

Complement factor I upregulates expression of matrix metalloproteinase-13 and -2 and promotes invasion of cutaneous squamous carcinoma cells

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Abstract

The incidence of cutaneous squamous cell carcinoma (cSCC) is increasing globally. Here, we have studied the functional role of complement factor I (CFI) in the progression of cSCC. CFI was knocked down in cSCC cells, and RNA-seq analysis was performed. Significant downregulation of genes in IPA biofunction categories *Proliferation of cells* and *Growth of malignant tumor*, in Gene Ontology (GO) terms *Metalloproteinase activity* and *Extracellular matrix component*, as well as Reactome *Degradation of extracellular matrix* was detected after CFI knockdown. Further analysis of the latter three networks, revealed downregulation of several genes coding for invasion-associated matrix metalloproteinases (MMPs) after CFI knockdown. The downregulation of MMP-13 and MMP-2 was confirmed at mRNA, protein and tissue levels by qRT-qPCR, Western blot and immunohistochemistry, respectively. Knockdown of CFI decreased the invasion of cSCC cells through type I collagen. Overexpression of CFI in cSCC cells resulted in enhanced production of MMP-13 and MMP-2 and increased invasion through type I collagen and Matrigel, and in increased ERK1/2 activation and cell proliferation. Altogether, these findings identify a novel mechanism of action of CFI in upregulation of MMP-13 and MMP-2 expression and cSCC invasion. These results identify CFI as a prospective molecular marker for invasion and metastasis of cSCC.

KEYWORDS

cancer, CFI, complement factor I, cSCC, cutaneous squamous cell carcinoma, invasion, matrix metalloproteinase, MMP

1 | INTRODUCTION

Keratinocyte carcinomas, basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC) are the most prevalent cancers globally.^{1,2} Invasive cSCC makes up 20% of keratinocyte carcinomas.³ The estimated metastasis rate of primary cSCC is 3–7%, and the prognosis of metastatic disease is poor.^{2–7} At present, the incidence of

cSCC is growing worldwide.^{1,2,8} In the process of cSCC development, premalignant lesion, actinic keratosis (AK), progresses to cSCC *in situ* (cSCCIS) and subsequently to invasive primary cSCC, which can be metastatic. Ultraviolet radiation is the most important risk factor for cSCC, and immunosuppression and chronic ulcers are other important predisposing factors.^{5,9} Furthermore, chronic inflammation plays a role in the development and progression of cSCC.⁹

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The complement system links innate and acquired inflammatory immune responses. It functions as a crucial component in host defense and consists of three distinctive pathways: classical, alternative and lectin pathways.¹⁰⁻¹³ Sequential activation of these pathways causes cleavage of central complement components C3 and C5 and the formation of small anaphylatoxic fragments C3a and C5a. Cleavage of C5 initiates activation of terminal lytic pathway and membrane attack complex-mediated cell lysis.¹⁰⁻¹² In order to prevent the lytic effect of complement system on normal host cells, its activation is strictly regulated by a series of soluble and membrane-bound proteins. These include complement factor I (CFI), complement factor H (CFH), complement receptor type 1 (CR1, CD35), membrane cofactor protein (MCP, CD46), CD55, C4b Binding Protein (C4BP), CD59, C1 inhibitor, clusterin and vitronectin.¹³⁻¹⁵

The serine proteinase CFI is a soluble 88 kDa glycoprotein with light (38 kDa) and heavy (50 kDa) subunits connected with a disulfide bond.¹³⁻¹⁵ CFI is one of the key negative regulators of the complement system that inhibits activation of all three pathways by cleaving C3b and C4b components of the C3- and C5-convertases. The activity of CFI is dependent on cofactors C4BP, CFH, CD35 or CD46.¹³⁻¹⁵ Recently, the role of complement system in cancer progression has become the focus of attention.^{10,16-19} In our previous studies, we have demonstrated significant upregulation of complement inhibitors CFI and CFH, as well as four complement activators CFB and C3 in alternative pathway, and C1r and C1s in classical pathway, in cSCC tumor cells *in vivo* and in culture.²⁰⁻²³ In the present study, we have further investigated the functional role and molecular mechanism of CFI in cSCC progression. The results indicate that CFI upregulates the expression of matrix metalloproteinase-13 (MMP-13) and MMP-2 and promotes invasion of cSCC cells providing evidence for it as a potential biomarker for invasion and metastasis of cSCC.

2 | METHODS

2.1 | Ethical issues

All studies with skin samples were approved by the Ethics Committee of the Hospital District of Southwest Finland. Written informed consent of patients was obtained before each procedure. The conduction of this research was approved by Turku University Hospital under the Declaration of Helsinki. All experiments with mice were carried out with permission of the animal test review board of the Southern Finland according to institutional guidelines.

2.2 | Cell cultures

Human cSCC cell lines were initiated from surgically removed cSCCs.²⁴ Two cSCC cell lines were derived from primary cSCC of face: UT-SCC-105 and UT-SCC-108. Two cSCC cell lines were from metastatic cSCCs of temporal skin: UT-SCC-7 and UT-SCC-59A. These cell

lines were authenticated by short tandem repeat (STR) DNA profiling.²⁴ The cell culture protocol was as previously described.^{24,25}

2.3 | CFI knockdown

Cultures of cSCC cells in 50% confluence were transfected with negative control siRNA (AllStars Negative Control siRNA, Qiagen) or two commercially available ones (120 nM, Qiagen) targeting different areas of CFI: CFI siRNA_1 (Hs_CFI_1; target sequence: 5'-AACTACCGTATCAGTGCCCAA-3'), CFI siRNA_2 (Hs_IF_2; target sequence: 5'-TAAGACAATGTTTCATATGCAA-3') using siLentFect Lipid Reagent (Bio-Rad Laboratories, Hercules, CA), as previously described.²⁰

2.4 | RNA-seq analysis

RNA was isolated from control siRNA- and CFI siRNA_1-transfected cSCC cell lines (UT-SCC-118, UT-SCC-7, UT-SCC-59A; 120 nM) 72 h post-transfection with miRNAeasy Mini kit (Qiagen, Germantown, MD, USA), and sequencing was done with Illumina HiSeq3000 system (Illumina, San Diego, CA) at the Finnish Functional Genomics Centre located in Turku, Finland. The reads were further aligned to the human reference genome (hg38) and trimmed mean of M values (TMM) was applied for data normalization (R version 3.3/Bioconductor package edgeR version 3.3). Statistical analysis was performed by Limma package version 3.10.²⁶ mRNA-seq data (accession number GSE166421) have been deposited in the public database GEO (Gene Expression Omnibus, NCBI; <http://www.ncbi.nlm.nih.gov/geo/>).

