MiR-130a Acts as a Tumor Suppressor MicroRNA in Cutaneous Squamous Cell Carcinoma and Regulates the Activity of the BMP/SMAD Pathway by Suppressing *ACVR1*

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Cutaneous squamous cell carcinoma (cSCC) is a malignant neoplasm of the skin resulting from the accumulation of somatic mutations due to solar radiation. cSCC is one of the fastest increasing malignancies, and it represents a particular problem among immunosuppressed individuals. MicroRNAs are short noncoding RNAs that regulate the expression of protein-coding genes at the post-transcriptional level. In this study, we identify miR-130a to be downregulated in cSCC compared to healthy skin and precancerous lesions (actinic keratosis). Moreoever, we show that its expression is regulated at the transcriptional level by HRAS and MAPK signaling pathway. We demonstrate that overexpression of miR-130a suppresses long-term capacity of growth, cell motility and invasion ability of human cSCC cell lines. We report that miR-130a suppresses the growth of cSCC xenografts in mice. Mechanistically, miR-130a directly targets *ACVR1* (*ALK2*), and changes in miR-130a levels result in the decreased activity of the BMP/SMAD pathway through ACVR1. These data reveal a link between activated MAPK signaling and decreased expression of miR-130a, which acts as a tumor-suppressor microRNA in cSCC and contribute to a better understanding of the molecular processes during malignant transformation of epidermal keratinocytes.

Journal of Investigative Dermatology (2021) **141,** 1922–1931; doi:10.1016/j.jid.2021.01.028

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is one of the most common human malignancies affecting more than 500,000 new patients per year throughout the world. The incidence of cSCC is increasing fast in the Caucasian population (Alam and Ratner, 2001; Que et al., 2018). The primary risk factor for cSCC is chronic exposure to solar UVR, which induces somatic mutations and epigenetic alterations resulting in the malignant transformation. cSCC can arise from actinic keratosis (AK), which are precancerous lesions commonly seen on sun-damaged skin, and patients diagnosed with AK have a 10% lifetime risk to develop cSCCs (Salasche, 2000). Although AKs are not malignant, multiple cSCC-associated genetic alterations are already present in them, including mutations in tumor suppressor *TP53* (Ashton et al., 2003). An additional risk factor for cSCC is immune suppression: organ-transplant recipients receiving life-long immunosuppression have approximately 65–250 times increased risk to develop cSCCs (Que et al., 2018). Moreover, cSCCs in organ-transplant recipients are often multiple, develop at a younger age, and are more aggressive, highlighting the importance of the immune system in controlling the expansion of transformed cell clones in the epidermis (Wells and Shirai, 2012). The massive incidence of cSCC makes this cancer type a major health concern. Although most of cSCCs are cured with surgical resection, prognosis is generally poor in patients who develop metastases, with an expected 5-year survival of 26–34%, indicating that development is needed in the treatment and management of patients with cSCC (Alam and Ratner, 2001; Li et al., 2015; Wells and Shirai, 2012).

Recent exome sequencing studies have shown that cSCC harbors a mutation burden greater than any other known cancer with metastatic potential (mean somatic mutation rate of 50 mutations per megabase pair of DNA) with key driver mutations in *TP53*, *CDNK2A*, *NOTCH1*, *NOTCH2*, and *HRAS* genes as well as frequent copy number gains in *EGFR*, *CCND1*, and *MYC* regions (Dotto and Rustgi, 2016). These genomic alterations ultimately lead to a transformed phenotype and drive cSCC growth and progression. However, to date, little is known about the association between the driver genes in cSCC and alterations in noncoding RNA expression.

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Abbreviations: AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; ERK, extracellular signal–regulated kinase; MEK, MAPK/extracellular signal–regulated kinase; miRNA, microRNA; NHEK, normal human epidermal keratinocyte; p-SMAD, phosphorylated SMAD; qRT-PCR, quantitative real-time reverse transcriptase–PCR; UTR, untranslated region

Received 13 August 2020; revised 22 December 2020; accepted 5 January 2021; accepted manuscript published online 22 March 2021; corrected proof published online 17 June 2021

MicroRNAs (miRNAs) are approximately ~22 nt non-coding RNAs that regulate gene expression at the post-transcriptional level primarily by sequence-specific base pairing to the 3′ untranslated region (UTR) of their target genes (Bartel, 2004). To date, more than 2,000 miRNAs have been identified in the human genome, and most protein-coding genes are thought to be regulated by miRNAs. Because one miRNA can regulate dozens if not hundreds of target genes, miRNAs can effectively regulate signaling pathways and play key roles in virtually every biological process, including stemness, development, inflammation, proliferation, and apoptosis (Alvarez-Garcia and Miska, 2005; Bartel, 2009). MiRNAs are frequently deregulated in cancer and may function as oncogenes (oncomiRs) or tumor suppressors (Calin and Croce, 2006; Iorio and Croce, 2012). We and others have identified several miRNAs whose expression is altered in cSCC, such as miR-203 (Benaich et al., 2014; Lohcharoenkal et al., 2016), miR-125b (Xu et al., 2012), and miR-31 (Wang et al., 2014). The functional characterization of these cSCC-associated miRNAs in in vitro and in vivo cancer models revealed their importance in the context of cancer-associated phenotypes such as proliferation, impaired terminal differentiation, invasiveness, colony formation, and angiogenesis-inducing capacity.

In this study, we aimed to investigate the function of miR-130a-3p in cSCC. We report that miR-130a expression is suppressed in invasive cSCCs but not in precancerous skin lesions. Moreoever, we find that activated MAPK pathway and increased HRAS expression can suppress miR-130a expression at the transcriptional level. Ectopic overexpression of miR-130a in cSCC cell lines resulted in the suppression of their long-term capacity of growth, cell motility, and invasiveness. In vivo, overexpression of miR-130a suppressed cSCC tumor growth on immunocompromised mice. Mechanistically, we identified *ACVR1* as a direct target of miR-130a and showed that miR-130a regulated the BMP/SMAD pathway through ACVR1. Taken together, our study identifies miR-130a as a tumor suppressor in cSCC, which can be suppressed by the activation of the MAPK pathway. Our results identify a potential cross-talk between MAPK and BMP/SMAD pathway in cSCC through miR-130a–mediated regulation of ACVR1.

RESULTS

MiR-130a is suppressed by the MAPK pathway in cSCC

Previously, we performed a global miRNA expression analysis in cSCC and found miR-130a to be downregulated in tumor tissues compared with healthy skin (Xu et al., 2012). To validate this observation, we performed TaqMan quantitative real-time reverse transcriptase–PCR (qRT-PCR) analysis of miR-130a in RNA isolated from a larger, independent cohort of healthy human skin, precancerous skin lesions (AK), and cSCC samples. Results from the qRT-PCR-analysis demonstrated that miR-130a expression was significantly decreased in cSCCs compared to both normal skin (Figure 1a) and AK, suggesting that decreased miR-130a expression is associated with the malignant phenotype. In situ hybridization analysis using locked nucleic acid probes in an independent cohort of healthy skin samples and cSCCs showed that the expression of miR-130a in normal skin was evenly expressed in all cell layers of the epidermal compartment (Figure 1b). In contrast, miR-130a expression was virtually undetectable in cSCC samples, suggesting the involvement of miR-130a in cutaneous squamous cell tumorigenesis.

