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RESEARCH ARTICLE

# Snail-Modulated MicroRNA 493 Forms a Negative Feedback Loop with the Insulin-Like Growth Factor 1 Receptor Pathway and Blocks Tumorigenesis

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**ABSTRACT** In this study, we have identified one microRNA, microRNA 493 (miR-493), which could simultaneously and directly regulate multiple genes downstream of the insulin-like growth factor 1 receptor (IGF1R) pathway, including IGF1R, by binding with complementary sequences in the 3′ untranslated region (UTR) of mRNAs of IGF1R, insulin receptor substrate 1 (IRS1), and mitogen-activated protein kinase 1 (MAPK1), thereby potentiating their inhibitory function at multiple levels in development and progression of cancers. This binding was further confirmed by pulldown of miR with AGO-2 antibody. Further, results from head and neck samples showed that miR-493 levels were significantly downregulated in tumors, with a concomitant increase in the expression of IGF1R and key downstream effectors. Functional studies from miR-493 overexpression cells and nude-mouse models revealed the tumor suppressor functions of miR-493. Regulation studies revealed that Snail binds to the miR-493 promoter and represses it. We found the existence of a dynamic negative feedback loop in the regulation of IGF1R and miR-493 mediated via Snail. Our study showed that nicotine treatment significantly decreases the levels of miR-493—with a concomitant increase in the levels of Snail—an indication of progression of cells toward tumorigenesis, reestablishing the role of tobacco as a major risk factor for head and neck cancers and elucidating the mechanism behind nicotine-mediated tumorigenesis.

**KEYWORDS** IGF1R, miR-493, Snail, feedback loop, nicotine, IGF1R

MicroRNAs (miRs) are a class of endogenously expressed, small noncoding RNA molecules that regulate the expression of the target mRNA. A single guide miR can regulate several mRNA targets, and conversely, multiple miRs can cooperatively regulate a single mRNA (1). Extensive base pairing between miR and mRNA target (10 or 11 bp, including the seed region) results in mRNA cleavage by AGO-2 and less complementarity in miR: mRNA binding leads to translation repression, which is reversible, as the target mRNA can be translated once the repression is eliminated (2). Through posttranscriptional regulation of expression of genes involved in different genetic pathways, including development, cell differentiation, proliferation, apoptosis, and metabolism (3–6), miRs have been demonstrated to play an important role in different cellular processes, all of which are often perturbed in tumors. The complex nature of miR expression and its mode of regulation make the miR classification as a tumor suppressor or oncogene a difficult task (7). The ability of an miR to regulate the expression of multiple mRNAs coupled with its tissue-specific expression pattern and

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mode of regulation of its expression makes a single miR a tumor suppressor and/or oncogenic in a tissue-specific manner (8). Growth factors were proven to have a role in the regulation of miRs (9, 10). A detailed study of the mode of regulation of miRs helps to understand the deregulated pathway in tumorigenesis.

Head and neck cancer is the sixth most common cancer worldwide, with an alarming increase in Asian countries (11). At the molecular level, it could be attributed to the overexpression of growth factor receptors like insulin-like growth factor 1 receptor (IGF1R), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGF-R) and deregulation of cell cycle regulators and tumor suppressors (12). IGF1R is an emerging target in head and neck cancer treatment, because of its reported role in tumor development, progression, and metastasis (13). In addition, the involvement of IGF1R pathway, including all its components—ligands (IGF1 and IGF2), ligand binding proteins, downstream molecules, including insulin receptor substrate 1 (IRS1), AKT, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) (14)—in tumorigenesis makes it a promising target for drug development. More than 10 IGF1/IGF1R targeting agents have entered clinical trials, sharing common effects on IGF1R signaling but with different modes of action, targets, and pharmacological characteristics (15). Most of the antibodies developed against the IGF1R were specific in their mode of action, but these downregulated IGF1R/insulin receptor (IR) hybrid formation (16). As IGF1R has extensive cross talk with other receptor tyrosine kinases, inhibition of IGF1R alone by a specific antibody may be compensated by other pathways (17). For instance, in a model of prostate cancer, following IGF1R inhibition, the IR was able to compensate for and mediate IGF1-induced mitogenic signaling (18). In an independent study on breast cancer, IGF1R small interfering RNA (siRNA) sensitized the cells to insulin-mediated activation of intracellular downstream signaling pathways (19). Recent studies have shown that IGF1R can behave like a functional receptor tyrosine kinase/G-protein related coupled receptor (GPCR) hybrid "borrowing" GPCR signaling components, such as different G proteins, and activating GPCR signaling (20). IGF1 ligand activity and its biological effects through its receptor are majorly dependent on a variety of adaptor proteins/signaling proteins through posttranslational modifications mediated via IGF1R (21). The use of potential drugs that can modify alternative downstream effects of the IGF1R or a combination therapy may help to avoid the complications due to cross signaling (22). The complexity of IGF1R signaling and its association with other signaling pathways made most of the therapies targeting IGF1R a failure *in vivo* (23). Owing to the limitations associated with the existing modalities of targeting agents for IGF1R, miR modulation could be explored as a novel therapeutic strategy against IGF1R in the future with the use of antagomirs (24) or miR mimics (25), depending on the oncogenic or tumor suppressor nature of miR (26). Considering the impact of overexpression of IGF1R in head and neck cancers, it is an ideal therapeutic target (27). Our present study aimed to identify and validate a microRNA which can downregulate IGF1R and its downstream effectors in head and neck cancer model system.

## RESULTS

**miR-493 is a direct target of Snail.** miR-493 has been shown to be downregulated in bladder, colon, gastric, and lung cancer in different independent experiments (28–31). To study the transcriptional regulation, we analyzed the upstream 2-kb region of the miR-493 gene for the presence of binding motifs for various transcription factors using the Consite program considering the fact that miR-493 is an intergenic miR located in chromosome 14 (100869060 to 100869148 [+]) and may have an independent transcriptional regulation (32). This analysis suggested the presence of two perfect consensus motifs for Snail binding within the −786 and −833 regions from the gene start site of miR-493 (Fig. 1a). Because Snail has been regarded as a universal transcriptional repressor, we decided to focus on Snail as a potential upstream modulator of miR-493 expression.

