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ROCK1 and *ROCK2* siRNAs Decrease Proliferation and Increase Apoptosis in Human Head and Neck Squamous Cell Carcinoma

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Abstract
Background: *ROCK* genes play an essential role in cancer development. The aim of the present study was to investigate a novel gene therapy approach by *ROCK1* and *ROCK2* genes silencing for head and neck squamous cell carcinoma (HNSCC) treatment.
Method: In this in-vitro study, two cell lines including HN5 for HNSCC and a normal cell line were treated with *ROCK1* and *ROCK2* siRNAs together or alone with or without cisplatin. MTS, the real time polymesrae chain reaction, 4′,6-diamidino-2-phenylindole (DAPI) staining, Enzyme-linked Immunosorbent Assay (ELISA) and flow cytometry tests were carried out.
Results: The highest level of apoptosis (88%) was seen in HN5 cells treated with *ROCK2* siRNA, while this treatment revealed 1.08% apoptosis in normal cells. Cell viability decreased significantly to 0.31 after *ROCK2* siRNA with cisplatin treatment and to 0.33 after *ROCK1 / ROCK2* siRNA with cisplatin treatment in HN5. *ROCK1 / ROCK2* genes silencing (especially *ROCK2*) increased apoptosis and decrease proliferation in HNSCC. The combination of *ROCK2* siRNA and cisplatin demonstrated the greatest effect on apoptosis of cancerous cells. However, adding cisplatin to *ROCK2* siRNA did not significantly increase apoptosis.
Conclusion: Based on our in-vitro data and due to the issue of cost and benefit, *ROCK2* siRNA alone represents a potent and selective agent for the induction of apoptosis and inhibition of proliferation in HNSCC. Our findings suggest that *ROCK1 / ROCK2* genes silencing (especially

ROCK2) warrants further investigation as potential treatment strategy for HNSCC. Further studies are essential to support the translational potential of *ROCK*-targeted siRNA therapy.

Keywords: Apoptosis, Gene silencing, Head and neck squamous cell carcinoma, Proliferation, *ROCK*

Introduction

Head and neck squamous cell carcinoma (HNSCC), one of the critical public health problems, is the 6th most common neoplasm with survival rates which have not raised considerably for several decades.[1, 2] In spite of improvements of radiotherapy, chemotherapy and surgical treatment methods, the life expectancy in patients has not significantly improved. Different biomarkers have been found for diagnosis and targeted therapy; however, no prominent oncogenic deriver has been introduced for HNSCC.[3] Therefore, there is a momentous need to discover novel targets in HNSCC for therapeutic purposes. Abnormal cellular proliferation exists in all cancers including HNSCC. Due to the decisive roles of apoptosis and proliferation in neoplasms, targeted inhibition of proliferation and apoptosis-associated genes is an important therapeutic goal.[4] In this regard, it is important to find out the effective molecular agents influencing the apoptosis and proliferation, as one of the top research priorities in the world.

The hallmark of all cancers, including HNSCC, is the dysregulation of cellular processes e.g., proliferation and apoptosis. The evasion of programmed cell death and uncontrolled cell division are fundamental for tumor development and progression. Therefore, targeted inhibition of genes critically involved in these cellular processes presents a promising therapeutic way. Processes Rho-associated protein kinase (*ROCK*) has two isoforms including *ROCK1* and *ROCK2* and has different functions in cardiovascular system, central nervous system, and various physiologic and pathologic processes. Growing evidence suggest the critical role of *ROCK1* in cardiomyocyte apoptosis, cardiac fibrosis and acute inflammation; while intrauterine growth retardation was shown in *ROCK 2* knockout mice as a result of limited growth of axons after central nervous system damage. *ROCKs* play important roles in modulating different functions such as growth and apoptosis.[4] Moreover, *ROCKs* have significant roles in progression of cancers e.g., breast, renal, lung and hematological malignancies.[5, 6] Small interfering RNA (siRNA), a short double-stranded, noncoding RNA (21-31 nucleotides) was used in this study for knocking down *ROCK1* and *ROCK2* genes in order to inhibit their activity. The potency of siRNA is that they can knock down the special sequences of genes which are responsible for specific function.[7] SiRNAs mediate post-transcriptional gene silencing through the RNA interference (RNAi) pathway. The siRNA guide strand directs the RNA-induced silencing complex to bind complementary target mRNA, leading to its cleavage and degradation, thereby preventing translation and reducing specific protein levels.

Studies have shown that targeting *ROCK* can inhibit proliferation and induce apoptosis in several cancer models. For example, the inhibition of *ROCK2* was found to be more effective than *ROCK1* in suppressing malignancy in Ewing sarcoma,[8] while *ROCK1* inhibition was synthetically lethal in certain renal cell carcinomas.[9] However, the specific roles and therapeutic potential of individually targeting *ROCK1* versus *ROCK2* in HNSCC remain poorly elucidated.

This approach can minimize off-target effects and provide insights into isoform-specific contributions to carcinogenesis.