Because *HRAS* is one of the driver genes for aggressive cSCC (Pickering et al., 2014), we hypothesized that the modulation of *HRAS* could affect the level of miR-130a. To test this hypothesis, we performed small interfering RNA–mediated knockdown of HRAS in UT-SCC-7 human cSCC cell line, which, as expected, resulted in decreased HRAS protein level and decreased phosphorylation of extracellular signal–regulated kinase (ERK), a downstream effector of HRAS (Figure 1c). qRT-PCR analysis demonstrated that the depletion of HRAS resulted in increased miR-130a expression in UT-SCC-7 cells (Figure 1c). In contrast, the transient overexpression of HRAS resulted in decreased miR-130a level in UT-SCC-7 cells (Figure 1d). To further investigate the potential involvement of the HRAS/MAPK/ERK kinase (MEK)/ERK1/2 pathway in the regulation of miR-130a expression, UT-SCC-7 cells were treated with U0126 (a selective inhibitor of MAPK kinases, MEK1 and MEK2 [Favata et al., 1998]) or SCH772984 (a specific inhibitor of ERK1 and 2 [Morris et al., 2013]), and the expression level of the primary transcript of miR-130a was measured by qRT–PCR. As expected, both treatments with MEK inhibitor and ERK inhibitor led to decreased phosphorylation of ERK (Figure 1e). qRT-PCR results demonstrated an increase in the miR-130a primary transcript following these treatments, strengthening the association between the MAPK pathway and the expression of miR-130a (Figure 1e). Taken together, these findings establish the decreased expression of miR-130a in invasive cSCC but not in precancerous skin lesions and suggest that the activation of MAPK pathway could provide a plausible explanation for decreased miR-130a in cSCC cells.

miR-130a suppresses primary tumor growth in vivo and long-term capacity of growth in vitro

Because miR-130a expression was decreased in cSCC but not in precancerous skin lesions, we hypothesized a role for it in cSCC. We therefore set out to explore the effect of miR-130a on cSCC tumor growth in in vivo xenograft model. To this end, UT-SCC-7 cells with stable miR-130a-overexpression were established by lentiviral transduction (Supplementary Figure S1a) and were subcutaneously injected into immunodeficient NOD SCID gamma mice. We observed that overexpression of miR-130a led to a significant reduction in tumor volume by week 4 and 5 (Figure 2a). cSCC cells with high miR-130a expression formed significantly smaller tumors as measured by tumor weight than the tumors formed by control cell line overexpressing a scrambled sequence (Figure 2b). Moreover, we observed a significant decrease in the number of Ki-67 positive cells in the xenografts formed by miR-130a–overexpressing cSCC cells (Supplementary Figure S1b). Thus, these in vivo results demonstrated the tumor-Suppressor potential of miR-130a in cSCC.

The ability of a single cell to grow into a colony is a critical characteristic of tumor cells, which is required for the expansion of transformed clones and for the establishment of metastases. Because miR-130a suppressed tumor growth in vivo, we hypothesized that it may regulate these

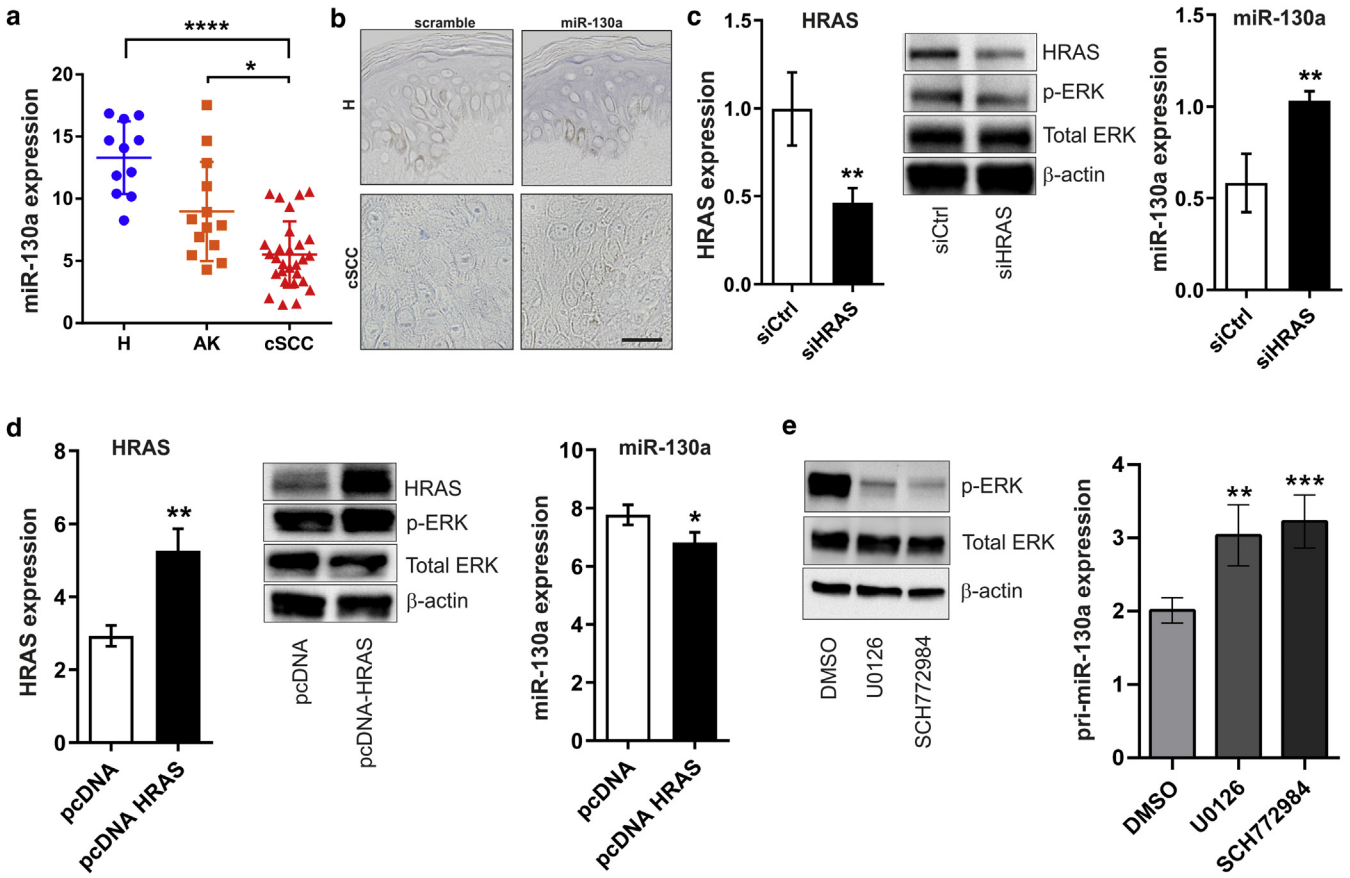

Figure 1. MiR-130a expression is decreased in cSCC and suppressed by the MAPK pathway. (**a**) TaqMan qRT-PCR analysis of miR-130a expression in H (n = 11), AK (n = 13), or cSCCs (n = 28). Mann–Whiney U-test. $*P < 0.05$, $****P < 0.0001$. (**b**) Representative images of LNA in situ hybridization analysis of miR-130a expression in tissues obtained from H and cSCCs. The positive signal for miR-130a is purple. Scrambled probe was used as a control. Scale bar = 100 μm. (**c**) The expression level of miR-130a in UT-SCC-7 cells after transfection with siHRAS or siCtrl. Transfection efficiency was confirmed by qRT-PCR and western blot (n = 3). $**P < 0.01$, Student's *t*-test. (**d**) The expression of miR-130a in UT-SCC-7 cells was analyzed by qRT-PCR after transfection with pcDNA-HRAS or empty vector (pcDNA). HRAS overexpression was confirmed by qRT-PCR and western blot (n = 3). $*P < 0.05$, $**P < 0.01$, Student's *t*-test. (**e**) The expression level of miR-130a primary transcript in UT-SCC-7 cells on the treatment with chemical inhibitors for MEK (U0126, 10 μM) or ERK (SCH772984, 0.1 μM). The effect of chemical inhibitors on ERK-phosphorylation was confirmed by western blot (n = 3). $**P < 0.01$, $***P < 0.001$, Student's *t*-test. Data are presented as mean ± SEM. AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; ERK, extracellular signal–regulated kinase; H, healthy human skin; LNA, locked nucleic acid; MEK, MAPK/extracellular signal–regulated kinase; p-ERK, phosphorylated extracellular signal–regulated kinase; qRT-PCR, quantitative real-time reverse transcriptase–PCR; siCtrl, small interfering RNA control; siHRAS, small interfering RNA HRAS.

processes in cSCC. To test this, we performed a colony formation assay using UT-SCC-7 and A431 cell lines. Ectopic overexpression of miR-130a (Supplementary Figure S2a) decreased the colony-forming ability of both UT-SCC-7 cells and in A431 cells (Supplementary Figure S2c and d). Furthermore, a sphere formation assay was performed to evaluate the role of miR-130a on cell long-term capacity of growth. We found that overexpression of miR-130a led to decreased sphere size in both UT-SCC-7 and A431 cells (Figure 2c and d). Conversely, inhibition of endogenous miR-130a in UT-SCC-7 and A431 cells resulted in the formation of larger spheres (Figure 2e and f and Supplementary Figure S2b). These results suggest that miR-130a modulates cSCC growth by regulating the long-term capacity of growth.

