ORIGINAL ARTICLE

Long Noncoding RNA PICSAR Promotes Growth of Cutaneous Squamous Cell Carcinoma by Regulating ERK1/2 Activity

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Keratinocyte-derived cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer, and its incidence is increasing globally. Long noncoding RNAs (lncRNA) are involved in various biological processes, and their role in cancer progression is emerging. Whole transcriptome analysis of cSCC cells (n = 8) and normal human epidermal keratinocytes (n = 4) revealed overexpression of long intergenic ncRNA (LINC00162) in cSCC cells. The expression of LINC00162 in cSCC cells was upregulated by inhibition of the p38α and p38δ mitogen-activated protein kinases. Analysis of tissue sections by RNA in situ hybridization showed that LINC00162 is specifically expressed by tumor cells in cSCCs but not by keratinocytes in normal skin in vivo. Knockdown of LINC00162 inhibited proliferation and migration of cSCC cells, and suppressed the growth of human cSCC xenografts in vivo. Furthermore, knockdown of LINC00162 inhibited extracellular signal-regulated kinase 1/2 activity and upregulated expression of dual specificity phosphatase 6 (DUSP6) in cSCC cells. Based on these observations, LINC00162 was named p38 inhibited cutaneous squamous cell carcinoma associated lincRNA (PICSAR). Our results provide mechanistic evidence for the role of PICSAR in promoting cSCC progression via activation of extracellular signal-regulated kinase 1/2 signaling pathway by down-regulating DUSP6 expression. These results also identify PICSAR as a biomarker and putative therapeutic target in cSCC.

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INTRODUCTION

Epidermal keratinocyte-derived cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer, the incidence of which is increasing worldwide (Madan et al., 2010; Ratushny et al., 2012). Solar UV radiation, chronic ulcers, and immunosuppression have been recognized as important risk factors for cSCC (Kivisaari and Kähäri, 2013). Primary cSCCs harbor a tendency for recurrence and metastasis, and the prognosis of metastatic cSCCs remains unfavorable in the absence of targeted therapies (Czarnecki et al., 2002). An important event in keratinocyte carcinogenesis is UV-induced mutation of tumor protein 53 gene (*TP53*), which is followed by marked accumulation of UV-induced simple mutations (Durinck et al., 2011). Another early event in the development of cSCC is loss-of-function mutation of *NOTCH1* (South et al., 2014; Wang et al., 2011). Transcription of *NOTCH1* is regulated by p53, and inactivation of p53 can lead to downregulation of NOTCH1 expression (Lefort et al., 2007). Mutations in *HRAS*, *KRAS*, and *EGFR* have also been detected in cSCC, emphasizing the role of EGFR and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in development of cSCC (Ratushny et al., 2012). Recently, previously unreported markers for progression of cSCC have been identified and characterized (Farshchian et al., 2011, 2015; Liu et al., 2013; Riihilä et al., 2014, 2015; Trimmer et al., 2012), but understanding of the molecular basis of cSCC progression from premalignant lesion (actinic keratosis, AK) to cSCC in situ (cSCCIS) and eventually to invasive cSCC remains incomplete.

Advances in whole genome sequencing have revealed that human genome is extensively transcribed into RNA, but only a fraction of the RNA transcripts encode proteins (Djebali et al., 2012). The sequence of protein coding genes comprise approximately 2% of the human genome and the majority of the remaining genomic sequences are transcribed into a wide range of noncoding RNAs, including microRNAs, small nucleolar RNAs, piwi-interacting RNAs, and long noncoding RNAs (lncRNAs) (International Human Genome Sequencing Consortium, 2004). LncRNAs are a diverse and largely uncharacterized group of noncoding transcripts. Currently, more than 18,000 annotated lncRNA transcripts have been identified in the human genome and they are

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Abbreviations: AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; cSCCIS, cSCC in situ; DUSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; FC, fold change; lncRNA, long noncoding RNA; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NHEK, normal human epidermal keratinocyte; PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; qRT-PCR, quantitative real-time reverse transcriptase-PCR; RNA-ISH, RNA in situ hybridization

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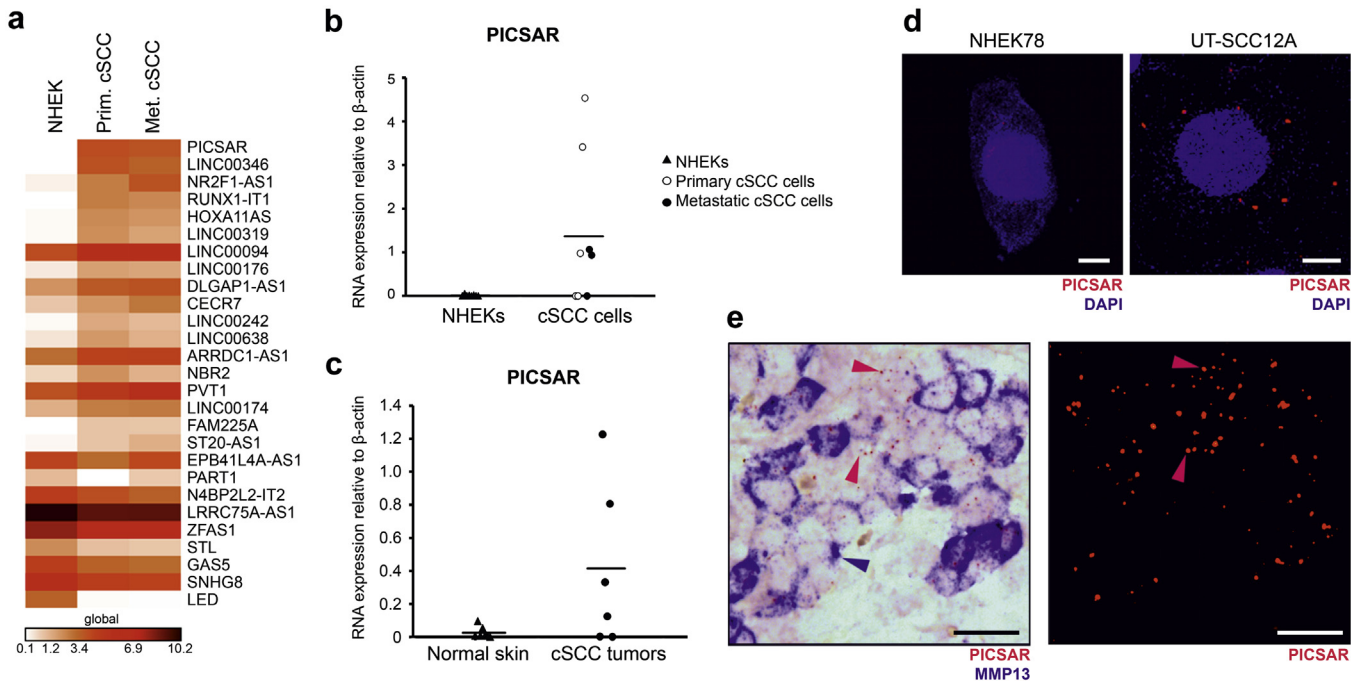

