EphB2 Promotes Progression of Cutaneous Squamous Cell Carcinoma

Mehdi Farshchian[1,2], Liisa Nissinen[1,2], Elina Siljamäki[1,3], Pilvi Riihilä[1,2], Mervi Toriseva[4], Atte Kivisaari[1,2], Risto Ala-aho[1,2], Markku Kallajoki[5], Esko Veräjänkorva[6], Hanne-Kaisa Honkanen[7], Ritva Heljasvaara[7], Taina Pihlajaniemi[7], Reidar Grénman[8], Juha Peltonen[4], Sirkku Peltonen[1] and Veli-Matti Kähäri[1,2]

Keratinocyte-derived skin cancer, cutaneous squamous cell carcinoma (cSCC), is the most common metastatic skin cancer. We have examined the role of Eph/ephrin signaling in the progression of cSCC. Analysis of the expression of *EPH* and *EFN* families in cSCC cells and normal epidermal keratinocytes revealed overexpression of *EPHB2* mRNA in cSCC cells and cSCC tumors *in vivo*. Tumor cell–specific overexpression of EphB2 was detected in human cSCCs and in chemically induced mouse cSCCs with immunohistochemistry, whereas the expression of EphB2 was low in premalignant lesions and normal skin. Knockdown of EphB2 expression in cSCC cells suppressed growth and vascularization of cSCC xenografts *in vivo* and inhibited proliferation, migration, and invasion of cSCC cells in culture. EphB2 knockdown downregulated expression of genes associated with biofunctions *cell viability*, *migration of tumor cells*, and *invasion of tumor cells*. Among the genes most downregulated by EphB2 knockdown were *MMP1* and *MMP13*. Moreover, activation of EphB2 signaling by ephrin-B2-Fc enhanced production of invasion proteinases matrix metalloproteinase-13 (MMP13) and MMP1, and invasion of cSCC cells. These findings provide mechanistic evidence for the role of EphB2 in the early progression of cSCC to the invasive stage and identify EphB2 as a putative therapeutic target in this invasive skin cancer.

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a keratinocyte-derived invasive and metastatic malignant tumor of skin (Madan *et al.*, 2010). The incidence of cSCC is increasing worldwide, making it the most common form of metastatic skin cancer (Rogers *et al.*, 2010). Important risk factors for cSCC include solar UV radiation, chronic ulcers, and

immunosuppression (Alam and Ratner, 2001). Primary cSCCs have a tendency for recurrence and metastasis, and treatment of metastatic cSCCs is challenging in the absence of targeted therapies (Rowe *et al.*, 1992; Czarnecki *et al.*, 2002). UV-induced inactivation of both alleles for *tumor protein 53* (*TP53*) gene is an early event in keratinocyte carcinogenesis resulting in marked expansion in simple mutations, making cSCCs one of the human cancers with highest mutation rate (Durinck *et al.*, 2011; Ratushny *et al.*, 2012). Another early event in the progression of cSCC is loss-of-function mutation of *NOTCH1* (Wang *et al.*, 2011; South *et al.*, 2014). However, at present, the knowledge on the molecular basis of cSCC progression from premalignant lesion (actinic keratosis (AK)) to cSCC *in situ* (cSCCIS) and eventually to invasive cSCC is incomplete (Ratushny *et al.*, 2012). Therefore, molecular markers for rapid progression and metastatic capacity of cSCCs, as well as new therapeutic targets for these tumors, are in need (Kivisaari and Kähäri, 2013).

Erythropoietin-producing hepatocellular (Eph) receptors represent the largest family of receptor tyrosine kinases (RTKs) (Pasquale, 2010). According to their structures and ligand-binding affinities, the human Eph RTKs and their ligands, ephrins, are divided into subclasses A and B (Pasquale, 2010; Nikolov *et al.*, 2013). Glycosylphosphatidylinositol-linked ephrin-A ligands (5 members) promiscuously bind to the EphA RTKs (9 members) and transmembrane ephrin-B ligands

[1]Department of Dermatology, University of Turku and Turku University Hospital, Turku, Finland; [2]MediCity Research Laboratory, University of Turku, Turku, Finland; [3]Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland; [4]Department of Cell Biology and Anatomy, University of Turku, Turku, Finland; [5]Department of Pathology, University of Turku and Turku University Hospital, Turku, Finland; [6]Department of Plastic and General Surgery, University of Turku and Turku University Hospital, Turku, Finland; [7]Biocenter Oulu and Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland and [8]Department of Otorhinolaryngology, Head and Neck Surgery, University of Turku and Turku University Hospital, Turku, Finland

Correspondence: Veli-Matti Kähäri, Department of Dermatology, University of Turku and Turku University Hospital, POB 52, Turku FI-20521, Finland. E-mail: veli-matti.kahari@utu.fi

(3 members) bind to EphB RTKs (5 members). As both receptor and ligand are located on the cell surface, cell–cell contact is usually needed for their interaction. Upon binding, both receptors and ligands can activate cellular signaling resulting in bidirectional reverse and forward signaling in adjacent cells (Pasquale, 2010; Nikolov et al., 2013). In addition, there is evidence for ligand-independent clustering and activation of Eph RTKs on cell surface (Nikolov et al., 2013). The role of Eph/ephrin signaling in numerous biological processes has been established (Lin et al., 2010, 2012; Nievergall et al., 2012; Gordon et al., 2013) and their role in skin homeostasis has been well documented (Egawa et al., 2009; Lin et al., 2010). In human epidermal keratinocytes, EphB2 acting as a ligand triggers reverse signaling, and consequently promotes epidermal differentiation (Walsh and Blumenberg, 2012). The role of Eph/ephrin signaling in tumor progression in general appears complex (Pasquale, 2010; Nievergall et al., 2012). Knowledge available on their role in the progression of skin cancer indicates that members of EphA subfamily serve as tumor suppressors in epidermal keratinocytes, and that loss of EphA2 enhances formation of chemically induced skin cancer in mouse model (Guo et al., 2006b). Furthermore, downregulation of EphA1 expression has been documented in human nonmelanoma skin cancer (Hafner et al., 2006). At present, the role of other members of Eph family in skin cancer is unclear.

Here, we provide evidence that EphB2 plays a role in the progression of cSCC. EphB2 is specifically overexpressed by tumor cells in cSCCs and EphB2 promotes proliferation, migration, and invasion of cSCC cells and growth of cSCC *in vivo* by regulating the expression of genes associated with cell migration and invasion. These results provide molecular background for the role of EphB2 in cSCC progression and identify EphB2 as a potential therapeutic target in this invasive cancer of skin.

RESULTS

Overexpression of *EPHB2* in cSCC cells and tumors

Expression of the entire *EPH* receptor and *EFN* ligand families in primary ($n = 5$) and metastatic ($n = 3$) cSCC cell lines and normal human epidermal keratinocytes (NHEKs) from 5 individuals was analyzed using microarray technique. Significant upregulation of *EPHA4* and *EPHB2* mRNA was noted in cSCC cells as compared with NHEKs (Figure 1a). Moreover, *EFNB1* mRNA levels were significantly lower in cSCC cell lines than in NHEKs. Expression levels of *EPH* and *EFN* mRNAs in cSCC cell lines and NHEKs were also determined by next-generation sequencing. This analysis confirmed specific upregulation of *EPHB2* mRNA and downregulation of *EFNB1* mRNA in cSCC cells as compared with NHEKs (Figure 1b).