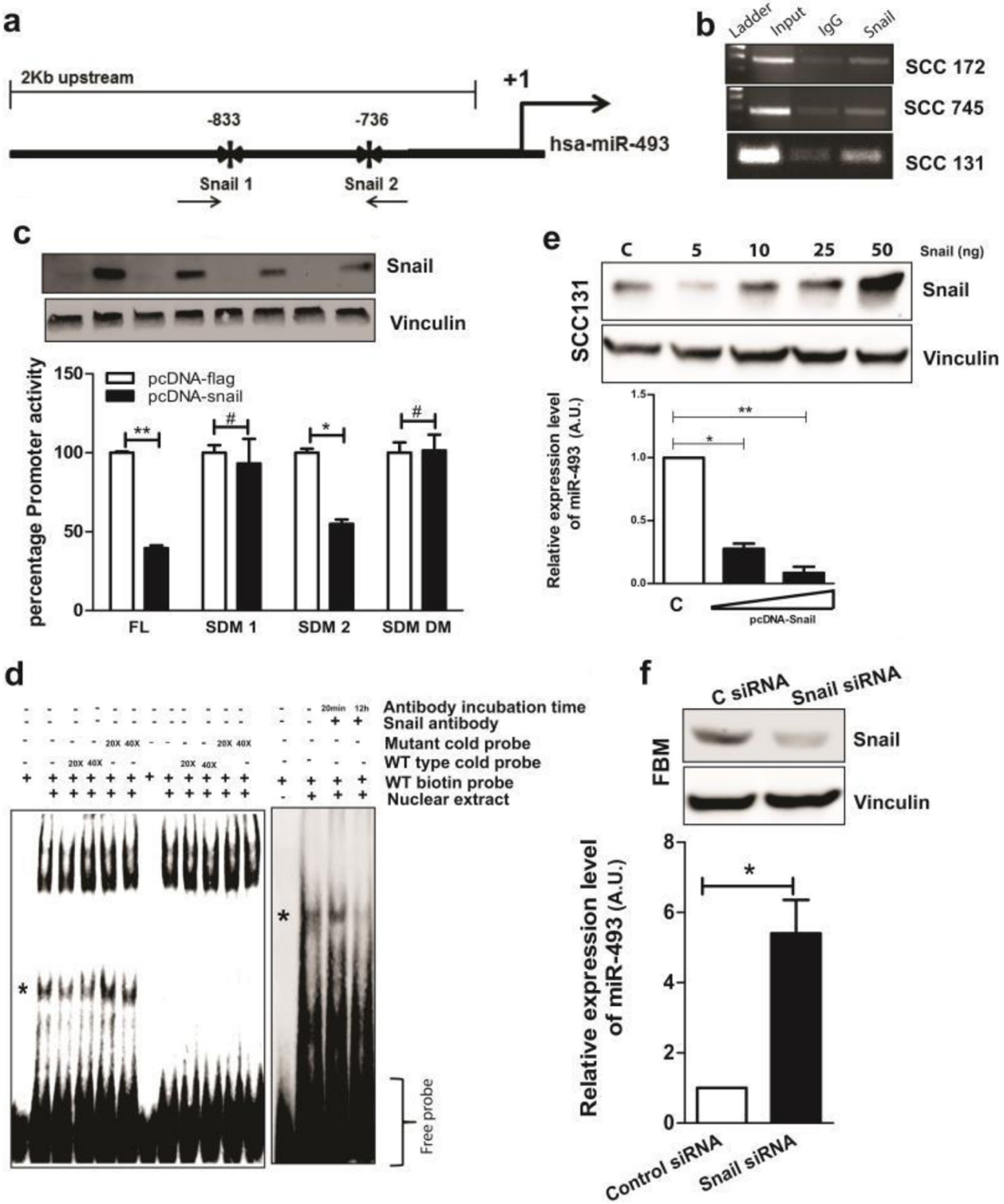


**FIG 1** miR-493 is a direct target of Snail. (a) Schematic representation of the miR-493 promoter with putative Snail binding sites using Consite as CAGGTG. (b) Recruitment of Snail to the miR-493 promoter was analyzed by ChIP. (c) Cells were cotransfected with the pGL3-miR-493 2Kb promoter and mutant miR-493 promoter constructs with Snail sites mutated on one site (as SDM1 and SDM2) or both sites mutated (SDM DM) along with pcDNA-Snail. After 24 h, the cells were lysed, and luciferase activity was measured ($n = 3$) and was normalized with $\beta$-galactosidase activity. Each value represents the mean $\pm$ SEM. Expression of pcDNA-Snail was confirmed by Western blotting of the same lysate. Vinculin was used as the loading control. #, no significant change compared with vector. (d) Electrophoretic mobility shift assay with biotin labeled and without biotin labeled (wild type [WT] as well as mutant) was performed with nuclear protein extract. Snail antibody was used to examine the specificity of the observed band. Asterisks indicate the bands of interest. (e) FBM cells were transfected with different concentrations of pcDNA-Snail, the level of expression of miR-493 was measured by qPCR, and expression of pcDNA-Snail was confirmed by Western blotting. (f) SCC131 was transfected siRNA against Snail and level of miR-493 expression was measured by qPCR. Knockdown of Snail was confirmed by Western blotting. *, $P < 0.05$; **, $P < 0.01$.

**TABLE 1** Characteristics of miR-493 having potential binding sites in IGF1R, IRS1, and MAPK1

| miR | Target | No. of predicted tools | No. of sites | No. of seed sequences | RNAhybrid ($\Delta G$) (kcal/mol) | PITA ($\Delta\Delta G$) |
|---|---|---|---|---|---|---|
| miR-493-3p | IGF1R | 9 | 3 | 13 | −30.9 | −14.37 |
| | | | | 7 | −16.9 | −8.05 |
| | | | | 7 | −21.1 | −10.55 |
| miR-493-3p | IRS1 | 2 | 1 | 6 | −19.4 | −7.96 |
| miR-493-3p | MAPK1 | 4 | 1 | 8 | −23.5 | −7.86 |

To study the effect of Snail on the expression of miR-493, the putative miR-493 promoter was cloned into a promoterless pGL3 basic vector and established the functionality of miR-493 in different head and neck cancer cell lines (see Fig. S1 in the supplemental material). Further, the result obtained from chromatin immunoprecipitation (ChIP) showed that Snail is recruited to the miR-493 promoter region encompassing −786 and −833 (280 bp), which included both Snail binding sites (Fig. 1b). Additionally, to validate the functional significance of the Snail binding regions on the miR-493 promoter, we cotransfected miR-493 promoter—full-length as well as mutant plasmid—for the single Snail binding sites alone or with both sites mutated, with pcDNA-Snail. The results showed that binding site1 is the functional Snail binding site (Fig. 1c). Further, Snail binding to the predicted site 1 was reconfirmed by electrophoretic mobility shift assay (EMSA), where in a shift was observed corresponding to the site1 and the observed complex formation was highly inhibited in the presence of Snail antibody (Fig. 1d). In support of this regulation aspect, we also observed a significant decrease in the level of miR-493 upon Snail overexpression in FBM cell line (Fig. 1e) and vice versa with Snail siRNA in SCC131 cells (Fig. 1f). Together, these observations suggest that Snail directly interacts with the putative miR-493 promoter and negatively regulate miR-493 expression.

**miR-493 is downregulated in head and neck cancer and directly targets the IGF1R pathway.** To identify mRNAs of molecules in IGF1R pathway that might be a target of miR-493, we have screened the public databases, like miR walk (33), Target scan (34), DIANA-microT 3.0 (35) and miRbase (36), for possible complementation of a minimum of 8 bp to the seed region of miRs (1). This search resulted in the identification of important effectors of IGF1R pathway as targets of miR-493 (miR-493-3p). This was supported by the bioinformatics prediction that miR-493 was expected to bind to the 3′ untranslated region (UTR) of IRS1, MAPK1, and IGF1R (validated target in colon cancer [37]) (Table 1; see also Fig. S2 in the supplemental material) and the miR binding sites in the target 3′ UTR was found to be conserved across different species (Fig. S3). Based on this, to evaluate the physiological and functional significance of miR-493 in clinical samples, expression of miR-493 was analyzed in 26 paired samples of human head and neck tumors and normal tissues by quantitative PCR (qPCR). We observed that miR-493 levels were significantly downregulated in significant number of tumor samples compared with normal tissues (Fig. 2a and b), with a concomitant increase in the expression of IGF1R and key downstream molecules such as MAPK1 and IRS1 in most of the tumor samples (Fig. S4). Further, we analyzed for the endogenous levels of miR-493 in head and neck cancer cell lines of different origins and in normal fetal buccal mucosa cell line (FBM, normal immortalized) by qPCR and observed that cancer cell lines have lower levels of miR-493 expression than the FBM cell line (Fig. 2c). This was supported by the finding that overexpression of the miR-493 mimic resulted in a decrease in the target protein level (Fig. 2d; see also Fig. S5) in a concentration- and time-dependent manner. Further, pulldown of miR with AGO-2, a key component of the RISC complex (38), showed enrichment of the selected targets along with miR-493, indicating that the selected mRNAs are physiologically relevant (Fig. 2e). This was supported by the fact that cotransfection of the miR-493 plasmid construct along with the 3′ UTR construct of the selected targets showed a reduction in luciferase activity