2.5 | Real-time quantitative PCR (RT-qPCR)

Total RNA extraction from control or CFI siRNA_1 transfected cSCC cells 72 h post-transfection, followed by cDNA synthesis was carried out according to the previous protocols.²¹ qPCR analysis was subsequently done by the QuantStudio 12 K Flex (Thermo Fisher Scientific) using specific primers and probes for MMP-13, MMP-2 and β -actin as described earlier.^{25,27,28} Samples were analysed in triplicates with threshold cycle values (Ct) below 5% of the mean Ct. β -actin mRNA expression was used as control.

2.6 | Western blot analysis

Media and lysates of CFI and control siRNA transfected cSCC cells, as well as CFI overexpressing and control expression vector (pcDNA3.1) transfected cSCC cultures were analysed with specific antibodies for CFI (OX-21, NBP1-02915, Novus Biologicals, Littleton, CO, USA) and MMP-13 (MAB3321, Millipore, Darmstadt, Germany) both in 1:500 dilution, and MMP-2 (gelatinase A, HPA001939, Sigma, St. Louis,

MO, USA), phospho-p44/42 MAPK (Thr204/Tyr204, p-ERK, 91015, Cell Signaling, Beverly, MA, USA) and p44/42 MAPK (ERK, 9102, Cell Signaling) in 1:1000 dilution 72 h post-transfection. For medium samples, TIMP-1 (MAB3300, Millipore; dilution: 1:1000) and TIMP-2 (Ab-1, IM11L, CalbioChem, Bad Soden, Germany) and for cell lysate samples, β -actin (AC-15, A-1978, Sigma; dilution: 1:4000) expression levels were used as loading control. Quantitation of protein expression was done by LI-COR Odyssey® CLx fluorescent imaging system Using fluorescent secondary antibodies in 1:15000 dilution (LI-COR Biosciences, Lincoln, NE, USA).

2.7 | Invasion assays

cSCC cells in 50% confluence were transfected with CFI expression construct (pcDNA3.1_CFI) or control expression vector (pcDNA3.1) and negative control siRNA or CFI siRNAs (1 and 2). 24 h post-transfection, cells were plated on an ImageLock 96-well plate coated with type I collagen ($5 \mu\text{g}/\text{cm}^2$, PureCol; Advanced BioMatrix, San Diego, CA) or Matrigel ($100 \mu\text{g}/\text{ml}$) (Corning, Corning, NY). After an overnight incubation period to allow the cells to attach, a scratch wound was made in the monolayer by IncuCyte® 96-well WoundMaker Tool (Essen Bioscience, Ann Arbor, MI) and another layer of collagen or Matrigel was applied to generate a three-dimensional (3D) matrix for the cell cultures. Type I collagen solution was prepared by mixing type I collagen (PureCol 3.1 mg/ml), $5\times$ Dulbecco's Modified Eagle Medium ($5\times$ DMEM), HEPES buffer and NaOH, and added on the cells and wounds. The proportion of PureCol to $5\times$ DMEM to HEPES buffer was 7:2:1, and 1 molar NaOH was added to the mixture to adjust the final PH to 7.4. Matrigel ($4 \text{ mg}/\text{ml}$) was added on the cells and wounds in Matrigel-coated plates. Following polymerization of type I collagen and Matrigel, cell culture medium containing 0.5% foetal calf serum (FCS) was added to the wells. Eventually, the process of wound closure was tracked using the IncuCyte S3 real-time cell imaging system (Essen Bioscience).^{29–32} The results were analysed via IncuCyte S3 software (Essen Bioscience). Invasion of cSCC cells through type I collagen and Matrigel was indicated by relative wound density.^{29–32}

2.8 | Human cSCC xenografts

Establishment of human cSCC xenografts was performed, as previously described.²⁰ CFI or control siRNA transfected UT-SCC-7 cells (5×10^6) were injected in the back of severe combined immunodeficiency (SCID/SCID) female mice (CB17/Icr-Prkdc^{scid}/IcrIcoCrI) (Charles River Laboratories, Wilmington, MA, USA) subcutaneously ($n = 8$ for control group and $n = 6$ for CFI siRNA xenograft tumors). After 18 days, tumors were harvested and processed for histological analysis with IHC, as previously described.²⁰ Immunostaining for MMP-13 and MMP-2 with mouse monoclonal MMP-13 antibody (MAB13424, Merck Millipore, Germany) and rabbit polyclonal MMP-2 antibody (HPA001939, Sigma, St. Louis, MO, USA) was

carried out, respectively, using Mayer's haematoxylin as counterstain.³³ The staining intensities of xenograft tumor cell cytoplasm were scored semiquantitatively as weak (+), moderate (++) or strong (+++).

2.9 | Overexpression of CFI

For generation of CFI expression vector, CFI cDNA fragment in pEX-A128 was obtained from Eurofins Genomics (Ebersberg, Germany) and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). To validate the integrity of cloned CFI segment, the construct was re-sequenced. Transfection of cSCC cells with the recombinant CFI expression construct (pcDNA3.1_CFI) or control expression empty vector (pcDNA3.1) was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA).³⁴

2.10 | Cell proliferation assay

cSCC cells cultured to 50% confluence were transfected with CFI expression construct (pcDNA3.1_CFI) or control negative expression vector (pcDNA3.1). 24 h after transfection, the cells were plated on a 96-well plate. Monitoring of the cell proliferation was accomplished with IncuCyte S3 real-time cell imaging system (Essen Bioscience, Ann Arbor, MI, USA) and the results were analysed with IncuCyte S3 software (Essen Bioscience). Relative confluence was used as the indicator of cSCC cells proliferation.