The expression profiling analyses with Affymetrix and RNA sequencing identified *EPHB2* as the only Eph receptor significantly overexpressed in cSCC cells with negligible expression in NHEKs (Figure 1a and b). In addition, high expression of *EFNB2*, which codes for ephrin-B2, a high-affinity ligand for EphB2, was detected in cSCC cells (Figure 1a and b). Therefore, *EPHB2* and *EFNB2* mRNA levels in

cSCC cell lines and NHEKs were determined with quantitative real-time reverse-transcriptase–PCR (qRT-PCR). The results showed significant upregulation of *EPHB2* mRNA in cSCC cells, whereas the expression was very low in NHEKs (Figure 1c). In contrast, abundant *EFNB2* mRNA expression was detected in both cSCC cells and NHEKs (Figure 1c). Next, *EPHB2* mRNA levels in cSCC tumors ($n = 6$) and normal skin samples ($n = 7$) *in vivo* were determined with qRT-PCR. The mean level of *EPHB2* mRNA expression was significantly higher in cSCC tumors compared with normal skin (Figure 1d). Abundant expression of *EFNB2* mRNA was detected in both cSCCs and normal skin (Figure 1d).
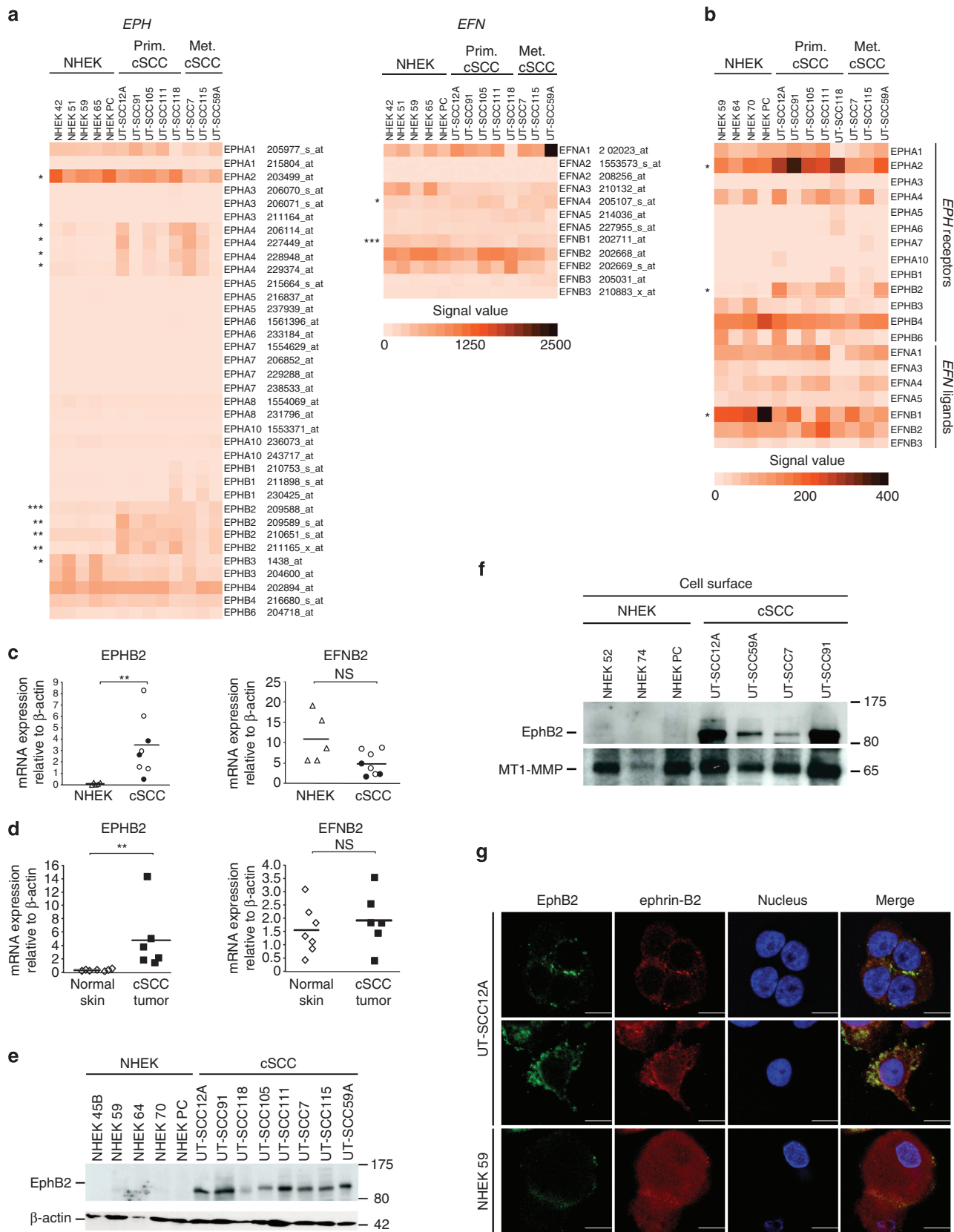
Overexpression of EphB2 on cSCC cell surface

Level of EphB2 production by NHEK and cSCC cell lines was determined by western blot analysis of the total cell lysate. Specific bands corresponding to EphB2 were detected in all cSCC cell lines, whereas EphB2 protein level was very low in NHEKs (Figure 1e). The levels of EphB2 on the surface of cSCC cells and NHEKs were determined by western blot analysis of the biotinylated cell surface proteins pulled down with avidin. Specific EphB2 bands were detected on cell surface in all cSCC cell lines examined, but not in NHEKs (Figure 1f). Cell surface EphB2 and ephrin-B2 on cSCC cells and NHEKs was also analyzed with immunofluorescence staining. Clustering of EphB2 was observed on the surface of individual cSCC cells and in cell–cell contact sites between adjacent cSCC cells (Figure 1g). Prominent staining for ephrin-B2 was noted on the surface of cSCC cells colocalizing with EphB2 clusters. In contrast, no labeling for EphB2 was detected on the surface of NHEKs and staining for ephrin-B2 on NHEKs was not clustered (Figure 1g).

Overexpression of EphB2 by tumor cells in human cSCCs *in vivo*

To study the role of EphB2 in the progression of cSCC *in vivo*, tissue microarrays consisting of normal skin ($n = 12$), UV-induced premalignant lesions (AK; $n = 69$), cSCCIS ($n = 56$), and cSCCs ($n = 68$) were analyzed by immunohistochemistry (IHC) using anti-EphB2 antibody. Positive EphB2 staining was observed on cell surface (Figure 2a) or in cytoplasm (Figure 2b and c) of tumor cells in invasive cSCCs and cSCCIS (Figure 2f) and the intensity of EphB2 labeling was in general stronger in cSCCs and cSCCIS than in premalignant lesions (Figure 2e) and normal skin (Figure 2d).

Semiquantitative analysis of EphB2 stainings (Figure 2g) revealed that the epidermal layer in normal skin (Figure 2d) was negative in 58% and weakly positive (+) in 42% of samples. In AK lesions (Figure 2e), EphB2 staining was mainly negative (48%) or weakly positive (+) (49%). Stronger EphB2 labeling was observed in cSCCIS tissue samples, as moderate (++) staining was observed in 19% of cSCCIS cases (Figure 2f). In invasive cSCCs, strong (+++) (Figure 2c) and moderate (++) (Figure 2b) tumor cell–associated EphB2 staining was noted in 24% of the tumors. In general, EphB2 staining was significantly stronger in cSCCIS and cSCCs than in normal skin and AK compared as groups (Figure 2g).

Overexpression of EphB2 by tumor cells in chemically induced mouse cSCCs

To obtain further evidence for the role of EphB2 in cSCC progression *in vivo*, we employed the well-characterized model of chemically induced mouse skin carcinogenesis (Abel *et al.*, 2009). Tissue samples from untreated normal mouse skin ($n = 5$), vehicle-treated skin ($n = 2$), hyperplastic mouse skin ($n = 6$), and mouse cSCCs ($n = 19$) were stained for EphB2 by IHC and semiquantitative analysis was performed based on staining intensity. Strong ($+++$) (Figure 3b and c) or moderate ($++$) (Figure 3a) EphB2 staining was noted in tumor cells in nearly all mouse cSCCs studied (95% of cases) and none of the tumors were negative. The majority of untreated (Figure 3d) and vehicle-treated (Figure 3e) mouse skin tissues were negative for EphB2 (86%) and weak ($+$) staining was noted in the epidermal layer of only 14% of samples. In hyperplastic mouse epidermal layer induced by 12-*O*-tetradecanoyl phorbol-13-acetate treatment, weak ($+$) staining for EphB2 (Figure 3f) was noted in 83% of cases and 17% were negative. Significantly stronger EphB2 staining was detected in cSCC tumors compared with all nonmalignant mouse skin tissues as a group (Figure 3g).

