pTRIPZ, and pLemiR nonsilencing controls.

293FT cells (Invitrogen) were infected with 250 mmol/L of CaCl₂ solution containing RhoC shRNA construct, 25 μmol/L chloroquine and viral particles (i.e., Gag, Pol, and Env) and grown overnight. The medium was changed after 12 h to remove chloroquine and fresh DMEM-10% FBS was added to the growing 293FT cells to produce the virus. The supernatants from the infected cells were collected and 1 mL of this solution was added to growing UM-SCC-11A and UM-SCC-1 lines. Cells were incubated at 37°C and the GFP expression was monitored after 48 h of infection. Positive (stable) clones were selected using puromycin antibiotic (1.6 and 2.0 μg/mL for UM-SCC-11A and UM-SCC-1, respectively). These were then analyzed using fluorescence microscopy which showed a strong green fluorescence in the majority of the cells, signifying a high efficiency of infection (Fig. 1A and B). Furthermore, flow cytometry analyses showed that the number of non-infected cells were significantly low (Fig. 1A and B).

**Flow Cytometry Analyses**

Approximately 70% to 80% confluent lentivirus-infected cells were harvested using trypsin-EDTA solution and resuspended in phosphate buffer saline containing 3% FBS, 0.5 mm EDTA, and 60 units/mL of DNase. Flow cytometry analysis was done using a BD FACS Aria III flow cytometer equipped with a 488 nm, 15 mW, air-cooled argon laser (Analytical Cytometry Laboratory, Ohio State University Comprehensive Cancer Centre). GFP-positive cells were sorted out and grown for subsequent experiments.

**qRT-PCRs**

Total RNA was isolated according to the standard procedure using TRIZol reagent (Invitrogen). qRT-PCR were conducted with a TaqMan probe system from Applied Biosystems by using the following products: cdc42, Hs03044122_g1; Rac1, Hs01025984_m1; Rac2, Hs01032884_m1; and RhoC, Hs00733980_m1. β-Actin and G3PDH were used as the data normalizers. Relative changes in gene expression were calculated using the 2^(-ΔΔCT) method (38).

**Cell Invasion and Motility Assay**

**Invasion Assay.** Cell invasion assays were done using BD BioCoat Matrigel Invasion Chamber which was obtained from BD Biosciences. The procedure was followed according to the instructions of the manufacturer. Briefly, ∼2.5 × 10⁵ cells in 2 mL of serum-free DMEM were added at the top of the insert and 1 mL of the medium was added in the bottom well of each insert. FBS albumin was added to the medium in the lower chamber (final concentration of FBS was 10%, v/v), which acted as a chemoattractant. Cells were incubated for 22 h in a humidified cell culture incubator at 37°C, 5% CO₂ atmosphere. Next, the noninvading cells at the top of the insert were scraped out with the help of cotton-tipped swab. The invading cells which were attached to the underside of the membrane were fixed in 100% methanol and stained with 1% Toluidine prepared in 100% methanol. After repeated washing of the membrane using distilled water, stained cells were allowed to air-dry at room temperature before it was visualized under a
microscope. A parallel experiment with control inserts (without Matrigel) was also run. Matrigel-invaded cells were counted microscopically at 40x and 100x magnifications.

**Motility Assay.** Cell motility assays were done in 100 mm Petri dishes. At ~80% confluence, cells were washed with PBS and a fine scratch in the form of a groove was made with the help of a sterile pipette tip and immediately photographed. We designated this time as the 0 h. Next, cells were supplemented with DMEM containing 10% FBS and allowed to grow. A migration of cells from the edge of the groove towards the center was monitored microscopically at 40x magnifications after 24 h to assess the extent of scratched area covered. The width of the scratch was measured at 0 h and after 24 h to calculate the percentage of the gape covered by the cells in a 24-h time period.

**Animal Xenograft**

Athymic SCID mice were obtained from the Jackson Laboratory. 6-wk-old mice were housed in cages of five animals each. Five animals per treatment were selected to receive parental, shRNA-scrambled sequence control and RhoC knockdown cells. At the end of the second week, the animals were euthanized under isoflurane anesthesia and mice were sacrificed by cervical dislocation. DMEM and injected thorough the tail vein and/or in the flank region of mice using a 0.5-inch, 27-gauge needle. Animals were sacrificed by cervical dislocation. RhoC GTPase expression modulates induction of angiogenic factors in breast cells. Neoplasia 2006;8:533.

**References**


**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Statistical Analysis**

Statistical analyses were done using SigmaGraphPad prism 4 software. The mean ± SD was reported. Differences were considered to be statistically significant at P < 0.05.
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