

MicroRNA-23b Plays a Tumor-Suppressive Role in Cutaneous Squamous Cell Carcinoma and Targets Ras-Related Protein RRAS2



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Cutaneous squamous cell carcinoma (cSCC) is one of the most common types of cancer with metastatic potential. MicroRNAs regulate gene expression at the post-transcriptional level. In this study, we report that miR-23b is downregulated in cSCCs and in actinic keratosis and that its expression is regulated by the MAPK signaling pathway. We show that miR-23b suppresses the expression of a gene network associated with key oncogenic pathways and that the miR-23b–gene signature is enriched in human cSCCs. miR-23b decreased the expression of *FGF2* both at mRNA and protein levels and impaired the angiogenesis-inducing ability of cSCC cells. miR23b overexpression suppressed the capacity of cSCC cells to form colonies and spheroids, whereas the CRISPR/Cas9-mediated deletion of *MIR23B* resulted in increased colony and tumor sphere formation in vitro. In accordance with this, miR-23b–overexpressing cSCC cells formed significantly smaller tumors upon injection into immunocompromised mice with decreased cell proliferation and angiogenesis. Mechanistically, we verify *RRAS2* as a direct target of miR-23b in cSCC. We show that *RRAS2* is overexpressed in cSCC and that interference with its expression impairs angiogenesis and colony and tumorsphere formation. Taken together, our results suggest that miR-23b acts in a tumor-suppressive manner in cSCC, and its expression is decreased during squamous carcinogenesis.

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INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a keratinocyte (KC)-derived skin cancer with an increasing global prevalence (Corchado-Cobos et al., 2020). The incidence of cSCC has rapidly increased over the last 30 years, reaching over one million new cases a year worldwide (Sung et al., 2021). cSCC develops primarily on sun-exposed skin, with cumulative UV exposure being the major etiological factor for its development (Ortonne, 2002). Immunosuppression is another important risk factor for cSCC; organ transplant recipients have over 100-fold risk for cSCC development (Hofbauer et al., 2010) and are prone to develop more

aggressive, metastatic, and recurrent cSCC. Although surgical excision is curative in most cases for indolent primary tumors, metastatic cSCCs have a poor long-term prognosis, with a dismal 10–20% 10-year survival rate (Clayman et al., 2005; Cranmer et al., 2010). To have more effective treatments, it is imperative to expand our knowledge of cSCC pathogenesis.

MicroRNAs (miRNAs) are a class of small (20–22 nucleotides long) noncoding RNAs that regulate gene expression at the post-transcriptional level. Through base pairing with miRNA recognition elements usually located in the 3′ untranslated region (3′ UTR) of mRNAs, they suppress their targets by inhibiting translation or inducing mRNA destabilization (Bartel, 2004). An increasing amount of evidence has emerged demonstrating that miRNAs regulate the majority of protein-coding genes (~60%), involved in almost every key cellular process such as proliferation, differentiation, and apoptosis (Brennecke et al., 2003; Friedman et al., 2009; Hatfield et al., 2005). Consequently, miRNAs have also been implicated in the pathogenesis of human diseases, including cancer (Lu et al., 2005). Depending on their transcriptional context, miRNAs may function both as oncogenes (such as miR-17-92 and miR-155) and as tumor suppressors (such as miR-15a or let-7) (Peng and Croce, 2016). Given their ability to regulate signaling pathways involved in cancer by concurrently regulating several genes at a time, miRNAs have emerged as potential therapeutic targets (Diener et al., 2022).

We have previously shown that the expression of a set of miRNAs is altered in cSCC (Lohcharoenkal et al., 2021, 2016; Xu et al., 2012). One of the most deregulated miRNAs identified in our screen was miR-23b-3p, an miRNA that has been

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Abbreviations: AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; ERK, extracellular signal–regulated kinase; HNSCC, head and neck squamous cell carcinoma; KC, keratinocyte; miRNA, microRNA; UTR, untranslated region

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shown to regulate KC differentiation (Barbollat-Boutrand et al., 2017); however, its role in cutaneous carcinogenesis has remained unexplored.

In this study, we report that miR-23b expression is decreased in cSCC and show that the suppression of miR-23b expression represents an early event during cutaneous carcinogenesis. Ectopic overexpression of miR-23b suppresses several cancer hallmarks both in vitro and in vivo, whereas CRISPR/Cas9-mediated knockout of *MIR23B* gene in cSCC cell lines increases angiogenesis-inducing, colony-formation, tumor spheroid formation, and proliferation abilities. Mechanistically, activation of the MAPK pathway suppresses miR-23b, which in turn leads to derepression of its direct target *RRAS2*. Our findings identify the previously unreported tumor-suppressive role of miR-23b in cSCC.

RESULTS

miR-23b is downregulated during squamous carcinogenesis and is suppressed by the activation of the MAPK/extracellular signal-regulated kinase pathway

Previously, we analyzed alterations of miRNA expression in cSCCs and identified miR-23b as one of the top downregulated

miRNAs in cSCC (Xu et al., 2012). To validate this observation, we performed TaqMan RT-PCR analysis on a larger, independent cohort of cSCC ($n = 20$), actinic keratosis (AK) ($n = 13$), and healthy skin ($n = 9$) samples. Results from qPCR confirmed our previous observation and demonstrated that miR-23b was over 3.5-fold downregulated in cSCCs compared with that in healthy skin (Figure 1a). miR-23b expression was already decreased by 1.8-fold in AK (Figure 1a), a precancerous lesion of the skin carrying many of the genetic abnormalities found in cSCC (Thomson et al., 2021). The suppression of miR-23b in cSCC was also confirmed by in situ hybridization (Supplementary Figure S1). Moreover, miR-23b expression was lower in cSCC cell lines than in normal human epidermal KCs (Figure 1b).

To gain further insights into the possible role of miR-23b in squamous carcinogenesis, we analyzed miR-23b expression in head and neck squamous cell carcinoma (HNSCC) (The Cancer Genome Atlas Network, 2015), revealing that miR-23b is also significantly downregulated in HNSCCs (Supplementary Figure S2a). Kaplan–Meier survival analysis revealed that high and moderate expression of miR-23b conferred longer overall survival than low miR-23b expression (Supplementary Figure S2b).