Alteration of gene expression profile in cSCC cells after EphB2 knockdown

To elucidate the molecular basis of the role of EphB2 in cSCC progression, microarray-based global gene expression profiling and pathway analysis were performed after small interfering RNA (siRNA) knockdown of EphB2 expression in cSCC cell lines ($n = 3$; Figure 4a). Analysis of the genes significantly regulated after EphB2 knockdown revealed that the differentially expressed genes were significantly associated with the biofunction categories *cell death*, *cellular movement*, *cell-to-cell signaling and interactions*, and *cellular growth and proliferation* (Figure 4b). Interestingly, based on the regulation z-score, *cell viability*, *invasion of tumor cells*, *migration of tumor cells*, *migration of cells*, and *cell movement* were the top biofunctions significantly downregulated following EphB2 knockdown (Figure 4b).

Analysis of the molecular network in the top biofunction *invasion of tumor cells* revealed downregulation of mRNAs for several matrix metalloproteinases (MMPs), especially *MMP1* (collagenase-1) and *MMP13* (collagenase-3) (Supplementary Figure S1 online). Interestingly, out of 2,460 differentially regulated probe sets, *MMP1* and *MMP13* were among the most downregulated genes after EphB2 knockdown (Figure 4c). In addition, comparison of the expression of the entire MMP family revealed that the expression of *MMP1* and

MMP13 was significantly downregulated following EphB2 knockdown (Figure 4d). *MMP1* and *MMP13* were also involved in other significantly downregulated biofunctions *migration of tumor cells* (Supplementary Figure S2 online), *migration of cells*, *cell movement*, and *cell viability* (data not shown).

EphB2 knockdown inhibits proliferation, migration, and invasion of cSCC cells

To explore the functional role of EphB2 in cSCC cells ($n = 3$), cell viability, cell migration, and invasion assays were performed following EphB2 knockdown. A significant reduction in the number of viable cSCC cells was noted 24, 48, and 72 hours after EphB2 knockdown (Figure 5a). Cell migration assay was performed 48 hours after transfection of the cSCC cells with control or EphB2 siRNA. Whereas the scratch wounds healed completely within 24 hours in the cSCC cell cultures transfected with control siRNA, knockdown of EphB2 expression clearly inhibited the motility of the cSCC cells (Figure 5b and c). Analysis of cSCC cell migration with time-lapse microscopy revealed effective and rapid migration of cSCC cells transfected with control siRNA (Supplementary Video S1 online). In contrast, a severe defect in the directional migration of cSCC cells was noted following EphB2 knockdown (Supplementary Video S2 online).

In addition, cSCC cells displayed significantly reduced invasion through collagen following knockdown of EphB2 as compared with the control siRNA-transfected cSCC cells (Figure 5d). EphB2 knockdown potently inhibited production of MMP1 and MMP13 in cSCC cell line with high basal expression level of *MMP1* and *MMP13* (Figure 5e). Furthermore, activation of EphB2 signaling by soluble EphB2 ligand, ephrin-B2-Fc (Figure 5f), enhanced invasion of cSCC cells through collagen (Figure 5g) and stimulated production of MMP13 (Figure 5h) and MMP1 (Supplementary Figure S3 online).

EphB2 knockdown suppresses growth of human cSCC xenografts

The role of EphB2 in cSCC growth *in vivo* was examined in xenograft model established with cSCC cells implanted subcutaneously in the back of severe combined immunodeficient (SCID) mice 72 hours after knockdown of EphB2 by transfection with siRNA. A significant delay in the growth of tumors established with EphB2 siRNA-transfected cells was noted already 4 days after implantation of the tumor cells as compared with the control siRNA group, and the difference

◄

Figure 1. Overexpression of *EPHB2* in cutaneous squamous cell carcinoma (cSCC). (**a**) Microarray and (**b**) next-generation sequencing-based analysis of the expression of *EPH* and *EFN* families in primary (Prim., $n = 5$) and metastatic (Met., $n = 3$) human cSCC cell lines and in primary normal human epidermal keratinocytes (NHEKs; $n = 5$ or $n = 4$, respectively). (**c**) *EPHB2* and *EFNB2* mRNA levels in the same cSCC cell lines and NHEKs were determined by quantitative real-time reverse-transcriptase–PCR (qRT-PCR). (**d**) *EPHB2* and *EFNB2* mRNA levels in cSCC tumors ($n = 6$) and normal human skin ($n = 7$) *in vivo* were determined by qRT-PCR. (**e**) EphB2 expression level was analyzed in cell lysates of NHEKs and cSCC cells. (**f**) Level of EphB2 protein on the cell surface of NHEKs and cSCC cells was determined with western blot analysis of biotinylated cell surface proteins. (**g**) EphB2 and ephrin-B2 on the cell surface of the cSCC cells and NHEKs were immunolabeled with primary and fluorescently label secondary antibodies. Yellow signal shows colocalization of the EphB2 and ephrin-B2. Scale bar = 10 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS, not significant; Mann–Whitney two-way *U*-test.
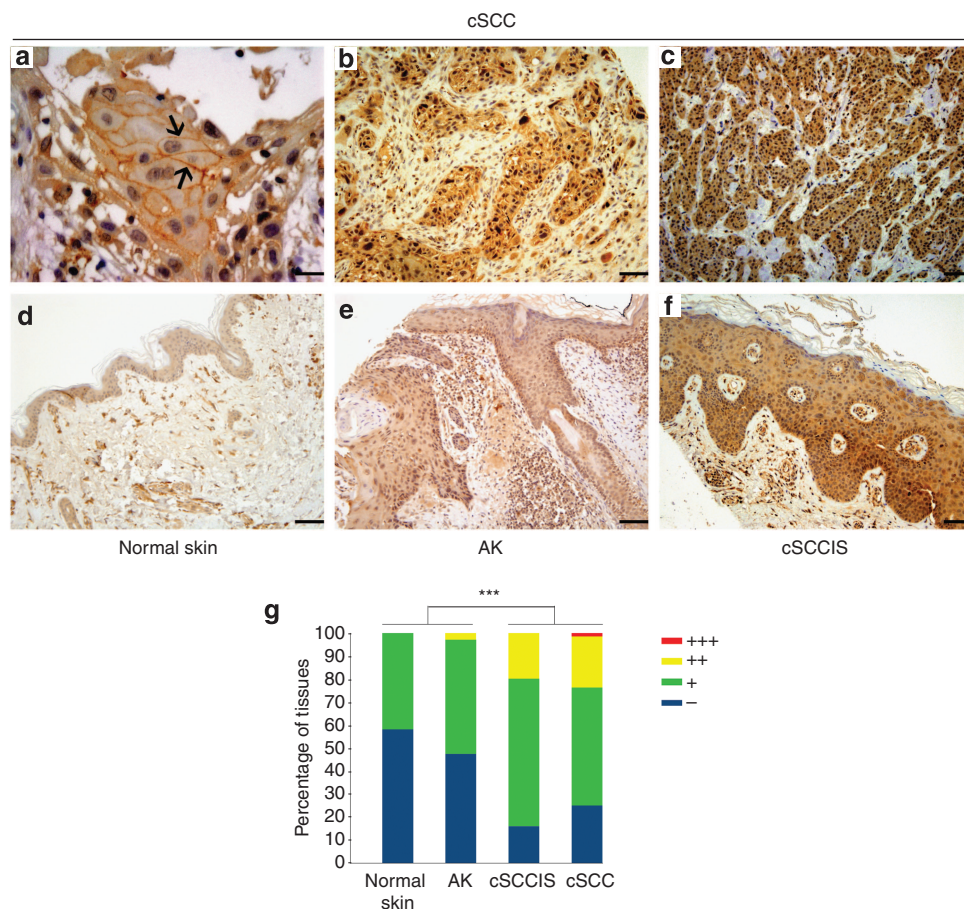
Figure 2. Overexpression of EphB2 in cutaneous squamous cell carcinoma (cSCC) tumor cells. (a–f) EphB2 expression was determined by immunohistochemistry (IHC) of tissue microarrays (TMAs) consisting of human normal skin ($n=12$), actinic keratosis (AK; $n=69$), cSCCs *in situ* (cSCCIS; $n=56$), and sporadic cSCCs ($n=68$). In sporadic, UV-induced human cSCCs, (a) cell surface (arrows) or (b and c) cytoplasmic EphB2 staining with (b) moderate (++) and (c) strong (+++) staining intensity was noted. In cSCCIS, staining intensity was mainly (f) moderate (++) or weak (+). EphB2 staining was (e) weak (+) or absent (−) in AK. (d) Negative (−) or weak (+) EphB2 staining was detected in epidermal layer of normal skin. (g) EphB2 staining intensity was significantly more abundant in cSCCs and cSCCIS compared with AK and normal skin. Scale bar = 25 µm (a) and 100 µm (b–f). ***$P<0.001$; Fisher's exact test.