Figure 1. Expression of PICSAR is specifically upregulated in cutaneous squamous cell carcinoma (cSCC) cells. (**a**) The heatmap of whole transcriptome analysis (SOLiD) showing significantly ($P < 0.05$) regulated lncRNAs in primary (Prim; n = 5) and metastatic (Met; n = 3) cSCC cell lines and in normal human epidermal keratinocytes (NHEK) (n = 4). LncRNA genes were identified based on the catalogue of annotated lncRNAs by The Human Genome Organization Gene Nomenclature Committee. Statistical analysis was performed with the Mann-Whitney *U*-test. (**b**) Expression of PICSAR in the same cSCC cell lines and in NHEKs (n = 8) was determined by qRT-PCR. β-Actin was used as a reference gene. (**c**) Expression of PICSAR in cSCC in vivo (n = 6) and normal skin (n = 7) was determined by qRT-PCR. β-Actin was used as a reference gene. (**d**) Expression of PICSAR in cSCC (UT-SCC12A) and NHEKs (NHEK78) was determined by RNA in situ hybridization (RNA-ISH) and visualized by fluorescence microscopy. Scale bar = 10 μm. (**e**) Left panel: expression PICSAR (red arrows) and matrix metalloproteinase-13 (MMP-13) (blue arrows) in xenograft tumors established in SCID mice with human UT-SCC12A cells was determined with RNA-ISH and visualized with two chromogens. Right panel: the fluorescence signal for PICSAR corresponds to the red chromogenic signal of the alkaline phosphatase (red arrow) in the same TYPE 6 probe set used for PICSAR in the left panel. Scale bar = 25 μm. PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; qRT-PCR, quantitative real-time reverse transcriptase-PCR; SCID, severe combined immunodeficient.

classified into long intergenic noncoding RNAs (lincRNAs), intronic lncRNAs, or natural antisense transcripts (Bhartiya et al., 2013; Moran et al., 2012). LncRNAs can function in cell regulation in several ways. They can modulate gene expression by regulating binding and activity of transcription factors, and they can regulate mRNA stability directly or by interacting with microRNAs (Geisler and Coller, 2013; Moran et al., 2012; Rinn and Chang, 2012). Recent observations have emphasized the role of lncRNAs in cancer progression, suggesting them as important biomarkers and therapeutic targets in different malignant tumors (Serviss et al., 2014; Wahlestedt, 2013; Yang et al., 2014).

In this study, we have identified a previously uncharacterized lincRNA, LINC00162, which is specifically overexpressed by cSCC cells in culture and in vivo. The expression of LINC00162 in cSCC cells was upregulated by inhibition of p38α and p38δ mitogen-activated protein kinases (MAPKs). Knockdown of LINC00162 inhibited ERK1/2 activity and proliferation and migration of cSCC cells, and markedly suppressed the growth of human cSCC xenograft tumors in vivo. On the basis of these observations and with the permission of Human Genome Organization Gene Nomenclature Committee, we have named this lincRNA P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA (PICSAR). These results indicate the oncogenic role for this lincRNA and identify PICSAR as a biomarker for progression of cSCC and a potential therapeutic target in this malignant tumor of skin.

RESULTS

Expression of PICSAR is specifically upregulated in cSCC cells

The whole transcriptome analysis of cSCC cell lines (n = 8) and normal human epidermal keratinocytes (NHEKs; n = 4) was performed with RNA-seq and lncRNA genes were identified based on the catalogue of annotated lncRNAs by The Human Genome Organization Gene Nomenclature Committee. The gene expression analysis revealed differential expression of several lncRNAs in cSCC cells compared with NHEKs (Figure 1a, Supplementary Figure S1 online). Among these, PICSAR (*LINC00162*) was the most upregulated lncRNA in cSCC cells compared with NHEKs (Figure 1a). Analysis with quantitative real-time reverse transcriptase-PCR (qRT-PCR) verified overexpression of PICSAR in cSCC cells lines (n = 8) and in cSCC tissues in vivo (n = 6), whereas the expression in NHEKs (n = 8) and normal skin (n = 7) was very low (Figure 1b and c). The analysis of PICSAR expression with RNA in situ hybridization (RNA-ISH) revealed specific mainly cytoplasmic signal for PICSAR in cSCC cells but not in NHEKs in culture (Figure 1d, Supplementary Figure S2 online). Furthermore, analysis of tissue sections of xenografts established with human cSCC cells (UT-SCC12A) with RNA-ISH

revealed specific expression of PICSAR in tumor cells (Figure 1e). Expression of matrix metalloproteinase-13 mRNA was detected in cSCC cells in the same xenografts as a positive control (Airola et al., 1997; Stokes et al., 2010).

PICSAR is expressed by cSCC tumor cells in vivo

To study the expression of PICSAR during cSCC progression in vivo, tissue microarrays consisting of tissue samples representing different stages of epidermal carcinogenesis, that is, normal skin (n = 9), UV-induced premalignant lesions (AK; n = 26), cSCCIS (n = 20), and invasive cSCCs (n = 21) were analyzed using RNA-ISH. Expression of PICSAR was detected in tumor cells in AK, cSCCIS, and cSCC, whereas no signal was detected in normal skin (Figure 2a–d). Analysis of the PICSAR positive tissue sections revealed that the number of positive sections increased with the progression from AK (23%) to cSCCIS (35%) and cSCC (43%), whereas all sections of normal skin were negative (Figure 2e).

Expression of PICSAR is downregulated by p38 MAPK pathway

Previous studies have demonstrated basal activation of ERK1/2 and p38 MAPKs in cSCC cells in culture and in vivo (Junttila et al., 2007; Kivisaari et al., 2010; Toriseva et al., 2012). To examine the regulation of basal PICSAR expression by ERK1/2 and p38 pathways, cSCC cells were treated for 24 hours with MAPK/ERK kinase 1/2 (MEK1/2) inhibitor (PD98059) and p38 inhibitors specific for p38α/β (SB203580) or all p38 isoforms (p38α/β/γ/δ) (BIRB796). Treatment of cSCC cells with BIRB796 significantly upregulated PICSAR expression, whereas treatment with PD98059 or SB203580 had no effect (Figure 3a). To verify the regulation of PICSAR by p38α and p38δ, cSCC cells were transfected with p38α and p38δ targeted siRNAs alone and in combination. Consistent with the results obtained with the chemical p38 inhibitors, knockdown of p38δ alone and in combination with p38α resulted in significant upregulation of PICSAR in cSCC cells (Figure 3b). In addition, adenoviral delivery of dominant negative mutant of MAP kinase kinase 3b (MKK3b) (RAdMKK3bA; Wang et al., 1998), the upstream activator of p38α and p38δ resulted in elevated expression of PICSAR (Figure 3c).