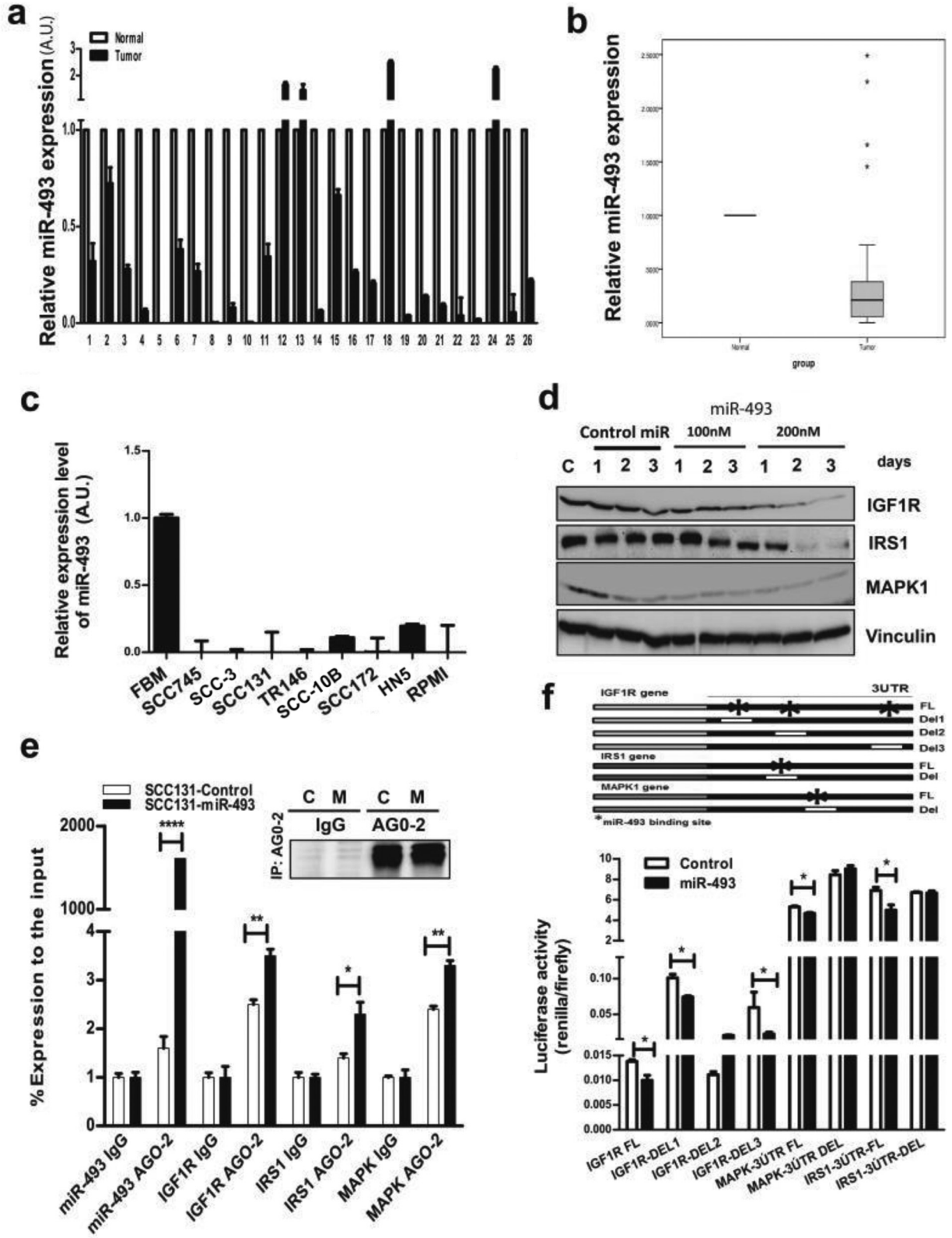


**FIG 2** miR-493 directly targets IGF1R pathway. (a) Expression of miR-493 was estimated in paired clinical samples by qPCR, and the fold change was analyzed. U6 was used as a loading control for RNA. (b) Relative expression of miR-493 in tumor and normal tissues from 26 patients. (c) Relative miR-493 expression levels in different head and neck cancer cell lines. FBM was used as the normal immortalized cell line. (d) miR-493-mimic overexpression reduced the level of expression of IGF1R, IRS1, and MAPK1. Vinculin was used as a loading control. (e) miR-493 was pulled down with AGO-2, showing enrichment of mRNAs of selected targets. Percent expression of the genes to the input was calculated. AGO-2 pulldown was confirmed using Western blotting. (f) Target 3′ UTR-psiCHECK-2 constructs; wild type and deletion

(Continued on next page)

and the reduction was abolished with the deletion of the miR binding site, indicating that miR-493 acts via the 3′ UTR of the target mRNAs (Fig. 2f) and the binding is specific to one of the three miR binding sites for IGF1R (site 2 alone) and the single miR-493 binding site for the other two target mRNAs.

**Effect of nicotine on miR-493 expression.** Usage of tobacco is one of the main risk factors for head and neck cancer (39), as well as bladder, lung, and other cancers. Nicotine is the major component in tobacco smoke, and $\alpha$-7 nicotinic receptor is shown to be present in oral epithelial cells (40). Nicotine is known to activate several key signaling pathways, such as the AKT, extracellular signal-regulated kinase (ERK), and JAK-STAT pathways (41–43), and to affect epithelial-mesenchymal transition (EMT) via the expression of many downstream genes, including the Snail gene (44). The role of $\alpha$-7 nicotinic receptor subunit, the principal mediator of tobacco signaling and nicotine-mediated toxicity in oral epithelial cells, prompted us to hypothesize that nicotine might affect the expression of miR-493 through Snail. To start with, we confirmed the presence of $\alpha$-7 nicotinic receptor subunit in the FBM cell line by semiquantitative reverse transcription-PCR (RT-PCR) (Fig. S6), and further, Snail expression was analyzed post-nicotine treatment at different concentrations and at different time points (Fig. 3a and b). We observed a significant increase in the recruitment of Snail to its consensus region in the miR-493 promoter *in vivo* and *in vitro* (Fig. 3c and d), with a resultant decrease in miR-493 expression (Fig. 3e) and a concomitant increase in the phosphorylation of AKT and glycogen synthase kinase 3$\beta$ (GSK-3$\beta$) (Fig. S7). The decrease in miR-493 expression was abolished with the knockdown of Snail using Snail siRNA (Fig. 3f), showing the role of Snail in mediating the effect of nicotine on miR-493.

**Tumor suppressor properties of miR-493 in cell line models.** miR-493 is reported to be a tumor suppressor in bladder, colon, lung and gastric cancer (28–31). Based on this, to study the functional role of miR-493 in head and neck cancers, we developed miR-493-overexpressing SCC131 cells using a retroviral system. Stable overexpression of miR-493 and subsequent downregulation of targets were confirmed by qPCR and Western blotting, respectively (Fig. 4a and b). Further, miR-493 overexpression significantly reduced the colony forming ability and anchorage-independent growth of SCC131 cells (Fig. 4c and d). The process of metastasis involves the detachment of cells from the primary site and subsequently their attachment at the secondary site, which finally leads to the migration and invasion of the cell. As the IGF1R pathway is known to play a significant role in the process of metastasis (45), we have studied the ability of miR-493-overexpressing SCC131 cells to attach and detach by conventional assays. Results showed that SCC131-miR-493 attached slowly compared to the control group, and the detachment was also observed to be a slow process (Fig. 4e), indicating that miR-493 is an antimetastatic miR capable of reducing invasion and migration and hence metastasis. This was further supported by decreased wound healing capacity and reduced migration and invasion competences of SCC131 stable cells overexpressing miR-493 compared to the control group (Fig. 4f and g).