increased throughout the observation period of 28 days (Figure 6a). Extended incubation of cSCC cells transfected with siRNA showed persistence of EphB2 knockdown up to 8 days (data not shown).

Histological analysis of the xenografts harvested at day 28 revealed that the tumors established with EphB2 siRNA-transfected cSCC cells contained more inert material and were less cellular as compared with the xenografts in the control siRNA group (Figure 6b, upper panels). Accordingly, the relative number of proliferating (Ki-67-positive) tumor cells was significantly lower in the xenografts established with cSCC cells transfected with EphB2 siRNA (17%) compared with control siRNA tumors (39%) (Figure 6b, middle panels, and Figure 6c). In addition, IHC analysis of the xenografts for vascular marker CD34 revealed significantly reduced density of CD34-positive blood vessels in EphB2 knockdown tumors compared with control siRNA xenografts (Figure 6b, lower panels, and Figure 6d).

DISCUSSION
The role of Eph/ephrin signaling in normal biological functions such as axon guidance, neural development, and cell migration and adhesion is well established, but their role in cancer progression is complex (Merlos-Suarez and Batlle, 2008; Coulthard et al., 2012). Distinct members of Eph/ephrin families have emerged both as tumor suppressors and as promoters of cancer progression (Merlos-Suarez and Batlle, 2008; Pasquale, 2010; Nievergall et al., 2012). Upregulation of EphA2 expression has been documented in prostate cancer (Walker-Daniels et al., 1999), breast cancer (Zelinski et al., 2001), melanoma (Udayakumar et al., 2011), and glioblastoma (Wykosky and Debinski, 2008), providing evidence for its role in tumor progression. Furthermore, EphB2 displays both tumor-suppressing and tumor-promoting effects. EphB receptor activation suppresses growth of colorectal cancer (Batlle et al., 2005). Loss-of-function mutations or deletion of *EPHB2* have been reported in prostate cancer
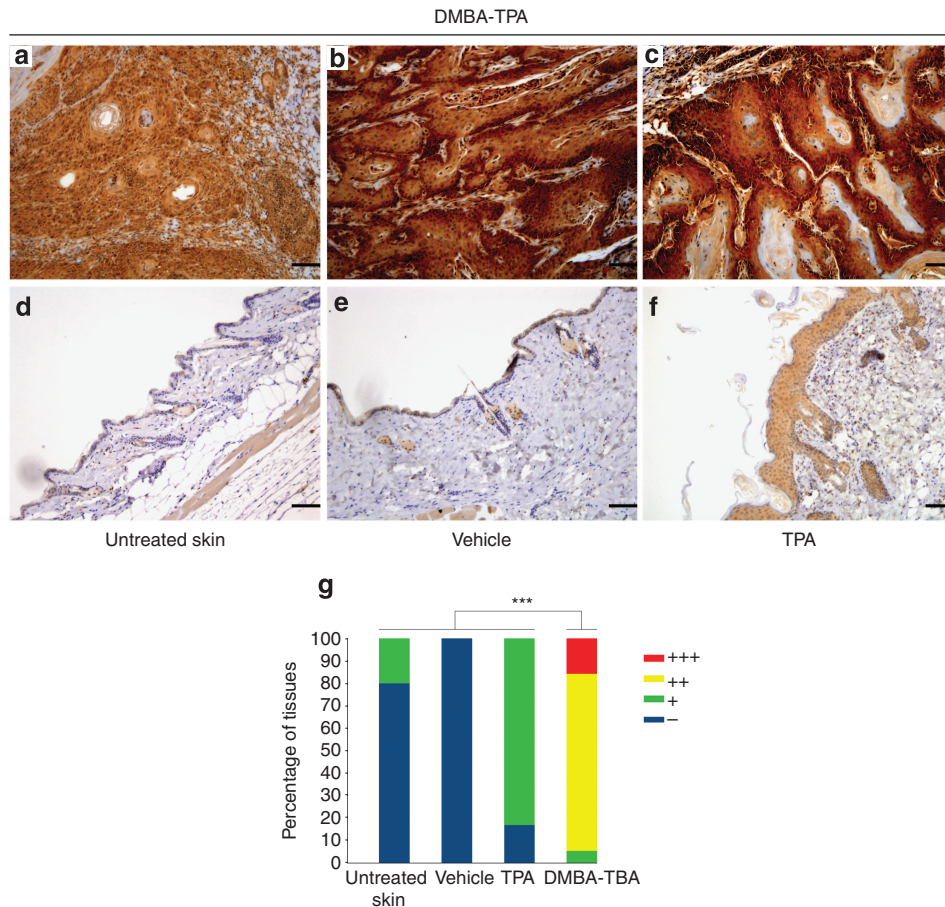
Figure 3. Overexpression of EphB2 in chemically induced mouse cutaneous squamous cell carcinoma (cSCC). (**a–f**) EphB2 expression was analyzed by immunohistochemistry (IHC) of normal (*n* = 5), vehicle-treated (*n* = 2), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)–treated mouse skin (*n* = 6), and chemically 7,12-dimethylbenz[α]anthracene (DMBA)/TPA–induced mouse skin SCCs (*n* = 19). (**a**) Moderate (++) and (**b** and **c**) strong (+++)cytoplasmic staining for EphB2 was detected in DMBA/TPA-induced mouse skin SCC. (**f**) Treatment of mouse skin with TPA-induced hyperplasia of the epidermis and a weak (+) EphB2 staining of keratinocytes. EphB2 staining was mainly negative (−) in epidermal layer of (**d**) untreated or (**e**) vehicle (acetone)-treated mouse skin. (**g**) Staining for EphB2 was significantly more abundant in DMBA/TPA-induced mouse cSCCs compared with nonmalignant samples as a group. Scale bar = 100 μm. ***$P < 0.001$; Fisher's exact test.