2.11 | Statistical analysis

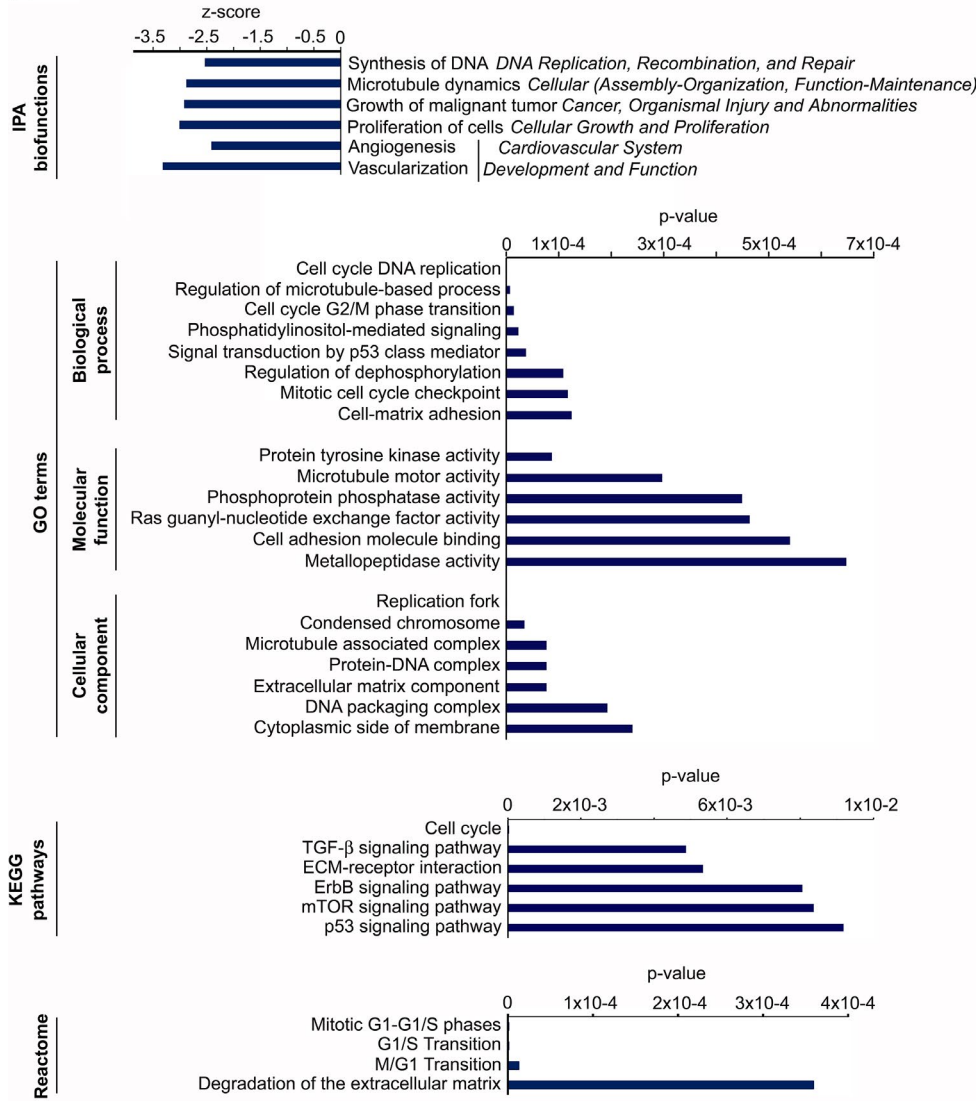
Statistical analysis between sample groups was calculated by either two-tailed Student's *t* test or Mann-Whitney *U* test.

3 | RESULTS

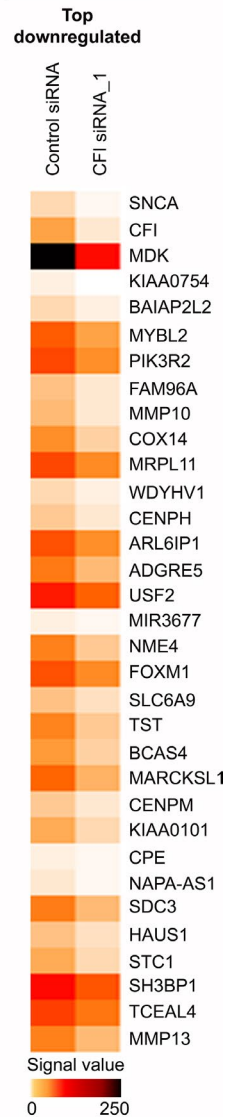
3.1 | Alteration of gene expression profile in cSCC cells after CFI knockdown

To examine the molecular mechanism of CFI in cSCC progression, the expression of CFI in cSCC cells was knocked down by transfection with CFI siRNA_1. mRNA-seq analysis was performed after CFI knockdown in three cSCC cell lines after a 72-h incubation (Figure 1). CFI knockdown significantly decreased the expression of CFI, its cofactor CFH, and complement components C1QL1 and C3 and upregulated the expression of CD55 (Complement decay-accelerating factor) and CFP (Properdin) (Figure S1). The expression of CFI cofactors CD46 and CR1 was not significantly altered (Figure S1). Ingenuity Pathway Analysis (IPA) revealed significant downregulation of genes in biofunction categories *Proliferation of cells* and *Growth of malignant tumor* after CFI knockdown. The