Given the established role of *ROCK* in oncogenesis and the need for new therapeutic targets in HNSCC, the present study was designed to investigate the effects of *ROCK1* and *ROCK2* gene silencing, both alone and in combination with the conventional chemotherapeutic agent cisplatin. We hypothesized that targeted knockdown of *ROCK1* and/or *ROCK2* would significantly inhibit proliferation and induce apoptosis in HNSCC cells. By employing siRNA-mediated knockdown in both cancerous and normal cell lines, we aimed to evaluate the efficacy and selectivity of this approach, and explore its potential as a novel gene therapy strategy for HNSCC treatment.

Materials and Methods

The research protocol of this in-vitro study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.845).

Cell lines and cultures

In this in-vitro study, HN5 (HNSCC cell line) and HUVEC (normal cell line) were obtained from the Pasteur Institute. HN5 cells were cultured in RPMI (Gibco, cat. no. 11875), 10% FBS and 1% antibiotics. HUVEC cell line was maintained in DMEM (Gibco, cat. no. 61965) media and supplemented with 20% FBS, 10 ng/ml endothelial cell growth factor and 1% antibiotics. Cells were cultured to about full cell confluency and transferred to the flasks with fresh medium.

siRNA treatment

SiDirect (http://design.RNAi. jp/) was used to design *ROCK1* and *ROCK2* siRNAs which were ordered from Invitrogen with these sequences:

ROCK1: 5'-AUAUUUUAGGAAUUUCUUCAG -3' (21nt guide), 5'-GAAGAAAUUCCUAAAAUAUUC -3' (21nt passenger)

ROCK2: 5'-ACAAAAGCUUUAGGAAUUGGG-3' (21nt guide), 5'-CAAUUCCUAAAGCUUUUGUUG -3' (21nt passenger)

Irrelevant siRNA served as nonspecific negative control. Furthermore, Cisplatin was used as positive control. Hiperfect reagent (QIAGEN, Inc., Mississauga, Ontario, Canada) was used to transfect the siRNAs with a concentration of 100 nM. After 48 h of transfection, experiment analyses were applied to all wells.

MTS assay

96-well plates at 1.2 × 104 cells per well were used to plate cancerous HN5 and normal HUVEC cells. CellTiter 96 AQueous One Solution Cell Proliferation Assay G3580 was used in order to evaluate the *ROCK1* and *ROCK2* siRNAs proliferative effects according to manufacturer's instructions. MTS reagent (10 μ) was poured to wells and incubated for 2 h at normal conditions.

The results represented the siRNA treated cells optical density / normal cells optical density × 100. Each experiment was carried out in triplicate.[10]

ELISA assay

6-well plates with a density of 5 × 105 cells for HN5 and HUVEC lines were used and were maintained in ordinary conditions for 24h. *ROCK1* and *ROCK2* siRNAs (100 nM) with/without cisplatin (90 Ml from 50 mg/ml vial) separately and together were used for cells treatment.

Briefly, wells were rinsed with phosphate buffered saline (PBS; pH 7.4) for two times after removing the medium.

Subsequently, ELISA lysis buffer (2 ml) was added to wells, cell lysates was prepared and the ELISA kit was used for all groups according to the manufacturer's instructions. Stop solution was added to all wells and after

10 min the microplates were read by ELISA microplate reader.[11]

Apoptosis analysis

Assessment of cellular apoptotic morphologies

DAPI (4', 6-diamidino-2-phenylindole) solvated in PBS (20pg/ml) was used to stain all treated cells for 20 min at dark conditions. Fluorescence microscope was used for scrutinizing the stained cells. The morphological changes of apoptotic cells were evaluated and photographs were captured with a computer-imaging system (Olympus, Tokyo, Japan).[12]

Flow cytometry

For analysis of apoptosis, the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was used for quantifying apoptotic cells with fluorescence-activated cell sorting (FACS)-flow cytometry. Trypsin- ethylenediaminetetraacetic acid (EDTA) was used for detachment of siRNA treated cancerous and normal cells. The centrifugation was done for 10 min at 900 rpm. The plates were washed in PBS and 1X binding buffer. Similar centrifugation conditions were applied for all plates. The Annexin V-FITC apoptosis detection kit was used according to the manufacturer's instructions. Data analysis was done using CELL Quest Pro software.

Real time polymerase chain reaction (PCR) analysis

We designed precise primers (for *ROCK1*: (5'->3') forward: 5'-GATGCAGTTGGCCAGCAAAG -3', reverse: 5'-TCTTGACTCTGGGAGGTTACCA -3' and for *ROCK2*: (5'->3') forward: 5' ACGACTTGGGAGAAATGGGG-3', reverse: 5'-GCTGCTGTCTATGTCACTGC-3' and for GAPDH: (5'->3') 5'-AAGCTCATTTCCTGGTATGACAACG-3', reverse: 5'-TCTTCCTCTTGTGCTCTTGCTGG-3'), in order to amplify *ROCK1* and *ROCK2* genes. Experiment mixtures (20 µl), containing 1 µl primer (20 pMol for each), 10 µl SYBR Green PCR master mix, 0.8 µl 6-carboxy-X-rhodamine, 1 µl cDNA (1 µg/µl), were exposed to ABI-step I plus. Triplicate reactions were performed for each sample. Thermal cycling steps were as follows: the first cycle was carried out at 95ºC for 5 min. The next forty cycles were passed at 95ºC for 20sec, 60ºC for 35 secs, and 72ºC for 10 secs. For normalizing the cycle values (Ct) we considered GAPDH as the reference gene and the Pfaffle method was applied for data analysis. The ratio of fold change target to fold change reference was used to report expression changes[13] and each experiment included negative controls.