MiR-130a suppresses the motility and invasiveness of cSCC cells

Next, we investigated the effect of miR-130a on cell motility and invasiveness, which are essential features of a metastatic disease. To this end, a scratch wound-healing assay was performed using miR-130a–overexpressing UT-SCC-7 and A431 cell lines. Measurement of the wound area revealed that miR-130a inhibited wound closure in scratch wound healing assays (Figure 3a). In line with this result, we observed that the overexpression of miR-130a inhibited both cell migration (Figure 3b) and the invasive capacity of cSCC cells (Figure 3c) in transwell migration and invasion assays.

In contrast to miR-130a-overexpression, the inhibition of endogenous miR-130a increased wound closure rate in comparison with control locked nucleic acid sequences in both cSCC cell lines (Figure 3d). Inhibition of miR-130a also greatly increased the migratory (Figure 3e) and invasive (Figure 3f) potential of cSCC cells. These results demonstrated that miR-130a can suppresse the motility and invasiveness of cSCC cells in vitro.

miR-130a regulates the BMP/SMAD signaling pathway by targeting *ACVR1* in cSCC

We next sought to determine the molecular basis for the observed effects of miR-130a in cSCC and to identify the biological pathways it regulates. To identify the functional

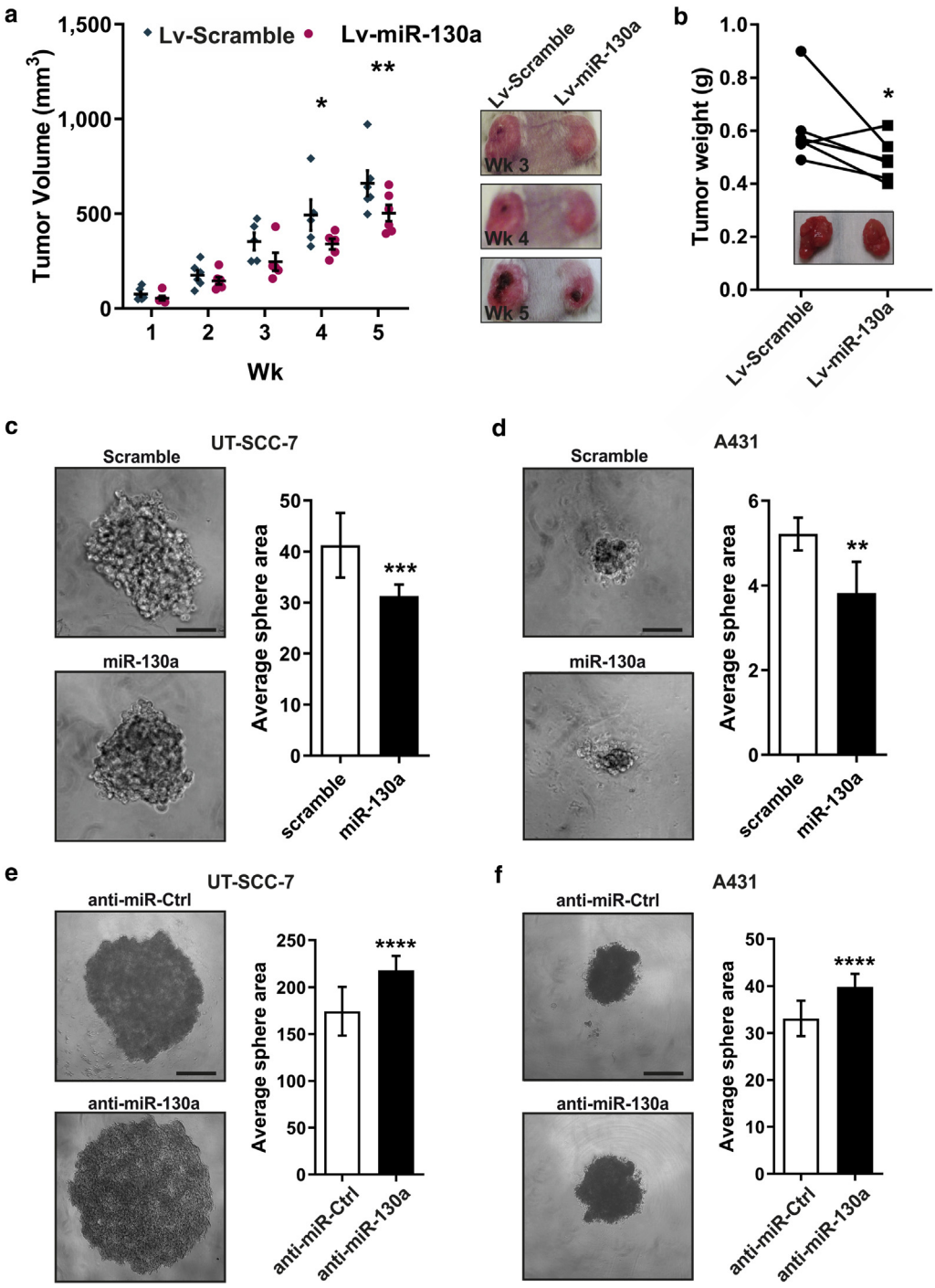

Figure 2. Overexpression of miR-130a suppresses primary tumor growth and long-term capacity of growth of cSCC. (**a**) Xenograft experiment was performed in NSG mice by subcutaneous injection of lentiviraly transduced stable miR-130a–overexpressing UT-SCC-7 cells (Lv-miR-130a) or control UT-SCC-7 cells (Lv-Scramble) into each flank of the mice (n = 6). $*P < 0.05$, $**P < 0.01$, ANOVA. Tumors were monitored, and tumor volume was calculated weekly. (**b**) At the end of the experiment, tumors were excised and weighed (n = 6). $*P < 0.05$, Student's *t*-test. (**c**, **d**) Tumor sphere formation assay was performed in miR-130a–overexpressing UT-SCC-7 and A431 cells (n = 3). $**P < 0.01$, $***P < 0.001$, Student's *t*-test. Scale bar = 100 μm. (**e**, **f**) Tumor sphere formation assay was performed using UT-SCC-7 and A431 cells transfected with miR-130a-inhibitor (anti-miR-130a) or control ODN (anti-miR-Ctrl; n = 3). $****P < 0.0001$, Student's *t*-test. Data are presented as mean ± SEM. Scale bar = 100 μm. cSCC, cutaneous squamous cell carcinoma; Ctrl, control; Lv, lentiviral; NSG, NOD SCID gamma mice; ODN, oligodeoxyribonucleotide; Wk, week.

targets of miR-130a in the context of cSCC, we interrogated our recent RNA-sequencing data from human cSCC (Das Mahapatra et al., 2020) for potential miR-130a–target genes on the basis of the prediction of miRNA-binding sites in their 3′UTRs using TargetScan (Supplementary Figure S3a). One of the identified candidate genes was *ACVR1*, which is a member of the BMP type 1 receptors, whose increased expression in cSCC was observed by RNAseq (Supplementary Figure S3b) and was confirmed by qRT–PCR (Figure 4a). *ACVR1* has been previously identified as an oncogene that could regulate proliferative signaling, invasion, and metastasis (Kim et al., 2017; Romero et al., 2010). Western blot analysis demonstrated decreased expression of ACVR1 protein on ectopic overexpression of miR-130a both in UT-SCC-7 cells and in A431 cells (Figure 4b). Furthermore, consistent with in vitro results and patient data, we observed a decreased ACVR1 abundance both at mRNA and protein level in miR-130a–overexpressing tumor xenografts (Figure 4c). The decreased expression of ACVR1 on overexpression of miR-130a was further confirmed by the immunohistochemical analysis of ACVR1 in tumor tissues from the xenograft experiment (Supplementary Figure S3c).