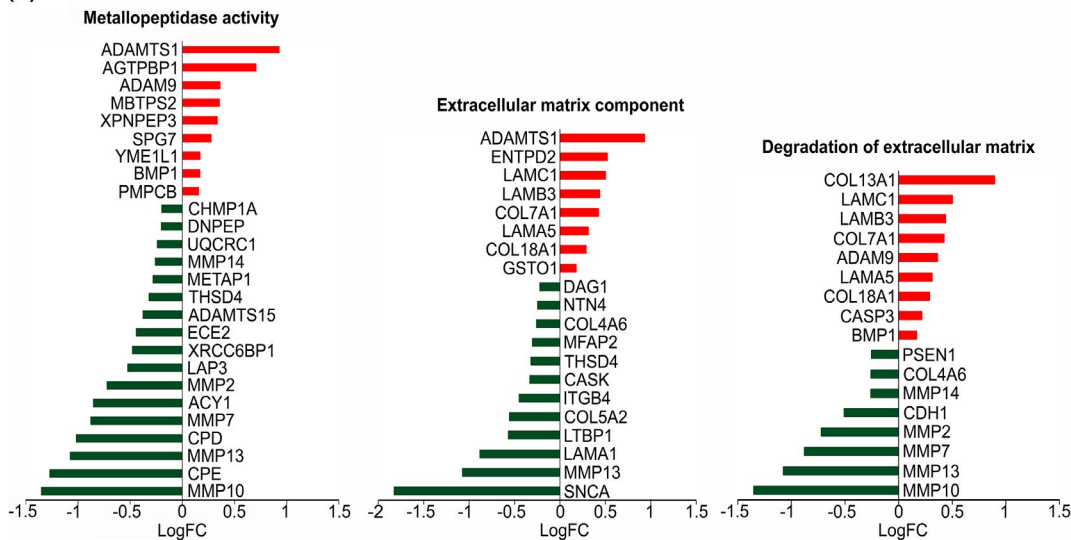
(A)



(B)



(C)



(D)

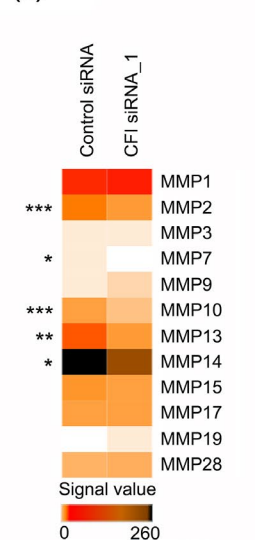


FIGURE 1 Alteration of gene expression profile in cSCC cells after CFI knockdown. cSCC cell lines (UT-SCC-118, UT-SCC-7 and UT-SCC-59A) were transfected with control siRNA or CFI siRNA_1 (120 nM), and mRNA sequencing was performed 72 h after transfection. (A) Ingenuity pathway analysis (IPA) biofunctions, gene ontology (GO) terms, Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome pathways related to CFI knockdown ($p < 0.05$, $|\log_2 FC| > 0.7$). (B) Heatmap showing the 33 top genes downregulated following knockdown of CFI ($|\log_2 FC| > 1$). (C) Significantly regulated genes belonging to the gene ontology (GO) terms *Metallopeptidase activity* and *Extracellular matrix component* as well as Reactome *Degradation of extracellular matrix* pathways are shown. (D) Heatmap showing the expression of MMP genes after CFI knockdown. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

genes significantly regulated following CFI knockdown were associated with Gene Ontology (GO) terms *Metallopeptidase activity* and *Extracellular matrix component* as well as Reactome *Degradation of extracellular matrix* (Figure 1A). *MMP13* and *MMP10* were found among the top 33 most downregulated genes (Figure 1B). In addition, detailed analysis of the GO terms and Reactome networks mentioned above, revealed genes coding for invasion-related proteases among the significantly downregulated genes after CFI knockdown (Figure 1C). Analysis of the mRNA-seq data indicated that genes for several MMPs, particularly, *MMP2*, *MMP7*, *MMP10*, *MMP13* and *MMP14* (p -value < 0.01 , $|\log_2 FC| \geq 0.7$) were downregulated following CFI knockdown (Figure 1D).

3.2 | Knockdown of CFI downregulates expression of MMP-13 and MMP-2 and attenuates invasion of cSCC cells

In the further experiments, we focused on MMP-13 (collagenase-3) and MMP-2 (gelatinase-A), both of which are expressed by tumor cells in cSCC and have been shown to promote invasion of cSCC cells.^{33,35-41} To further examine the molecular function of CFI, cSCC cells were transfected with CFI targeted siRNAs (CFI siRNA_1, CFI siRNA_2) and negative control siRNA. qRT-qPCR showed significantly reduced mRNA levels for *MMP13* and *MMP2* in primary cSCC cell line (UT-SCC105) and in metastatic cSCC cell line (UT-SCC-7) after CFI knockdown compared with control siRNA transfected cells (Figure 2A, Figure S2). Western blot analysis of the conditioned media confirmed downregulation of MMP-13 and MMP-2 production at protein level following CFI knockdown in two metastatic cSCC cell lines (UT-SCC-7 and UT-SCC-59A) (Figure 2B). Moreover, invasion of cSCC cells (UT-SCC-59A) through 3D type I collagen was significantly attenuated following CFI knockdown (Figure 2C). Similar results on the invasion capacity of cSCC cells were obtained with another cSCC cell line (UT-SCC-105) (Figure S3).

3.3 | Knockdown of CFI inhibits expression of MMP-13 and MMP-2 in cSCC *in vivo*

The effect of CFI on MMP expression in cSCCs *in vivo* was studied in xenograft tumors generated with cSCC cells after silencing CFI expression with specific siRNA (CFI siRNA_1).²¹ To investigate the effect of CFI knockdown on the production of MMP-13 and MMP-2, cSCC xenografts were stained with MMP-13 and MMP-2 antibodies

and analysed with IHC. Weaker staining intensities for MMP-13 and MMP-2 were detected in tumor cells in the invasive front of CFI siRNA_1 xenograft tumors (Figure 3A). Also, a decreased number of MMP-13 and MMP-2 positive cells was noted as compared with control siRNA tumors (Figure 3A). Semiquantitative analysis of MMP-13 expression revealed strong (+++) staining intensity in 63% and moderate (++) staining intensity in 37% of the tumors in the control group, whereas no weak staining was observed (Figure 3B). On the contrary, among the CFI knockdown tumors, 50% were scored strong (+++), 33% were moderate (++) and 17% were weak (+) for MMP-13 expression (Figure 3B). MMP-2 staining intensity was strong (+++) in 57% and moderate (++) in 43% of the control siRNA tumor cases, with no weak (+) staining detected (Figure 3C). In contrast, in CFI siRNA_1 tumors no strong (+++) staining was noted, while 60% of cases were moderately (++) and 40% weakly (+) stained (Figure 3C). Incubation of UT-SCC-7 cultures in parallel showed that knockdown of CFI mRNA persisted up to 15 days after siRNA transfection (data not shown).

