

Cancer Stem-like Cells Act via Distinct Signaling Pathways in Promoting Late Stages of Malignant Progression

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Abstract

Cancer stem-like cells (CSC) play key roles in long-term tumor propagation and metastasis, but their dynamics during disease progression are not understood. Tumor relapse in patients with initially excised skin squamous cell carcinomas (SCC) is characterized by increased metastatic potential, and SCC progression is associated with an expansion of CSC. Here, we used genetically and chemically-induced mouse models of skin SCC to investigate the signaling pathways contributing to CSC function during disease progression. We found that CSC regulatory mechanisms change in advanced SCC, correlating with aggressive tumor growth and enhanced metastasis. β -Catenin and EGFR signaling,

induced in early SCC CSC, were downregulated in advanced SCC. Instead, autocrine FGFR1 and PDGFR α signaling, which have not been previously associated with skin SCC CSC, were upregulated in late CSC and promoted tumor growth and metastasis, respectively. Finally, high-grade and recurrent human skin SCC recapitulated the signaling changes observed in advanced mouse SCC. Collectively, our findings suggest a stage-specific switch in CSC regulation during disease progression that could be therapeutically exploited by targeting the PDGFR and FGFR1 pathways to block relapse and metastasis of advanced human skin SCC. *Cancer Res*; 76(5); 1245–59. ©2015 AACR.

Introduction

Skin squamous cell carcinoma (SCC) is the second most common nonmelanoma skin cancer in humans. Eight percent to 10% of patients suffer tumor relapse after surgical excision, which is associated with an enhanced propensity to metastasize and with poor survival (1). SCC development in humans and mice is a multistage process ending in the generation of invasive tumors (2). Most invasive tumors conserve some epithelial traits and are considered to be well-differentiated

SCCs (WD-SCCs). However, some tumors have poorly differentiated features (PD-SCCs) and are eventually spindle-shaped, these traits being associated with enhanced recurrence and metastasis (1, 3). Currently, advanced and metastatic skin SCCs are mostly treated with radiotherapy or classical chemotherapy, which have limited clinical benefits (4). Furthermore, the mechanisms that control SCC growth and metastasis at different stages of progression remain unclear, limiting the possibilities for targeted therapy.

Solid tumors may be hierarchically organized and contain cancer stem cells (CSC), which drive long-term tumor growth and disease progression (5, 6) and are responsible for relapse after therapy (7, 8). CSCs are also the most likely candidates for metastasis-initiating activities, as they may induce epithelial-to-mesenchymal transition (EMT), which promotes tumor cell migration (9, 10). Mouse skin SCC cells expressing CD34 and $\alpha 6$ -integrin are enriched in tumor-initiating and long-term tumor-propagating cells compared with the bulk of tumor cells, and they are able to recapitulate the phenotypic cell diversity of parental tumors in engraftment assays (11, 12). Sox2 expression is induced in CSCs of SCCs to promote CSC self-renewal and skin SCC growth (13, 14). Wnt/ β -catenin, TGF β , and VEGF signaling pathways regulate CD34⁺-CSC features at early stages of progression (11, 15, 16). Moreover, skin SCC progression is associated with an expansion of the CD34⁺-CSC population (12, 16). However, it is not known whether signaling pathways regulating CSC function switch during progression. In this study, we demonstrate that CSC features and regulatory mechanisms change during late stages of skin SCC progression to promote aggressive tumor growth and metastasis.

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Mouse models

K14-HPV16^{Tg/+} mice (FVB/C57/Bl6 F1; ref. 17) were used as models of SCC development. K14-HPV16^{Tg/+} mice were treated with 7,12—dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA), as previously described (18). To generate ortho-SCC (OT-SCC) lineages, small pieces of spontaneous or DMBA/TPA-induced tumor (2–4 mm³) were implanted in the back skin of nude mice (Harlan Laboratories). Tumor sizes were measured every week ($V = \pi/6 \times L \times W^2$). When they reached a critical size, they were surgically excised and a small piece was serially engrafted in each new immunodeficient mouse. Resected mice were checked daily until they presented symptoms of poor health, whereupon they were sacrificed and checked for the presence of metastatic lesions. Animal housing, handling, and all procedures involving mice were approved by the Bellvitge Biomedical Research Institute (IDIBELL) Ethics Committee (Barcelona, Spain), in accordance with the Spanish national regulations.