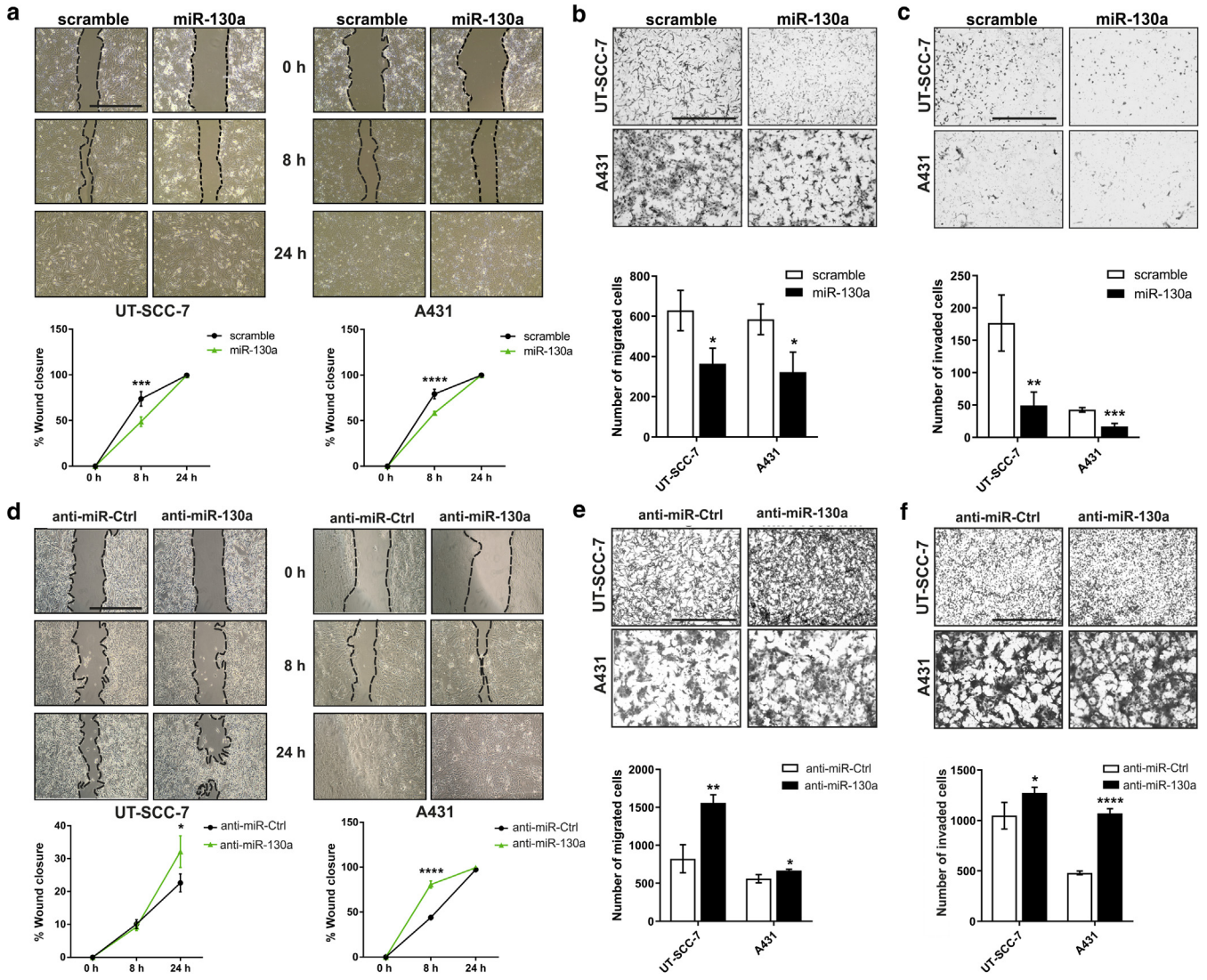

Figure 3. MiR-130a inhibits cell motility of cSCC cell lines. (**a**) Scratch wound healing experiments were performed with UT-SCC-7 (left) and A431 (right) cell lines upon ectopic overexpression of miR-130a or scramble ODNs. Wound closure was calculated relative to the wound size at 0 h (n = 3). ***$P < 0.001$, ****$P < 0.0001$, ANOVA. Scale bar = 1 mm. (**b**) Transwell migration and (**c**) Transwell invasion experiment of miR-130a-overexpressing or control UT-SCC-7 and A431 cSCC cell lines (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test. Scale bar = 1 mm. (**d**) Scratch wound-healing experiments were performed with UT-SCC-7 (left) and A431 (right) cell lines on transfection with miR-130a inhibitor or negative Ctrl. Healing percentage was calculated on the basis of the wound size at 0 h (n = 3). *$P < 0.05$, ****$P < 0.0001$, ANOVA. Scale bar = 1 mm. (**e**) Transwell migration and (**f**) Transwell invasion experiment of UT-SCC-7 and A431 SCC cell lines transfected with miR-130a inhibitor or negative control (n = 3). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, Student's *t*-test. Data are presented as mean ± SEM. Scale bar = 1 mm. cSCC, cutaneous squamous cell carcinoma; Ctrl, control; h, hour; ODN, oligodeoxyribonucleotide.

Because of the presence of a predicted miR-130a–binding site in the 3′UTR of *ACVR1* and because miR-130a regulated *ACVR1* expression both in cSCC cell lines and in tumor xenografts, we hypothesized that *ACVR1* may be a direct target of miR-130a. To test this hypothesis, we next performed a 3′UTR luciferase assay with wild-type and mutated *ACVR1* 3′UTR sequences (Figure 4d). We found that miR-130a suppressed the luciferase activity of wild-type *ACVR1* 3′UTR plasmid significantly both in normal human epidermal keratinocytes (NHEKs) and in UT-SCC-7 cells (Figure 4e and Supplementary Figure S3d). In contrast, the effect of miR-130a was abolished by the site-specific mutation of the predicted miR-130a–binding site within the *ACVR1* 3′UTR construct, demonstrating a sequence-specific interaction between miR-130a and *ACVR1* (Figure 4e and Supplementary Figure S3d).

Because ACVR1 elicits its cellular effects by activating specific SMAD proteins, including SMAD1, SMAD5, and SMAD8 (Omi et al., 2019), we next investigated the effect of miR-130a on SMAD1, SMAD5, and SMAD8 signaling by detecting nuclear translocation of phosphorylated SMAD (p-SMAD) 1 in primary human epidermal NHEKs. NHEKs were treated with 100 ng/ml recombinant human BMP7 for 45 minutes. Western blot analysis revealed that miR-130a overexpression decreased ACVR1 protein level and suppressed SMAD1 and SMAD5 phosphorylation in NHEKs, whereas its inhibition increased the level of ACVR1 and augmented SMAD1 and 5 phosphorylation (Figure 4f).