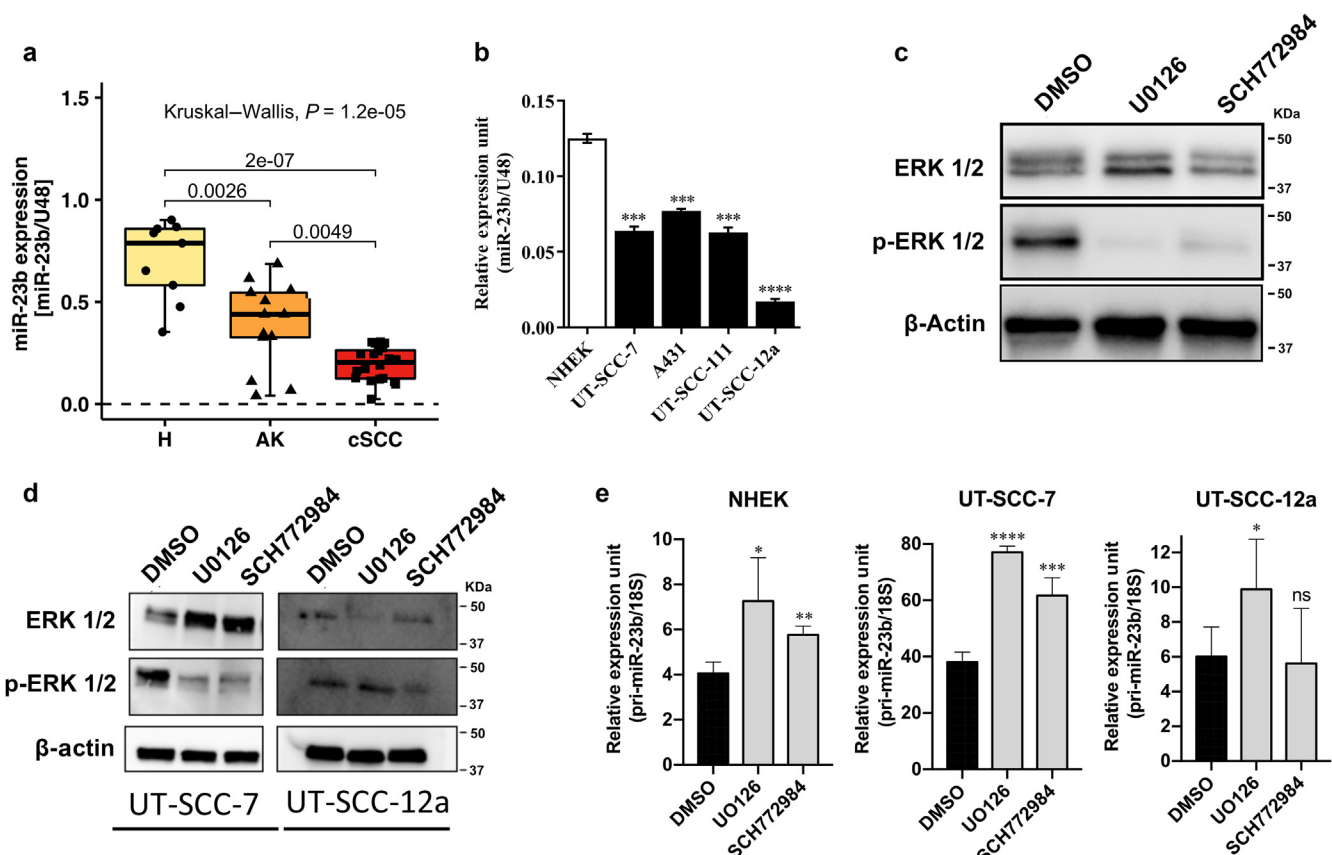


Figure 1. miR-23b is downregulated in cutaneous squamous cell carcinoma. (a) qPCR analysis of miR-23b expression in healthy skin ($n = 9$), AK ($n = 13$), and cSCC ($n = 20$). Analysis was performed with Kruskal–Wallis test. (b) qPCR analysis of miR-23b expression in a panel of human cSCC cell lines and NHEKs ($n = 3$). $***P < 0.001$ and $****P < 0.0001$. Analysis was performed with Student's *t*-test. (c) The effect of MEK (U0126) and ERK (SCH772984) inhibitors on ERK phosphorylation in NHEK cells was analyzed by western blotting ($n = 3$). (d) The effect of inhibitors for MEK (U0126, 10 μ M) or ERK (SCH772984, 0.1 μ M) on ERK phosphorylation in UT-SCC-7 and UT-SCC-12a cSCC cell lines was confirmed by western blot analysis ($n = 3$). (e) qPCR analysis of miR-23b primary transcript after treatment with chemical inhibitors for MEK and ERK on cSCC and NHEK cells ($n = 3$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Analysis was performed with Student's *t*-test. Data are presented as mean \pm SEM. H denotes healthy skin. AK, actinic keratoses; cSCC, cutaneous squamous cell carcinoma; ERK, extracellular signal-regulated kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; NHEK, normal human epidermal keratinocyte; ns, not significant; p-ERK, phosphorylated extracellular signal-regulated kinase.

Considering the central role of the MAPK cascade in cSCC pathogenesis (Di Nardo et al., 2020), we hypothesized that miR-23b expression was regulated by this pathway. To test this hypothesis, we treated normal human epidermal KCs and two cSCC cell lines with U0126 or SCH772984 (specific inhibitors of MAPK/extracellular signal-regulated kinase [ERK] kinase MEK1/2 [Favata et al., 1998] and ERK1/2 [Morris et al., 2013], respectively). Both inhibitors decreased phosphorylation of ERK1/2 in normal human epidermal KCs (Figure 1c) and UT-SCC-7 but not in UT-SCC-12a (Figure 1d) cell lines. qPCR analysis demonstrated a significant increase in the level of pri-miR-23b in normal human epidermal KCs and UT-SCC-7 upon treatment; a significant increase was only achieved in UT-SCC-12a when using U0126 (Figure 1e).

These data demonstrate that miR-23b expression is decreased at an early stage during squamous carcinogenesis in the skin because its expression is downregulated in AK and is further decreased in cSCC.

Transcriptomic analysis of miR-23b–overexpressing cSCC cells reveals enrichment of transcripts belonging to key oncogenic pathways

To obtain insights into the biological processes and signaling pathways regulated by miR-23b, we performed a transcriptomic analysis of a human cSCC cell line (UT-SCC-12a) stably overexpressing miR-23b (Supplementary Figure S3). Analysis of gene expression data revealed that miR-23b–overexpressing cSCC and control cell lines form distinct clusters on the basis of their mRNA profiles (Figure 2a) and identified 199 downregulated and 267 upregulated ($P < 0.01$) genes in miR-23b–overexpressing cells (Figure 2b and c and Supplementary Table S1). Intriguingly, gene set enrichment analysis found that the gene set suppressed by miR-23b in UT-SCC-12a was significantly enriched among genes overexpressed in cSCC tumors (Figure 2d and e and Supplementary Figure S4). Pathways with key importance in tumor development such as angiogenesis, MAPK signaling pathway, regulation of cell proliferation, VEGFR pathway, and cell–cell communication were enriched among miR-23b-regulated transcripts (Supplementary Figure S5a–d). Of particular interest, miR-23b overexpression decreased the expression of several proangiogenic cytokines and GFs (VEGFC, FGF2, CXCL8/IL-8, PDGFA, IL-A, and IL-B) (Supplementary Figure S6) (Li et al., 2003; Geindreau et al., 2022). To investigate the role of endogenous miR-23b on FGF2-expression, we generated miR-23b–knockout (*MIR23B*^{KO}) UT-SCC-7 and UT-SCC-12a cell lines (Supplementary Figure S7a–c). *FGF2* expression was significantly higher in *MIR23B*^{KO} cells both at the mRNA and protein levels (Supplementary Figure S8b and d). In accordance, miR-23b overexpression significantly suppressed *FGF2* at the mRNA and protein levels (Supplementary Figure S8b and c).