Knockdown of PICSAR inhibits proliferation and migration of cSCC cells

To study the cellular functions of PICSAR, cSCC cells were transfected with two PICSAR targeted siRNAs and control siRNA and harvested 3 days after transfection. Effective knockdown of PICSAR expression in cSCC cells was detected using RNA-ISH (Figure 4a, lower panel; Supplementary Figure S3 online). Analysis with qRT-PCR indicated that PICSAR expression was decreased by 95–97% with siRNA1 and by 74–91% with siRNA2 (Figure 4a, upper panel; Supplementary Figure S4a online), as compared with control siRNA transfected cultures. Analysis of cell proliferation revealed that the number of viable cSCC cells was significantly diminished after PICSAR knockdown compared with control siRNA-transfected cultures using two different cSCC cell lines (Figure 4b, Supplementary Figure S4b). Analysis of the corresponding cell lysates showed that PICSAR knockdown resulted in a marked decrease in the levels of p-ERK1/2 and marker of proliferation Ki-67 (Figure 4c, Supplementary Figure S4c). In contrast, knockdown of PICSAR had no effect on the activity of p38, c-Jun N-terminal kinase (JNK), or acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (Akt) (data not shown).

Analysis of cell migration after knockdown of PICSAR expression was performed with two different PICSAR targeting siRNAs with two cSCC cell lines. The wound healing assay showed that knockdown of PICSAR decreased cell migration significantly in comparison with the control siRNA transfected cells (Figure 4d and e, Supplementary Figure S4d and e).

Alteration of gene expression profile of cSCC cells after PICSAR knockdown

To elucidate the molecular mechanisms of the effects of PICSAR on cSCC cells, expression profiling of cSCC cells (UT-SCC12A, UT-SCC59A, and UT-SCC118) transfected with PICSAR siRNA1 and control siRNA was performed with RNA-seq. Ingenuity pathway analysis of the RNA-seq expression data showed a significant ($P < 0.05$; fold change [FC] log2 > 0.5) decrease in biofunctions *M-phase of tumor cell lines*, *transformation of tumor cell lines*, and *phosphorylation of L-serine* (Figure 5a, upper panel). In addition, canonical pathway *ERK/MAPK signaling* was significantly regulated ($P < 0.0001$) in PICSAR knockdown cSCC cells (Supplementary Figure S5 online).

Differentially expressed genes in PICSAR knockdown cSCC cells were also significantly associated with specific gene ontology terms and Kyoto Encyclopedia of Genes and Genomes pathways (Figure 5a, lower panel). In PICSAR knockdown cSCC cells, significantly enriched gene ontology terms included biological processes *cell proliferation* ($P = 5.5 \times 10^{-7}$), *response to wounding* ($P = 4.0 \times 10^{-6}$), and *regulation of cell migration* ($P = 1.4 \times 10^{-5}$). Molecular functions significantly regulated included *laminin binding* ($P = 4.1 \times 10^{-4}$), *extracellular matrix binding* ($P = 9.0 \times 10^{-4}$), and *peptidase regulator activity* ($P = 1.1 \times 10^{-3}$) (Figure 5a). Significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways included *complement and coagulation cascades* ($P = 6.6 \times 10^{-3}$), *hematopoietic cell lineage* ($P = 2.3 \times 10^{-2}$), and *extracellular matrix-receptor interaction* ($P = 2.4 \times 10^{-2}$) (Figure 5a). These findings are consistent with functional assays in culture showing decreased ERK1/2 phosphorylation, cell proliferation, and cell migration after PICSAR knockdown in cSCC cells. The top most upregulated and downregulated genes ($P < 0.05$, FC log2 > 0.7) after PICSAR knockdown in cSCC cells are shown in Figure 5b and Supplementary Tables S1 and S2 online.

Regulation of mitogen-activated protein kinase phosphatases by PICSAR

Among the top 50 most upregulated genes by knockdown of PICSAR ($P < 0.05$) was *DUSP1* (dual-specificity phosphatase 1) (Figure 5b, Supplementary Table S1). DUSP1 belongs to the mitogen activated protein kinase phosphatase family and it dephosphorylates p38, JNK, and ERK (Patterson et al., 2009). Analysis of the mitogen activated protein kinase phosphatase family in detail revealed upregulation of *DUSP1* and *DUSP6* (FC log2 = 1.27 and 1.09, respectively) after PICSAR knockdown (Figure 5c). As knockdown of PICSAR had no effect on the activity on p38 or JNK, we focused on DUSP6, a specific negative regulator of ERK2. Significant upregulation of *DUSP6* mRNA levels by PICSAR knockdown in cSCC cells was verified by qRT-PCR (Figure 5d). Western