3.4 | Overexpression of CFI promotes ERK1/2 activation and proliferation of cSCC cells

To obtain direct evidence for the role of CFI in cSCC cell growth and invasion, we generated a recombinant CFI expression construct. To study the effect of CFI on cSCC proliferation, cells were transfected with the CFI expression construct (pcDNA3.1_CFI) or control expression vector (pcDNA3.1). A significant increase in proliferation was detected in CFI overexpressing cSCC cells (UT-SCC-7 and UT-SCC-59A) compared with control expression vector-transfected cultures (Figure 4A). Activation of ERK1/2 was detected after CFI overexpression in UT-SCC-59A cells (Figure 4B).

3.5 | Overexpression of CFI up-regulates expression of MMP-13 and MMP-2 and promotes invasion of cSCC cells

To further elucidate the mechanistic role of CFI in cSCC cell invasion, cSCC cells were transfected with the recombinant CFI expression construct (pcDNA3.1_CFI) or empty control vector (pcDNA3.1). Markedly elevated levels of CFI were detected in the conditioned media of cSCC cells transfected with CFI expression construct (Figure 4C). In addition, elevated levels of MMP-13 and MMP-2 were detected in conditioned media of CFI overexpressing cSCC cells in

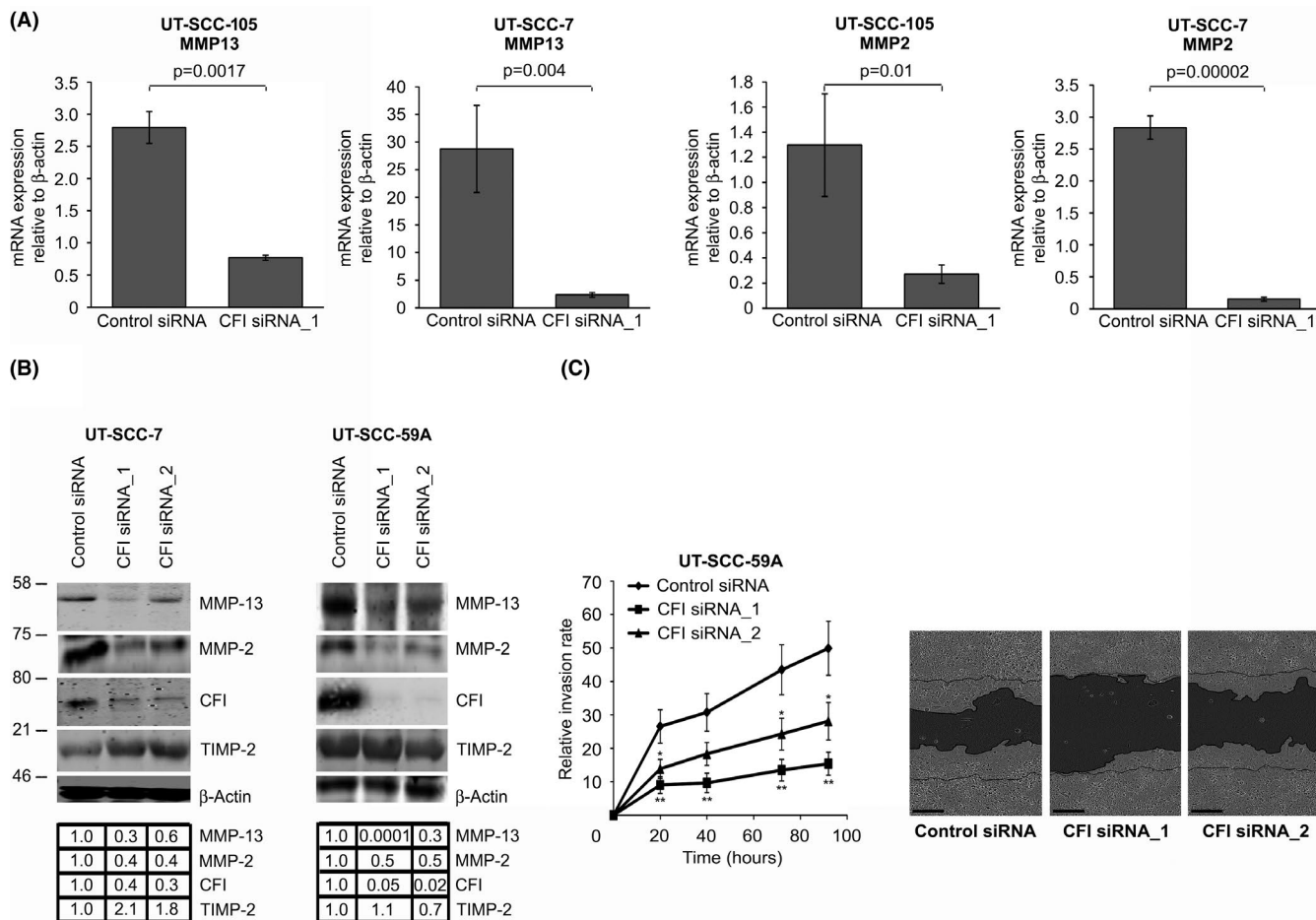


FIGURE 2 Knockdown of CFI downregulates expression of matrix metalloproteinase-13 (MMP-13) and MMP-2 and attenuates invasion of cSCC cells. (A) Primary cSCC cell line (UT-SCC-105) and metastatic cSCC cell line (UT-SCC-7) were transfected with control siRNA or CFI siRNA₁ (120 nM), and RNA was isolated 72 h following transfection. Levels of MMP13 and MMP2 mRNAs were determined with RT-qPCR. Mean \pm S.D. is shown; two-tailed t test. (B) Levels of MMP-13, MMP-2, CFI and TIMP-2 in conditioned media of cSCC cells (UT-SCC-7, UT-SCC-59A) were determined by Western blot analysis 72 h after CFI knockdown. β -actin was used as a sample control reference. (C) cSCC cells (UT-SCC-59A) were transfected with control siRNA or two distinct CFI siRNAs (CFI siRNA₁, CFI siRNA₂) and plated on type I collagen 24 h later. After scratching the cell monolayer, type I collagen solution was added into the wells and allowed to polymerize. Cell culture medium containing 0.5% foetal calf serum was added and cell invasion through 3D-collagen was monitored with IncuCyte S3 real-time cell imaging system. Result of the analysis of cell invasion assay is shown. Representative images of the cell invasion assay are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed t test

comparison to empty control vector-transfected cells (Figure 4C). Furthermore, significant increase in the invasion of cSCC cells through 3D type I collagen (Figure 4D) and 3D Matrigel (Figure 4E) was noted following CFI overexpression.