**miR-493 affects key signaling molecules regulated by IGF1R.** The canonical activation of IGF1R signaling leads to the phosphorylation and activation of several key downstream signaling effectors (46–48). As miR-493 targets IGF1R, we were interested in determining whether miR-493 expression abolished phosphorylation of downstream molecules of IGF1R in our SCC131 stable cells overexpressing miR-493. The results showed that there was a significant reduction in the phosphorylation status of MAPK1, AKT, STAT3, GSK-3$\beta$, and NF-$\kappa$B (Fig. 4h). These data indicate that the inhibitory effects of miR-493 on IGF1R affect cell growth, survival, motility, and invasion via affecting the phosphorylation of its downstream molecules.

**miR-493 expression modulates EMT in head and neck cancer cells.** It is clearly evident from the attachment and detachment, migration, and invasion assays that

**FIG 2** Legend (Continued)
constructs were cotransfected with miR-493 expression plasmid in HEK-293, and luciferase activity was examined by a *Renilla*/firefly luciferase assay system. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

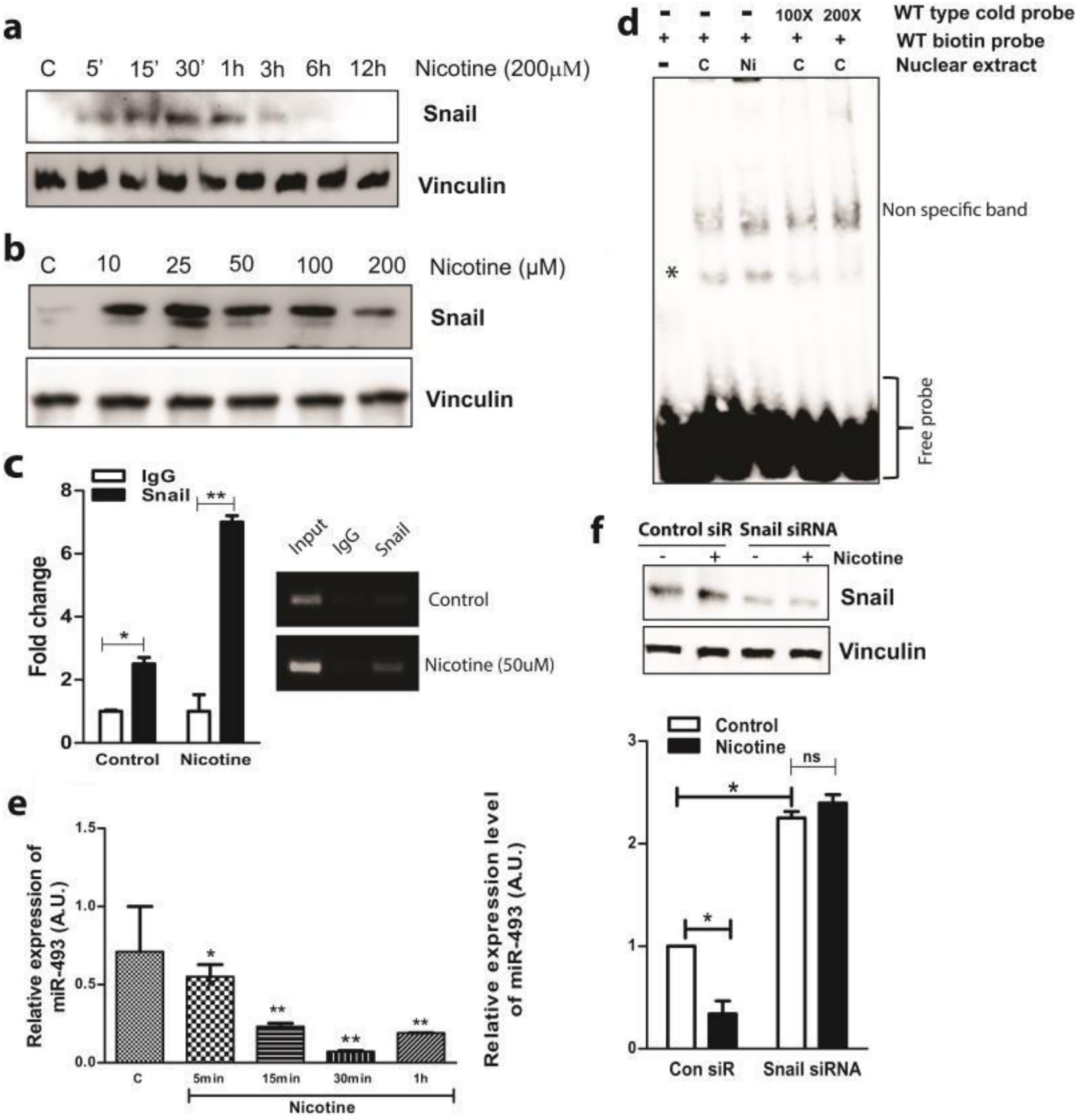


**FIG 3** Effect of nicotine on miR-493 expression. (a and b) Levels of Snail protein were measured with nicotine treatment by varying the time (a) and concentration (b). (c and d) The enhanced recruitment of snail to the miR-493 promoter with nicotine treatment was validated by chromatin immunoprecipitation (c) and electrophoretic mobility shift assay using nuclear protein extract from control (C) and nicotine treated cells (Ni) (d). (e) The effect of nicotine on relative expression of miR-493 was examined by qPCR. (f) miR-493 expression was analyzed with nicotine treatment after knockdown of snail using siRNA. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

high-level miR-493 expression is associated with decreased invasive and metastatic potential of head and neck cancer cells. It was shown that IGF1R promotes EMT and invasiveness of the cells via upregulation of transcription factor Snail (49). Snail affects EMT by reducing the level of the cell adhesion molecule E-cadherin (50). This raised the possibility that miR-493 modulates EMT in head and neck cancer cells via repressing IGF1R and its downstream effector molecules, which, in turn, reduces Snail levels. To