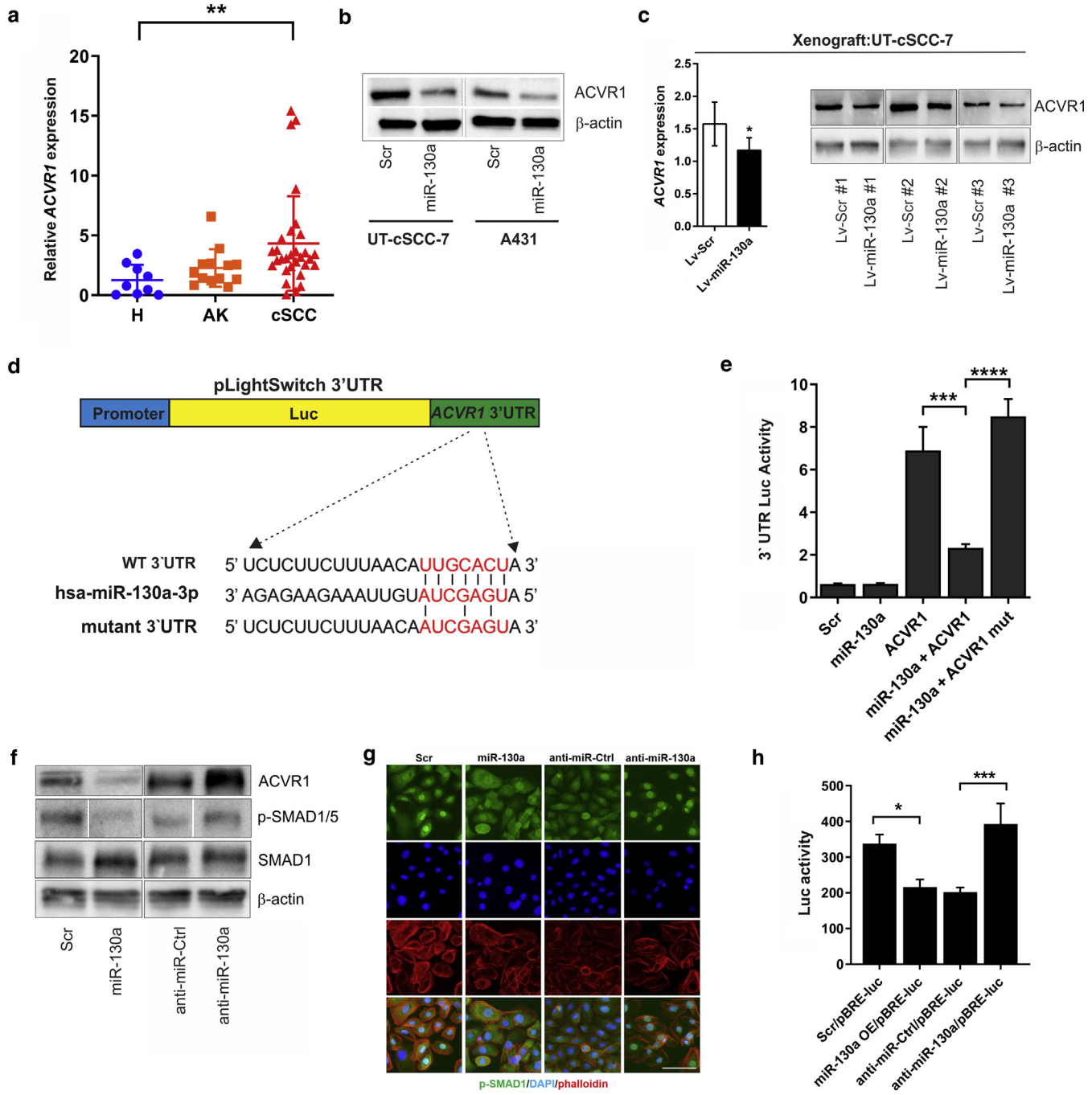

Figure 4. MiR-130a targets *ACVR1* and dampens phosphorylation of SMAD1in keratinocytes. (**a**) mRNA expression of *ACVR1* in healthy skin samples (n = 11), AK (n = 13), and cSCC (n = 28) by qRT-PCR. Mann–Whiney U-test. $^{**}P < 0.01$. (**b**) ACVR1 protein level was determined in cSCC cell lines after transfection with miR-130a mimic or Scr ODNs by western blot. (**c**) ACVR1 protein level was determined in tumors harvested from mice injected with miR-130a or Scr ODNs-overexpressing UT-SCC-7 cells by qRT-PCR and western blot (n = 6). $^{*}P < 0.05$, Student's *t*-test. (**d**) Schematic representation of the wild type and mutated binding site of miR-130a in the *ACVR1*-3′UTR luciferase assay. (**e**) *ACVR1* 3′UTR and mutant *ACVR1* 3′UTR were constructed, and Luc reporter assay was performed in NHEK cells (n = 3). $^{***}P < 0.001$, $^{****}P < 0.0001$, Student's *t*-test. (**f**) Western blot analysis of ACVR1 and p-SMAD1/5 levels in NHEK cells transfected with Scr ODNs, miR-130a mimic, miRNA inhibitor Ctrl (Neg), or miR-130a inhibitor. (**g**) p-SMAD1 nuclear translocation was analyzed by immunofluorescence with p-SMAD1/5 antibodies in NHEK cells after the overexpression or knockdown of miR-130a. Nucleus and cytoskeleton were stained by DAPI and phalloidin. Scale bar = 50 μm. (**h**) p-SMAD1/5/8-dependent promoter activity was determined by Luc reporter assay in NHEK cells after cotransfection of pBRE-luc plasmid and miR-130a mimic (miR-130a), control mimic (Scr), miR-130a inhibitor (anti-miR-130a) or control inhibitor (anti-miR-Ctrl) (n = 3). $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t*-test. Data are presented as mean ± SEM. AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; Ctrl, control; H, healthy human skin; Luc, luciferase; Lv, lentiviral; Neg, negative; NHEK, normal human epidermal keratinocyte; miRNA, microRNA; mut, mutated; ODN, oligodeoxyribonucleotide; OE, overexpression; p-SMAD, phosphorylated SMAD; qRT-PCR, quantitative real-time reverse transcriptase–PCR; Scr, scrambled; UTR, untranslated region; WT, wild-type.