Statistical analysis

Data analysis was carried out using independent t-test one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The statistical package for the social sciences (version 22.0) was used for statistical analysis. The level of significance was set at 0.05 and the obtained data was presented as mean ± standard deviation values.

Results

MTS assay

In comparison with the control group, in all HN5 groups the cell viability reduced significantly to 52 %, 42 %, 45%, 37%, 41 %, 36% and 70% after treatment with *ROCK1*, *ROCK1*/cisplatin, *ROCK1*/*ROCK2*, *ROCK1*/*ROCK2*/cisplatin, *ROCK2*, *ROCK2*/cisplatin, and cisplatin within 24h, respectively. In HUVEC cell line, the cell viability decreased to 83.05%, 79.98%, 83.56%, 86.73%, 96.92%, 81.79% and 87% with similar mentioned treatments as compared with the control group (Figure 1).

Apoptosis assay

According to the results of DAPI staining, some minor morphological changes were

observed in *ROCK1* and *ROCK2* siRNAs treated HUVEC cells and these treated cells were approximately similar to the control group (Figure 2). Chromatin condensation as an early apoptosis morphologic change was detected in the cisplatin treated HUVEC cells (Figure 3). DNA condensation and fragmentation, and the apoptotic morphological changes were observed in all siRNA transfected treated groups of HN5 cells (Figure 2). More than 95 % viable cells for control groups in both normal and cancerous cell lines were seen in flow cytometric analyses (Figures 3 and 4). In siRNAs treated normal cells no significant raise was seen in apoptosis (Figure 3). Significant amplification in apoptosis of treated HN5 groups was seen as mentioned: *ROCK1* (72.6 %); *ROCK1*/cisplatin (90.3%); *ROCK2* (88%); *ROCK2*/cisplatin (90.7 %); *ROCK1*/ROCK2(82.1%); *ROCK1*/*ROCK2*/cisplatin (84.2%), cisplatin (76.1%) (Figure 4, B-G).

ROCK1 and ROCK2 genes expression assay

Real time PCR method was used to asses *ROCK1* and *ROCK2* siRNAs effects on the expression of specific genes in cancerous and normal cell lines. In comparison with the control group (treated with non-specific siRNA), the expression ratios of *ROCK1* gene in HN5 and HUVEC lines decreased: 0.04, 0.08 for *ROCK1*; 0.09, 0.19 for *ROCK1*/cisplatin; 0.13, 0.04 for *ROCK1*/*ROCK2*; 0.06, 0.13 for *ROCK1*/*ROCK2*/cisplatin; 0.31, 0.83 for *ROCK2*/cisplatin and 0.49, 0.88 for *ROCK2* groups, respectively. In comparison with the control group, the expression ratios of *ROCK2* gene in HN5 and HUVEC lines decreased to 0.98, 0.44 for *ROCK1*; 0.16, 0.26 for *ROCK1*/cisplatin; 0.20, 0.01 for *ROCK1*/*ROCK2*; 0.23, 0.18 for *ROCK1*/*ROCK2*/cisplatin; 0.18, 0.08 for *ROCK2*/cisplatin and 0.01, 0.03 for *ROCK2* groups, respectively (Figure 5).

ROCK1 and ROCK2 proteins ELISA assay

The *ROCK1* protein concentrations in HUVEC cell lines treated with *ROCK1*siRNA/cisplatin, *ROCK1*siRNA/ *ROCK2* siRNA, cisplatin, and *ROCK1*siRNA/ *ROCK2*siRNA/cisplatin were significantly lower in comparison with HN5 cells. The concentration of ROCK1 protein in the HN5 groups was significantly decreased in comparison with the control group, mostly in the *ROCK1*siRNA/cisplatin treated group ($P < 0.001$) (Figure 6). The concentration of *ROCK2* protein was reduced significantly in all treated groups in both cell lines ($P < 0.001$), but a significant difference in the *ROCK2* protein concentrations was not observed between HUVEC and HN5 lines. The lowest *ROCK2* protein levels in HN5 and HUVEC lines were observed in *ROCK1* siRNA + *ROCK2* siRNA treated group (Figure 6).