(Huusko *et al.*, 2004), colorectal cancer (Alazzouzi *et al.*, 2005), and gastric cancer (Davalos *et al.*, 2007). Reduction in the expression of EphB2 is associated with invasion, metastasis, and poor prognosis in colorectal tumors (Lugli *et al.*, 2005; Guo *et al.*, 2006a), and high level of EphB2 expression in colorectal cancer is associated with longer survival (Jubb *et al.*, 2005). In contrast, activation of EphB2 has been shown to promote invasion of glioblastoma *in vivo* (Wang *et al.*, 2012). In transitional cell carcinoma of the bladder, loss of EphB2 and gain of EphB4 expression have been reported to be associated with the tumor progression, and EphB4 inhibitors have been shown to have antitumor properties in xenograft model of this tumor (Li *et al.*, 2014). Previous studies on the role of Eph/ephrin signaling in the progression of skin cancer have identified members of EphA subfamily, especially EphA2, as tumor suppressor in epidermal keratinocytes (Guo *et al.*, 2006b).

Here, we have examined the role of EphB2 in the progression of cSCC, the most common metastatic skin cancer (Alam and Ratner, 2001; Czarnecki *et al.*, 2002; Madan *et al.*, 2010; Rogers *et al.*, 2010). Overexpression of *EPHB2* was noted in primary and metastatic cSCC cell lines compared with NHEKs using microarray and next-generation sequencing techniques and qRT-PCR. Remarkably more EphB2 was noted on the cell surface of cSCC cells as compared with NHEKs with western blot analysis of cell surface proteins and with immunofluorescence staining. Immunofluorescence staining also revealed clustering of EphB2 and colocalization with its ligand, ephrin-B2, on cSCC cell surface. Moreover, analysis of *EPHB2* mRNA levels by qRT-PCR revealed significant overexpression of *EPHB2* in cSCC tumors *in vivo* as compared with normal skin. In addition, IHC analysis of a large panel of tissue samples from normal skin, premalignant lesions (AKs), and cSCCs revealed stronger tumor cell–associated labeling for ephB2 in cSCCIS and cSCCs than in normal skin and AK lesions. Prominent tumor cell–associated staining for EphB2 was also detected in chemically induced murine skin SCCs, whereas the EphB2
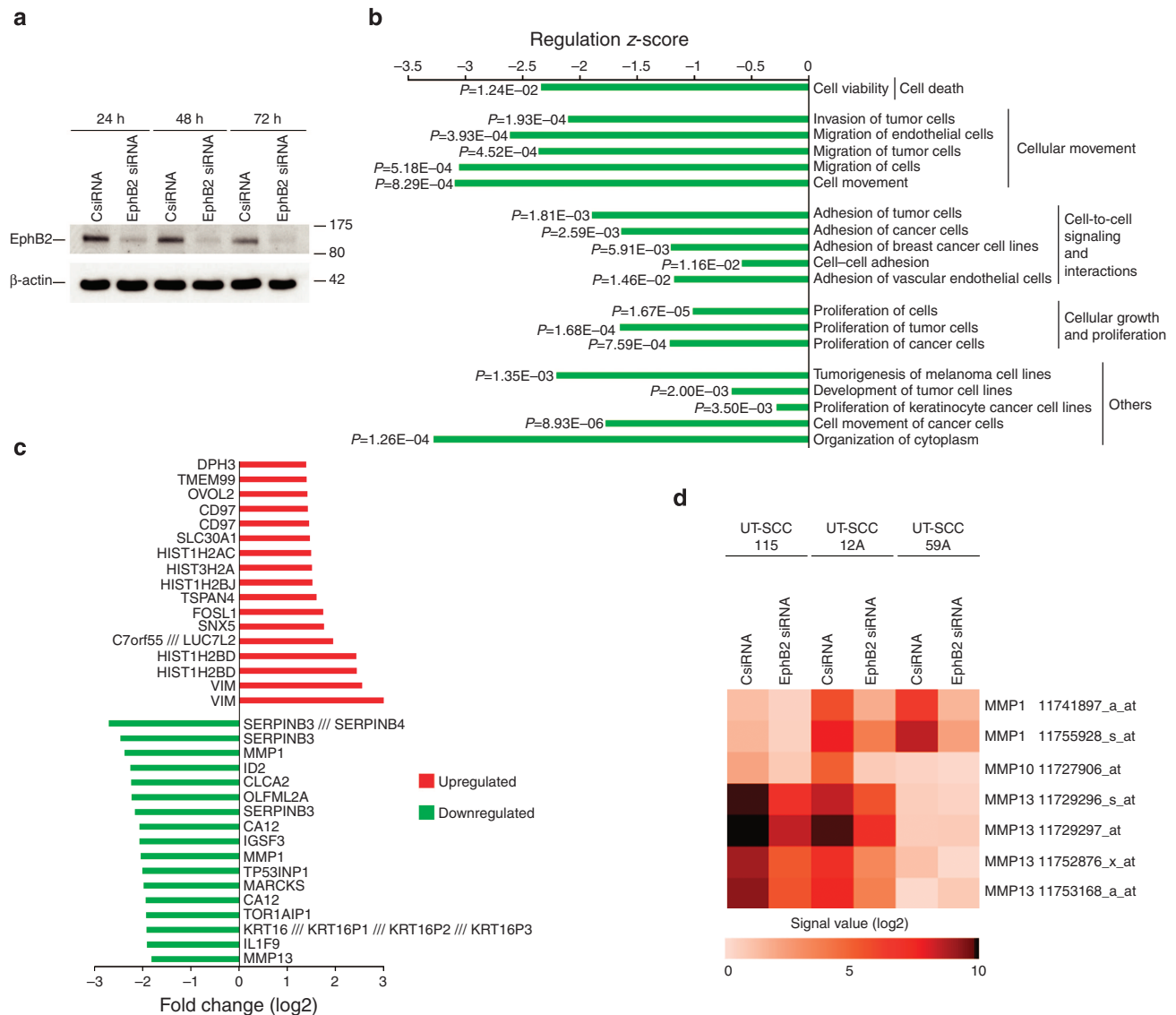
Figure 4. Alteration of gene expression profile in cutaneous squamous cell carcinoma (cSCC) cells after EphB2 knockdown. (**a**) UT-SCC12A cells were transfected with EphB2 or control small interfering RNA (siRNA) and transfection efficiency was verified by western blotting. (**b**) Summary of significantly regulated biofunctions associated with the differentially expressed molecules in three cSCC cell lines 72 hours after EphB2 knockdown as compared with the corresponding control siRNA-transfected cultures. Categories of related biofunctions are shown on the right. Others include categories: cancer, cellular development, and organismal development. (**c**) The most significantly ($P < 0.05$) upregulated and downregulated genes based on fold changes following EphB2 knockdown in cSCC cell lines ($n = 3$). (**d**) The expression of the *MMP* genes that were significantly ($P < 0.05$) regulated by EphB2 knockdown is shown as heatmap.

staining in hyperplastic and normal mouse skin was either weak or absent. Similar to what has been observed in other tumor cells (Wu *et al.*, 2004), labeling for EphB2 was noted predominantly in the cytoplasm of the cSCC tumor cells. Internalization of the full-length EphB2 by endocytosis upon activation is the possible explanation for the cytoplasmic localization of EphB2 *in vivo* (Zimmer *et al.*, 2003; Pasquale, 2010) Altogether, these results show that the expression of EphB2 is very low in normal intact skin and is specifically induced in tumor cells in cSCCs, suggesting EphB2 as a biomarker for cSCC. Furthermore, expression of EphB2 in cSCCIS provides evidence that the induction of EphB2

expression is an early event in cSCC development and may play a role in the progression of *in situ* cSCC to the invasive stage.