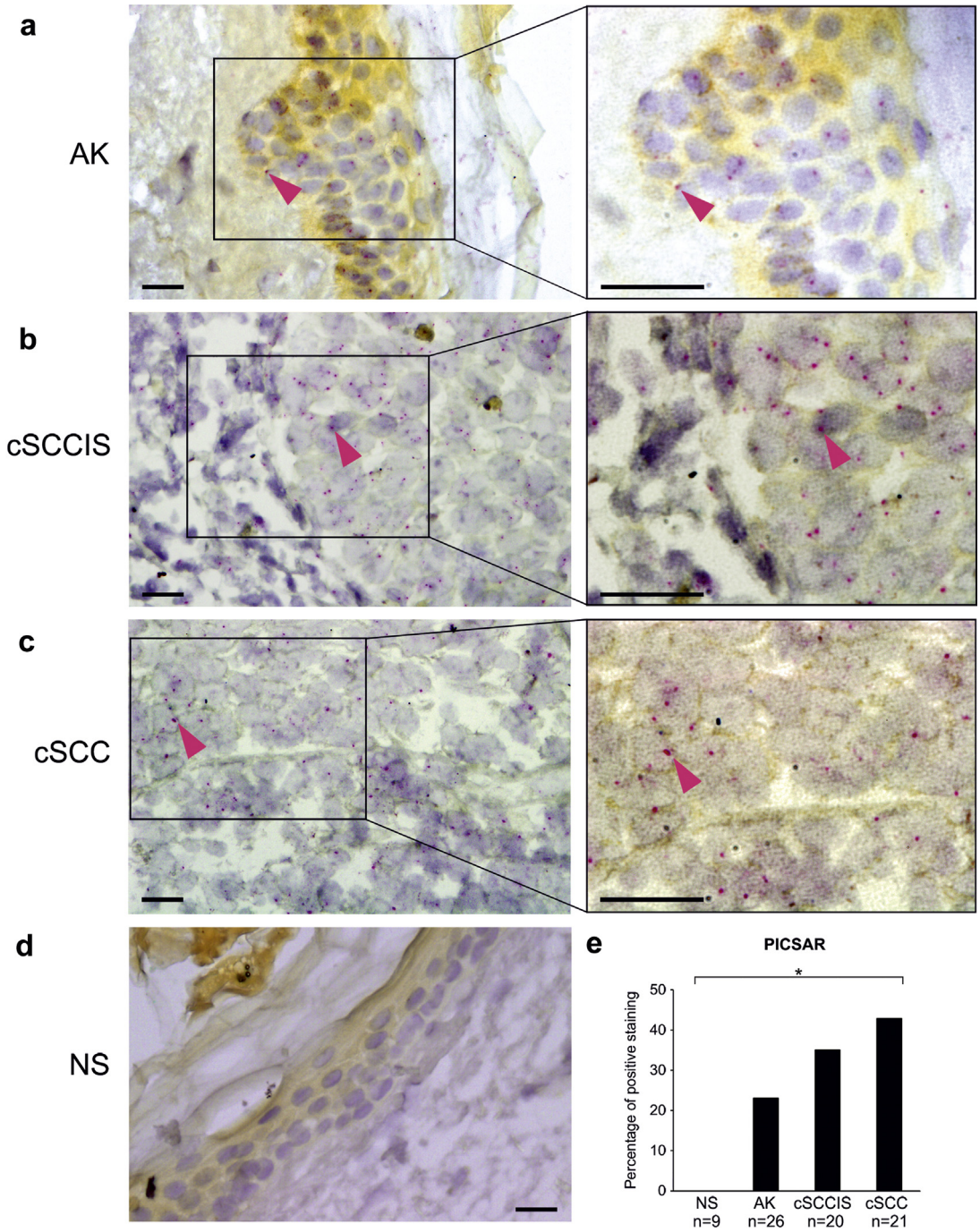

Figure 2. PICSAR is specifically expressed by tumor cells in cSCC in vivo. (**a**–**d**) Expression of PICSAR in paraffin-embedded tissue sections of AK (n = 26), cSCCIS (n = 20), cSCC (n = 21), and normal skin (n = 9). PICSAR (red arrow) was analyzed with RNA-ISH. Specific signal for PICSAR was detected in tumor cells in cSCC, cSCCIS, and AK, but not in epidermal keratinocytes in normal skin. Scale bar = 20 μm. (**e**) Percentage of tissue sections with PICSAR positive tumor cells in each group. *$P < 0.05$, Fisher's exact test. AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; cSCCIS, cSCC in situ; NS, normal skin; PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; RNA-ISH, RNA in situ hybridization.

blot analysis of cSCC cell lysates also showed upregulation of DUSP6 at protein level after PICSAR knockdown (Figure 5e, Supplementary Figure S6 online). Treatment of cSCC cells with DUSP6 inhibitor (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one increased phosphorylation of ERK1/2 and potently abrogated a decrease in ERK1/2 phoshorylation after PICSAR knockdown providing further evidence for DUSP6 as the regulatory link between PICSAR and ERK1/2 (Figure 5f).

Knockdown of PICSAR suppresses growth of cSCC xenografts in vivo

The role of PICSAR in cSCC progression in vivo was investigated using a cSCC xenograft model in severe combined immunodeficient mice. UT-SCC12A cells were transfected with PICSAR siRNA1 and control siRNA, and incubated for 72 hours. Transfected cells were injected subcutaneously into the back of the severe combined immunodeficient mice and tumor growth was measured twice a week. Knockdown of PICSAR