In summary, these results suggest that the miR-23b–regulated gene network is enriched in cSCC where miR-23b expression is decreased, suggesting that the decreased expression of miR-23b in cSCC may contribute to the pathogenesis of the disease.

miR-23b suppresses the angiogenesis-inducing capacity of cSCC cells

To determine whether the observed effect of miR-23b on angiogenesis-related genes translates into function, we next analyzed the effect of miR-23b on the angiogenesis-inducing capacity of cSCC cells. Human umbilical vein endothelial cells were treated with conditioned medium from miR-23b–overexpressing cSCCs. We found that both miR-23b–overexpressing cSCC cell lines had a significantly reduced ability to induce tube formation (Figure 3a). In accordance, the ablation of *MIR23B* gene resulted in significantly enhanced angiogenesis-inducing capacity in both cSCC cell lines (Figure 3b). These data demonstrate that miR-23b can regulate the angiogenesis-inducing ability of cSCC cells and suggest that the decreased expression of miR-23b in cSCCs may contribute to neoangiogenesis.

miR-23b suppresses proliferation, long-term growth ability, and tumor spheroid-forming capacity of cSCC

Subsequently, we investigated the effect of miR-23b on cell proliferation using 5-ethynyl-2'-deoxyuridine–proliferation assays in miR-23b–overexpressing cSCCs. FACS analysis of 5-ethynyl-2'-deoxyuridine–positive cells demonstrated that miR-23b overexpression resulted in a consistent and significant decrease in the number of cells in the S-phase (Supplementary Figure S9a). In accordance, *MIR23B*^{KO} resulted in a higher percentage of 5-ethynyl-2'-deoxyuridine–positive cells in both cell lines (Supplementary Figure S9b).

Next, we conducted colony-formation assays using UT-SCC-12a and UT-SCC-7. Quantification of well-formed colonies revealed that miR-23b overexpression leads to a significant reduction in the number of colonies in both cell lines (Figure 3c). Conversely, *MIR23B*^{KO} resulted in a significantly increased number of colonies formed by both cell lines (Figure 3d). Moreover, we found that miR-23b overexpression significantly decreased the size of spheroids formed by UT-SCC-12a and UT-SCC-7 (Figure 3e). In line with these results, *MIR23B*^{KO} resulted in the formation of larger tumor spheroids (Figure 3f). These data suggest that the decreased expression of miR-23b during cSCC development may contribute to higher levels of cellular proliferation and growth.

miR-23b suppresses primary cSCC tumor growth in vivo

Our in vitro assay results demonstrated that miR-23b has a suppressive effect on cell growth, sphere formation, and angiogenesis-inducing capacity. Given these results, we performed a tumor xenograft experiment with miR-23b–overexpressing UT-SCC-12a cells in NOD SCID gamma mice. The measurement of tumor growth revealed that miR-23b–overexpressing cSCC cells formed significantly smaller tumors (Figure 4a). Moreover, analysis of the tumor weight revealed that tumors derived from miR-23b–overexpressing cells were significantly lighter (Figure 4b and c). In line with in vitro results, quantification of Ki-67⁺ and CD31⁺ cells in xenograft tumors through immunofluorescence revealed that tumors formed by miR-23b–overexpressing cells had significantly fewer proliferating and CD31⁺ cells (Figure 4d and e). In summary, miR-23b overexpression could decrease primary

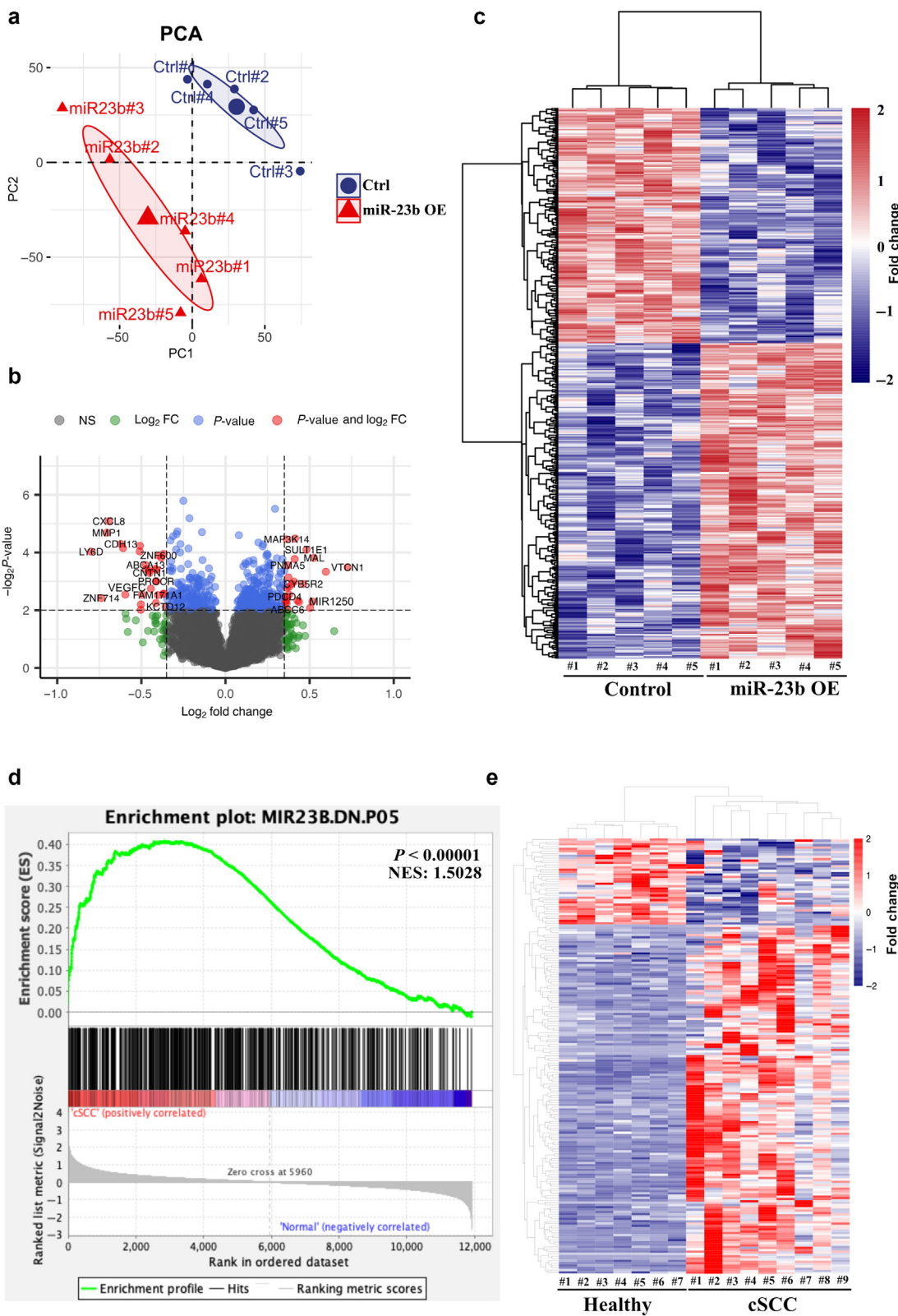


Figure 2. miR-23b regulates the pathways involved in oncogenic transformation and inhibits angiogenesis. (a) Visualization of an unsupervised PCA of log-transformed mean-centered mRNA expression levels for all transcripts deregulated by miR-23 overexpression in UT-SCC-12a cells. (b) Volcano plot of differently expressed genes between control and miR-23b-overexpressing cells. (c) Heat map displaying the expression of miR-23b-associated genes in control UT-SCC-12a cells (left) and UT-SCC-12a-overexpressing miR-23b cells (right). (d) GSEA on RNA-sequencing data from healthy skin and cSCCs using a list of miR-23b-suppressed genes. (e) Heat map displaying the expression of transcripts suppressed by miR-23b in seven healthy skin and nine cSCC samples. For additional information, see [Supplementary Figure S4](#). cSCC, cutaneous squamous cell carcinoma; GSEA, gene set enrichment analysis; NES, normalized enrichment score; PC1, principal component 1; PC2, principal component 2; PCA, principal component analysis.