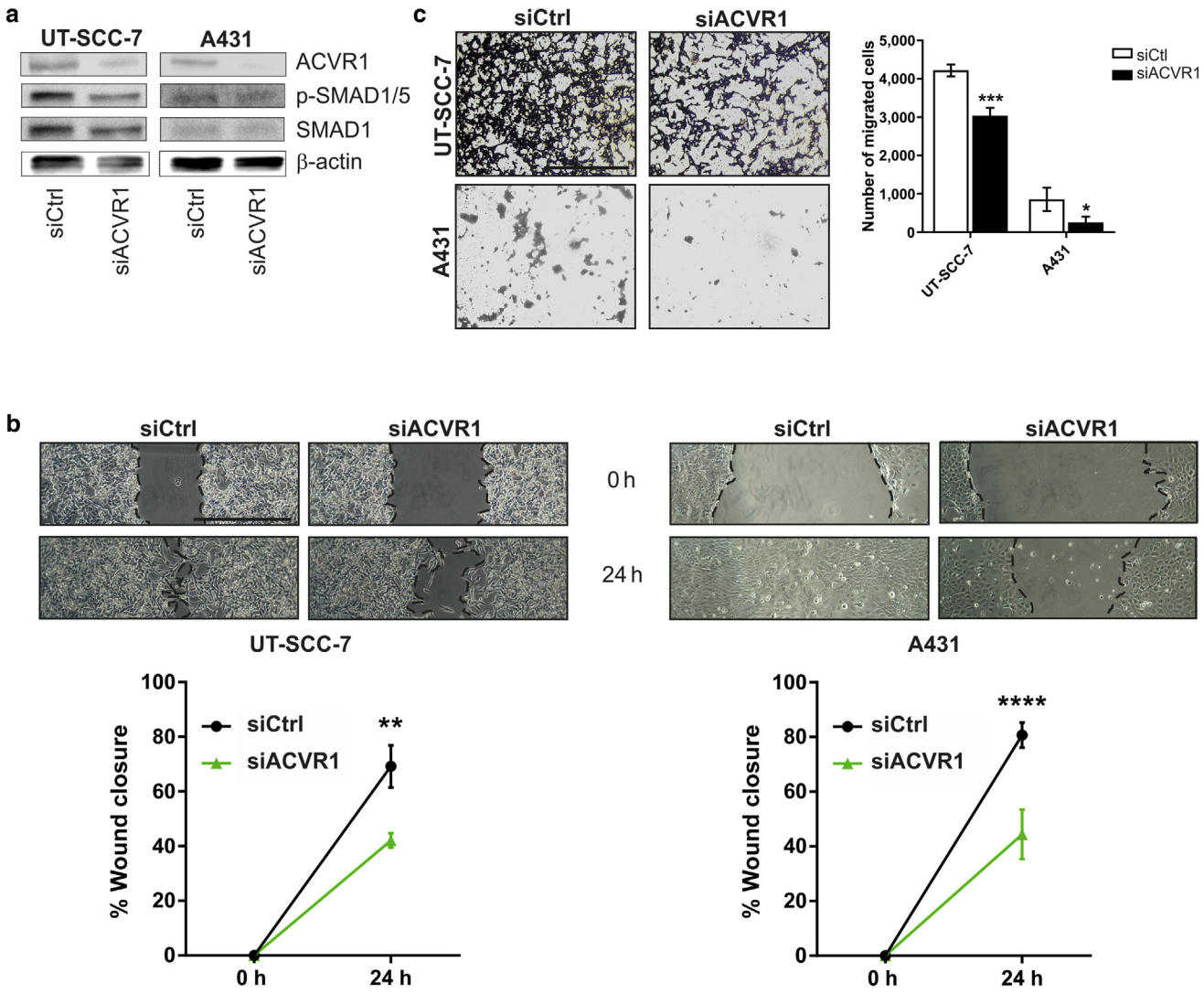

Figure 5. Knockdown of *ACVR1* phenocopies the effect of miR-130a-overexpression on cSCC motility. (**a**) Western blot analysis of ACVR1 protein level in cSCC cell lines after siACVR1 transfection (n =3). (**b**) Scratch wound-healing experiments were performed with UT-SCC-7 and A431 cell lines after siACVR1-transfection. Percentage of wound closure was calculated relative to the wound size at 0 h (n = 3). $^{**}P < 0.01$, $^{****}P < 0.0001$, ANOVA. Scale bar = 1 mm. (**c**) Transwell migration experiment with UT-SCC-7 and A431 cSCC cell lines transfected with siACVR1 or control (siCtrl). The number of migrated cells (mean ± SEM) is shown (n = 3). $^{*}P < 0.05$, $^{***}P < 0.001$, Student's *t*-test. Scale bar = 1 mm. cSCC, cutaneous squamous cell carcinoma; h, hour; p-SMAD, phosphorylated SMAD; siACVR1, small interfering RNA ACVR1; siCtrl, small interfering RNA control.

Consistent with these findings, immunofluorescence analysis demonstrated that miR-130a overexpression inhibited the translocation of p-SMAD1 into the nucleus (Figure 4g). In contrast, depletion of miR-130a resulted in an increased nuclear p-SMAD1 staining (Figure 4g). To analyze whether miR-130a can modulate SMAD-dependent DNA-binding activity in cells, we next performed a luciferase reporter assay using pGL3-BRE-luc plasmid, a BMP-responsive luciferase reporter construct known to respond to p-SMAD1, p-SMAD5, and p-SMAD8 (Korchynskyi and ten Dijke, 2002). Results of promoter-luciferase reporter assays demonstrated that overexpression of miR-130a significantly decreased SMAD-dependent promoter activity in NHEKs. In contrast, depletion of miR-130a resulted in a significantly increased SMAD-dependent promoter activity (Figure 4h), thus demonstrating that miR-130a can regulate BMP/SMAD signaling in NHEKs.

To further corroborate this conclusion and to determine whether ACVR1 can mediate the effects of miR-130a in cSCC, scratch wound-healing and cell-migration assays using UT-SCC-7 and A431 cells were carried out after small interfering RNA–mediated knockdown of *ACVR1*. Knockdown of ACVR1 was confirmed by western blot (Figure 5a), which resulted in a decrease in the level of p-SMAD1/5 (Figure 5a). Furthermore, similar to the effect of miR-130a-overexpression, knockdown of *ACVR1* significantly inhibited migration ability of both UT-SCC-7 and in A431 cells (Figure 5b). Moreover, recapitulating the effect of miR-130 overexpression, silencing of *ACVR1* significantly reduced the number of migrating cells through Transwell inserts (Figure 5c).

Next, we investigated whether the ectopic expression of ACVR1 could counteract the effect of miR-130a-

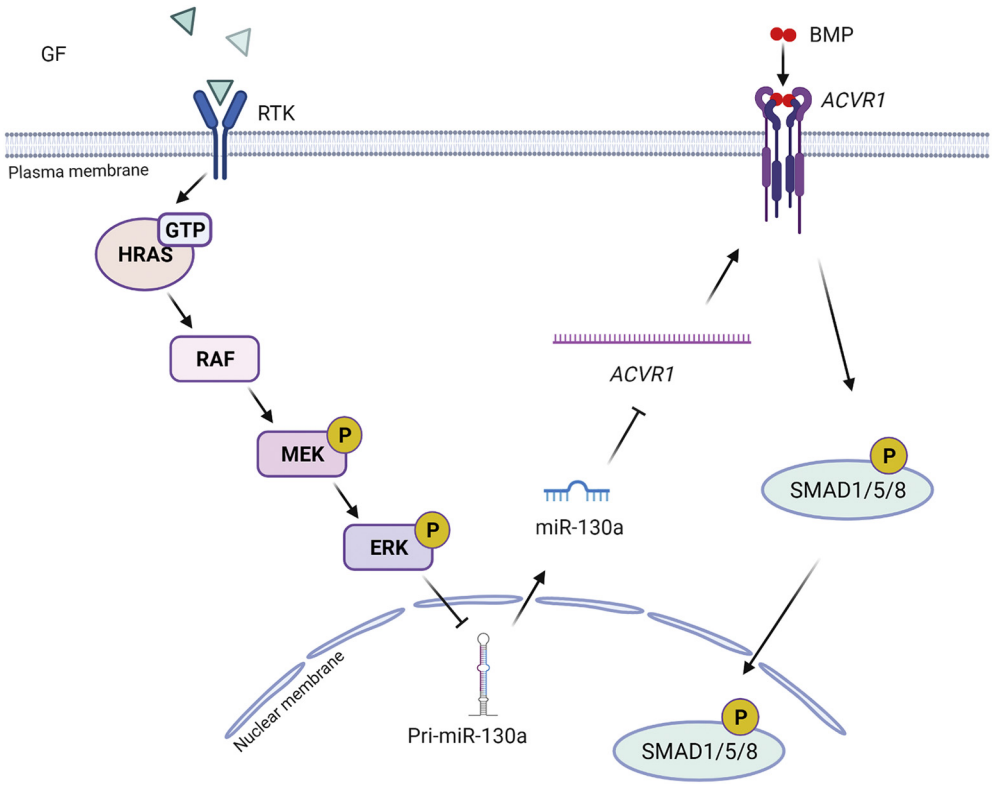

Figure 6. Schematic model about the regulation and mode of action of miR-130a in cSCC. Activation of the MAPK pathway can suppress miR-130a transcription through HRAS–MEK–ERK1/2, leading to decreased miR-130a level in cSCC. Decreased intracellular level of miR-130a may lead to increased expression of *ACVR1*, the direct target of miR-130a, which in turn activates SMAD signaling and cancer-associated phenotypes. cSCC, cutaneous squamous cell carcinoma; ERK, extracellular signal–regulated kinase; GTP, guanosine triphosphate; MEK, MAPK/extracellular signal–regulated kinase kinase.

overexpression. To this end, cSCC cell lines were transfected with miR-130a and an ACVR1-plasmid containing ACVR1 open reading frame without its 3′UTR (pCMV-ACVR1), which we refer to as miR-130a-resistant ACVR1. Ectopic expression of miR-130a–resistant ACVR1 (Supplementary Figure S4a) could reverse miR-130a–mediated suppression of SMAD1/5 phosphorylation in UT-SCC-7 cells (Supplementary Figure S4b) and reverse the miR-130a–mediated suppression of cell migration and invasion both in UT-SCC-7 and in A431 cells (Supplementary Figure S5a and b).

Taken together, our data suggest that activation of the MAPK signaling pathway in cSCC results in suppression of miR-130a expression through HRAS–MEK–ERK1/2. In turn, decreased level of miR-130a may lead to increased ACVR1 protein level and increased activity of the BMP/SMAD pathway, which may contribute to the malignant phenotype of cSCC (Figure 6).