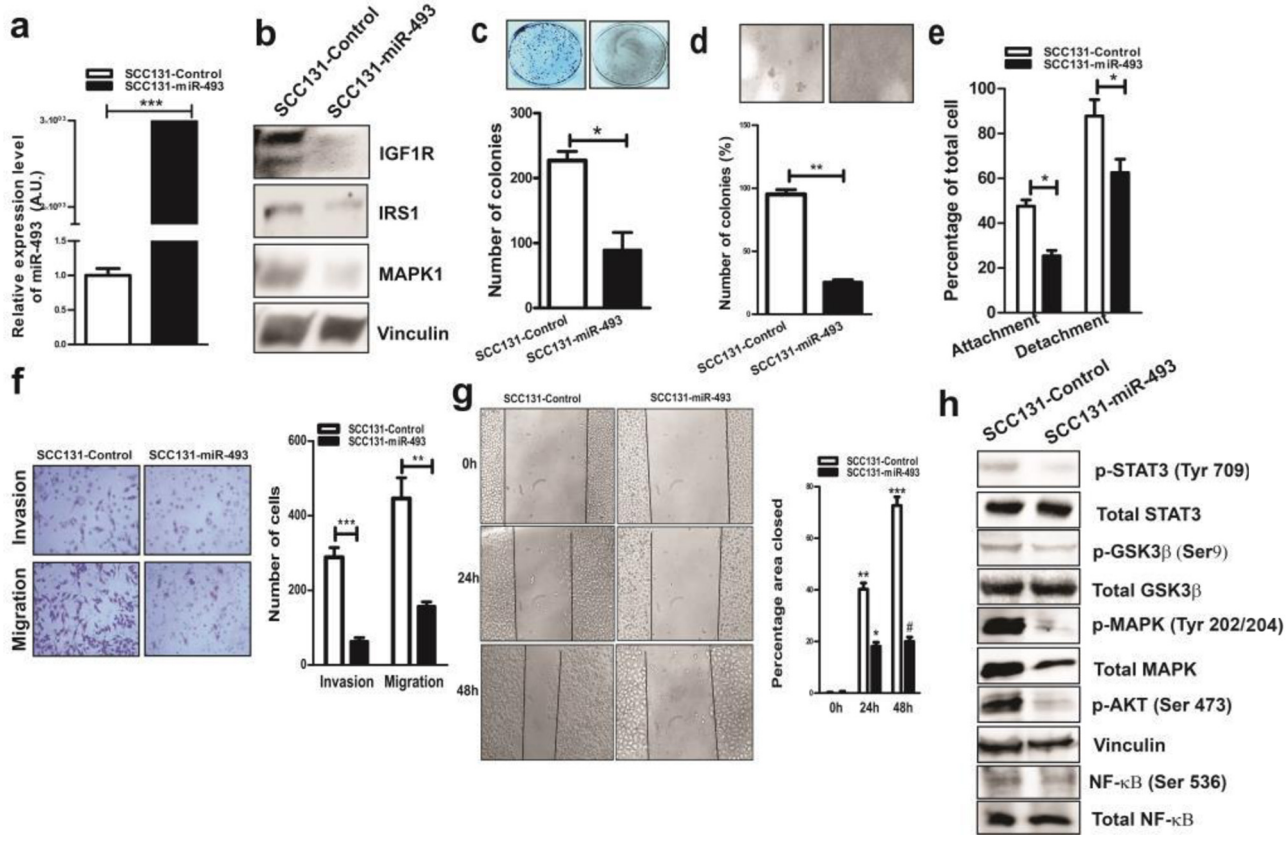


**FIG 4** Tumor suppressor properties of miR-493 in cell line models. (a) The relative expression level of miR-493 was examined in SCC131-Control as well as SCC131-miR-493 by qPCR. (b) Expression of IGF1R, IRS1 and MAPK1 was determined by Western blotting. (c to h) miR-493 overexpression reduced colony formation (c), anchorage independence (d), ability to attach and detach (e), invasion and migration (f), wound healing (g), and major cell signaling mediated through IGF1R (h). All the experiments were performed in triplicates, and representative images are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

test our hypothesis, we checked for the expression of EMT markers in miR-493 stable overexpression clones of SCC131 cells by Western blotting. Convincingly, we noticed that E-cadherin expression was upregulated in miR-493 stable overexpression clones, with a corresponding decrease in the expression of mesenchymal markers vimentin, fibronectin, Snail, and slug (Fig. 5a; see also Fig. S8). We further analyzed for the mRNA levels of two typical indicators of EMT, E-cadherin and vimentin, in miR-493 stable overexpression clones of SCC131 cells by qPCR. The results showed that E-cadherin mRNA was significantly upregulated in miR-493 overexpression clones. Similarly, there is a significant decrease in the levels of vimentin mRNA with miR-493 overexpression (Fig. 5b). Consistently, immunofluorescence studies showed an inverse correlation between E-cadherin and vimentin levels in miR-493 overexpression clones (Fig. 5c). Taken together, these results suggested that miR-493 plays a significant role in modulating EMT in head and neck cancer cells by regulating the expression of EMT markers.

**miR-493 expression inhibits tumor growth in nude-mouse models.** Consistent with the above-described *in vitro* results, nude mice injected with miR-493 overexpression clones showed a significant reduction in tumor burden compared to the SCC131 control (SCC131-Control) cells (*P* value, 0.006) The growth kinetics recorded at three different time points postinoculation and the tumor volumes (in cubic millimeters) measured are shown in Fig. 6a to c and Table 2. Additionally, we also measured the tumor weights at the end of the study period, and the mean tumor weight (standard error of the mean [SEM]) for the miR-493 was determined to be 0.258 g (0.049 g), compared to 0.898 g (0.38 g) for the mice xenografted with SCC131-Control cells. The *in vivo* study clearly demonstrated that miR-493 expression suppressed tumor forma-

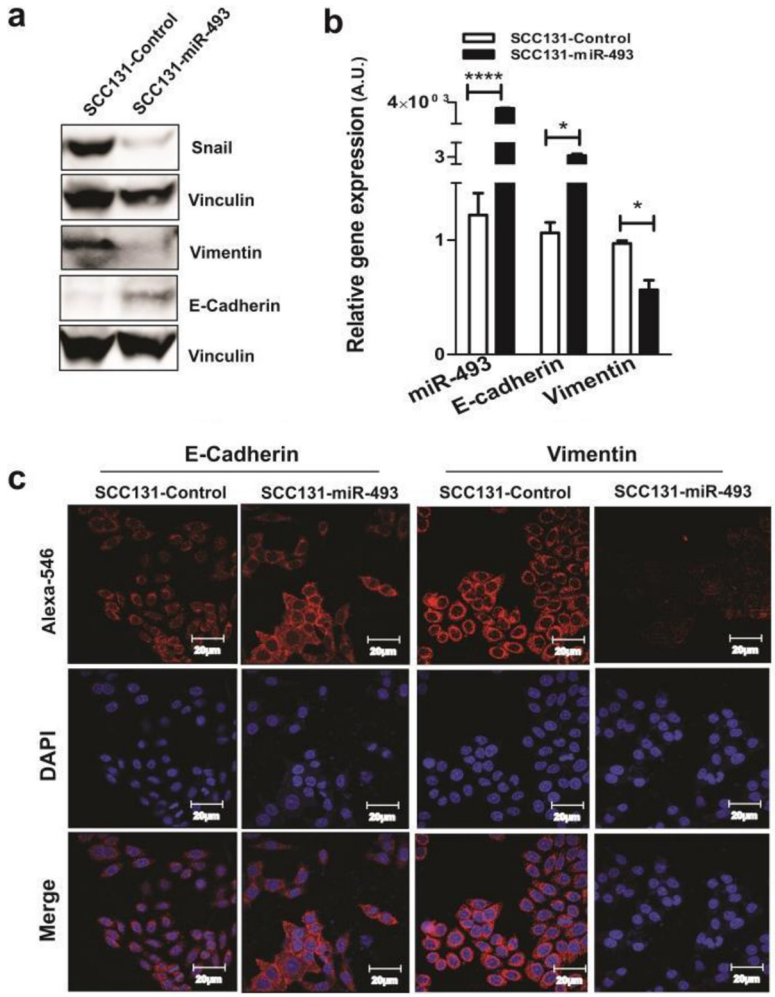


**FIG 5** miR-493 expression modulates epithelial-mesenchymal transition (EMT) in head and neck cancer cells. (a) Western blot analysis of major EMT markers was performed. (b and c) The expression level of E-cadherin and vimentin was checked by qPCR (b) and immunofluorescence (c). *, $P < 0.05$; ****, $P < 0.0001$.

tion of SCC131. Further, Western blot analysis for miR-493 targets as well as Snail in excised tumors revealed that IGF1R, IRS1, MAPK1, and Snail were low in the miR-493 overexpression group, with a concomitant increase in the levels of miR-493 (Fig. 6d and e). These results revealed the *in vivo* tumor suppressor activity of miR-493.

**Regulation of Snail by miR-493.** IGF1 and IGF1R are known to increase the levels of Snail. Likewise, it was shown that Snail expression was significantly reduced upon silencing of IGF1R (51). Based on both of these independent studies, we hypothesized that there might be a feedback loop that regulates the expression of these two molecules via miR-493. While testing this, we found that Snail levels were significantly reduced in our miR-493 stable overexpression clones of SCC131 cells (Fig. 7a), with a concomitant increase in the levels of miR-493 (Fig. 4a). This was supported by the finding that there was significant reduction in the levels of Snail recruitment to the miR-493 promoter in miR-493-overexpressing SCC131 cells, as analyzed by ChIP and EMSA (Fig. 7b and c), and the reduction in miR-493 resulted in the increased level of Snail (Fig. 7d). All the above-described observations and results showed the existence of a negative feedback loop of IGF1R/Snail/miR-493 which is required for the maintenance of epithelial characteristics of cells, and disruption of this feedback loop may be one of the factors leading to tumorigenesis (Fig. 7e).