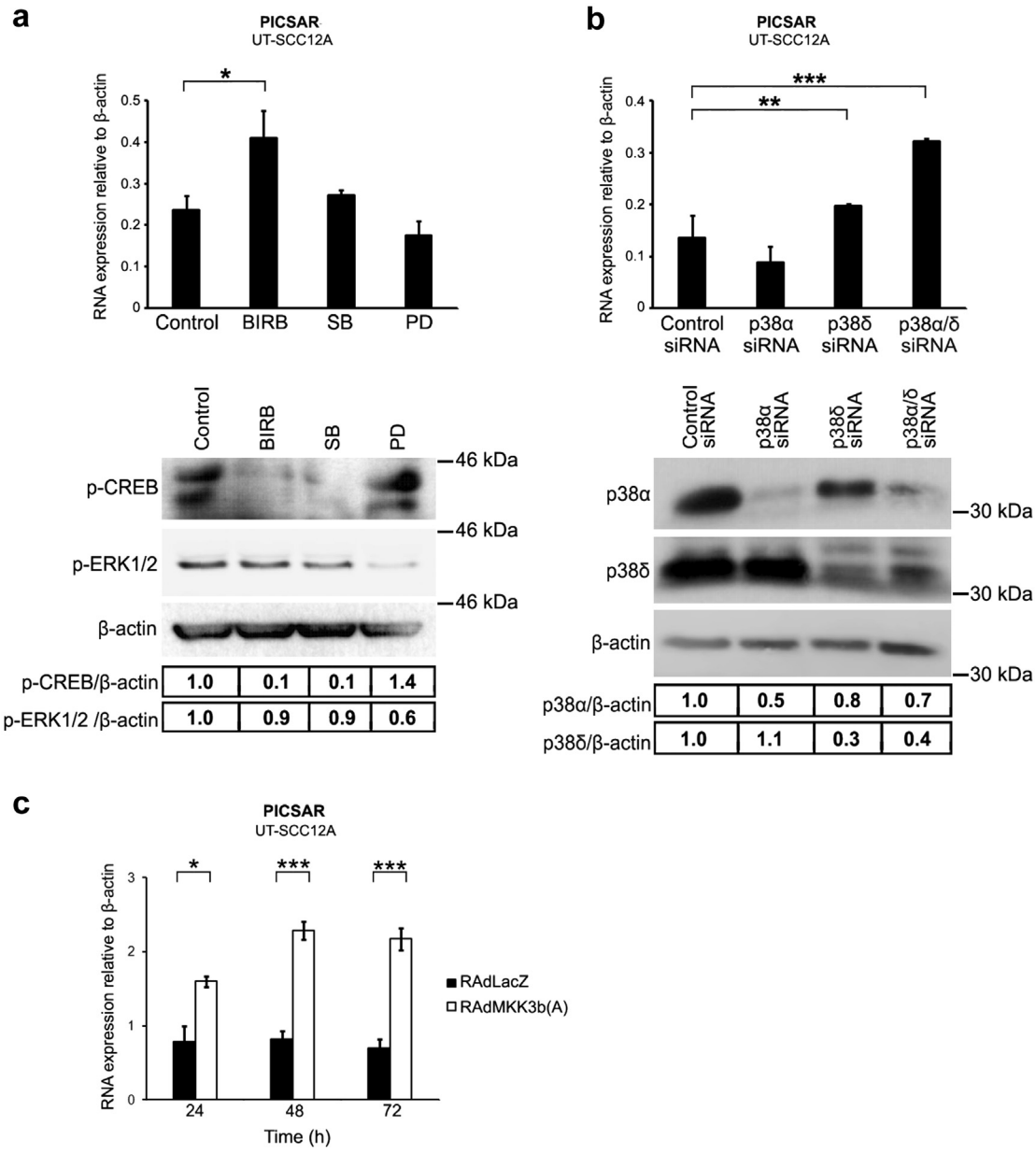

Figure 3. Expression of PICSAR is downregulated by p38 MAPK pathway. (**a**) Upper panel: UT-SCC12A cells were treated for 24 hours with MEK1/2 inhibitor (PD98059, 30 μM) and p38 inhibitors specific for p38α/β (SB203580, 10 μM) or all p38 isoforms (p38α/β/γ/δ) (BIRB796, 10 μM). Expression of PICSAR was measured by qRT-PCR and corrected for the levels of β-actin mRNA in the same samples (mean ± SD; control n = 2, BIRB n = 4, PD n = 4, SB n = 2). Lower panel: levels of phosphorylated CREB (p-CREB), a downstream mediator of the p38 MAPK pathway, and ERK1/2 (p-ERK1/2) were determined by western blot analysis of the corresponding cell lysates to verify the effect of p38 and MEK1/2 inhibitors. (**b**) Upper panel: UT-SCC12A cells were transfected with negative control siRNA, and with p38α or p38δ siRNA alone or in combination (p38α/δ) (75 nM). Expression of PICSAR was determined with qRT-PCR 72 hours after transfection (mean ± SD; n = 3). Lower panel: Cell lysates were analyzed for p38α and p38δ protein levels by western blotting. Levels of p38α and p38δ quantitated densitometrically and corrected for β-actin levels in the same samples are shown below the western blots relative to levels in untreated control cells (1.0). (**c**) UT-SCC12A cells were infected with control adenovirus (RAdLacZ) and with adenovirus containing the dominant negative MKK3b (RAdMKK3bA). PICSAR expression was determined by qRT-PCR 24, 48, and 72 hours after infection (mean ± SD; n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Student's *t*-test. CREB, CRE-binding protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK3b, MAP kinase kinase 3b; PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; qRT-PCR, quantitative real-time reverse transcriptase-PCR.

expression resulted in significant inhibition in the growth of the tumors compared with the control tumors noted already at 4 days (Figure 6a). Tumors were harvested 18 days after implantation and weighed. The mass of PICSAR knockdown tumors was significantly lower compared with the control tumors (Figure 6b). Extended incubation of PICSAR siRNA transfected UT-SCC12A cells showed that the expression of PICSAR was still reduced by 55% after 14 days (data not shown). The relative number of proliferating (Ki-67 positive) tumor cells was significantly lower in xenografts established with cSCC cells transfected with PICSAR siRNA, as compared with the control siRNA tumors (Figure 6c and d).

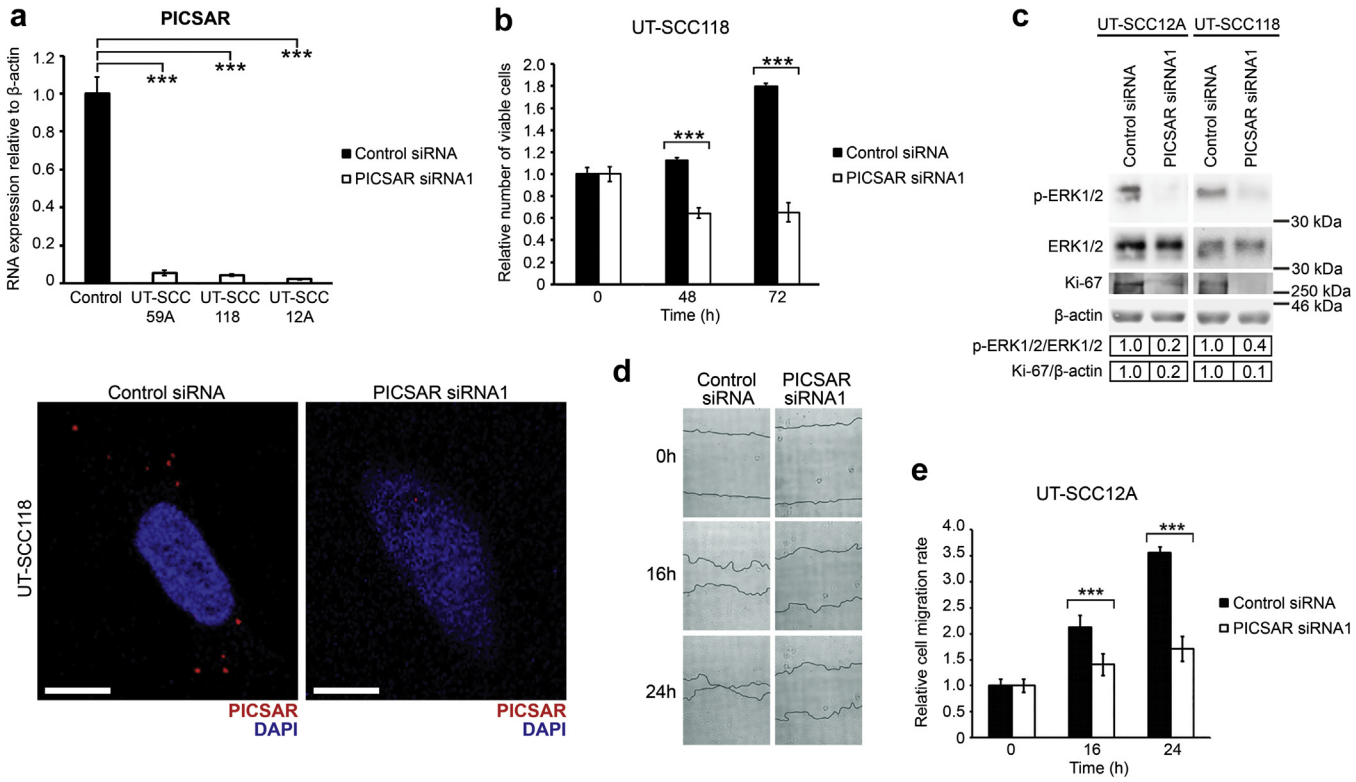

Figure 4. Knockdown of PICSAR inhibits proliferation and migration of cSCC cells. (**a**) Upper panel: cSCC cells were transfected with negative control siRNA or PICSAR siRNA1 (75 nM). PICSAR expression was determined 72 hours after transfection by qRT-PCR and corrected for β-actin mRNA levels in the same samples (mean ± SD; n = 3). Lower panel: expression of PICSAR in cSCC cells was determined with RNA-ISH after transfection with negative control siRNA or PICSAR siRNA1. Scale bar = 10 μm. (**b**) The number of viable cSCC cells (UT-SCC118) was determined with WST-1 assay 48 and 72 hours after transfection with PICSAR and control siRNA1 (75 nM) (n = 3−8). (**c**) Cell lysates of cSCC cells (UT-SCC12A and UT-SCC118) were analyzed by western blotting for the levels of phosphorylated ERK1/2 (p-ERK1/2) and Ki-67 proliferation marker 72 hours after transfection with PICSAR siRNA1 and control siRNA. Levels of p-ERK1/2 and Ki-67 quantitated densitometrically and corrected for total ERK1/2 and β-actin levels, respectively, in the same samples are shown below the western blots relative to levels in untreated control cells (1.0). (**d**) cSCC cells (UT-SCC12A) were transfected with PICSAR siRNA1 or control siRNA (75 nM both). Forty-eight hours after transfection, cells were incubated for 6 hours with hydroxyurea (1 mM) to prevent cell proliferation. Cell monolayer was scratched with a sterile pipette tip and incubation was continued in DMEM and 1% fetal calf serum with 0.5 mM hydroxyurea. Photomicrographs were taken at 0, 16, and 24 hours from three parallel wells (3−5 images per well). (**e**) Quantitation of the relative cell migration rates (n = 3−5). Mean ± SD is shown; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Student's *t*-test. cSCC, cutaneous squamous cell carcinoma; ERK, extracellular signal-regulated kinase; Ki-67, marker of proliferation Ki-67; PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; qRT-PCR, quantitative real-time reverse transcriptase-PCR; RNA-ISH, RNA in situ hybridization; WST, water-soluble tetrazolium salt.