4 | DISCUSSION

In this study, we have elucidated the functional role and molecular mechanism of complement inhibitor CFI in cSCC progression in detail. Using mRNA-seq analysis, alteration of gene expression profile in cSCC cells after CFI knockdown was evaluated and significant downregulation of the biofunction category *Proliferation of cells* was revealed by IPA, which is in accordance with our previous observations showing the role of CFI in cSCC cell proliferation.²⁰ In

addition, genes significantly regulated after CFI knockdown were related to GO terms *Metallopeptidase activity* and *Extracellular matrix component* along with Reactome *Degradation of extracellular matrix*, suggesting a role for CFI in cSCC cell invasion. Further analysis of the above-mentioned GO terms and Reactome pathways, revealed significant downregulation of the expression of several genes coding for invasion-associated MMPs, including MMP13, MMP2, MMP10 and MMP-7 following CFI knockdown. In the further studies, we focussed on MMP-13 (collagenase-3) and MMP2 (gelatinase-A), both of which are expressed by tumor cells in cSCC and have been shown to promote invasion of cSCC cells.^{33,35-41} Significant decrease in MMP13 and MMP2 mRNA levels after CFI knockdown in primary and metastatic cSCC cells was confirmed with RT-qPCR. Furthermore, knockdown of CFI resulted in reduced production of MMP-13 and MMP-2 and in significant inhibition in the invasion capacity of cSCC

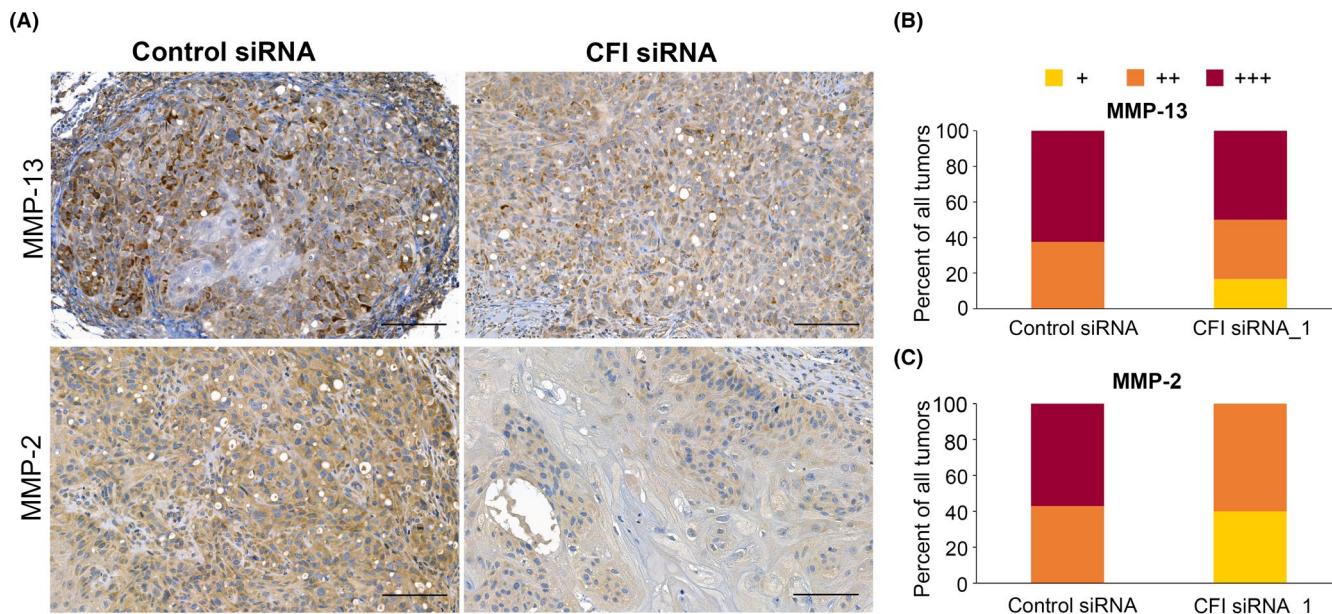


FIGURE 3 Knockdown of CFI suppresses the expression of MMP-13 and MMP-2 in cutaneous squamous cell carcinoma (cSCC) *in vivo*. (A) Human metastatic cSCC cells (UT-SCC-7) were transfected with control and CFI siRNA_1 for 72 h, and the cSCC cells (5×10^6) were implanted subcutaneously into the back of SCID/SCID female mice ($n = 8$ for control group and $n = 6$ for CFI siRNA xenograft tumors). Xenograft tumors were harvested after 18 days, and the expression of MMP-13 and MMP-2 was analysed by immunohistochemistry using MMP-13 and MMP-2 antibodies. The staining intensities for MMP-13 and MMP-2 were weaker in CFI siRNA_1 xenograft tumors and relatively decreased number of positive cells was noted as compared with the control siRNA group. Representative stainings from each group are shown. Scale bars = 100 μ m. Semiquantitative analysis of immunostainings for MMP-13 (B) and MMP-2 (C) IHC were scored as weak (+), moderate (++) and strong (+++). (B) The MMP-13 IHC analysis revealed strong (+++) staining intensity in 63% and moderate (++) staining intensity in 37% of the control group tumors; No weak staining was observed in this category. On the contrary, among the CFI knocked-down tumors, 50% were scored strong (+++), 33% moderate (++) and 17% weak (+) for MMP-13. (C) MMP-2 staining intensity was detected strong (+++) in 50% and moderate (++) in 43% of the control siRNA tumor cases, with no weak (+) staining observed. In contrast, there was no strong (+++) staining in CFI siRNA_1 tumors, while 60% of tumors were moderately (++) and 40% weakly (+) stained

cells through 3D type I collagen. These observations were confirmed using an inverse approach of CFI overexpression using a recombinant CFI expression construct (pcDNA3.1_CFI) generated in this study. In accordance with our previous findings with CFI knock-down,²⁰ overexpression of CFI resulted in activation of ERK1/2 signalling pathway and significantly increased the proliferation of cSCC cells. Overexpression of CFI also resulted in enhanced production of MMP-13 and MMP-2 and in significantly increased invasion capacity of cSCC cells through 3D type I collagen and Matrigel. Taken together, these results provide evidence for the mechanistic role of CFI as an autocrine stimulator of cSCC invasion.