Knockdown of EphB2 expression in cSCC cells resulted in significant delay in the growth of cSCC xenograft tumors in SCID mice already at the early stage compared with control siRNA group. These results provide further evidence that EphB2 plays a role in implantation and early growth of cSCC tumor cells *in vivo*. Furthermore, our results showed that EphB2 knockdown inhibited vascularization of the cSCC xenografts, suggesting a role for EphB2 in cSCC tumor angiogenesis. Functional studies with cSCC cells indicated
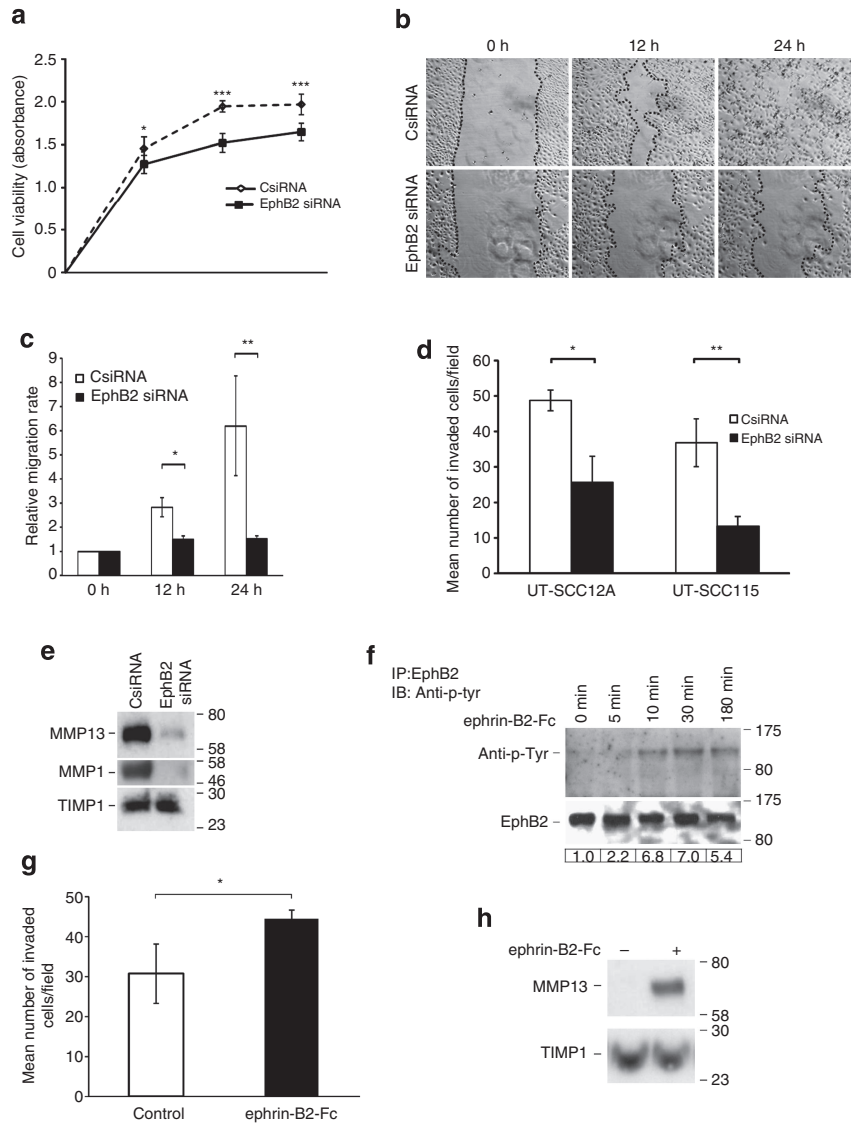
Figure 5. EphB2 knockdown inhibits proliferation, migration, and invasion of cutaneous squamous cell carcinoma (cSCC) cells. (a) The number of viable cSCC cells (UT-SCC59A) was determined with cell viability assay 24, 48, and 72 hours after transfection with EphB2 and control small interfering RNA (siRNA; mean ± SD, $n = 8$). **(b)** UT-SCC12A cells were transfected with EphB2 and control siRNA (CsiRNA). At 48 hours after transfections, cultures were treated for 6 hours with hydroxyurea (2 mM) and scratch wounds were created in monolayer cultures. **(c)** The images of migration were analyzed with ImageJ (mean ± SEM, $n = 3$). **(d)** cSCC cells were seeded to the inserts coated with type I collagen 24 hours after transfection with EphB2 and control siRNA. The number of invaded cells was counted after 48 hours (mean ± SD, $n = 3$). **(e)** Level of matrix metalloproteinases-13 (MMP13) and MMP1 in conditioned media of cSCC cells (UT-SCC12A) were determined by western blot analysis 72 hours after EphB2 knockdown. Tissue inhibitor of metalloproteinase-1 (TIMP1) was used as marker for equal loading. **(f)** UT-SCC59A cells were incubated with ephrin-B2-Fc (0.05 μM) for 5, 10, 30, and 180 minutes. EphB2 was immunoprecipitated from cell lysates and analyzed by western blot with anti-phosphotyrosine antibody. The blot was reprobed to examine total EphB2. Levels of p-EphB2 were quantitated by densitometry and corrected for the level of total EphB2 (values below the blots). IB, immunoblotting; IP, immunoprecipitation. **(g)** UT-SCC115 cells were treated with ephrin-B2-Fc (0.05 μM) for 5 minutes and seeded to the inserts coated with collagen I. The number of invaded cells was counted 48 hours later. (mean ± SD, $n = 3$). **(h)** UT-SCC59A cells were treated with ephrin-B2-Fc (0.05 μM) for 48 hours. MMP13 levels were determined in conditioned media by western blot analysis. TIMP1 was used as loading control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Student's t-test.

that knockdown of EphB2 expression markedly inhibited proliferation and migration of cSCC cells. In addition, analysis of cell migration by time-lapse microscopy showed that knockdown of EphB2 potently inhibited directional migration of cSCC cells. Eph signaling can mediate cell contact–dependent cell repulsion that can guide directional migration of cells (Poliakov *et al.*, 2004; Lin *et al.*, 2008). Our results

suggest that reduction in cell repulsion as a result of EphB2 knockdown leads to defective directional migration of cSCC cells and may this way impair their invasion capacity.

The molecular background for the role of EphB2 in cSCC growth and migration and invasion was examined by global gene expression profiling of cSCC cells following EphB2 knockdown. Pathway analysis showed that significantly
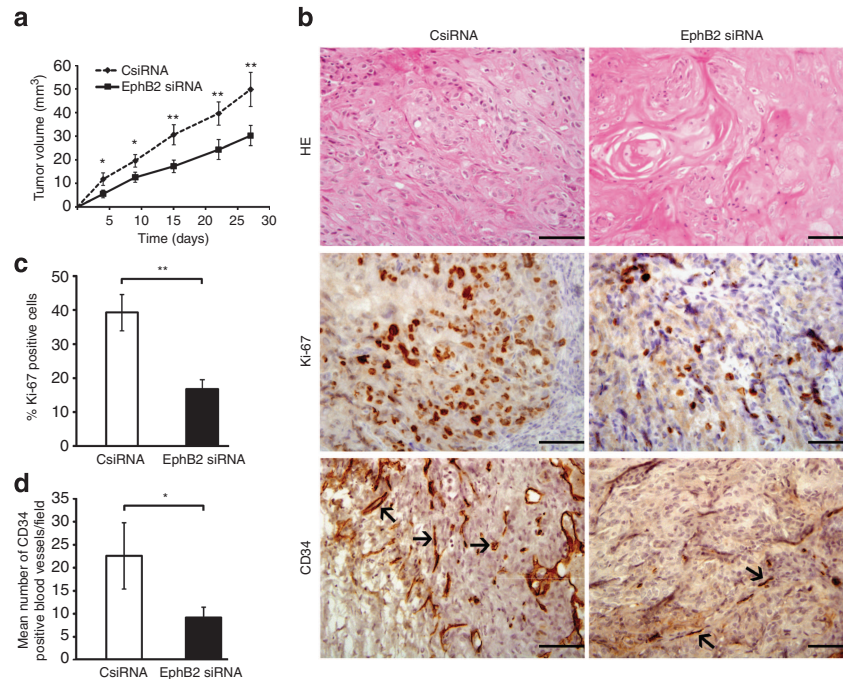
Figure 6. EphB2 knockdown suppresses growth of human cutaneous squamous cell carcinoma (cSCC) xenografts. (a) UT-SCC7 cells (5×10^6) were injected subcutaneously into the back of severe combined immunodeficient (SCID) mice 72 hours after transfection with EphB2 siRNA ($n = 8$) or control siRNA (CsiRNA; $n = 7$) (mean ± SEM). **(b)** Tumors were harvested at day 28 and stained with hematoxylin and eosin (HE; upper panels), for proliferation marker Ki-67 (middle panels), and for vascular endothelial marker CD34 (lower panels). Arrows indicate CD34-positive blood vessels. **(c)** The percentages of Ki-67-positive tumor cells in xenografts were counted (mean ± SEM). **(d)** The density of CD34-positive blood vessels in xenografts was analyzed (mean ± SEM). Scale bar = 100 μm. *$P < 0.05$, **$P < 0.01$; Student's *t*-test.