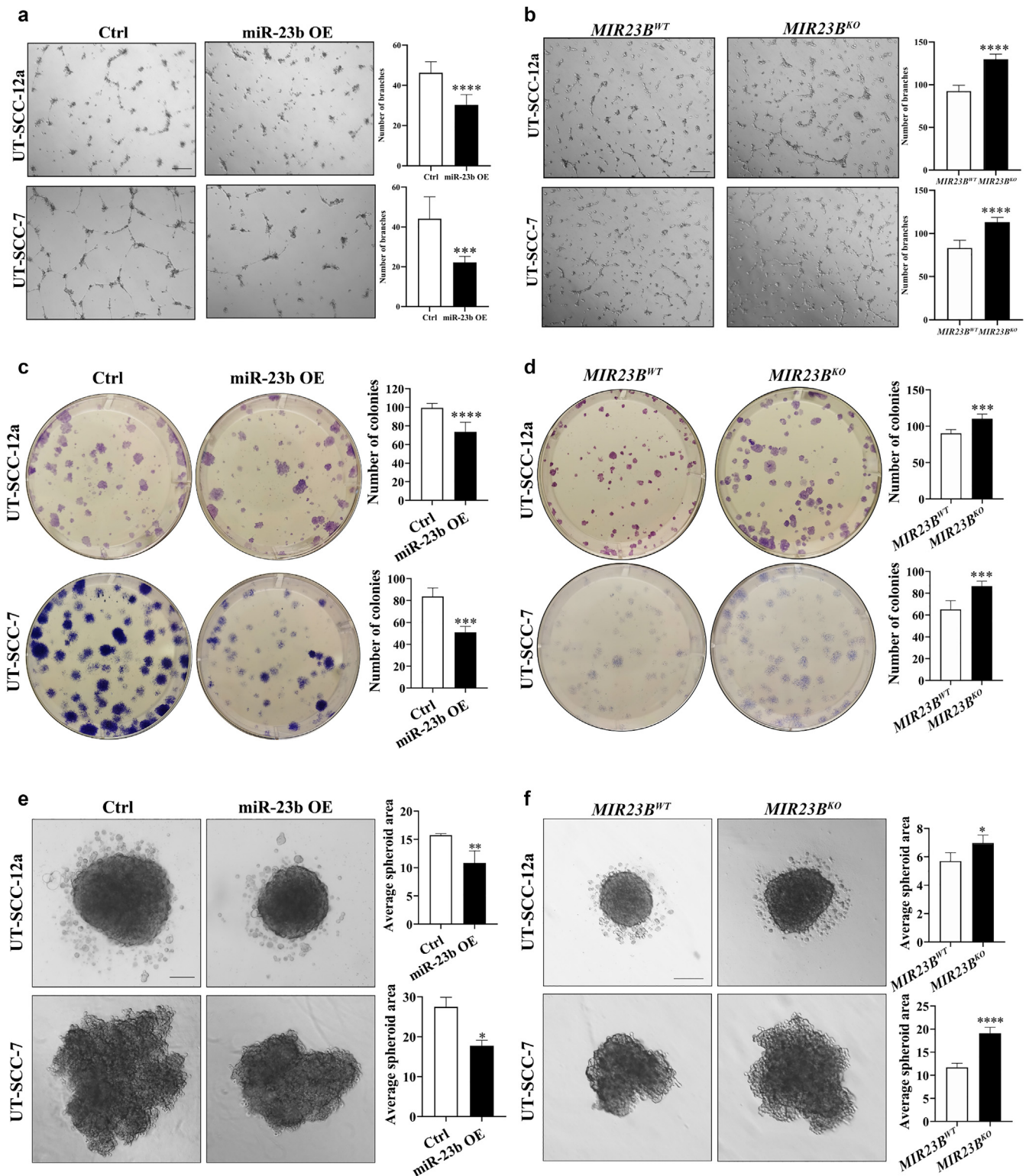


Figure 3. miR-23b impairs the angiogenesis-inducing capacity of cSCC and their capacity to form colonies and tumor spheroids. (a) Representative images of tube formation assays performed by treatment of HUVEC cells with conditioned supernatant of cSCC cells (UT-SCC-12a and UT-SCC-7) stably overexpressing miR-23b or a Ctrl nontargeting RNA. Photos were taken, and the number of branches formed was counted after 8 hours of incubation (n = 3). ***P < 0.001 and ****P < 0.0001. Analysis was performed with Student's *t*-test. Bar = 200 μ m. (b) Tube formation assays with conditioned supernatant of *MIR23B*^{WT} or WT cSCC cells (UT-SCC-12a and UT-SCC-7). Photos were taken, and the number of branches formed was counted after 8 hours of incubation (n = 3). ****P < 0.0001. Analysis was performed with Student's *t*-test. Bar = 200 μ m. (c) The effect of miR-23b OE on the colony-formation ability of UT-SCC-12a and UT-SCC7 human cSCC cell lines. Representative images of colonies are shown (left panel, n = 3). The number of colonies was quantified using ImageJ (right panel). ***P < 0.001 and ****P < 0.0001. Analysis was performed with Student's *t*-test. (d) The effect of *MIR23B* gene deletion on the colony-formation ability of UT-SCC-12a and UT-SCC7 human cSCC cell lines. Representative images of colonies are shown (left panel, n = 3). The number of colonies was quantified using ImageJ (right panel). ***P < 0.001. Analysis was performed with Student's *t*-test. (e) The effect of miR-23b OE on the tumorsphere-