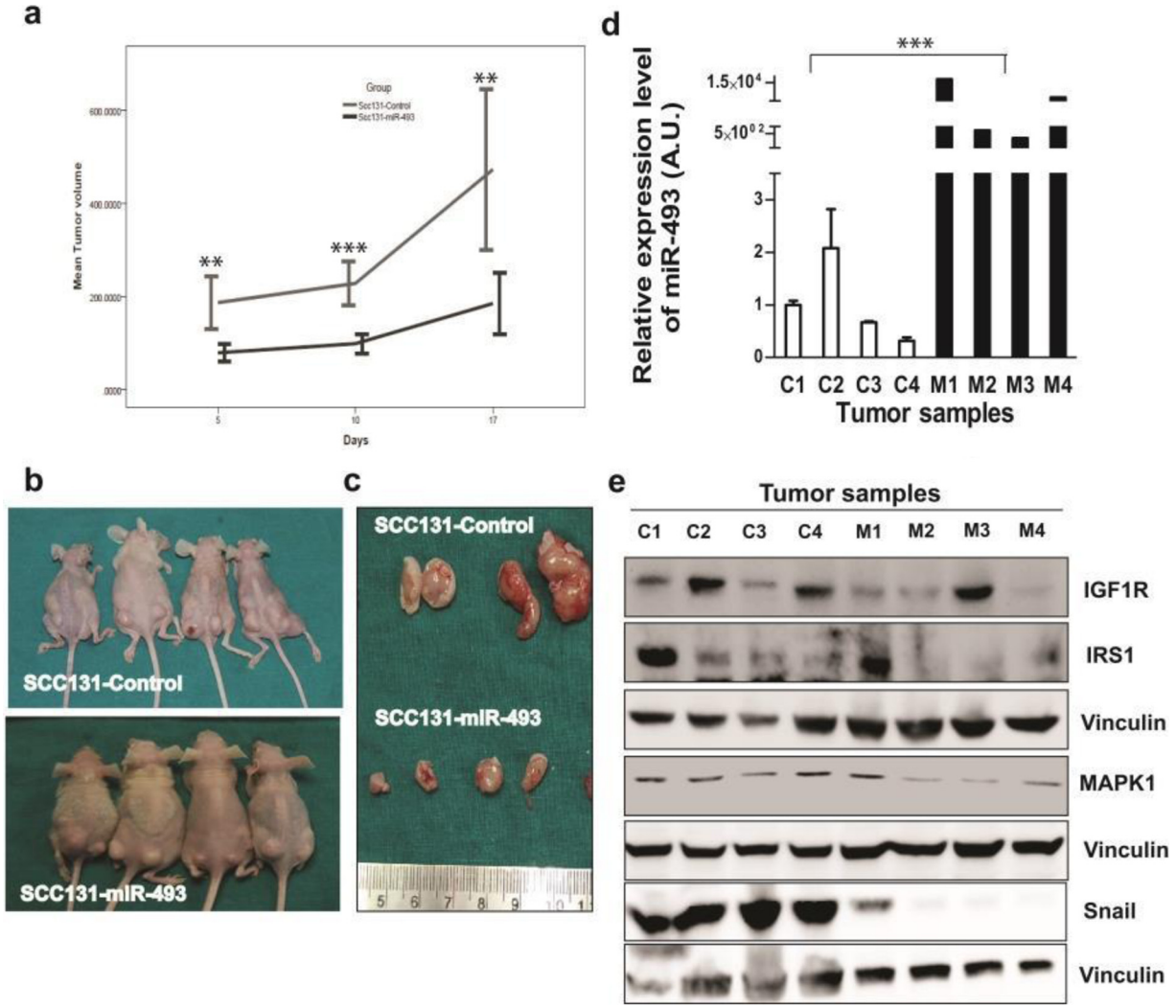


**FIG 6** miR-493 expression inhibits tumor growth in nude-mouse models. (a to c) miR-493 overexpression reduced the tumor growth in nude mice at different time points (a), with reduced tumor burden in SCC131-miR-493 cells (b and c). (d and e) Relative expression of miR-493 in the excised tumor was analyzed (d) along with target expression as well as Snail expression by Western blotting (e). Samples were run separately for various proteins and are represented with corresponding vinculin. **, $P < 0.01$; ***, $P < 0.001$.

## DISCUSSION

The maintenance of balance between cellular proliferation and differentiation plays a vital role in many physiological processes, organ formation, and development (52). Any conditions which disturb the equilibrium lead to impaired wound healing, tumorigenesis, and many other pathologies. The IGF1 signaling pathway mediates signals for DNA synthesis, cell proliferation, differentiation, and survival (53). It has also been shown to significantly contribute to tumorigenesis both *per se* and by allowing tumor

**TABLE 2** Growth kinetics of tumor formation in nude mice with SCC131 xenografts

| Group | Day | No. of tumors | Tumor vol ($mm^3$) | | *P* value |
|---|---|---|---|---|---|
| | | | Median | Mean (SEM) | |
| SCC131-Control | 5 | 8 | 155.10 | 186.86 (28.04) | 0.001 |
| SCC131-miR-493 | | 8 | 80.615 | 78.94 (9.07) | |
| SCC131-Control | 10 | 8 | 218.81 | 228.16 (23.83) | 0.0005 |
| SCC131-miR-493 | | 8 | 98.64 | 98.62 (10.4) | |
| SCC131-Control | 17 | 7 | 459.21 | 473.07 (86.53) | 0.006 |
| SCC131-miR-493 | | 8 | 172.59 | 184.93 (32.96) | |