DISCUSSION

In this study, we report that miR-130a is downregulated in cSCC and is involved in key cellular functions related to long-term capacity of growth, cell motility, and cell invasion of cSCC in vitro as well as in impaired primary tumor growth in vivo. Our results suggest that decreased miR-130a expression may be a consequence of the activated MAPK pathway in cSCC. We identify *ACVR1* as a target of miR-130a and demonstrate that through regulating ACVR1, it regulates BMP/SMAD1 signaling pathway and acts as a tumor suppressor in cSCC.

Our results showing that miR-130a is suppressed in cSCC but not in precancerous skin lesions suggested that it may be related to the malignant phenotype of cSCC. Interestingly, activation of the MAPK pathway was recently identified as one of the key molecular differences between AKs and cSCCs (Lambert et al., 2014), and we found that the expression of miR-130a was regulated by the MAPK pathway. Aberrant activation of driver genes and loss-of-function alterations in tumor suppressor genes have been reported in cSCC pathogenesis. MAPK pathway is one of the most commonly activated pathways that contribute to cSCC pathogenesis (Di Nardo et al., 2020). It can be activated by overexpression of growth factors and amplification or mutations of receptor tyrosine kinases such as *EGFR* as well as mutation of RAS guanosine triphosphatases (Braicu et al., 2019; Katz et al., 2007). This pathway is also the molecular target of drugs that are used to treat cSCCs (Maubec et al., 2011). In humans, cSCCs harbor activated mutations in *HRAS* at a frequency of 3–20% (Li et al., 2015; Pickering et al., 2014) and *HRAS* has been demonstrated to both directly and indirectly contribute to cSCC initiation and progression (Maurelli et al., 2016; Pickering et al., 2014; White et al., 2011). The impact of oncogenic *HRAS* on the miRNA landscape of NHEKs has been previously reported by Riemondy et al. (2015) who by performing quantitative miRNA sequencing in $HRAS^{G12V}$-transformed murine NHEKs could identify 15 differentially expressed miRNAs on $HRAS^{G12V}$ expression. However, to our knowledge, the regulation of miR-130a by *HRAS* oncogene has not been reported before our study. Further studies will be needed to clarify whether the suppression of miR-130a by oncogenic MAPK pathway acts as a downstream effector of Ras-mediated transformation and whether drugs targeting the MAPK pathway can also regulate miR-130a-expression in cSCC.

Our findings suggest that miR-130a acts as a tumor suppressor miRNA in cSCC. We observed decreased tumor growth in vivo after miR-130a-overexpression, which was in accordance with our in vitro findings about that miR-130a suppressed several important cancer hallmarks, including the long-term capacity of growth, cell migration, and cell invasion. Altered miRNA expression has been implicated in the initiation and progression of virtually all human cancers (Peng and Croce, 2016). In particular, miR-130a has been reported as a tumor suppressor in several cancer types, such as in hepatocellular carcinoma, prostate carcinoma, breast cancer, and non–small cell lung cancer cells, and it has been shown to suppresses cell viability, apoptosis, migration, and invasion in these diseases (Li et al., 2014; Pan et al., 2015; Wang et al., 2018a). Interestingly, decreased miR-130a level has been linked to docetaxel resistance in head and neck squamous cell carcinoma (Dai et al., 2011).

In our study, we identified *ACVR1* as a direct target of miR-130a. ACVR1, a member of BMP type-I receptors, has been implicated in multiple types of cancers, including breast cancer (Wang et al., 2018b) and diffuse intrinsic pontine glioma (Taylor et al., 2014). Of note, a clinical trial of oral ACVR1 inhibitor is currently being conducted in patients with advanced solid tumors. The BMP signaling cascade regulates diverse processes across multiple organ systems, including cell proliferation, tissue stem cell dynamics, organogenesis, tissue remodeling, and physiologic homeostasis (Waite and Eng, 2003). Dysregulation of this signaling pathway has been implicated in a variety of human diseases, including vascular disorders, skeletal defects, congenital heart disease, and cancers (Massagué, 2008). BMP ligands transduce their signals through type-I and type-II serine-threonine kinase transmembrane receptors. Activated BMP type-I receptors initiate intracellular signaling through phosphorylating specific SMAD proteins, including SMAD1, SMAD5, and SMAD8 (Korchynskyi and ten Dijke, 2002; Omi et al., 2019). In this study, we showed that miR-130a regulated the phosphorylation and nuclear translocation of SMAD1/5 by regulating ACVR1 expression. No miRNA functions through the regulation of a single target gene, and miR-130a probably also regulates several target genes in cSCC in addition to ACVR1. Of relevance to our study, miR-130a has also been reported to regulate the SMAD pathway by directly targeting SMAD4 and mediate gemcitabine sensitivity in hepatoma cells (Liu et al., 2016). Furthermore, miR-130a has been shown to suppress migration and invasion by targeting RAB5B in human breast cancer stem cell–like cells (Kong et al., 2018). Moreover, miR-130a has been shown to regulate cell growth by targeting TNF-α and forms a feedback loop with NF-κB in cervical cancer (Zhang et al., 2014).

Collectively, our results suggest that miR-130a acts as a tumor suppressor in cSCC and that it regulates the activity of the BMP/SMAD pathway. In conclusion, we report that miR-130a expression is suppressed by the activation of the MAPK pathway. Thus, this study reveals an important role for miR-130a in cSCC tumorigenesis and suggests that the restoration of miR-130a, by modulating miRNAs or by modulating the activity of the MAPK pathway, may be relevant for the treatment of cSCC.

MATERIALS AND METHODS

Clinical samples

After obtaining written informed consent, biopsies were taken from the skin of healthy donors and the skin of patients with cSCC at the Dermatology and Venereology Unit, Karolinska University Hospital, Stockholm, Sweden and at the Department of Dermatology, Heinrich Heine University, Düsseldorf, Germany. The clinical diagnosis was made by a dermatologist and confirmed by histopathological evaluation. The formalin-fixed, paraffin-embedded normal skin and cSCC biopsies were obtained from Karolinska University Hospital Biobank. The study was approved by the Regional Ethics Committees and was conducted according to the Declaration of Helsinki Principles. RNA was extracted from frozen biopsies or formalin-fixed, paraffin-embedded tissue sections using the method described previously (Xu et al., 2011) or miRNeasy FFPE Kit (Qiagen, Sollentuna, Sweden), respectively.

Further details for cell culture, transfections and transductions, qRT-PCR, locked nucleic acid in situ hybridization, tumor xenograft experiment, immunostaining, western blot, in vitro functional assay, luciferase reporter assay, and statistical analysis are available in Supplementary Materials and Methods online.

Data availability statement

Data sharing is not applicable to this article because no data sets were generated or analyzed during the study. Data supporting the findings of this study are available within the article and in its Supplementary Materials.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

The study was supported by the Swedish Skin Research Foundation (Hudfonden/Welander and Finsens Foundation), the Swedish Cancer Society (Cancerfonden, Stockholm, Sweden), the LEO Foundation (Ballerup, Denmark), Swedish Research Council (Vetenskapsrådet, Stockholm, Sweden), the Stockholm County Council (Sweden) (SLL), Avtal om Läkarutbildning och Forskning (ALF Medicin), Deutsche Forschungsgemeinschaft (DFG) (FOR2690 Ho 2092/7-1 and ALF Medicine 20190367).

AUTHOR CONTRIBUTIONS

Conceptualization: AP, ES, WL; Data Curation: WL, CL, KDM; Formal Analysis: WL, CL, KDM, AP; Funding Acquisition: AP, ES; Investigation: WL, CL, KDM; Project Administration: AP, ES; Resources: JL, BH, ES, AP; Supervision: AP, ES; Validation: WL, CL; Visualization: WL, CL; Writing - Original Draft Preparation: WL, CL; Writing - Review and Editing: WL, CL, KDM, JL, BH, ES, AP

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.2021.01.028.

REFERENCES

Alam M, Ratner D. Cutaneous squamous-cell carcinoma. N Engl J Med 2001;344:975–83.

Alvarez-Garcia I, Miska EA. MicroRNA functions in animal development and human disease. Development 2005;132:4653–62.

Ashton KJ, Weinstein SR, Maguire DJ, Griffiths LR. Chromosomal aberrations in squamous cell carcinoma and solar keratoses revealed by comparative genomic hybridization. Arch Dermatol 2003;139:876–82.