DISCUSSION

More than 18,000 annotated lncRNA transcripts have been identified in the human genome, but the function of most lncRNAs is still unknown. Dysregulation of specific lncRNAs has been documented in different cancer types and they have been proposed as biomarkers and therapeutic targets in cancer (Serviss et al., 2014; Wahlestedt, 2013; Yang et al., 2014). For example, a widely characterized lncRNA, HOTAIR has been suggested as a prognostic marker in different cancers (Yao et al., 2014). Several lncRNAs with oncogenic functions have been characterized, such as HOTAIR, MALAT1, and ANRIL (Geisler and Coller, 2013; Rinn and Chang, 2012). On the other hand, certain lncRNAs have been shown to possess tumor suppressive functions, adding to the complexity of the role of lncRNAs in cancer progression (Huarte et al., 2010; Mourtada-Maarabouni et al., 2009).

Here, we have characterized the role of lincRNA PICSAR (*LINC00162*) in the progression of cSCC, the most common metastatic skin cancer (Madan et al., 2010; Ratushny et al., 2012). The whole transcriptome expression analysis identified PICSAR as one of the differentially expressed lncRNAs with the highest mean expression level in cSCC cells compared with NHEKs. Overexpression of PICSAR was noted in primary and metastatic cSCC cell lines compared with NHEKs using RNA-seq analysis, qRT-PCR, and RNA-ISH. Moreover, analysis of PICSAR levels by qRT-PCR revealed overexpression of PICSAR in cSCC tumors in vivo, as compared with normal skin. In addition, RNA-ISH analysis of a panel of tissue samples from normal skin, premalignant lesions (AKs), and cSCCs revealed tumor cell-associated labeling for PICSAR in cSCCs, but not in normal skin. These results show that the expression of PICSAR is specifically induced in tumor cells in cSCCs, suggesting PICSAR as a biomarker for cSCC. Furthermore, expression of PICSAR in a subset of AKs and cSCCIS provides evidence that the induction of PICSAR expression is an early event in keratinocyte carcinogenesis and may play a role in the progression of AKs to cSCCIS and eventually to invasive cSCC. However, it is evident that further analysis of a larger panel of cSCCs