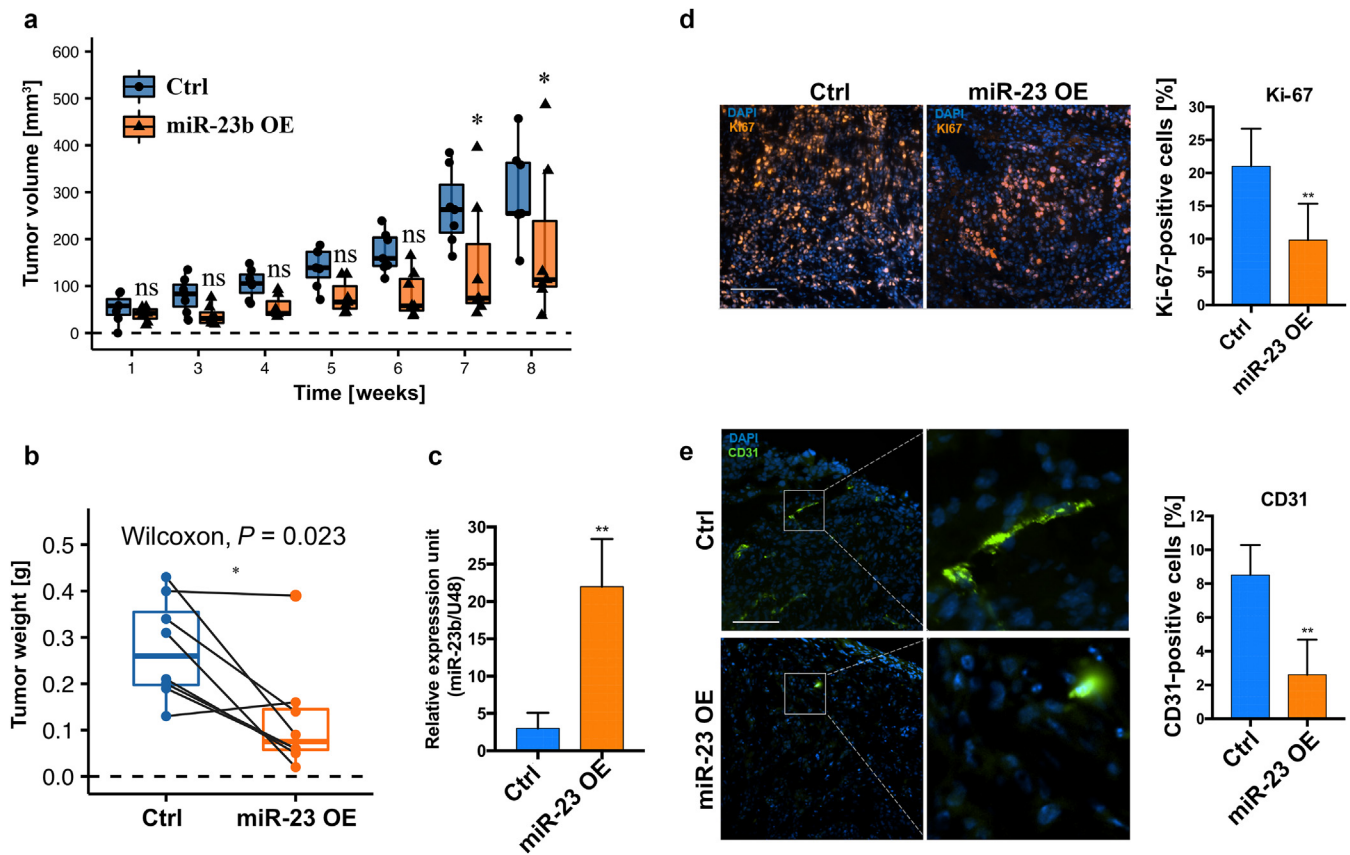


Figure 4. OE of miR-23b suppresses primary tumor growth in xenograft model of cSCC. (a) Changes in tumor volume of xenografts formed by miR-23b–overexpressing UT-SCC-12a cells (miR-23b OE) or their Ctrl (n = 8). **P* < 0.05. Analysis was performed with two-way ANOVA with Bonferroni correction. (b) The effect of miR-23b OE on the weight of tumors formed by miR-23b–overexpressing and Ctrl cSCC cells. The graph shows the weight of tumors harvested after 8 weeks (n = 8). **P* < 0.05. Analysis was performed with Student’s *t*-test. (c) qPCR analysis of miR-23b expression in xenograft tumors formed by Ctrl or miR-23b–overexpressing UT-SCC-12a cells. ***P* < 0.0001. Analysis was performed with Student’s *t*-test. (d) Immunofluorescence analysis of Ki-67⁺ cells in tumor xenografts formed by miR-23b–overexpressing or Ctrl human cSCC cells. Bar = 200 μm. (e) Immunofluorescence analysis of CD31⁺ cells in tumor xenografts formed by miR-23b–overexpressing or Ctrl human cSCC cell lines. Bar = 200 μm. Representative images were taken from at least four samples. Data are presented as mean ± SEM. cSCC, cutaneous squamous cell carcinoma; Ctrl, control; ns, not significant; OE, overexpression.

tumor growth and angiogenesis in the xenograft model of cSCC, suggesting that this miRNA could play a tumor-suppressive role in cSCC.

miR-23b regulates several genes involved in oncogenic transformation and targets *RRAS2*

To investigate the molecular mechanisms for the observed tumor-suppressive effects of miR-23b in cSCC, we interrogated our transcriptomic data. Confirming the validity of this approach to capture miR-23b–specific changes, miR-23b overexpression in cSCC cells resulted in a preferential suppression of transcripts harboring seed matches to miR-23b in their 3’UTRs (Supplementary Table S2). To identify and prioritize miR-23b targets of functional relevance to cSCC, we performed an intersection analysis of (i) genes downregulated

by miR-23b in cSCC cells, (ii) genes significantly upregulated in cSCC tumors identified by our whole transcriptome sequencing analysis (Das Mahapatra et al., 2020), and putative miR-23b–target genes predicted by (iii) TargetScan and (iv) miRDB (Figure 5a and Supplementary Figure S10). Of the top predicted targets, we analyzed the expression of three, *RRAS2*, *FBN2*, and *TOP1*, by qPCR. This confirmed that all three were significantly suppressed by miR-23b overexpression (Supplementary Figure S11). One of the potential miR-23b targets that fulfilled all of the criteria was *RRAS2* (*Ras-Related Protein R-Ras2, a.k.a Teratocarcinoma Oncogene*) - a gene poorly characterized in the context of cSCC. Further supporting a possible miR-23b::*RRAS2* interaction in vivo, qPCR analysis of *RRAS2* revealed a significantly increased expression of *RRAS2* (Figure 5b) in cSCC compared to healthy skin

forming capacity of human cSCC cell lines UT-SCC-12a and UT-SCC-7. Representative images of tumor spheroids are shown (left panel, n = 3). The tumor spheroids were quantified using ImageJ and plotted as the average spheroid area (right panel). **P* < 0.05 and ***P* < 0.01. Analysis was performed with Student’s *t*-test. Bar = 200 μm. (f) The effect of *MIR23B* gene deletion by CRISPR/Cas9 genetic editing on the tumorsphere-forming capacity of human cSCC cell lines UT-SCC-12a and UT-SCC-7. Representative images of tumor spheroids are shown (left panel, n = 3). The tumor spheroids were quantified using ImageJ and plotted as the average spheroid area (right panel). **P* < 0.05 and *****P* < 0.0001. Analysis was performed with Student’s *t*-test. Bar = 200 μm. The number of areas of spheroids and the number of colonies were quantified using ImageJ. Data are presented as mean ± SEM. cSCC, cutaneous squamous cell carcinoma; Ctrl, control; HUVEC, human umbilical vein endothelial cell; OE, overexpression; WT, wild type.