Overexpression of several MMPs by tumor and stromal cells in keratinocyte carcinomas has been reported.⁴² MMP-13 has been shown to be specifically expressed by tumor cells and stromal fibroblasts in cutaneous and head and neck SCCs (HNSCC), but not by keratinocytes in normal skin, AKs or cSCCISs.^{33,35,36} MMP-13 promotes the invasion and survival of cSCC cells and growth of cSCC xenografts *in vivo*.³⁷ In HNSCC, high MMP-13 expression is associated with local invasion of the tumor and correlates with poor prognosis.^{27,43} Upregulation of MMP-2 (gelatinase A) expression has been detected in the invasive margin of cSCC, and the expression is lower in AK and SCCIS.^{38–40} MMP-2 expression is detected also in the peritumoral epidermal layer in cSCC, suggesting association

with UV-induced damage.⁴¹ Latent MMP-2 is specifically activated by MMP-14 (MT1-MMP) and the expression of MMP-14 and MMP-2 correlates with the invasive capacity of cSCC cells.^{44,45} Accordingly, upregulation of MMP-2 expression is associated with tumor invasion in cSCC.⁴⁶ MMP-2 can also activate latent MMP-13, and may this way promote invasion of cSCC.⁴⁷ Interestingly, elevated expression of CFI has also been noted in other invasive cancers, including glioblastoma, pancreatic adenocarcinoma and stomach adenocarcinoma.¹⁸

CFI is a serine protease that hampers all three complement pathways through preventing the formation and promoting the destruction of C3- and C5-convertases by cleaving their major subunits C3b (alternative pathway) and C4b (classical and lectin pathway). Cleavage of C3b has also other major functional consequences, because it inhibits the amplification cascade of complement activation, while simultaneously generating iC3b. Due to the fundamental and strong activity of C3b in various biological processes, its generation and inactivation are strictly regulated. The activity of CFI requires other complement components (CFH, CD35 and CD46) as cofactors and for cleaving C4b, cofactors (C4BP, CD35 and CD46) are also needed.^{13–15} The activity of CFI *in vivo* is regulated by the availability of its substrates, as CFI has no endogenous inhibitors.¹⁵ Our results show that cSCC cells express cofactors of CFI (CFH and CD46) providing basis for its activity under cell culture conditions (Figure S1).