downregulated genes were associated with biofunctions *cell viability*, *migration of cells*, *cell movement*, *migration of tumor cells*, and *invasion of tumor cells*. Among the genes most downregulated by EphB2 knockdown were *MMP1* and *MMP13* that code for collagenase-1 and collagenase-3, respectively, the two metalloproteinases that are associated with cancer invasion. Among these, MMP13 has previously been identified as an SCC-specific proteinase, as it is expressed by tumor cells in SCCs of the skin and head and neck, but not by keratinocytes in normal skin and oral mucosa (Airola *et al.*, 1997; Johansson *et al.*, 1997). MMP13 also promotes invasion of cSCC cells and growth of cSCC *in vivo* (Ala-aho *et al.*, 2004), and its expression in head and neck SCCs correlates with local invasion (Stokes *et al.*, 2010). Our results showed that EphB2 knockdown potently inhibited the production of MMP13 and MMP1 by cSCC cells, resulting in inhibition of cSCC cell invasion through collagen. On the other hand, activation of EphB2 with soluble ligand ephrin-B2-Fc induced the expression of MMP13 and MMP1, and increased cSCC cell invasion through collagen. Together, these observations show that EphB2 specifically regulates the expression of two important invasion proteinases, MMP13 and MMP1, and may this way promote cSCC cell invasion and implantation into tissue. Interestingly, both MMP13 and MMP1 can cleave ephrin-A1 and in this way regulate its signaling function (Beauchamp *et al.*, 2012) that may play a role in cSCC progression (Hafner *et al.*, 2006; Guo *et al.*, 2006b).

In conclusion, our findings demonstrate specific over-expression of EphB2 by cSCC cells in culture and *in vivo*. These results identify EphB2 as a biomarker for cSCC and propose a role for EphB2 in the early stage of tumor progression to invasive cSCC. In addition, knockdown of EphB2 expression suppresses growth of cSCC tumors *in vivo* and inhibits proliferation, migration, and invasion of cSCC cells. Altogether, these results suggest EphB2 as a therapeutic target in cSCCs, especially in patients with unresectable, recurrent, or multiple tumors, and in patients with high risk for cSCC (e.g., immunosuppressed patients).

MATERIALS AND METHODS
Ethical issues
Approval for the use of archival tissue specimens and the collection of normal skin and cSCC tissues was obtained from the Ethics Committee of the Hospital District of Southwest Finland, Turku, Finland. The study was performed in accordance with the Declaration of Helsinki Principles. Each patient gave their informed written consent. The experiments with mice were approved by the State Provincial Office of Southern Finland and conducted according to institutional guidelines.

Cell culture
Human cSCC cell lines ($n = 8$) were established from surgically removed cSCCs (Farshchian *et al.*, 2011). NHEK cultures were established from normal skin of patients ($n = 10$) undergoing surgery

for mammoplasty (Farshchian *et al.*, 2011) and purchased from PromoCell ($n = 1$) (Heidelberg, Germany). cSCC cells and NHEKs were cultured as previously described (Farshchian *et al.*, 2011; Riihilä *et al.*, 2014b).

Biochemical and molecular biology techniques

Protocols for gene expression profiling, qRT-PCR, western blot analysis, analysis of cell surface protein, immunofluorescence staining, and analysis of phosphorylated EphB2 are provided in Supplementary Materials and Methods online.

Tissue samples

Cutaneous SCC tumor samples ($n = 6$) were obtained from the Turku University Hospital after surgery of primary tumors. Normal skin samples ($n = 7$) were collected from the mammoplasty surgery (Farshchian *et al.*, 2011). Tissue microarray blocks consisting of samples from normal sun-protected skin ($n = 12$), AK ($n = 69$), cSCCIS ($n = 56$), and UV-induced cSCC ($n = 68$) were generated from the archival paraffin blocks from the Department of Pathology, Turku University Hospital (Farshchian *et al.*, 2011; Riihilä *et al.*, 2014b).

Mouse skin chemical carcinogenesis

Tissue sections of normal mouse skin ($n = 5$), acetone-treated ($n = 2$), 12-*O*-tetradecanoyl phorbol-13-acetate–treated hyperplastic skin ($n = 6$), and 7,12-dimethylbenz[α]anthracene/12-*O*-tetradecanoyl phorbol-13-acetate–induced mouse skin SCC ($n = 19$) were prepared from the skin of FVB/N HanHsd mice as described previously (Brideau *et al.*, 2007; Farshchian *et al.*, 2011).

Immunohistochemistry

Detailed protocol for IHC staining and analysis are given in Supplementary Materials and Methods online.

EphB2 knockdown and functional studies

The detailed protocols for EphB2 knockdown with siRNA, cell viability, migration and invasion assay, and MMP1 and MMP13 production analysis are described in the Supplementary Materials and Methods online.

Human cSCC xenografts

SCID female, 6-week-old mice (CB17/Icr-*Prkdc*^scid^/IcrIcoCrl) (Charles River Laboratories, Wilmington, MA) were randomly allocated into EphB2 ($n = 8$) and control siRNA group ($n = 7$). UT-SCC7 cells (5×10^6) were injected subcutaneously into the back of mice 72 hours after the transfection with EphB2 or control siRNA (Ala-aho *et al.*, 2004). Tumors were measured and their volumes were calculated using the formula: $V = \pi 4/3((L+W)/4)$ (Riihilä *et al.*, 2014a). Tumors were excised after 28 days, embedded in paraffin, and stained with hematoxylin and eosin. Proliferating cells were detected with IHC using mouse anti-Ki-67 antibody (Dako, Glostrup, Denmark). The percentages of Ki-67-positive cells were counted in four distinct microscopic fields at ×20 magnification in areas with the highest proportion of Ki-67 staining. Vascularization of the xenograft tumors was assessed by IHC with anti-CD34 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blood vessel density was evaluated in each sample by counting the number of CD34-positive blood vessels in four defined microscopic fields at ×20 magnification.