Human skin SCC samples

Samples of human skin SCCs were supplied by the Plastic Surgery and Pathology Units of the Hospital Universitario de Bellvitge (IDIBELL, Barcelona Spain) and the Spanish Hospital Platform Biobank Network (RetBioH; www.redbiobancos.es). The protocol of sample collection was supervised and approved by the Ethical Committee of Clinical Research of Hospital Universitario de Bellvitge (IDIBELL). All patients were informed beforehand and their signed consent to participate was obtained.

Isolation of SCC cells and flow cytometry analysis

Tumor cells were isolated from SCC and analyzed for flow cytometry as previously described (19). For detailed protocols, see Supplementary Methods.

Cell cultures and treatments

Tumor cells (hematopoietic lineage and endothelial-negative cells) isolated from PD/S-SCCs (PD/S cells) were grown in basic medium composed of DMEM-F12 medium (Gibco, Life Technologies) with B27 (Gibco, Life Technologies) and penicillin/streptomycin (PAA Laboratories) in a humidified 37°C, 5% CO₂ incubator. Tumor cells isolated from WD-SCCs (WD cells), were grown in basic medium with EGF (20 ng/mL; Sigma). Protocols for determining cell proliferation and lentiviral transduction are described in detail in Supplementary Methods.

Tumor cell grafting and *in vivo* treatments

To limiting dilution assays, tumor cells isolated from WD-SCCs and PD/S-SCCs were serially diluted, mixed with 1×10^6 newborn dermal fibroblasts, and subcutaneously injected in nude mice (Harlan Laboratories). Tumor growth was monitored for 8 to 12 weeks. The tumor-initiating cell frequency was calculated using ELDA software (<http://bioinf.wehi.edu.au/software/elda>). A similar frequency of tumor-initiating cells was obtained when Matrigel (BD Biosciences) was coinjected with tumor cells (1:1 v/v) instead of dermal fibroblasts (data not shown). To determine the effect of PDGFR α knockdown on tumor growth and metastasis, 4×10^3 sh-control and sh-PDGFR α PD/S cells were subcutaneously injected in immunodeficient mice, as described above. Tumors were excised when they reached a critical size, and the mice were sacrificed 20 days later. Lungs were recovered to

quantify metastasis lesions. For pharmacologic inhibition of FGFR1 and PDGFR α , mice carrying orthotopically engrafted PD/S-SCCs were randomly assigned to a control or inhibitor treatment group. Imatinib (150 mg/kg; diluted in water; LC Laboratories) was orally administrated daily, and PD173074 (25 mg/kg; diluted in 50 mmol/L lactic acid; LC Laboratories) was daily intraperitoneally injected. Control groups were treated with the respective vehicle. OT-SCCs were excised, and resected mice were treated with their respective control or inhibitor solution and sacrificed 8 to 10 days later. Lungs and other organs were collected for the assessment of metastasis development.

In vitro invasion assays

Invasion assays were performed in CIM-16 plates (ACEA Biosciences) coated with 5% Matrigel (Factor-Reduced; BD Biosciences). Bottom chamber wells with DMEM 10% FBS and top chamber wells with serum-free medium were assembled and equilibrated for 1 hour at 37°C. PD/S cells (8×10^4), previously treated without (control) or with imatinib (4 μ mol/L) for 48 hours, and sh-control and PDGFR α -knockdown PD/S cells were seeded onto the top chamber and placed in the xCELLigence system. The cell index represents the capacity for cell invasion. In addition, sh-control, PDGFR α knockdown, and PD/S cells treated for 48 hours with different doses of imatinib were included in Matrigel and seeded in replicates on previously Matrigel-coated 96-well plates with 100 μ L of basic medium without or with imatinib. Images were captured 24 hours later using an inverted phase microscope, and spheres containing ≥ 4 invasion structures longer than 25 μ m were quantified.

Histology, IHC, and immunoblotting assays

Tumor samples were fixed in 4% formaldehyde overnight at 4°C, paraffin-embedded, and sectioned at 4 μ m. For immunofluorescence or immunohistochemical staining, we followed the protocols previously described in ref. 19. Whole-cell extracts for Western blot assays were prepared from tumors and isolated cells as previously described (see Supplementary Methods; ref. 20).