Discussion

Based on the study results, *ROCK1* and *ROCK2* siRNAs had useful effects on amplification of cell apoptosis and diminishing proliferation and final protein production of the genes. Both *ROCK1* and *ROCK2* siRNAs reduced cell viability and induced apoptosis in HN5 cell line, while exhibiting lower cytotoxic effect on normal cells. *ROCK2* knockdown produced the largest cancer-selective effect; and importantly, *ROCK2* siRNA produced minimal apoptosis in normal HUVEC cells. These results indicate that isoform-specific targeting of the *ROCK* pathway — and particularly *ROCK2* — is a promising strategy to reduce HNSCC cell viability.

In the present study, *ROCK2* siRNA/cisplatin in HN5 cells increased apoptosis up to 90.7% and had the lowest apoptosis rate in the normal HUVEC cells. If inhibiting the *ROCK* pathway promotes apoptosis, targeting this pathway could serve as a goal to inhibit the proliferation cancerous cells. *ROCK* gene controls a wide range of cellular activities,

such as cell morphology and apoptosis.[8, 14] The induction of apoptosis, as evidenced by DAPI staining and flow cytometry, is consistent with the known function of *ROCK* in regulating membrane blebbing and the formation of apoptotic bodies, key morphological events in programmed cell death. A study by Pinca et al. found that when Ewing Sarcoma cells were exposed to *ROCK* inhibitors, cell growth, proliferation and differentiation were significantly reduced. The direct association between *ROCK2* expression and cell growth was seen.[8] In a study by Thompson et al., the inhibition of *ROCK1* expression by selective siRNA reduced the ability of proliferation and colonies formation in CC-RCCs (renal cell carcinoma) while reducing *ROCK2* had no effect.[14]

The superior efficacy of *ROCK2* siRNA over *ROCK1* was observed in our HNSCC model that highlights the importance of isoform-specific signaling, which may be cancer-type dependent. While Thompson et al. found *ROCK1* to be critical in clear cell renal cell carcinoma,[14] our data parallels the findings of Pinca et al. in Ewing sarcoma.[13] This divergence can be explained by tissue-specific expression patterns, differential engagement with upstream regulators, or distinct subcellular localizations of the two isoforms. *ROCK2* has been more strongly linked to inflammation and *NF-κB* activation via its interaction with IKKβ,[15, 16] a pathway often hijacked in HNSCC for survival. Therefore, our results suggest that in HNSCC, the *ROCK2-IKK-NF-κB* axis may be a dominant survival pathway, making its silencing particularly lethal.

The high levels of apoptosis following *ROCK2* siRNA treatment (88%) that was observed in this study can be explained by the disruption of key survival signals. The *RhoA/ROCK* pathway is known to promote cell survival through activation of the *NF-κB* pathway and by inhibiting the pro-apoptotic protein Bim.[9, 15-17, 14, 15] By silencing *ROCK2*, we likely disrupted these antiapoptotic signals, tipping the balance towards programmed cell death. This is strongly supported by our flow cytometry and DAPI staining results, which showed massive apoptotic morphology specifically in HN5 cells. The fact that *ROCK2* knockdown was more potent than *ROCK1* in triggering apoptosis suggests a more critical or non-redundant role for *ROCK2* in maintaining survival signaling in HNSCC, a finding that aligns with a study in Ewing sarcoma where *ROCK2* was identified as the primary oncogenic isoform.[8]

The significant reduction in cell viability, as measured by the MTS assay, directly reflects the role of *ROCK* in regulating the cell cycle and cytokinesis. *ROCK* activity is essential for the formation of the actomyosin-based contractile ring during cytoplasmic division. The inhibition of *ROCK* prevents successful cytokinesis, leading to multinucleated cells and eventual cell cycle arrest or death.[14] Our phenotypic observations likely included such multinucleated cells, a direct consequence of disrupting this fundamental *ROCK*-dependent pathway. Our molecular data provide direct evidence for the disruption of the *ROCK* pathway. The significant knockdown of both *ROCK1* and *ROCK2* mRNA and protein levels confirms the on-target effect of our siRNA strategy. The reduction in protein levels, as confirmed by ELISA, is the definitive link between gene silencing and the observed phenotypic changes. Without functional *ROCK* protein, the downstream signaling cascade—including myosin light chain phosphorylation and cofilin inactivation—cannot be sustained, leading to the collapse of cytoskeleton-based functions and the initiation of apoptosis.

In this study, when cisplatin was added, the rate of vital cancer and normal cells was reduced. Decreased vital cells was not