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.

Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215–33.

Benaich N, Woodhouse S, Goldie SJ, Mishra A, Quist SR, Watt FM. Rewiring of an epithelial differentiation factor, miR-203, to inhibit human squamous cell carcinoma metastasis. Cell Rep 2014;9:104–17.

Braicu C, Buse M, Busuioc C, Drula R, Gulei D, Raduly L, et al. A comprehensive review on MAPK: a promising therapeutic target in cancer. Cancers (Basel) 2019;11:1618.

Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.

Dai Y, Xie CH, Neis JP, Fan CY, Vural E, Spring PM. MicroRNA expression profiles of head and neck squamous cell carcinoma with docetaxel-induced multidrug resistance. Head Neck 2011;33:786–91.

Das Mahapatra K, Pasquali L, Søndergaard JN, Lapins J, Nemeth IB, Baltás E, et al. A comprehensive analysis of coding and non-coding transcriptomic changes in cutaneous squamous cell carcinoma. Sci Rep 2020;10:1–12.

Di Nardo L, Pellegrini C, Di Stefani A, Del Regno L, Sollena P, Piccerillo A, et al. Molecular genetics of cutaneous squamous cell carcinoma: perspective for treatment strategies. J Eur Acad Dermatol Venereol 2020;34:932–41.

Dotto GP, Rustgi AK. Squamous cell cancers: a unified perspective on biology and genetics. Cancer Cell 2016;29:622–37.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al. Identification of a novel inhibitor of mitogen-activated protein kinase. J Biol Chem 1998;273:18623–32.

Iorio MV, Croce CM. microRNA involvement in human cancer. Carcinogenesis 2012;33:1126–33.

Katz M, Amit I, Yarden Y. Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochim Biophys Acta 2007;1773:1161–76.

Kim M, Choi O, Pyo S, Choi SU, Park CH. Identification of novel ALK2 inhibitors and their effect on cancer cells. Biochem Biophys Res Commun 2017;492:121–7.

Kong XZ, Zhang J, Li J, Shao J, Fang L. MiR-130a-3p inhibits migration and invasion by regulating RAB5B in human breast cancer stem cell-like cells. Biochem Biophys Res Commun 2018;501:486–93.

Korchynskyi O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 2002;277:4883–91.

Lambert SR, Mladkova N, Gulati A, Hamoudi R, Purdie K, Cerio R, et al. Key differences identified between actinic keratosis and cutaneous squamous cell carcinoma by transcriptome profiling. Br J Cancer 2014;110:520–9.

Li BH, Huang P, Qiu J, Liao Y, Hong J, Yuan Y. MicroRNA-130a is down-regulated in hepatocellular carcinoma and associates with poor prognosis. Med Oncol 2014;31:230.

Li YY, Hanna GJ, Laga AC, Haddad RI, Lorch JH, Hammerman PS. Genomic analysis of metastatic cutaneous squamous cell carcinoma. Clin Cancer Res 2015;21:1447–56.

Liu YL, Li Y, Wang R, Qin S, Liu J, Su F, et al. MiR-130a-3p regulates cell migration and invasion via inhibition of Smad4 in gemcitabine resistant hepatoma cells. J Exp Clin Cancer Res 2016;35:19.

Lohcharoenkal W, Harada M, Lovén J, Meisgen F, Landén NX, Zhang L, et al. MicroRNA-203 inversely correlates with differentiation grade, targets c-MYC, and functions as a tumor suppressor in cSCC. J Invest Dermatol 2016;136:2485–94.

Massagué J. TGFbeta in cancer. Cell 2008;134:215–30.

Maubec E, Petrow P, Scheer-Senyarich I, Duvillard P, Lacroix L, Gelly J, et al. Phase II study of cetuximab as first-line single-drug therapy in patients with unresectable squamous cell carcinoma of the skin. J Clin Oncol 2011;29:3419–26.

Maurelli R, Tinaburri L, Gangi F, Bondanza S, Severi AL, Scarponi C, et al. The role of oncogenic Ras in human skin tumorigenesis depends on the clonogenic potential of the founding keratinocytes. J Cell Sci 2016;129:1003–17.

Morris EJ, Jha S, Restaino CR, Dayananth P, Zhu H, Cooper A, et al. Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. Cancer Discov 2013;3:742–50.

Omi M, Kaartinen V, Mishina Y. Activin A receptor type 1–mediated BMP signaling regulates RANKL-induced osteoclastogenesis via canonical SMAD-signaling pathway. J Biol Chem 2019;294:17818–36.

Pan Y, Wang R, Zhang F, Chen Y, Lv Q, Long G, et al. MicroRNA-130a inhibits cell proliferation, invasion and migration in human breast cancer by targeting the RAB5A. Int J Clin Exp Pathol 2015;8:384–93.

Peng Y, Croce CM. The role of microRNAs in human cancer. Signal Transduct Target Ther 2016;1:15004.

Pickering CR, Zhou JH, Lee JJ, Drummond JA, Peng SA, Saade RE, et al. Mutational landscape of aggressive cutaneous squamous cell carcinoma. Clin Cancer Res 2014;20:6582–92.

Que SKT, Zwald FO, Schmults CD. Cutaneous squamous cell carcinoma: incidence, risk factors, diagnosis, and staging. J Am Acad Dermatol 2018;78:237–47.

Riemondy KW, Wang XJ, Torchia EC, Roop DR, Yi R. MicroRNA-203 represses selection and expansion of oncogenic Hras transformed tumor initiating cells. Elife 2015;4:e07004.

Romero D, Terzic A, Conley BA, Craft CS, Jovanovic B, Bergan RC, et al. Endoglin phosphorylation by ALK2 contributes to the regulation of prostate cancer cell migration. Carcinogenesis 2010;31:359–66.

Salasche SJ. Epidemiology of actinic keratoses and squamous cell carcinoma. J Am Acad Dermatol 2000;42:4–7.

Taylor KR, Mackay A, Truffaux N, Butterfield Y, Morozova O, Philippe C, et al. Recurrent activating ACVR1 mutations in diffuse intrinsic pontine glioma. Nat Genet 2014;46:457–61.

Waite KA, Eng C. From developmental disorder to heritable cancer: it's all in the BMP/TGF-beta family. Nat Rev Genet 2003;4:763–73.

Wang A, Landén NX, Meisgen F, Lohcharoenkal W, Ståhle M, Sonkoly E, et al. MicroRNA-31 is overexpressed in cutaneous squamous cell carcinoma and regulates cell motility and colony formation ability of tumor cells. PLoS One 2014;9:e103206.

Wang SH, Han H, Hu Y, Yang W, Lv Y, Wang L, et al. MicroRNA-130a-3p suppresses cell migration and invasion by inhibition of TBL1XR1-mediated EMT in human gastric carcinoma. Mol Carcinog 2018a;57:383–92.

Wang YZ, Zhang Z, Wang J. MicroRNA-384 inhibits the progression of breast cancer by targeting ACVR1. Oncol Rep 2018b;39:2563–74.

Wells JL 3rd, Shirai K. Systemic therapy for squamous cell carcinoma of the skin in organ transplant recipients. Am J Clin Oncol 2012;35:498–503.

White AC, Tran K, Khuu J, Dang C, Cui Y, Binder SW, et al. Defining the origins of Ras/p53-mediated squamous cell carcinoma. Proc Natl Acad Sci USA 2011;108:7425–30.

Xu N, Brodin P, Wei T, Meisgen F, Eidsmo L, Nagy N, et al. MiR-125b, a microRNA downregulated in psoriasis, modulates keratinocyte proliferation by targeting FGFR2. J Invest Dermatol 2011;131:1521–9.

Xu N, Zhang L, Meisgen F, Harada M, Heilborn J, Homey B, et al. MicroRNA-125b down-regulates matrix metallopeptidase 13 and inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion. J Biol Chem 2012;287:29899–908.

Zhang J, Wu H, Li P, Zhao Y, Liu M, Tang H. NF-κB-modulated miR-130a targets TNF-α in cervical cancer cells. J Transl Med 2014;12:155.