Reverse transcription, qPCR, and microarray analysis

Total RNA was extracted using TRIzol (Invitrogen), and reverse transcription and qPCR were carried out as previously described (see Supplementary Methods; ref. 19). cDNA amplification by picoprofiling was performed as previously described (21). Seven micrograms cDNA was used for hybridization in an Affymetrix Mouse Genome 430 PM Strip Array. Data array analyses are described in Supplementary Methods.

Sequencing analysis

Mutations in Hras (Q61L) and Kras (G12V, G12A, G12D, G13R, G13D, Q61L, and Q61H) were analyzed by pyrosequencing assays. The set of primers for PCR amplification and sequencing were designed with PyroMark Assay Design Software (Qiagen). PCR products were pyrosequenced and allele mutations were quantified with the Pyromark Q24 System (Qiagen), following the manufacturer's instructions.

Accession number

The gene expression data described in this study have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE59439.

Results

Generation and characterization of lineages of skin SCC progression

To determine whether the aggressive growth and enhanced metastasis of advanced SCCs are associated with changes in CSC features, we generated mouse models of skin SCC progression based on tumors developed in K14-HPV16 mice. K14-HPV16 mice express E6 and E7 oncoproteins from the HPV16 papillomavirus in basal keratinocytes, and 30% of them develop spontaneous SCCs during their first year (3, 19), 27% of which were undifferentiated and/or spindle tumors. To promote SCC development, these mice were treated with DMBA/TPA. At the end of the treatment, all mice developed multiple invasive SCCs, most of them showing epithelial traits, while spindle-shaped tumors were more infrequent (4.65%), as previously reported in other mouse strains (22). Single small pieces from several SCC samples (spontaneous or DMBA/TPA-induced) were orthotopically grafted onto the back skin of nude mice. Samples of each OT-SCCs were serially engrafted in nude mice over several passages, generating SCC lineages (Supplementary Table S1). After the first engraftment, OT-SCCs recapitulated the histopathologic features of the parental tumors (Supplementary Fig. S1A). However, after serial engraftments, 62.5% of the WD-SCCs showing epithelial differentiation features (tumor cells organized in nests containing keratin pearls) progressed to moderated SCCs (MD-SCC), which evolved to PD-SCCs that frequently contained focal spindle regions, and finally to mesenchymal-shaped spindle tumors, in which the epithelial phenotype was completely lost (Supplementary Fig. S1A; Supplementary Table S1). Therefore, PD and spindle SCCs can be generated by the malignant advance of WD-SCCs.

K14 expression was strongly reduced and K8 expression was upregulated in PD-SCCs and spindle tumors (Supplementary Figs. S1C and S1D; ref. 3). On the basis of keratin expression and histopathologic features, SCCs from each lineage were classified as WD-SCCs or PD and spindle SCCs (PD/S-SCCs). PD/S-SCCs grew significantly faster than their respective WD-SCC precursors in all tumor lineages (Supplementary Fig. S1E). Nude mice carrying PD/S-SCCs showed reduced survival due to more frequent lower-latency metastasis occurring mainly in the lungs and occasionally in regional lymph nodes, kidney, and liver (Supplementary Figs. S1F–S1H).

Analysis of *Ras* genes showed that *Hras* was frequently mutated in primary DMBA/TPA-induced SCCs (*Hras* Q61L; 75% of tumors; refs. 23, 24). This mutation was infrequent in spontaneous SCCs, but 57.14% of them instead exhibited a distinct percentage of alleles with activating mutations in the *Kras* gene (G13R, G12A, G12D, and Q61L; Supplementary Fig. S1B; ref. 25). The intratumor genetic heterogeneity was frequently maintained in the respective OT-SCCs (data not shown), and some WD-SCCs (OT14 lineage) were able to progress to PD/S-SCCs in the absence of previously described *Hras*- or *Kras*-activating mutations.