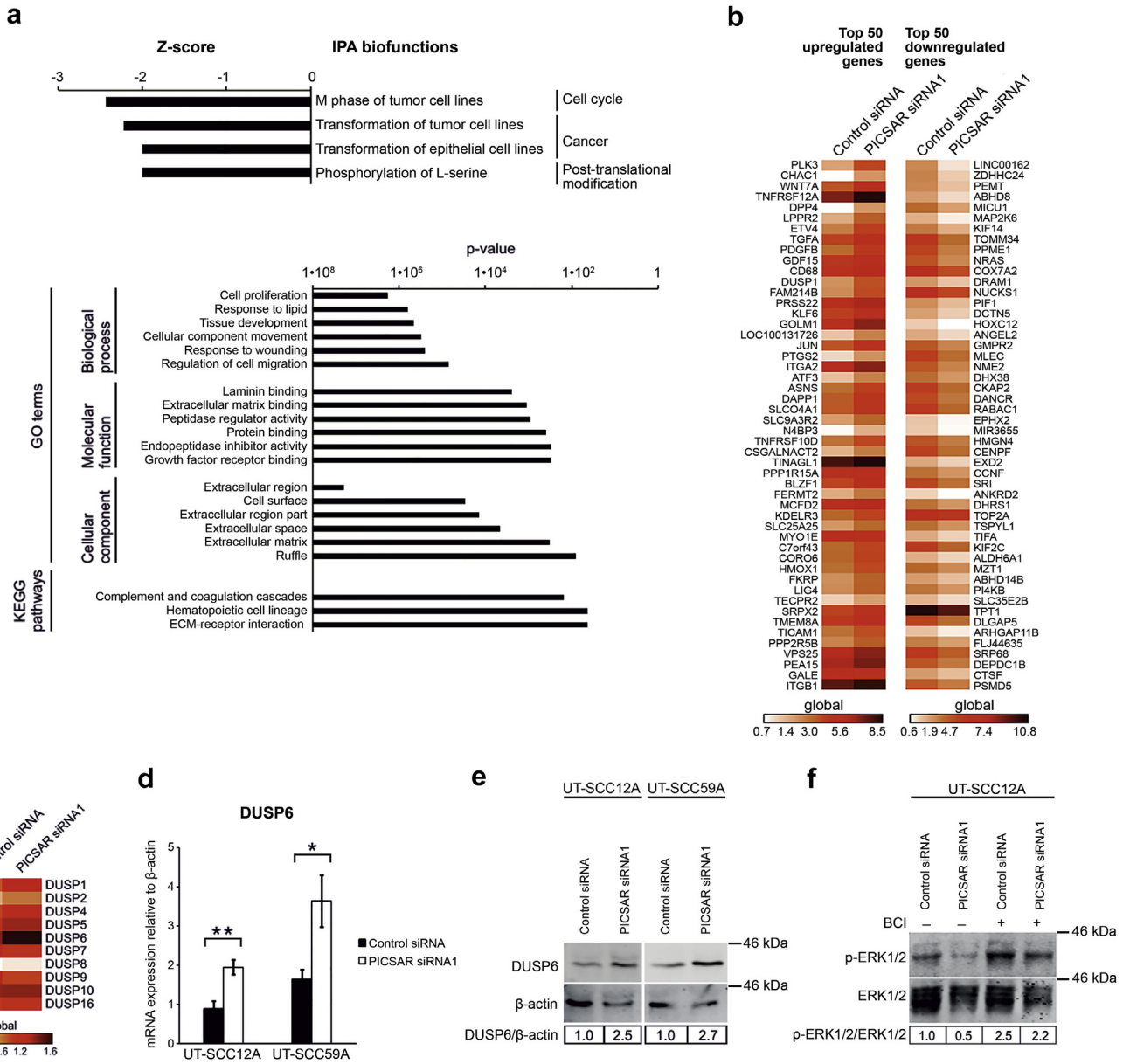

Figure 5. Alteration of gene expression profile in cSCC cells after knockdown of PICSAR. (**a**–**c**) Three cSCC cell lines (UT-SCC12A, UT-SCC59A, and UT-SCC118) were transfected with PICSAR siRNA1 or control siRNA. Seventy-two hours after transfection, whole transcriptome analysis was performed with RNA-seq. The results were examined as log2 fold change (FC) values between the mean expression values of two sample groups. (**a**) Summary of ingenuity pathway analysis (IPA) biofunctions (upper panel), gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (lower panel) related to PICSAR knockdown ($P < 0.05$, FC log2 > 0.7). GO and KEGG results shown are average *P*-values of three cSCC cell lines ($P < 0.05$). (**b**) Heatmap showing the 50 most upregulated and downregulated genes after PICSAR knockdown ($P < 0.05$, FC log2 > 0.7). (**c**) Heatmap showing mitogen-activated protein kinase phosphatases regulated by knockdown of PICSAR in cSCC cells. (**d**) Expression of *DUSP6* mRNA was determined by qRT-PCR in cSCC cells 72 hours after PICSAR knockdown. β-Actin mRNA levels were determined as a reference gene. (**e**) DUSP6 protein levels were determined by western blotting analysis in cSCC cells 72 hours after PICSAR knockdown. Levels of DUSP6 quantitated densitometrically and corrected for β-actin levels in the same samples are shown below the western blots relative to the levels in control cells (1.0). (**f**) DUSP6 inhibitor BCI was added to cSCC cells 24 hours after PICSAR knockdown and incubation was continued for 24 hours. Levels of phosphorylated and total ERK1/2 were determined by western blot analysis. Levels of p-ERK1/2 quantitated densitometrically and corrected for ERK1/2 levels in the same samples are shown below the western blots relative to the levels in control cells (1.0). $^*P < 0.05$, $^{**}P < 0.01$; Student's *t*-test. BCI, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one; cSCC, cutaneous squamous cell carcinoma; DUSP6, dual specificity phosphatase 6; ERK, extracellular signal-regulated kinase; PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; qRT-PCR, quantitative real-time reverse transcriptase-PCR.

and stratification of the tumor material based on clinical parameters is required to fully evaluate the role of PICSAR as a biomarker for cSCC progression.

Solar UV-radiation is the most common environmental carcinogen causing skin cancer (Kivisaari and Kähäri, 2013; Ratushny et al., 2012). There is evidence that UVA activates ERK1/2 signaling and proliferation in keratinocytes, and may in this way promote cutaneous carcinogenesis (Bachelor and Bowden, 2004; He et al., 2004). Activation of MAPK signaling is associated with cSCC progression and basal activation of ERK1/2 is detected in tumor cells in cSCC (Kivisaari et al., 2010; Lambert et al., 2014; Toriseva et al.,

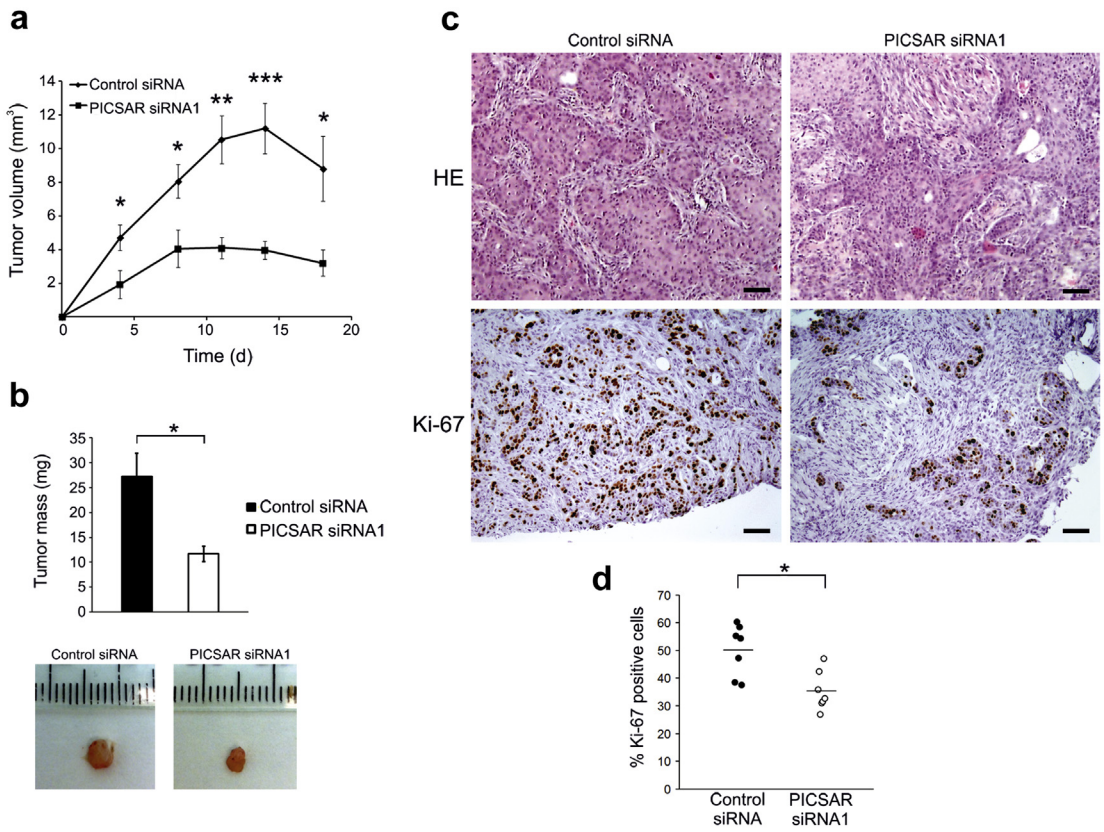

Figure 6. Knockdown of PICSAR suppresses growth of cSCC xenografts. (**a**) UT-SCC12A cells were transfected with PICSAR targeted siRNA1 or negative control siRNA. Seventy-two hours after transfection, cells (7×10^6) were injected subcutaneously into the back of SCID mice and the growth of tumors was measured twice a week (n = 7 in both groups). (**b**) Xenografts were harvested 18 days after inoculation and weighed. Mean ± SEM is shown; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Student's *t*-test. (**c**) Histology of the tumors was analyzed in hematoxylin and eosin (H&E) stained tumor sections. Ki-67 was detected in xenografts by immunohistochemistry. Hematoxylin was used as a counterstain. Scale bar = 100 μm. (**d**) The relative number of Ki-67 positive cells was determined by counting 500–2600 cells in all tumor sections (n = 7 in both groups) at ×20 magnification. $*P < 0.05$, Mann-Whitney *U*-test. cSCC, cutaneous squamous cell carcinoma; Ki-67, marker of proliferation Ki-67; PICSAR, P38 Inhibited Cutaneous Squamous cell carcinoma Associated lincRNA; SCID, severe combined immunodeficient; SEM, standard error of mean.

2012). Moreover, activation of p38 MAPK pathway, especially p38α and p38δ, plays a role in invasion of cSCC cells and growth of cSCC in vivo (Junttila et al., 2007; Schindler et al., 2009). It has been shown that cSCC cells predominantly express p38α and p38δ MAPK isoforms and that p38 is activated in cSCC cells at basal level (Junttila et al., 2007). In this context, we examined the role of MAPK signaling in the regulation of PICSAR expression. Inhibition of the activity of p38α and p38δ with BIRB796 or with specific siRNAs resulted in upregulation of PICSAR expression. Inhibition of ERK1/2 activation with MEK1/2 inhibitor PD98059 had no effect on PICSAR expression. These results identify PICSAR as a target for p38 signaling pathway in cSCC cells.