unexpected due to the proven effects of cisplatin. The highest reduction of viability in both cancerous and normal cells was seen in *ROCK2* siRNA / cisplatin and *ROCK1*/ *ROCK2* siRNA / cisplatin treatment groups. Due to the cost-effectiveness and not to use extra materials, *ROCK2* siRNA / cisplatin appears to be a better treatment option. Another advantage of this compound is its significant effect on apoptosis. The combination of *ROCK2* siRNA and cisplatin yielded the highest levels of apoptosis, though the increase over *ROCK2* siRNA alone was not substantial. This can be interpreted through the lens of convergent pathways. Cisplatin primarily induces DNA damage, triggering apoptosis via the *p53* pathway. It is plausible that *ROCK2* knockdown pre-sensitizes the cells by lowering their apoptotic threshold, making the subsequent DNA damage from cisplatin more lethal. However, the fact that *ROCK2* siRNA alone was almost as effective is an important finding. It suggests that targeting the *ROCK2* pathway is a powerful monotherapy that potentially bypasses mechanisms of cisplatin resistance, which are common in HNSCC. The dramatic selectivity for cancer cells over normal cell line further underscores its potential as a safe and worthy strategy, as normal cells may be less dependent on hyperactive *ROCK* signaling for survival. Whatcott et al. have reported, *ROCK1*, which is enhanced in both tumor epithelial and stromal cells, is associated with proliferation and migration. The inhibition of *ROCK* kinase activity has resulted in the inhibition of proliferation and migration of cancerous cells. *ROCK* plays a significant role in the development of pancreatic malignancy.[17] A study by Inaba et al. found that increased *ROCK* expression was the main cause of uncontrollable glioma neoplastic lesions. In previous studies, the researchers noted the role of *ROCK* in the cell proliferation, and their recent studies found that the *ROCK1* inhibition was useful for the treatment of glioma malignancy.[18] A study by Yao et al. Points found out that the *RohA / ROCK* pathway was involved in the inhibition of cell growth and motility through *TEAα*, and by inhibiting *RhoA / ROCK* signaling, growth of colon cancer cells significantly reduced. Increased *ROCK* levels have been reported in liver, skin, ovarian, bladder, stomach, breast, and testicular cancers as well. *ROCK* and *RhoA* expression and activity are associated with poor prognosis and cancer recurrence and epidermal hyper-proliferation.[19, 20] Saito et al. examined the effects of inhibiting *ROCK* activity in cell-to-cell adhesion maintenance and showed that CAR, as a negative regulator, had a substantial effect on the growth and survival of oral SCC cells.[21] Some studies, including Tilson et al., have shown that selective inhibitors of *ROCK*, Y27632, and Fasudil have been used to maintain survival and proliferation in cancerous cells in in-vitro experiments.[22] The final results of the study by Miyazaki et al. showed that PARG1 plays a decisive role in the development of human RCC (renal cell carcinoma), increasing proliferation, and the ability to invade by inhibiting the *RhoA-ROCK* axis.[23] Tilson et al. showed that the inhibition of *ROCK* at non-lethal concentrations inhibited apoptosis in glioblastoma cells.[22] Zand Vakili et al. suggested that GTPases are definitive signaling transmitters in multiple pathways and could be useful as antineoplastic targets. Several studies on the animal models have confirmed that *ROCK1* and *RhoA* were antitumor targets for treating tumors. Other studies have suggested that, at least in specific samples, individual Rho GTPase inhibition might paradoxically lead to proneoplastic effects in primary leukemia / lymphoma and gastric cancer.[24]

In the present study, the antiproliferative and apoptotic outcomes of *ROCK1* and *ROCK2*

siRNAs and their mixtures were quantified by Annexin-V staining and flow cytometry and studied qualitatively by DAPI staining. In the fluorescent microscopic view, the treatment groups were able to have the most apoptotic effect on the cancerous cells and least on the normal cell groups. This is a good result for cancer treatment with the least damage to normal cells. These results also showed that increasing the apoptosis in cancer cells by treatment with *ROCK* siRNAs is one of the main mechanisms for the degradation of cancerous cells. Furthermore, according to the results of experiments conducted with *ROCK1* primer, it was shown that the reduction in expression occurred in both treated cancerous and normal cells compared with the control groups and the reduction was mostly seen in *ROCK1* and *ROCK1*/*ROCK2* siRNA treatment groups in HN5 and HUVEC cells, respectively. As well as *ROCK1* studies, treatment with the *ROCK2* primer have shown that the reduction in expression occurred in both cancerous and normal cells compared with the control group. The groups treated with *ROCK2* and *ROCK1*/*ROCK2* siRNAs in cancerous and normal lines showed the maximum reduction, respectively. Small-molecule inhibitors of *ROCK*, such as Y27632 and Fasudil, have demonstrated significant anti-tumor effects across various cancer types, functionally validating genetic knockdown approaches. For example, in oral squamous cell carcinoma—a major subtype of HNSCC—the *ROCK* inhibitor Y27632 has been shown to suppress cancer cell growth and survival.[21] Similarly, studies in pancreatic cancer[17] and glioma models[18] have reported that pharmacological *ROCK* inhibition leads to reduced proliferation and migration. The concordance between these independent pharmacological studies and our genetic silencing data provides compelling corroborating evidence that the *ROCK* pathway is a robust and druggable therapeutic target for HNSCC. Future work should directly compare the efficacy and specificity of siRNA-based strategies with established *ROCK* inhibitors in HNSCC models to determine the optimal clinical approach.