Limiting dilution assays showed that the frequency of tumor-initiating cells was dramatically increased in OT7 and OT14 PD/S-SCCs compared with their WD-SCC precursors (Fig. 1A). SCCs with an epithelial and mesenchymal phenotype were respectively generated by tumor-initiating cells of parental WD-SCCs and PD/S-SCCs (Fig. 1B), suggesting that tumor-initiating cells of WD-SCCs, but not those of PD/S-SCCs, retained the ability to differentiate into an epithelial shape. As reported in other mouse models (11, 12), $\alpha 6$ -integrin⁺/CD34⁺ cells (hematopoietic and

endothelial lineage negative cells) isolated from orthotopic WD-SCCs were significantly enriched in tumor-initiating cells relative to $\alpha 6$ -integrin⁺/CD34⁺ cells or to the overall tumor cell population (Fig. 1A and Supplementary Fig. S2A). Moreover, tumors derived from PD/S-SCC $\alpha 6$ -integrin⁺/CD34⁺ grew significantly faster than tumors generated from WD-SCC $\alpha 6$ -integrin⁺/CD34⁺ cells (Supplementary Fig. S2B). These results indicate that the ability of CD34⁺-CSCs to promote tumor growth changes significantly during progression. Although a similar tumor-initiating capability and impaired epithelial differentiation was observed in $\alpha 6$ -integrin⁺/CD34⁺ and $\alpha 6$ -integrin⁺/CD34⁺ cells isolated from PD/S-SCCs (Supplementary Fig. S2A; data not shown), previous reports demonstrated that, in contrast to CD34⁺ cells, CD34⁺-CSCs present long-term self-renewal capability in advanced SCCs and are considered as long-term tumor-propagating cells (12). Therefore, we compared the frequency and molecular features of the $\alpha 6$ -integrin⁺/CD34⁺ cells from advanced tumors and from their respective WD-SCC precursors. We found that PD/S-SCCs of different lineages, derived from spontaneous or DMBA/TPA-induced WD-SCCs, showed a significant increase in the percentage of $\alpha 6$ -integrin⁺/CD34⁺ cells as compared with their respective precursors (Fig. 1C and D). A similar expansion in this cell population was observed in spontaneous PD/S-SCCs (Supplementary Fig. S2C).