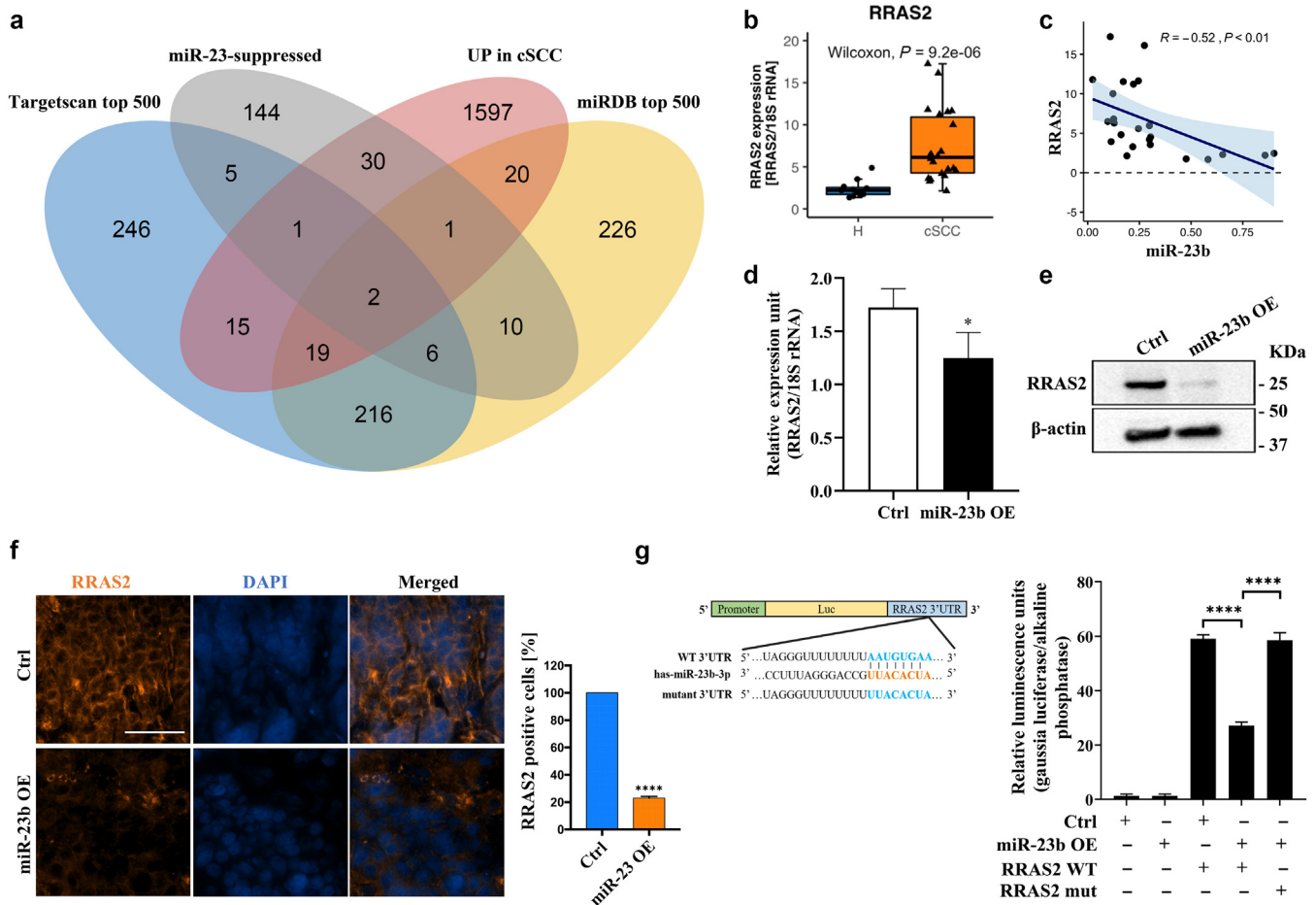


Figure 5. miR-23b regulates RRAS2 in cSCC. (a) Overlap between downregulated genes in miR-23b–overexpressing UT-SCC-12a cell line (miR-23 suppressed), predicted miR-23 target genes (TargetsScan top 500), and list of upregulated genes in cSCC cohort (up in cSCC) and miRDB predictions to identify potential target genes of miR-23b (miRDB top 500). (b) qPCR analysis of RRAS2 expression in healthy skin and cSCCs. Analysis was performed with Wilcoxon test. (c) Correlation between miR-23b and RRAS2 in healthy tissues and primary cSCC tumors. Spearman $r = -0.6502$ and $P = 0.0001$. (d) qPCR analysis of RRAS2 expression in Ctrl or miR-23b shMIMIC-transduced UT-SCC-12a cells ($n = 3$). $*P < 0.05$. Analysis was performed with Student’s t -test. (e) Western blot analysis of RRAS2 in Ctrl or miR-23b shMIMIC-transduced UT-SCC-12a cells ($n = 3$). (f) RRAS2 expression was analyzed by immunofluorescence staining (in orange) in xenograft tumor tissues. Nuclei were stained by DAPI (in blue). Bar = 50 μm . (g) Schematic diagram showing Watson–Crick base pairing between the miR-23b–seed and the WT or mutated miR-23b–binding site within RRAS2 3’UTR and a bar chart depicting the relative luciferase activity from Luciferase reporter assay of cells with RRAS2 3’UTR and mutant RRAS2 3’UTR ($n = 3$). $****P < 0.0001$. Analysis was performed with Student’s t -test. Data are presented as mean \pm SEM. H denotes healthy skin, and mut denotes mutant. 3’ UTR, 3’ untranslated region; cSCC, cutaneous squamous cell carcinoma; Ctrl, control; OE, overexpression; WT, wild type.

and a significant negative correlation between miR-23b and RRAS2 (Figure 5c). Similar to our observations in cSCC, an increased expression of RRAS2 and a negative correlation between miR-23b and RRAS2 were observed in The Cancer Genome Atlas HNSCC cohort (Supplementary Figure S12a and b). Notably, miR-23b overexpression resulted in decreased RRAS2 mRNA and protein levels as demonstrated by qPCR and western blot analyses (Figure 5d and e). In accordance with this, increased RRAS2 protein was observed in *MIR23B*^{KO} cSCC cells (Supplementary Figure S13). Consistent with these observations, immunofluorescence staining demonstrated that RRAS2 was significantly decreased in xenograft tumors formed by miR-23b–overexpressing cells in vivo (Figure 5f), suggesting that miR-23b may target RRAS2 in cSCC.

To experimentally test whether RRAS2 is a direct target of miR-23b in cSCC, we performed 3’ UTR luciferase reporter assays using constructs containing the wild-type RRAS2

3’UTR or an RRAS2 3’ UTR with a alteration introduced to the predicted miR-23b–binding site (Figure 5g). miR-23b overexpression resulted in the suppression of luciferase activity in cells transfected with the wild-type RRAS2 3’ UTR plasmid compared with that in control cells (Figure 5g). In contrast, the luciferase activity in miR-23b–overexpressing cells was restored upon RRAS2 3’ UTR sequence mutation demonstrating the interaction between miR-23b and 3’ UTR of RRAS2 (Figure 5g).

In summary, these results establish RRAS2 as a direct target of miR-23b in cSCC and demonstrate that the overexpression of RRAS2 as well as the negative correlation between RRAS2 and miR-23b is a common feature of cSCC and HNSCC.

RRAS2 regulates colony-formation ability and angiogenesis-inducing capacity of cSCC cells

Because RRAS2 had been poorly characterized in the context of cSCC and our results suggested that miR-23b may at least