It is important to note that our study is limited to in-vitro models and do not fully recapitulate the complexity of the in-vivo tumor microenvironment. The promising selectivity and efficacy observed here warrant future validation in animal models. It is recommended to evaluate the effects of other genes affecting apoptosis and proliferation in HNSCC and compare them with *ROCK1* and *ROCK2* siRNAs. Furthermore, it would be useful to further investigate the downstream signaling pathways affected by *ROCK* silencing to fully elucidate the mechanism of action.

Conclusion

The combination of *ROCK2* siRNA and cisplatin demonstrated the highest effect on the apoptosis of cancerous cells compared with other study groups. However, adding cisplatin to *ROCK2* siRNA did not significantly increase apoptosis. Therefore, based on our in-vitro data and due to the issue of cost and benefit, *ROCK2* siRNA alone represents a potent and selective agent for the induction of apoptosis and inhibition of proliferation in HNSCC cells. Our findings suggest that *ROCK1* / *ROCK2* genes silencing (especially *ROCK2*) warrants further investigation as a potential treatment strategy for HNSCC. Further studies are essential to support the translational potential of *ROCK*-targeted siRNA therapy.

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Authors' Contribution

M.V.S., investigation, methodology, writing - original draft; YMG, investigation, methodology, writing - original draft; A.Y.K., supervision, project administration, formal analysis, writing - review; M.K., supervision, conceptualization, project administration, writing – review, editing; All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

None declared.

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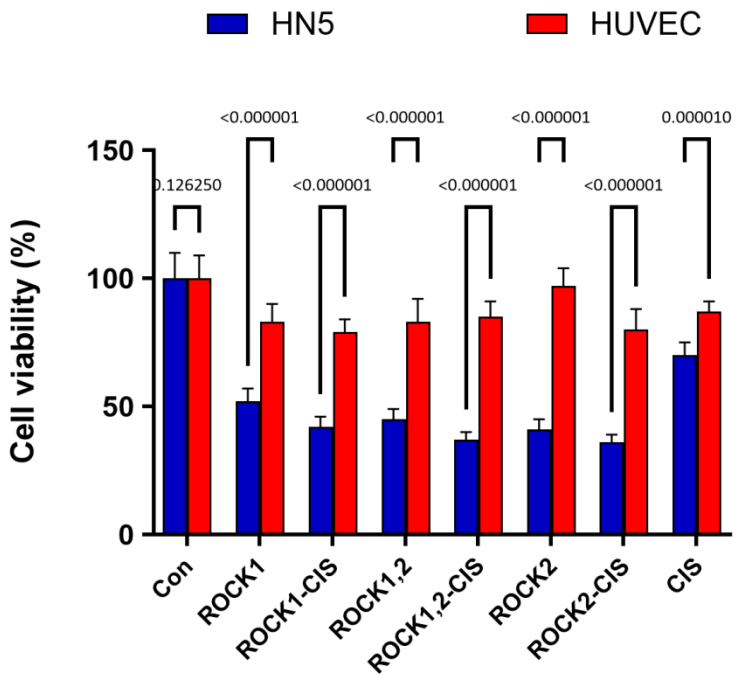

Figure 1. *ROCK1* and *ROCK2* siRNAs and CIS effects on HN5 and HUVEC cell lines viability reported as mean viability ± standard deviation values. Irrelevant siRNA served as nonspecific negative con.

CIS: Cisplatin; con: Control

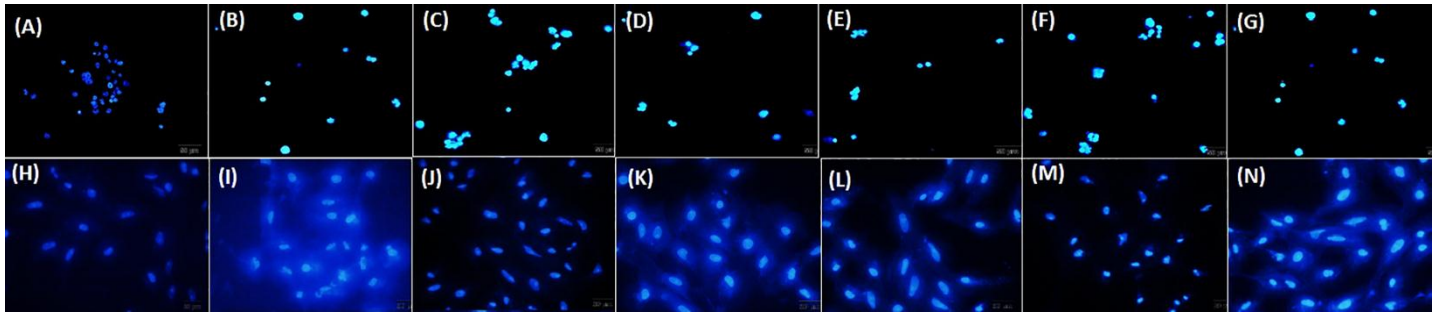

Figure 2. 4′,6-diamidino-2-phenylindole (DAPI) staining of HN5 cells (treated with: A, non-specific siRNA; B, *ROCK1* siRNA+ *ROCK2* siRNA+ cisplatin; C, *ROCK1* siRNA+ cisplatin; D, *ROCK2* siRNA+ cisplatin; E, *ROCK1* siRNA+ *ROCK2* siRNA; F, *ROCK1* siRNA; G, *ROCK2* siRNA) and HUVEC (treated with: H, non-specific siRNA; I, *ROCK1* siRNA+ *ROCK2* siRNA+ cisplatin; J, *ROCK1* siRNA+ cisplatin; K, *ROCK2* siRNA+ cisplatin; L, *ROCK1* siRNA+ *ROCK2* siRNA; M, *ROCK1* siRNA; ROCK2 siRNA).