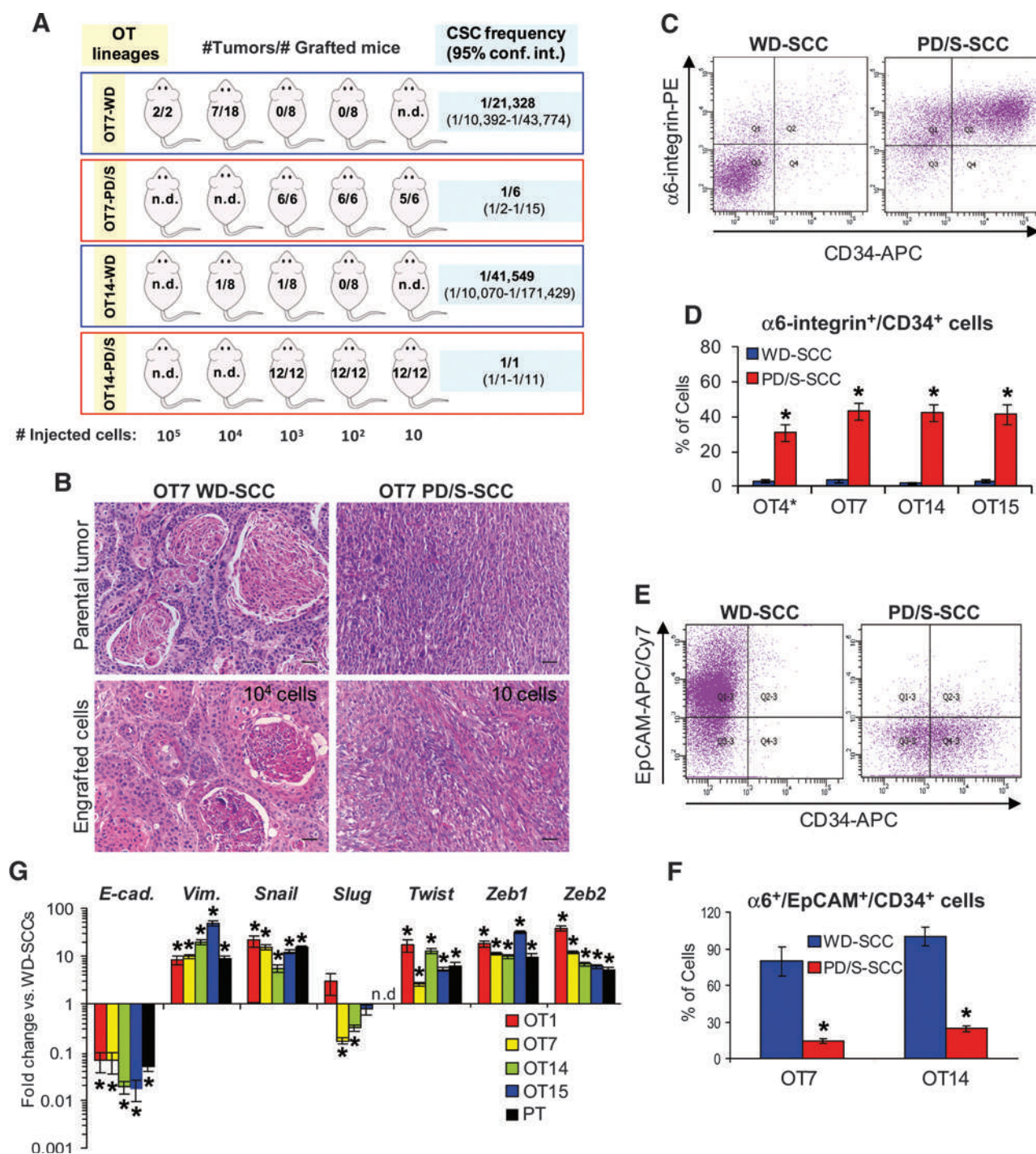
In accordance with the loss of epithelial traits, the expression of the EpCAM epithelial marker was significantly downregulated in $\alpha 6$ -integrin⁺/CD34⁺ cells of PD/S-SCCs (Fig. 1E and F and Supplementary Fig. S2C), implying that CSC features change during tumor progression. In addition, diminished expression of *Cdh1* (E-cadherin), and upregulation of *vimentin* and the EMT-inducer transcription factors *Snail*, *Twist*, *Zeb1*, and *Zeb2* were detected in PD/S-SCCs from different lineages and in spontaneous PD/S-SCCs (Fig. 1G and Supplementary Figs. S2D and S2E), as reported in other mouse models (22). Thus, an expansion of CSCs and robust induction of EMT occur during OT-SCC progression, along with a switch from epithelial-to-mesenchymal features and enhanced metastasis capability.

It is important to highlight that the tumors that did not progress to PD/S-SCC during serial engraftment did not exhibit an expansion of CSCs or further induction of EMT (OT9 in Supplementary Fig. S2F), indicating that these events are not a consequence of long-term growth in immunodeficient mice. These results validate the OT-SCC lineages as models for characterizing CSC alterations during tumor progression.