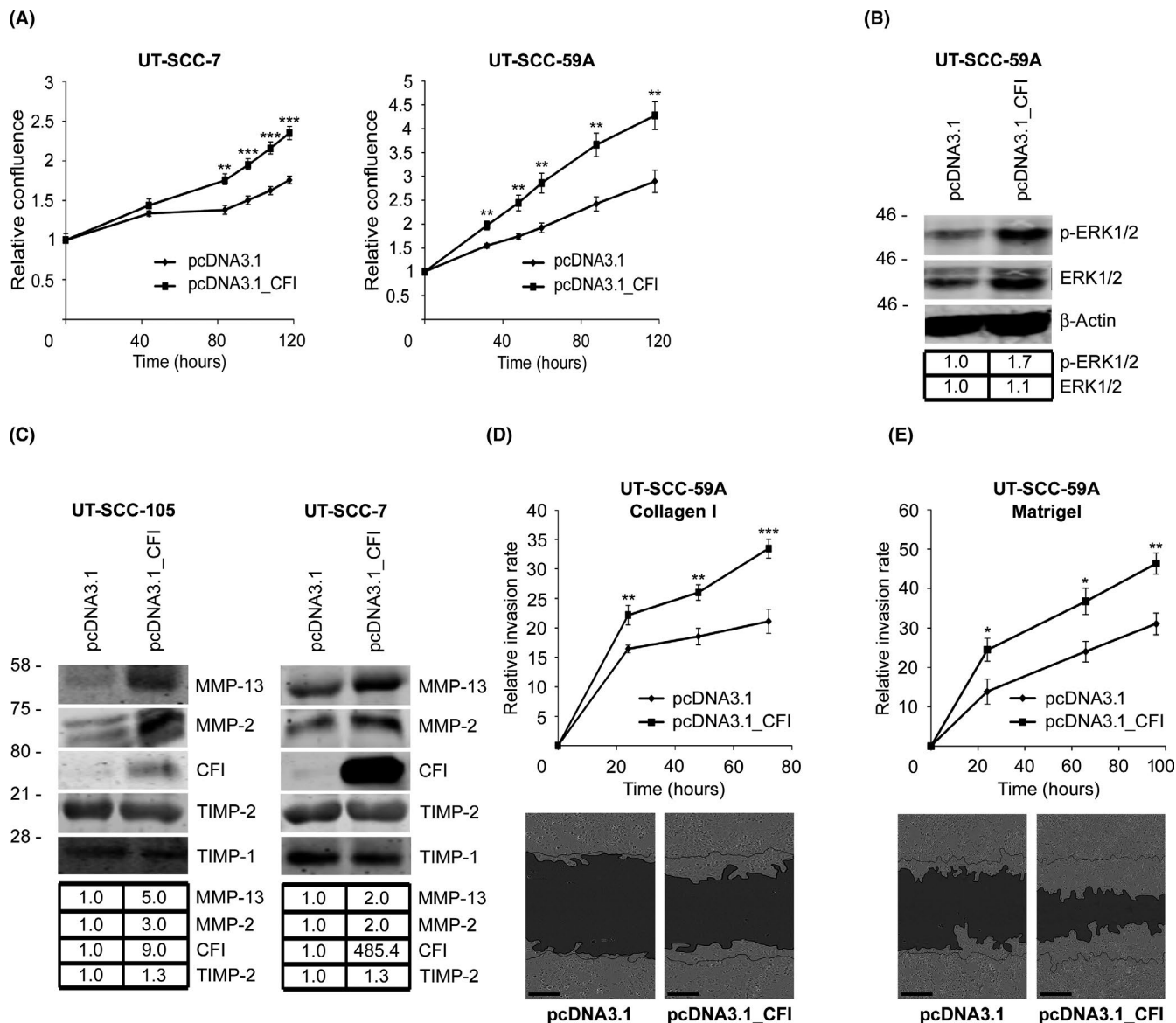


FIGURE 4 Overexpression of CFI upregulates expression of MMP-13 and MMP-2 and promotes invasion of cSCC cells. (A) Metastatic cSCC cells (UT-SCC-7 and UT-SCC-59A) were transfected with negative control expression vector (pcDNA3.1) or CFI expression construct (pcDNA3.1_CFI). Cell proliferation was determined by IncuCyte S3 real-time cell imaging system. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed t test. (B) Protein levels for phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2 in negative control expression vector (pcDNA3.1) transfected and CFI overexpressing cSCC cell lysates were determined by Western blot analysis 72 h following transfection. β -actin was used as a loading reference. (C) Levels of MMP-13, MMP-2, TIMP-2 and TIMP-1 in conditioned media of cSCC cells transfected with negative control expression vector (pcDNA3.1) or CFI expression construct (pcDNA3.1_CFI) were determined by Western blot analysis 72 h post-transfection. TIMP-1 was used as a reference. (D,E) cSCC cells were transfected with negative control expression vector (pcDNA3.1) or CFI expression construct (pcDNA3.1_CFI) and plated on type I collagen (D) or Matrigel (E). The cell monolayer was scratched and type I collagen or Matrigel solution was added into the wells. Following polymerization of collagen (D) or Matrigel (E), cell culture medium with 0.5% foetal calf serum was added. Invasion of cells through 3D-collagen and Matrigel was imaged live with the IncuCyte S3 real-time cell imaging system. The results of cell invasion assay and representative images are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed t test

This notion is supported by our previous studies showing, that, the C3 produced by cSCC cells in culture is cleaved and inactivated, indicating that the CFI produced by these cells is active.²¹ In addition, our previous study shows that knockdown of CFI expression results in activation of C3 and accumulation of C3b on tumor cell surface in cSCC xenografts *in vivo*.²¹ It is possible that the autocrine effect of CFI on cSCC cell proliferation and invasion involves C3. In

this context, it is interesting that cSCC tumor cells also produce C3, which has been shown to function intracellularly and be cleaved to C3a and C3b.^{48,49} However, the function of intracellular C3 in cSCC cells still remains to be explored.

Previous studies have reported expression of several complement components and inhibitors, including CFI and the components of the terminal lytic pathway, by human epidermal keratinocytes.⁵⁰⁻⁵³

Our previous studies and the results of the present study show that cSCC cells do not express components of the terminal pathway (C6, C7, C8 and C9). It is, therefore, likely that in contrast to normal epidermal keratinocytes, cSCC cells are this way to certain extent less sensitive to complement-mediated cell lysis.

In conclusion, the results of this study show that CFI upregulates the expression of invasion-associated MMPs, MMP-13, capable of cleaving fibrillar collagens, and MMP-2, which can cleave several other components of ECM and BM, and activate latent MMP-13.⁴² Increased production of these MMPs by cSCC cells generates a potent proteolytic network in peritumoral environment of cSCC, capable of cleaving the surrounding ECM. Based on these findings we suggest, that CFI promotes progression of cSCC in an autocrine manner by enhancing invasion of cSCC cells and by stimulating proliferation of cSCC cells via ERK1/2 signalling. Our results provide evidence for a novel mechanistic role of CFI as an autocrine stimulator of cSCC invasion. Accordingly, CFI may serve as a prospective molecular marker for invasive and metastatic cSCC.

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CONFLICT OF INTEREST

The authors have declared no conflicting interest.

AUTHOR CONTRIBUTIONS

LN and V-MK supervised and conceived the study. PRN, LN and V-MK designed the study. PRN, LN and PR performed the experiments. LN and MP prepared the CFI expression construct. PRN, LN, PR, MK and V-MK analysed and interpreted the data. PRN, LN, PR and V-MK wrote the original draft. PRN, LN, PR, SM, and V-MK edited the manuscript. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Fig S1. Alteration of complement-related gene expression profile in cSCC cells after CFI knockdown. cSCC cells (UT-SCC-118, UT-SCC-7, and UT-SCC-59A) were transfected with control siRNA or CFI siRNA_1 (120 nM) and mRNA sequencing was performed 72 h after transfection. Heatmap shows the expression of complement-related genes after CFI knockdown. (* $p < 0.05$, ** $p < 0.01$).

Fig S2. Knockdown of CFI down-regulates expression of matrix metalloproteinase-13 (MMP13) and MMP2. Metastatic UT-SCC-7 cells were transfected with control siRNA or CFsiRNA_2 (120 nM) and RNA was isolated 72 h following transfection. Levels of MMP13

and MMP2 mRNAs were determined with RT-qPCR. Mean \pm SD is shown; two-tailed *t*-test.

Fig S3. Knockdown of CFI attenuates the invasion of cSCC cells. UT-SCC-105 cells were transfected with control siRNA or two specific CFI siRNAs (CFI siRNA_1, CFI siRNA_2) and plated on collagen type I 24 h after transfection. After scratching the cell monolayer, collagen I solution was added into the wells and allowed to get polymerized. Cell culture medium including 0.5% fetal calf serum was finally added and cell invasion was tracked using the IncuCyte S3 real-time cell imaging system. Result of the analysis of cell invasion assay is shown. **p* < 0.05, ***p* < 0.01, ****p* < 0.001; two-tailed *t*-test.

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