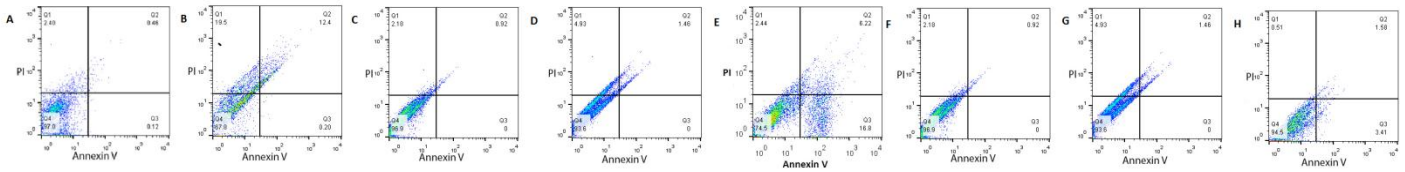

Figure 3. Flow cytometry analysis of apoptosis in HUVEC cells treated with: A, siRNA (non-specific); B, *ROCK1* siRNA+ *ROCK2* siRNA+ cisplatin; C, *ROCK1* siRNA+ cisplatin; D, *ROCK2* siRNA+ cisplatin; E, cisplatin; F, *ROCK1* siRNA; G, *ROCK2* siRNA; H, *ROCK1* siRNA+ *ROCK2* siRNA.

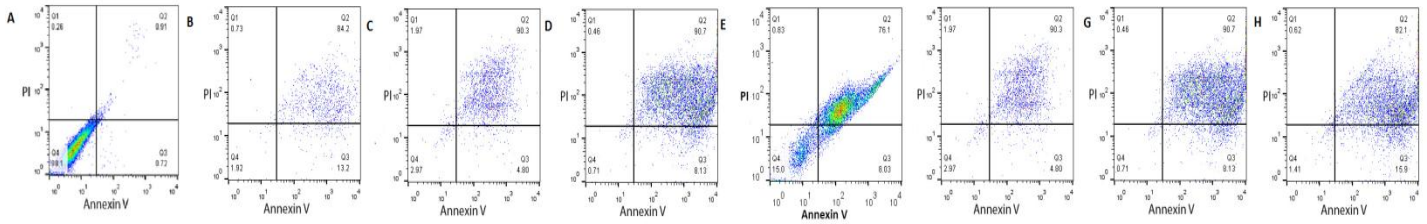

Figure 4. Flow cytometry analysis of apoptosis in HN5 cells treated with: A, siRNA (non-specific); B, *ROCK1* siRNA+ *ROCK2* siRNA+ cisplatin; C, *ROCK1* siRNA+ cisplatin; D, *ROCK2* siRNA+ cisplatin; E, cisplatin; F, *ROCK1* siRNA; G, *ROCK2* siRNA; H, *ROCK1* siRNA+ *ROCK2* siRNA.