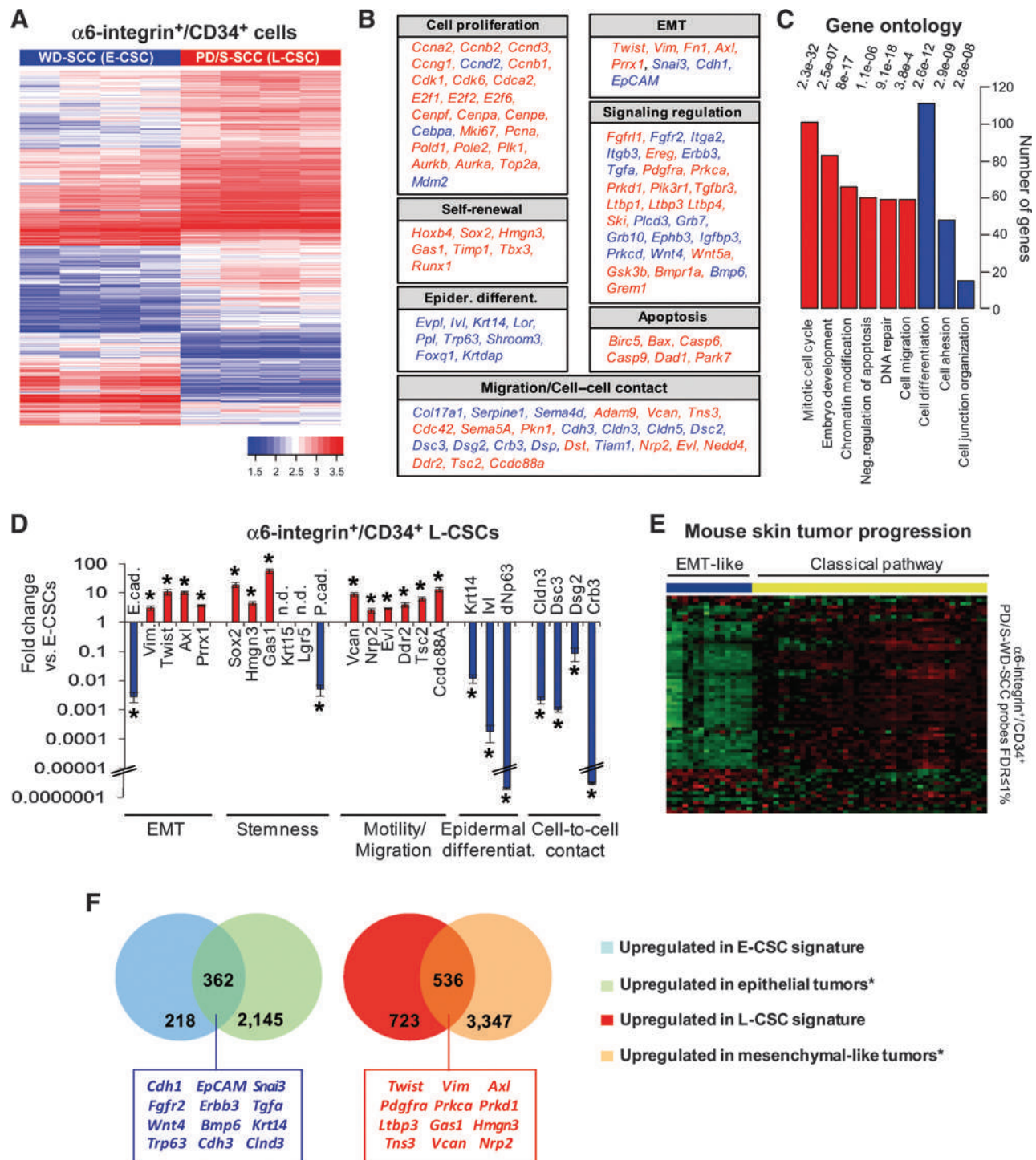
$\alpha 6$ -integrin⁺/CD34⁺ CSC features change during SCC progression

To determine whether alterations in features of CSCs can contribute to tumor progression, we compared the global gene expression profiles of $\alpha 6$ -integrin⁺/CD34⁺ cells isolated from PD/S-SCCs (late CSC, L-CSC) and their respective precursor WD-SCCs (early CSC, E-CSC) from different lineages. This analysis revealed 1,839 genes differentially expressed (by 2-fold or more; FDR < 5%) in L-CSCs compared with E-CSCs, giving rise to a gene signature for CSCs in advanced and mesenchymal SCCs (Fig. 2A; Supplementary Table S2). Genes overexpressed in this signature were mainly those associated with proliferation, morphogenesis, negative regulation of apoptosis, cytoskeleton organization, motility, and metastasis, whereas underexpressed genes were linked to cell differentiation, cell adhesion, and tight junction