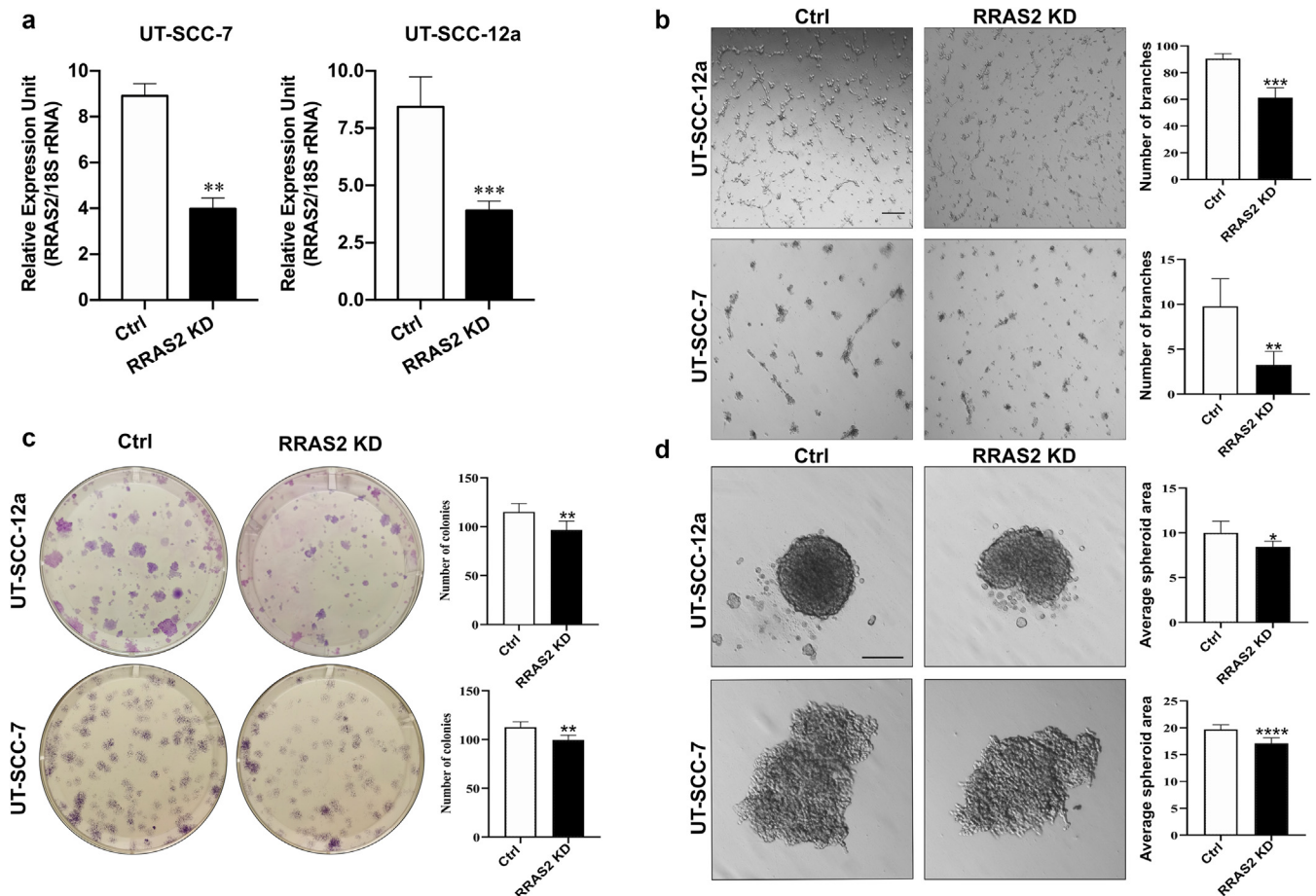


Figure 6. RRAS2 is a regulator of cSCC colony-formation, tumorsphere formation, and tube formation-inducing capacities. (a) qPCR analysis of RRAS2 expression in UT-SCC-12a and UT-SCC-7 cells transfected by Ctrl or RRAS2 siRNAs ($n = 3$). $**P < 0.01$ and $***P < 0.001$. Analysis was performed with Student's *t*-test. (b) Representative images of HUVECs tube formation assay performed by resuspending HUVECs in supernatant collected from cSCC cells (UT-SCC-12a and UT-SCC-7 knocked down for RRAS2). Photos were taken, and the number of branches formed was counted after 8 hours of incubation ($n = 3$). $**P < 0.01$ and $***P < 0.001$. Analysis was performed with Student's *t*-test. Bar = 200 μm . (c) The effect of siRNA-mediated KD of RRAS2 on the colony-formation ability of UT-SCC-12a and UT-SCC-7 cSCC cell lines. Representative images of colonies are shown (left panel, $n = 3$). The number of colonies was quantified using ImageJ (right panel). $**P < 0.01$. Analysis was performed with Student's *t*-test. (d) The effect of RRAS KD on the tumorsphere-forming capacity of human cSCC cell lines UT-SCC-12a and UT-SCC-7. Representative images of tumor spheroids are shown (left panel, $n = 3$). The tumor spheroids were quantified using ImageJ and plotted as the average spheroid area (right panel). $*P < 0.05$ and $****P < 0.0001$. Analysis was performed with Student's *t*-test. Bar = 200 μm . Data are presented as mean \pm SEM. cSCC, cutaneous squamous cell carcinoma; Ctrl, control; HUVEC, human umbilical vein endothelial cell; KD, knockdown; rRNA, ribosomal RNA; siRNA, small interfering RNA.

partially mediate its function through its regulation, we next investigated the role of RRAS2 on the phenotypes observed upon modulation of miR-23b expression. First, the effect of RRAS2 knockdown was observed on the angiogenesis-inducing capacity of cSCC cell lines. It was observed that conditioned supernatant from RRAS2-knockdown cell lines significantly decreased the formation of angiogenic branches (Figure 6a and b). Furthermore, the knockdown of RRAS2 resulted in a significant reduction in the number of colonies formed by UT-SCC-12a and UT-SCC-7 cells (Figure 6c). Moreover, results from tumor spheroid formation experiments revealed that RRAS2 knockdown in cSCC cell lines resulted in a decreased spheroid size (Figure 6d).

In summary, these results suggest that RRAS2 plays a role in angiogenesis, colony-formation, and tumor spheroid formation ability of cSCC, suggesting that its dysregulation due to decreased miR-23b expression may contribute to the disease (Supplementary Figure S14).

DISCUSSION

In this study, we report the decreased expression of miR-23b in cSCC and the increased expression of a gene network suppressed by miR-23b in the disease. We found that miR-23b regulated the angiogenesis-inducing, colony-formation, and tumor spheroid formation abilities and proliferation in cSCC cells as well as cSCC tumor growth in vivo. We identify RRAS2, a relatively poorly characterized member of the Ras family, as a direct target of miR-23b and report its increased expression in cSCC. Our results suggest that miR-23b may have a tumor-suppressive role in cSCC and that its expression may be dysregulated owing to alternations in the MAPK-signaling pathway.

miR-23b is an evolutionarily ancient miRNA that appeared in the vertebrate lineage (Bartel, 2018) and is abundant in KCs (Hildebrand et al., 2011; Srivastava et al., 2019). miR-23b is a member of a miRNA cluster that consists of three highly conserved miRNAs (miR-23b, miR-27b, and miR-24-1)

transcribed as one polycistronic transcript on chromosome 9q22 (+) (Liang et al., 2014). It has been reported that the miR-23b cluster has altered expression in various diseases and regulates multiple biological functions, including the cell cycle, proliferation, differentiation, and angiogenesis (Barbollat-Boutrand et al., 2017; Chhabra et al., 2010; Hua et al., 2018; Liu et al., 2018; Zhou et al., 2011). In healthy skin, miR-23b has been shown to promote KC differentiation by activating the TGF β –SMAD2 signaling pathway (Barbollat-Boutrand et al., 2017). Interestingly, miR-23b has been shown to be the most highly upregulated miRNA in primary KCs upon UV-A irradiation (Kraemer et al., 2013). This finding together with our results suggests that miR-23b play a tumor-suppressive role in the skin by potentially preventing UV-damaged cells from proliferating. Although previous studies have elucidated tumor-suppressive roles for miR-23b in other cancers (An et al., 2015; Majid et al., 2012; Zhang et al., 2011), miR-23b had remained completely uncharacterized in cSCC.