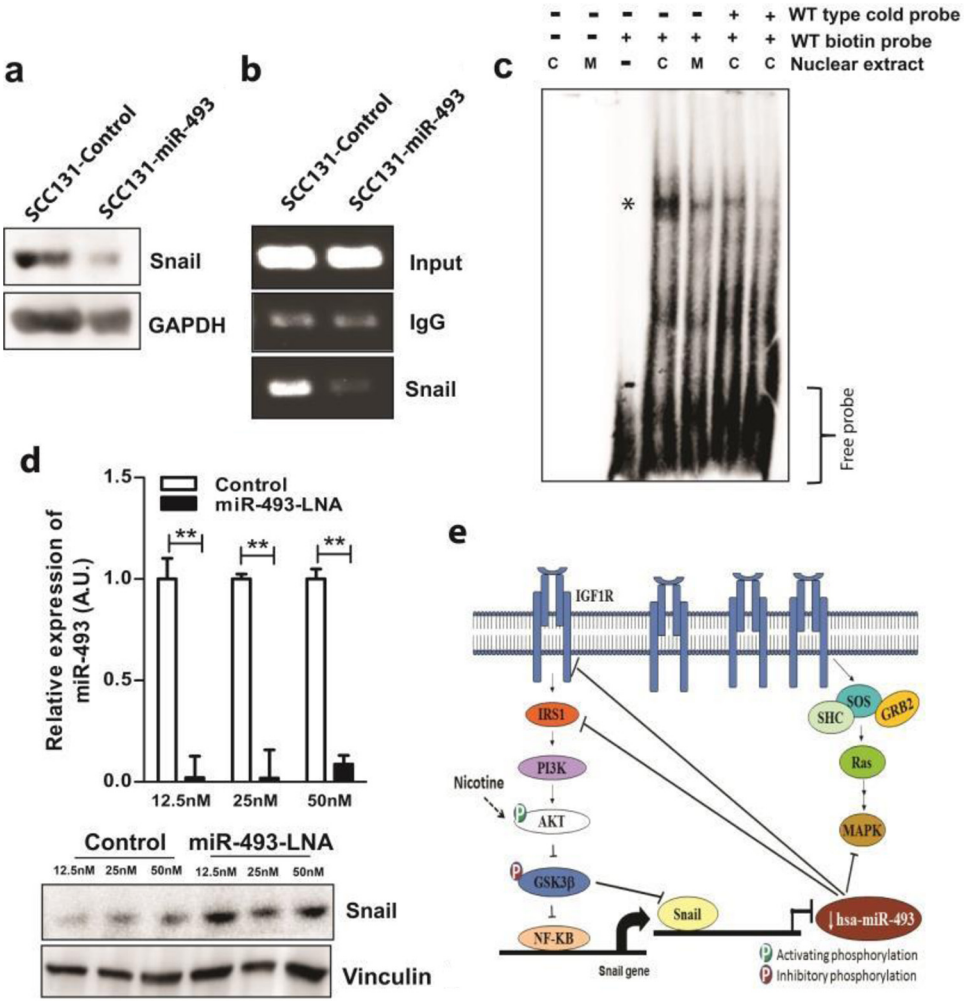


**FIG 7** Regulation of Snail by miR-493. (a) The level of expression of Snail was analyzed in SCC131 stably expressing miR-493. (b and c) Binding of Snail to miR-493 promoter was examined both in SCC131-Control and in SCC131-miR-493 cell lines *in vivo* by ChIP (b) and *in vitro* by EMSA (c) using nuclear protein extract from SCC131-control (C) and SCC131-miR-493 (M). An asterisk indicates the band of interest. (d) miR-493 was knocked down using miR-493-specific inhibitor (miR-493-LNA), and Snail protein expression was analyzed. **, $P < 0.01$. (e) Schematic representation of IGF1R/Snail/miR-493 feedback loop.

cells to bypass the EGFR pathway and maintain survival functions (54). Studies on prostate cancer showed a critical role of the IGF1R axis in the regulation of normal and cancer tissue homeostasis (55). Recent reports have shown the role of miR in the regulation of IGF1R and existence of an equilibrium between the expression of IGF1R and the functions associated with it (56). For head and neck cancer, in which tobacco is one of the major causative agents for the tumorigenesis, deregulation of miR was reported in many studies (57). Nicotine, an important component of tobacco smoke, is shown to be the reason for the altered miR expression which leads to tobacco addiction (58), tobacco-induced cadiomyocyte damage (59), and cancer (60, 61). In our study, we have observed a reduced expression of miR-493 with nicotine addition. Nicotine is known to affect the expression of different genes, including those for transcription factors such as Snail (40), c-Fos, and c-Jun (62). Treatment with nicotine is observed to increase the expression of Snail in time- and concentration-dependent manner, with an increased activating phosphorylation of AKT (Ser473) and inhibitory phosphorylation of GSK-3$\beta$ (Ser9). This aberrant signaling leads to downstream activation of a number of important cellular signaling pathways that mediate proliferation, survival, invasion/metastasis, and angiogenesis, all of which contribute to the malignant phenotype and epithelial-mesenchymal transition (63, 64). Of the several miRs that were shown to be

deregulated in head and neck cancers (65), miR-493 was found to be the prospective candidate, as it can directly target multitude genes downstream of IGF1R pathway, thereby giving less scope for the cancer cells to bypass and activate the alternate signaling pathway. Even though miR-493 was proven to reduce metastasis in colon cancer, its transcriptional regulation aspect and tumor suppressor role have not been studied in head and neck cancers. Data from the tumor samples in this study showed an increase in the expression of IGF1R as well as its downstream effector molecules—IRS1 and MAPK1 in most of the samples. This could be partly ascribed to a decrease in the levels of miR-493, as evident in these tumors. The reduced expression can be attributed to the deregulated transcription of miR in cancer or can be due to the loss of chromosomal deletion or loss of heterogeneity in chromosome 14q32, as observed in the case of miR-376a and miR-376 in melanoma cells (66) where miR-493 is located (chromosome 14q31). Therefore, targeting IGF1R alone may not have complete impact on tumors where the downstream effector expression is significantly higher than the level required for normal functioning of cells. Hence, combination therapy will be an effective way to treat cancers for which overexpression of multiple critical genes (67) is the major cause. Considering cancer as a heterogenous disease with different characteristics (68) and multiple genes playing a critical role in development and progression toward metastasis, targeting multiple molecules may help to increase disease free survival of patients. From this perspective, it was interesting that miR-493 simultaneously regulates a variety of target genes of IGF1R pathway by binding with partially complementary sequences in the 3′ UTR of mRNAs involved in this pathway, thereby potentiating their inhibitory function at multiple levels in the development and progression of human cancers.

Further, functional studies from cell lines and animal models revealed the tumor suppressor functions of miR-493, which prompted us to study about how miR-493 itself is regulated. The facts that the levels of miR-493 are reduced in tumors and Snail—the transcriptional repressor—bound to the miR-493 promoter provided us clues to further understand that Snail mediated miR-493 regulation and subsequently IGF1R regulation. However, the IGF1R pathway by itself was shown to have a role in regulating Snail level through IGF1R/AKT/GSK-3$\beta$/NF-$\kappa$B pathway (49). Growth factors such as IGF1, EGF, and transforming growth factor $\beta$ (TGF-$\beta$) (69) alone or together have been shown to increase Snail levels (70). Our results along with all these studies point out the fact that there exists a dynamic negative feedback loop in the regulation of IGF1R and miR-493, and this is mediated via Snail. Prospectively, Snail binds to the miR-493 promoter and represses its expression, whereas miR-493 downregulates the expression of Snail through regulation of the IGF1R signaling pathway. Snail is an important transcriptional factor that plays a role in epithelial-mesenchymal transition (71). By reducing the level of Snail, miR-493 increases the epithelial characteristic of the cell by increasing the level of E-cadherin, an important cell adhesion molecule and reduces metastasis. This feedback loop regulation can be either positive or negative and has been shown to be involved in the regulation of miR-1269 by TGF-$\beta$ (72), miR-146b by NF-$\kappa$B (73), miR-34a by STAT3 (74), miR-146 by FOXP3 (75), miR-17/20 by E2F1-3 (76), and miR-130a by YAP (77).

There are reports of involvement of carcinogens such as nicotine, a major component of tobacco, in the positive modulation of Snail in lung cancer (78). In tobacco-induced head and neck cancers, nicotine may be the stimulative factor for the Snail overexpression, which, in turn, reduces miR-493 expression by acting as a transcriptional repressor, resulting in the overexpression of its targets, IGF1R, IRS1, and MAPK1. This also could be one of the reasons for reduced miR-493 expressions in head and neck cancers. Considering all the above-described observations, miR-493 can be considered one of the important factors which help to maintain cells in their epithelial condition so the cells can grow in a controlled manner. Our study also helped to delineate one of the pathways which explain how IGF1R can modulate its own pathway using Snail as a mediator, by repressing miR-493 and playing its role in tumorigenesis. Use of miR-493 and its targeted delivery to the tumor can be explored as a therapy in the

future for head and neck cancers, in which IGF1 signaling pathway is the major cause of tumorigenesis.