Knockdown of PICSAR expression in cSCC cells resulted in significant inhibition in the growth of cSCC xenograft tumors already at the early stage compared with control siRNA group. These results provide evidence that PICSAR plays a role in implantation and early growth of cSCC tumor cells in vivo. Functional studies with cSCC cells indicated that knockdown of PICSAR expression suppressed cellular functions important in cancer progression, that is, proliferation and migration of cSCC cells. Moreover, knockdown of PICSAR significantly decreased the levels of p-ERK1/2, suggesting that the functional effect of PICSAR involves regulation of ERK1/2 pathway.

The molecular background for the role of PICSAR in cSCC growth and migration was examined by RNA-seq analysis of three distinct cSCC cell lines after PICSAR knockdown. Pathway analysis of the gene expression profile of cSCC cells revealed that significantly downregulated genes after PICSAR knockdown were associated with biofunctions *M-phase of tumor cell lines*, *transformation of tumor cell lines*, *transformation of epithelial cell lines*, and *phosphorylation of L-serine*. In addition, differentially expressed genes were associated with significantly enriched gene ontology terms *cell proliferation*, *regulation of cell migration*, and *extracellular matrix binding*. Among the genes most upregulated by PICSAR knockdown was *DUSP1*, which codes for dual specificity phosphatase 1 (Patterson et al., 2009). As decreased activation of ERK1/2 was noted after PICSAR knockdown, we wanted to examine the role of dual specificity phosphatases in detail. Further analysis revealed upregulation of DUSP6, a specific ERK2 phosphatase at mRNA and protein level in cSCC cells after PICSAR knockdown. In addition, in the presence of DUSP6 inhibitor, PICSAR knockdown had no effect on ERK1/2 activation providing further evidence for DUSP6 as regulatory link between PICSAR and ERK1/2 signaling pathway. Expression of DUSP6 in cSCC cells has previously been shown to be upregulated by keratinocyte

growth factor (KGF), which suppresses the malignant phenotype of these cells (Toriseva et al., 2012). However, keratinocyte growth factor had no effect on the expression of PICSAR in cSCC cells (data not shown). DUSP6 is activated on binding to ERK2 and it specifically dephosphorylates and inactivates ERK2, but not JNK or p38 (Groom et al., 1996; Muda et al., 1998). This is in accordance with our finding that ERK1/2 activity is decreased after PICSAR knockdown but not the levels of active JNK and p38. LncRNAs can regulate gene expression both at transcriptional and posttranscriptional level (Geisler and Coller, 2013). RNA-seq analysis of cSCC cells after PICSAR knockdown revealed significantly altered expression of numerous protein and nonprotein coding genes, which could mediate the effects of PICSAR. The cytoplasmic localization of PICSAR in cSCC cells suggests that it may regulate the stability of DUSP6 mRNA directly or indirectly. Nevertheless, these findings provide evidence that PICSAR knockdown decreases ERK1/2 activation in cSCC cells, which in turn may lead to decreased cell proliferation, viability, and migration. These observations also identify PICSAR as a regulatory link between p38 and ERK1/2 signaling pathways.

At present, there is little information available on the expression of PICSAR in human tissues outside central nervous system (Kawashima et al., 2006; Mills et al., 2013). The gene coding for PICSAR is located in 21q22.3, between protein coding genes *ADARB1* (adenosine deaminase, RNA-specific, B1) and *FAM207A* (family with sequence similarity 207, member A). Interestingly, single nucleotide polymorphisms, copy-number alterations, and loss of heterozygosity in 21q22.3 associated with certain cancers have been found by genome-wide association studies (Boyd et al., 2012; Hwang et al., 2008; Vékony et al., 2007; Wu et al., 2011). It remains to be elucidated whether any of these genomic alterations involve PICSAR gene.

In summary, we have identified PICSAR as a lincRNA specifically overexpressed in cSCC cells in culture and in vivo. In addition, we show that PICSAR regulates proliferation and migration of cSCC cells and growth of cSCCs in vivo. Our results also provide mechanistic evidence that PICSAR increases activity of ERK1/2 pathway via inhibition of MAPK phosphatase DUSP6. The results of this study implicate PICSAR in cSCC progression, and identify this lincRNA as a biomarker and putative therapeutic target in cSCC.

MATERIALS AND METHODS

Ethical issues

The use of tumor and normal skin samples was approved by the Ethics Committee of the Hospital District of Southwest Finland. All participants gave their written informed consent and the study was carried out with the permission of Turku University Hospital, according to the Declaration of Helsinki. The experiments with mice were performed with the permission of the State Provincial Office of Southern Finland.

Tissue samples

Tissue samples of cSCC tumors (n = 6) and normal skin (n = 7) were collected in Turku University Hospital (Riihilä et al., 2014). Tissue microarrays consisting of samples from normal sun-protected skin (n = 9), AK (n = 26), cSCCIS (n = 20), and cSCC (n = 21) were generated from the archival paraffin blocks from the Department of Pathology, Turku University Hospital (Farshchian et al., 2015; Riihilä et al., 2015). Formalin-fixed paraffin-embedded xenograft tumors were established with human UT-SCC12A cell line in severe combined immunodeficient mice.

Cell cultures

cSCC cell lines were established from surgically removed SCCs of the skin in Turku University Hospital (Lansdorf et al.,1999; Stokes et al., 2010). The authenticity of all cSCC cell lines has been verified by short tandem repeat profiling (DDC Medical, Fairfield, OH). NHEKs were established from skin of healthy individuals undergoing mammoplasty (Farshchian et al., 2011) and NHEK-PC was purchased from PromoCell (Heidelberg, Germany). cSCC cells and NHEKs were cultured as previously described (Farshchian et al., 2015; Riihilä et al., 2015). For inhibition of MAPKs, cSCC cells were treated for 24 hours with p38 inhibitors BIRB796 and SB203580 (10 μM each), and MEK1/2 inhibitor PD98059 (30 μM) (all from Calbiochem, La Jolla, CA). For inhibition of DUSP6, 24 hours after PICSAR knockdown, cSCC cells were treated with 5 μM (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (Calbiochem, San Diego, CA) for 6 hours and incubation was continued for 18 hours with 1 μM (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one.

RNA analysis and RNA in situ hybridization

Protocols for RNA sequencing analysis, quantitative real-time PCR, and RNA-ISH are provided in Supplementary Materials and Methods online.

Knockdown of PICSAR and functional studies

Protocols for siRNA transfection, adenoviral gene delivery, and assays for cell proliferation, migration and western blotting are provided in Supplementary Materials and Methods.

Human cSCC xenografts

Protocols for establishing human cSCC xenografts and tumor immunohistochemistry are provided in Supplementary Materials and Methods.

Statistical analysis

Statistical analyses were performed using SPSS Statistics for Windows, v. 20.0 (IBM, Armonk, NY). Student's *t*-test and Mann-Whitney *U*-test were used to compare the means between two groups. Statistical analysis for immunohistochemistry stainings was performed with Fisher's exact test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.03.028.

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