Statistical analysis

Statistical analysis between groups was performed with Mann-Whitney *U*-test and Student's *t*-test. Statistical analysis of the IHC results was performed with Fisher's exact test.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Abel EL, Angel JM, Kiguchi K *et al.* (2009) Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *Nat Protoc* 4:1350–62

Airola K, Johansson N, Kariniemi AL *et al.* (1997) Human collagenase-3 is expressed in malignant squamous epithelium of the skin. *J Invest Dermatol* 109:225–31

Ala-aho R, Ahonen M, George SJ *et al.* (2004) Targeted inhibition of human collagenase-3 (MMP-13) expression inhibits squamous cell carcinoma growth in vivo. *Oncogene* 23:5111–23

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

Alazzouzi H, Davalos V, Kokko A *et al.* (2005) Mechanisms of inactivation of the receptor tyrosine kinase EPHB2 in colorectal tumors. *Cancer Res* 65: 10170–3

Batlle E, Bacani J, Begthel H *et al.* (2005) EphB receptor activity suppresses colorectal cancer progression. *Nature* 435:1126–30

Beauchamp A, Lively MO, Mintz A *et al.* (2012) EphrinA1 is released in three forms from cancer cells by matrix metalloproteases. *Mol Cell Biol* 32: 3253–64

Brideau G, Mäkinen MJ, Elamaa H *et al.* (2007) Endostatin overexpression inhibits lymphangiogenesis and lymph node metastasis in mice. *Cancer Res* 67:11528–35

Coulthard MG, Morgan M, Woodruff TM *et al.* (2012) Eph/Ephrin signaling in injury and inflammation. *Am J Pathol* 181:1493–503

Czarnecki D, Sutton T, Czarnecki C *et al.* (2002) A 10-year prospective study of patients with skin cancer. *J Cutan Med Surg* 6:427–9

Davalos V, Dopeso H, Velho S *et al.* (2007) High EPHB2 mutation rate in gastric but not endometrial tumors with microsatellite instability. *Oncogene* 26: 308–11

Durinck S, Ho C, Wang NJ *et al.* (2011) Temporal dissection of tumorigenesis in primary cancers. *Cancer Discov* 1:137–43

Egawa G, Osawa M, Uemura A *et al.* (2009) Transient expression of ephrin b2 in perinatal skin is required for maintenance of keratinocyte homeostasis. *J Invest Dermatol* 129:2386–95

Farshchian M, Kivisaari A, Ala-Aho R *et al.* (2011) Serpin peptidase inhibitor clade A member 1 (SerpinA1) is a novel biomarker for progression of cutaneous squamous cell carcinoma. *Am J Pathol* 179:1110–9

Gordon K, Kochkodan JJ, Blatt H *et al.* (2013) Alteration of the EphA2/Ephrin-A signaling axis in psoriatic epidermis. *J Invest Dermatol* 133:712–22

Guo DL, Zhang J, Yuen ST *et al.* (2006a) Reduced expression of EphB2 that parallels invasion and metastasis in colorectal tumours. *Carcinogenesis* 27:454–64

Guo H, Miao H, Gerber L *et al.* (2006b) Disruption of EphA2 receptor tyrosine kinase leads to increased susceptibility to carcinogenesis in mouse skin. *Cancer Res* 66:7050–8

Hafner C, Becker B, Landthaler M *et al.* (2006) Expression profile of Eph receptors and ephrin ligands in human skin and downregulation of EphA1 in nonmelanoma skin cancer. *Mod Pathol* 19:1369–77

Huusko P, Ponciano-Jackson D, Wolf M *et al.* (2004) Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer. *Nat Genet* 36:979–83

Johansson N, Airola K, Grenman R *et al.* (1997) Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. *Am J Pathol* 151:499–508

Jubb AM, Zhong F, Bheddah S *et al.* (2005) EphB2 is a prognostic factor in colorectal cancer. *Clin Cancer Res* 11:5181–7

Kivisaari A, Kähäri VM (2013) Squamous cell carcinoma of the skin: Emerging need for novel biomarkers. *World J Clin Oncol* 4:85–90

Li X, Choi WW, Yan R *et al.* (2014) The differential expression of EphB2 and EphB4 receptor kinases in normal bladder and in transitional cell carcinoma of the bladder. *PLoS One* 9:e105326

Lin KT, Sloniowski S, Ethell DW *et al.* (2008) Ephrin-B2-induced cleavage of EphB2 receptor is mediated by matrix metalloproteinases to trigger cell repulsion. *J Biol Chem* 283:28969–79

Lin S, Gordon K, Kaplan N *et al.* (2010) Ligand targeting of EphA2 enhances keratinocyte adhesion and differentiation via desmoglein 1. *Mol Biol Cell* 21:3902–14

Lin S, Wang B, Getsios S (2012) Eph/ephrin signaling in epidermal differentiation and disease. *Semin Cell Dev Biol* 23:92–101

Lugli A, Spichtin H, Maurer R *et al.* (2005) EphB2 expression across 138 human tumor types in a tissue microarray: high levels of expression in gastrointestinal cancers. *Clin Cancer Res* 11:6450–8

Madan V, Lear JT, Szeimies RM (2010) Non-melanoma skin cancer. *Lancet* 375:673–85

Merlos-Suarez A, Batlle E (2008) Eph-ephrin signalling in adult tissues and cancer. *Curr Opin Cell Biol* 20:194–200

Nievergall E, Lackmann M, Janes PW (2012) Eph-dependent cell-cell adhesion and segregation in development and cancer. *Cell Mol Life Sci* 69:1813–42

Nikolov DB, Xu K, Himanen JP (2013) Eph/ephrin recognition and the role of Eph/ephrin clusters in signaling initiation. *Biochim Biophys Acta* 1834:2160–5

Pasquale EB (2010) Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer* 10:165–80

Poliakov A, Cotrina M, Wilkinson DG (2004) Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev Cell* 7:465–80

Ratushny V, Gober MD, Hick R *et al.* (2012) From keratinocyte to cancer: the pathogenesis and modeling of cutaneous squamous cell carcinoma. *J Clin Invest* 122:464–72

Riihilä P, Nissinen L, Farshchian M *et al.* (2014a) Complement Factor I promotes progression of cutaneous squamous cell carcinoma. *J Invest Dermatol* 135:579–88

Riihilä PM, Nissinen LM, Ala-aho R *et al.* (2014b) Complement factor H: a biomarker for progression of cutaneous squamous cell carcinoma. *J Invest Dermatol* 134:498–506

Rogers HW, Weinstock MA, Harris AR *et al.* (2010) Incidence estimate of nonmelanoma skin cancer in the United States, 2006. *Arch Dermatol* 146:283–7

Rowe DE, Carroll RJ, Day CL Jr (1992) Prognostic factors for local recurrence, metastasis, and survival rates in squamous cell carcinoma of the skin, ear, and lip. Implications for treatment modality selection. *J Am Acad Dermatol* 26:976–90

South AP, Purdie KJ, Watt SA *et al.* (2014) NOTCH1 mutations occur early during cutaneous squamous cell carcinogenesis. *J Invest Dermatol* 134:2630–8

Stokes A, Joutsa J, Ala-Aho R *et al.* (2010) Expression profiles and clinical correlations of degradome components in the tumor microenvironment of head and neck squamous cell carcinoma. *Clin Cancer Res* 16:2022–35

Udayakumar D, Zhang G, Ji Z *et al.* (2011) EphA2 is a critical oncogene in melanoma. *Oncogene* 30:4921–9

Walker-Daniels J, Coffman K, Azimi M *et al.* (1999) Overexpression of the EphA2 tyrosine kinase in prostate cancer. *Prostate* 41:275–80

Walsh R, Blumenberg M (2012) Eph-2B, acting as an extracellular ligand, induces differentiation markers in epidermal keratinocytes. *J Cell Physiol* 227:2330–40

Wang NJ, Sanborn Z, Arnett KL *et al.* (2011) Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proc Natl Acad Sci USA* 108:17761–6

Wang SD, Rath P, Lal B *et al.* (2012) EphB2 receptor controls proliferation/migration dichotomy of glioblastoma by interacting with focal adhesion kinase. *Oncogene* 31:5132–43

Wu Q, Suo Z, Risberg B *et al.* (2004) Expression of Ephb2 and Ephb4 in breast carcinoma. *Pathol Oncol Res* 10:26–33

Wykosky J, Debinski W (2008) The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting. *Mol Cancer Res* 6:1795–806

Zelinski DP, Zantek ND, Stewart JC *et al.* (2001) EphA2 overexpression causes tumorigenesis of mammary epithelial cells. *Cancer Res* 61:2301–6

Zimmer M, Palmer A, Kohler J *et al.* (2003) EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. *Nat Cell Biol* 5:869–78