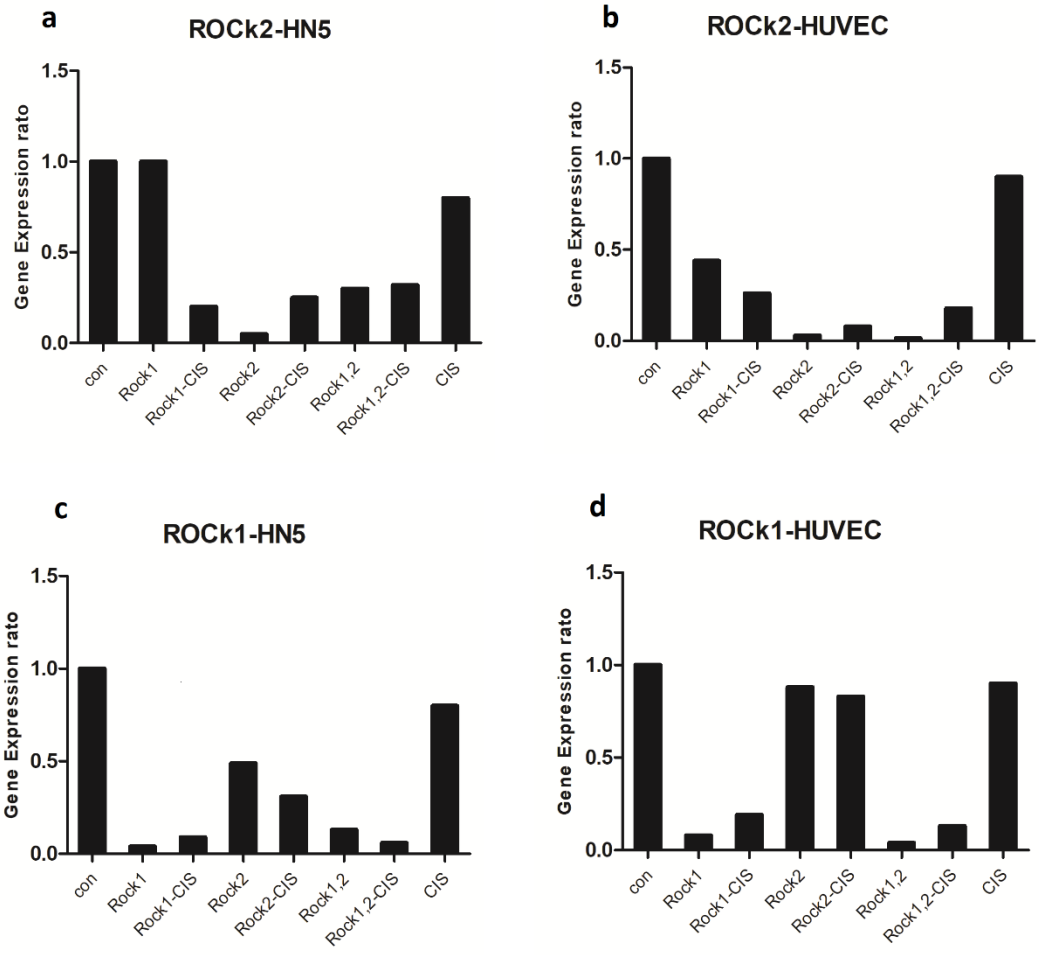

Figure 5. Real time PCR results for *ROCK1* and *ROCK2* genes expressions in HUVEC and HN5 cells treated with *ROCK1*siRNA / *ROCK2* siRNA / CIS / non-specific siRNA (con).
PCR: Polymerase chain reaction; CIS: Cisplatin; con: Control

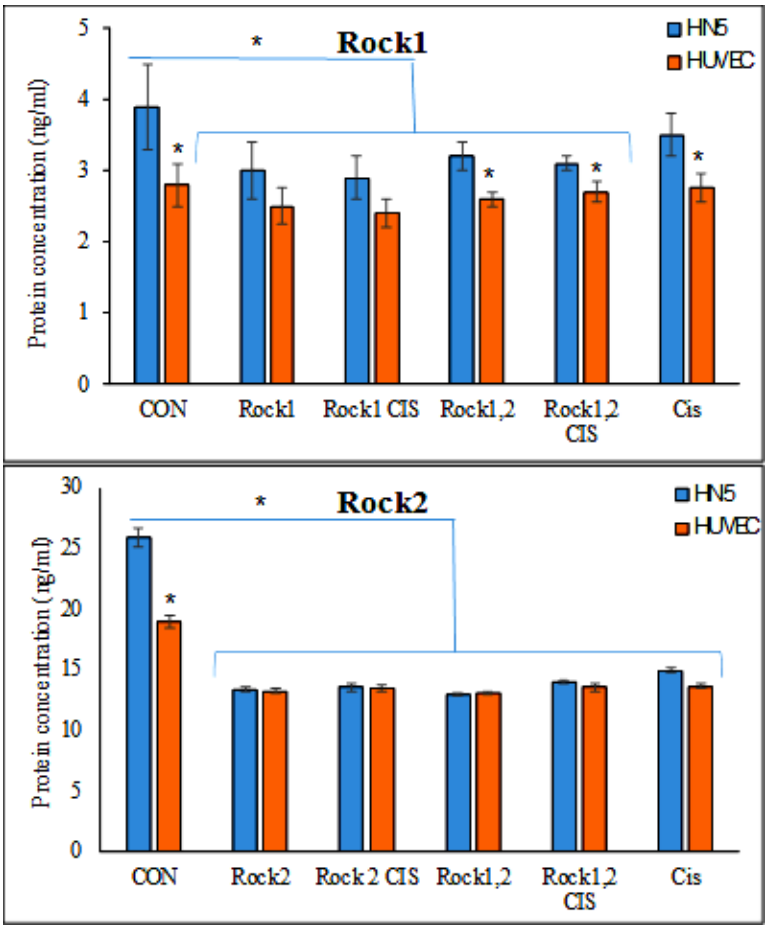

Figure 6. Quantification of protein levels in HN5 (a) and HUVEC (b) cells treated with *ROCK1* siRNA, *ROCK2* siRNA, Cis and non-specific siRNA by ELISA assay. Asterisks signify statistically significant differences. Irrelevant siRNA served as nonspecific negative con.

Cis: Cisplatin; ELISA: Enzyme-linked Immunosorbent Assay; con: Control