The decreased expression of miR-23b in AK indicates that a decrease in miR-23b level may represent an early event in cSCC carcinogenesis. AK contains several of the key driver mutations in cSCC (e.g., *TP53*, *NOTCH1*, and *NOTCH2*) and also shows partial disruption of the epidermal differentiation program (Thomson et al., 2021; Cockerell, 2000; Kubo et al., 1994). Our results regarding decreased miR-23b expression in AK together with its role in KC differentiation suggest that the decreased miR-23b expression at an early stage of cutaneous carcinogenesis may contribute to the perturbation of the epidermal differentiation program.

Of note, the expression of miR-23b is further decreased in cSCC compared with that in precancerous lesions. The MAPK pathway regulates critical cancer-associated traits and its activation has been suggested to contribute to the progression from AK to invasive SCC (Lambert et al., 2014). Thus, our results showing that miR-23b expression is under the regulation of the MAPK pathway could explain the decreased expression of miR-23b during cSCC carcinogenesis. In other cancers, miR-23b has been shown to be directly induced by p53 (Bisio et al., 2013); in accordance with this, miR-23b is increased after UV exposure in human KCs (Kraemer et al., 2013) and is decreased in precancerous skin lesions and cSCC in which TP53 is the most commonly mutated gene.

In this study, we verify RRAS2 as a direct target of miR-23b in cSCC. RRAS2 is a member of the R-Ras subfamily of Ras-like small GTPases that shares downstream signaling elements with Ras protooncogene, including phosphatidylinositol 3-kinase, Ral GDP dissociation stimulator, and Raf kinase (Larive et al., 2014). In line with this, RRAS2 has been shown to be able to drive tumorigenesis through enhanced activation of the MAPK pathway (Flex et al., 2014). Of note, *Rras2*-knockout mice displayed impaired ERK activation (Capri et al., 2019; Larive et al., 2012). Furthermore, heterozygous germline activating mutations in RRAS2 gene have been linked to Noonan syndrome, a hereditary disease, resulting in the permanent activation of ERK (Ceremsak et al., 2016; Niihori et al., 2019). In agreement with the findings of Zhang et al. (2011) in human colon cancer cell lines, we show in this study that miR-23b directly targets RRAS2 in cSCC. The fact that RRAS2 has been identified by five independent studies (including ours) (Barbollat-Boutrand et al., 2017; Kraemer et al., 2013; Yu et al.,

2015; Zhang et al., 2011) as a target of miR-23b suggests that it is indeed one of the important downstream effectors of miR-23b. Despite the importance of RRAS2 and its overexpression in various cancer types (Lee et al., 2011), its increased expression in cSCC had been previously unreported. Our results show that the knockdown of RRAS2 can affect several cancer hallmarks, suggesting that RRAS2 is an important player in cSCC and that miR-23b-mediated targeting of RRAS2 may contribute to squamous carcinogenesis.

miRNAs act through a network of target genes, and evolutionarily conserved miRNAs can have up to 400 target genes (Friedman et al., 2009). Indeed, in addition to RRAS2, our study has identified that miR-23b regulates several predicted miR-23b-target genes (e.g., *VEGFC*, *FBN2*, *FGF2*, *TOPI1*, and others), suggesting that the effects of miR-23b are mediated through a cohort of these genes. Several of these genes have been reported to regulate cell proliferation and cancer progression, in agreement with our results showing that modulation of intracellular miR-23b activity by overexpression or genetic ablation of *MIR23B* regulates cSCC cell proliferation colony-formation ability as well as their capacity to form tumor spheres. Interestingly, several miR-23b-regulated genes in cSCC cell lines were known to be involved in the regulation of angiogenesis (e.g., *FGF2*, *PDGFA*, *IL8*, and *VEGFC*). In line with these findings, miR-23b overexpression significantly inhibited the expression of angiogenesis-related genes and suppressed the angiogenesis-inducing ability of cSCC, whereas deletion of *MIR23B* resulted in the opposite phenotype. This finding is also consistent with observations in other cancer types establishing a tumor-suppressive role for miR-23b across a broad range of cancers (Campos-Viguri et al., 2020; Wei et al., 2018).

In conclusion, we report that the expression of miR-23b is decreased in cSCC; that it is under the regulation of the MAPK pathway; and that miR-23b regulates several cancer hallmarks, including angiogenesis, proliferation, and colony and tumor spheroid formation. Our analysis of The Cancer Genome Atlas HNSCC showing that a low level of miR-23b is associated with shorter patient survival supports an important role for miR-23b in squamous cell carcinomas with a clinical implication. Our findings suggest a model in which the activation of MAPK pathway can decrease the expression of miR-23b, leading to derepression of RRAS2 and other miR-23b targets, which in turn contributes to tumor progression during squamous carcinogenesis (Supplementary Figure S14).

MATERIALS AND METHODS

Patient samples

Healthy donors (n = 9) and patients with cSCC (n = 20) were recruited at Karolinska University Hospital (Stockholm, Sweden); patients with AK (n = 13) were recruited at Heinrich-Heine University (Düsseldorf, Germany). The study was approved by the local ethics committees, and written informed consent was obtained from all participants. The diagnosis of cSCC was confirmed through histopathology.

Stable miR-23b-overexpressing cSCC cell lines

UT-SCC-7 and UT-SCC-12a cell lines were transduced with shMIMIC Lentiviral miRNAs (Horizon, Stockholm, Sweden); control

cells were treated with scrambled RNA according to the manufacturer's instructions, with a multiplicity of infection of 20, in the presence of 6 µg/ml polybrene. Cells were selected using puromycin (1 µg/ml) for 2 weeks. miR-23b overexpression was validated by qRT-PCR.

Tumor xenografts

NOD SCID gamma mice (Jackson Laboratory, Bar Harbor, ME) were used for xenograft experiments, which were approved by The Swedish Board of Agriculture (Jordbruksverket). Briefly, 10⁷ tumor cells were injected subcutaneously; mice were monitored for 8 weeks.

miR-23b—knockout cSCC cell lines

A pair of single-guide RNAs were designed using the UCSC Genome Browser (<https://genome.ucsc.edu>). UT-SCC-7 and UT-SCC-12a were cotransfected with single-guide RNAs and SpCas9 2NLS Nuclease (Synthego, Redwood City, CA) through nucleofection with a Human Keratinocyte Nucleofector Kit (Lonza, Solna, Sweden). *MIR23B*^{KO} was validated using PCR.

Colony formation assay

A total of 250 cells per well of a six-well plate were seeded in a complete medium for 7–10 days. Colonies were stained with 0.2% crystal violet and photographed. Clones were counted using ImageJ (<https://imagej.nih.gov/ij/>).

ELISA

FGF2 secretion in media was measured using FGF2 ELISA kit (KHG0021, Invitrogen, Waltham, MA) according to the manufacturer's instructions. Results were normalized to the corresponding cell numbers.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). The statistical methods employed were Wilcoxon, Mann–Whitney *U* test, Kruskal–Wallis, two-way ANOVA, and Student's *t*-test. *P* < 0.05 was considered significantly different.

Further details regarding cell culture, experiments, and statistical analyses are available in [Supplementary Materials and Methods](#).

Data availability statement

The microarray data from this study have been submitted to the National Center for Biotechnology Information and are available under the accession number GSE206584. Datasets related to this article can be found at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206584>.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: ES, AP; Data Curation: CS, JE, CL, KDM; Formal Analysis: CS, JE, CL, KDM; Investigation: CS, JE, CL, KDM; Supervision: ES, AP; Writing - Original Draft Preparation: JE, AP, CS, KDM, CL, ES

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2023.05.026>.

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