## MATERIALS AND METHODS

**Cell culture and clinical samples.** Head and neck cancer cell lines UPCI:SCC131 and SCC172 (both are kind gifts from Susanne M. Gollin, Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA), RPMI 2650 (NCCS, Pune, India), SCC745 and SCC969 (both are generous gifts from Robert Mandic, Department of Otolaryngology, Philipps University Marburg, Germany) and HEK-293T were grown in Dulbecco modified Eagle medium (DMEM)–F-12 (Hi-medium); SCC-10B, SCC-3 (University of Michigan), HN5 (ICR, UK), and TR146 (Sigma-Aldrich, USA) were grown in RPMI 1260, and immortalized fetal buccal mucosa (FBM) cells (a kind gift from Milind Vaidya, ACTREC, Tata Memorial Centre, Navi Mumbai, India) were maintained in Iscove's modified Dulbecco's medium (IMDM) with the supplements (79). SCC131 was used as the model system for our study. Patient samples were procured during surgery with their consent, with ethics committee approval (Cancer Institute, Womens India Association; Protocol 1 Head and Neck Co- operative Oncology Group and IEC-NI/16/NOV/56/70 for samples from Sri Ramachandra University).

**Antibodies and reagents.** IGF1R$\beta$ (sc-713) antibody was purchased from Santa Cruz Biotechnology (USA); IRS1 (2382), AKT (9272), phospho-AKT (9271), STAT3 (9139), pSTAT3 (9131), MAPK (9107), pMAPK (9106), Slug (9385), E-cadherin (3195), phospho-NF-$\kappa$B (3033), total NF-$\kappa$B, phospho-GSK-3$\beta$, and total GSK-3$\beta$ were purchased from Cell Signaling, USA. Snail (ab85931; Abcam, Cambridge, MA), fibronectin (BD Biosciences, Bedford, MA), vimentin (V9; Pathn Situ Biotechnologies, CA), and vinculin (V9131; Sigma-Aldrich, USA) were used in this study. MicroRNA mimics, both control (CN-001000-01-50) and miR-493-3p (C-300858-03-0050), and siRNAs, both control (sc-37007) and Snail siRNA (J-010847-09-0005), were used for our studies (Dharmacon, Inc., USA). miRCURY LNA, a powerful microRNA inhibitor (4100001-4109334)-101 (DTI catalog no. E48917), was purchased from Exiqon (Denmark). Nicotine bitartrate (Sigma-Aldrich, USA) was used in our study. All other chemicals and reagents used in the present study were of analytical grade.

**Plasmid construction, transfection, and luciferase assay.** Promoter luciferase assay was done using pGL3 basic vector (Promega, Madison, WI) with the 2-kb region upstream of the miR-493 gene amplified from genomic DNA. Mutations in the Snail binding sites were made using site-directed mutagenesis (Stratagene, La Jolla, CA). pcDNA–Snail-Flag was a generous gift from Stephen Weiss (Life Sciences Institute, University of Michigan). Luciferase assay was performed according to manufacturer protocol (Promega, Madison, WI). $\beta$-Galactosidase ($\beta$-Gal) was used for normalization. 3′ UTRs of the selected targets as well as miR binding site deletion constructs were cloned in the XhoI-NotI site of the psiCHECK-2 vector (Promega), and the Dual-Luciferase assay was performed using the respective assay buffers. Firefly luciferase was used for normalization. Transfections were performed in triplicates for each plasmid construct.

**AGO-2 pulldown.** SCC131 cells, both control and miR overexpression cells, were used for the pulldown studies; the procedure was performed as described earlier (38).

**qPCR and Western blotting.** Total RNA obtained by the TRIzol method was used for cDNA synthesis. Stem-loop primer for miR-493-3p (see Table S1 in the supplemental material) was used for cDNA synthesis using a PrimeScript RT reagent kit (TaKaRa Bio, Inc., India) according to manufacturer protocol. Real-time reaction was done using sybr green mastermix (TaKaRa Bio, Inc.), and the reaction was performed in an ABI thermocycler. U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls. Primer details are provided in the Table S1 in the supplemental material. Equal amounts of protein lysates were run on 8% SDS-PAGE, transferred to a nitrocellulose membrane, and probed for different proteins. Vinculin or GAPDH was used as the internal control.

**Stable cell line generation.** The miR-493 insert was subcloned from pMIR-miR-493 (OriGene) into pbabe-puro vector digested with XhoI. HEK-293 cells were transfected with pbabe (control as well as miR-493) along with the package plasmids (80). Medium was collected and centrifuged at 25,000 rpm for 1.5 h, and the pellet was dissolved in 100 $\mu$l of sterile phosphate-buffered saline (PBS). SCC131 was treated with different viral titers, and selection was done using puromycin. Clones were confirmed by real-time PCR, and targets were checked by immunoblotting.

**ChIP assay.** Cells were cross-linked with 1% formaldehyde (Sigma-Aldrich, St. Louis, MO) and processed for chromatin immunoprecipitation (ChIP) analyses using the ChIP assay kit (EMD Millipore, MA) according to manufacturer protocol. PCRs were performed with the primers specific for the miR-493 promoter containing both Snail sites. Primer details are given in Table S1 in the supplemental material.

**EMSA.** Nuclear protein extract was prepared from SCC131 cells, biotin-labeled probes corresponding to Snail binding sites were annealed, and electrophoretic mobility shift assay (EMSA) was performed according to manufacturer protocol (Pierce Biotechnology, Rockford, IL).

**Attachment and detachment assay.** SCC131-Control as well as SCC131-miR-493 cells were plated on a 24-well plate, and the assay was performed as described earlier (81).

**Anchorage-independent colony formation assay.** The control and miR-493 were suspended in complete medium with 0.5% agarose. Cells were overlaid onto the bottom layer of solidified 0.8% agarose in medium at a concentration of $5 \times 10^3$ per well. Cells were incubated for 2 weeks, stained, and counted.

**Invasion and migration assay.** Cells ($2.5 \times 10^4$) were suspended in serum-free medium and added to the upper chamber of an insert (Biocoat; BD Biosciences, Bedford, MA), and the insert was placed in a 24-well plate containing medium supplemented with 10% fetal bovine serum. The migrated/invaded cells were stained and scored.

**Immunofluorescence.** Immunofluorescence testing for vimentin and E-cadherin was performed on SCC131-Control as well as SCC131-miR-493 cells as described earlier (82).

**MicroRNA target prediction based on free energy.** MicroRNAs having PITA (83) ΔΔG scores of less than or equal to −7 and RNAhybrid (84) ΔG scores of less than or equal to −15 kcal/mol were selected for experimental validation. ΔΔG is an energetic score; the lower the ΔΔG value, the stronger the binding of the microRNA to the given site is expected to be. In RNAhybrid, minimum free energy hybridization of long and short RNA is calculated.

**Animal studies.** Ten million each of SCC131-Control and SCC131-miR-493 cells were injected into both flanks of nude mice (4 to 6 weeks old, male *nu/nu*), and tumor volume was measured over a period of 17 days. After 3 weeks, the tumors were excised from the mice and confirmed for the expression of miR-493 by real-time PCR and targets by Western blotting.

**Statistical analysis.** Data were expressed as means ± standard errors of the means (SEMs) by Prism (GraphPad Software, Inc., San Diego, CA). The mean difference between the groups was analyzed by the Student unpaired *t* test or analysis of variance (ANOVA) using GraphPad Prism 5.0 software. A *P* value of ≤0.05 was considered statistically significant. Box plot analysis was performed using SYSTAT 11 (SPSS Inc., Chicago, IL) to find the significance in the reduction of miR-493 in cancer samples.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MCB.00510-16.

**TEXT S1**, PDF file, 3.5 MB.

**TEXT S2**, PDF file, 0.08 MB.

**TEXT S3**, PDF file, 0.09 MB.

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We declare no conflict of interest.

A.S.K. performed experiments and analyzed data; S.J. performed experiments; R.S.P. performed statistical analysis; V.R., K.V., and G.V. analyzed data; and S.K.R. conceived the idea, analyzed the data, and wrote the paper.

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