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**Figure 1.**

Strong induction of EMT and expansion of CSCs is observed in advanced SCCs of different lineages of progression. A, serial dilutions of tumor cells isolated from WD-SCCs and PD/S-SCCs from the indicated lineages (OT) were injected into immunodeficient mice (6–18 mice per dilution). The number of mice developing tumors, frequency of CSCs, and confidence intervals (conf. int.) for each condition is shown (n.d., not determined). B, representative images showing histopathologic features of parental and regenerated tumors after the engraftment of the indicated number of tumor cells. Scale bar, 30 μ m. C and D, quantification of $\alpha 6$ -integrin⁺/CD34⁺ cells in OT WD-SCCs and PD/S-SCCs by flow cytometry. D, means ($\pm$ SE) of the percentages of $\alpha 6$ -integrin⁺/CD34⁺ cells in the indicated tumors. OT4* is SCC lineage derived from a spontaneous WD-SCC. E and F, mean $\pm$ SE of the percentages of $\alpha 6$ -integrin⁺/EpCAM⁺/CD34⁺ cells in WD-SCCs and PD/S-SCCs (6–8 tumors per group), as quantified by flow cytometry. G, qRT-PCR results showing the levels (mean $\pm$ SE) of the indicated mRNAs in PD/S-SCCs relative to WD-SCCs (six samples per group) in different lineages (OT) and in primary SCCs (PT) spontaneously developed in K14-HPV16 mice (four samples per group). *, significant differences between WD-SCCs and PD/S-SCCs (*t* test; *P* < 0.05). E-cad., E-cadherin; Vim., vimentin.

**Figure 2.**

CSC gene signature changes during SCC progression. A, hierarchical gene-cluster analysis of genes differentially expressed ($\log_2$ FC ≥ 1 ; FDR $P < 0.05$) between $\alpha 6$ -integrin⁺/CD34⁺ CSCs of PD/S-SCCs (L-CSC) and their respective WD-SCC precursors (E-CSC; four CSC samples per group) from three lineages. B, selected set of genes overexpressed (red) or underexpressed (blue) in L-CSCs relative to E-CSCs. C, gene ontology enrichment analysis of genes differentially expressed in L-CSCs compared with E-CSCs. D, mean ($\pm$ SE) mRNA levels relative to *Gapdh* expression of the indicated genes in L-CSCs and E-CSCs (three samples per group). n.d., the expression of these genes was not detected in E-CSCs or L-CSCs. *, significant differences between groups (t test; $P < 0.05$). E, heatmap representation of differentially expressed genes in L-CSCs that identify genes in the previously reported EMT-like mouse SCC signature (22). F, Venn diagrams showing the overlap of genes from the L-CSC signature and those associated with epithelial-like SCCs and mesenchymal-like SCCs (22). A selected number of these overlapping genes is indicated.

maintenance (Fig. 2B–D). *Sox2*, *Hmgn3*, and *Gas1* stem cell markers were overexpressed, whereas *Cdh3* (P-cadherin) was underexpressed in L-CSCs compared with E-CSCs. *Krt15* and *Lgr5* was undetectable in both populations of CSCs (Fig. 2B and D). In addition, E-cadherin expression was strongly downregulated and *vimentin*, *Twist*, and *Axl* significantly overexpressed in L-CSCs, indicating that stemness and EMT were enhanced in these CSCs relative to E-CSCs. The overexpression of self-renewal-promoting genes and the underexpression of differentiation-related genes (Fig. 2D), together with the impaired differentiation capability of tumor-initiating cells of advanced tumors, suggest that an imbalance between self-renewal and differentiation is produced in CSCs during late stages of progression. Some of the altered genes described in this signature were also similarly deregulated in unrelated skin PD-SCCs, as unsupervised clustering correctly identified skin SCCs with an EMT-like status in other mouse SCC models (Fig. 2E; ref. 22). Indeed, a large subset of genes overexpressed in L-CSCs (536/1,259 genes) and in E-CSCs (362/578 genes) was also upregulated in EMT-like SCCs and in epithelial-like SCCs, respectively, in this DMBA/TPA-induced SCC model (Fig. 2F).

Furthermore, the expression of key factors related to WNT, FGFR, PDGFR, and EGFR pathways, which were previously associated with progression in other tumor types (26–28), was also signifi-

cantly altered in our L-CSC signature (Fig. 2B). This suggests that alterations in these pathways may be associated with CSC expansion, aggressive tumor growth, and metastasis in advanced SCCs.

β-Catenin signaling is downregulated in advanced skin SCCs

β-Catenin signaling is essential for sustaining CSC features in the early stages of skin SCCs (11), therefore we compared β-catenin expression in WD-SCCs and PD/S-SCCs. Most epithelial cells showed β-catenin labeling at the cell surface and more than 25% showed β-catenin nuclear staining in WD-SCCs (Fig. 3A and B). In contrast, there was considerable reduction of β-catenin overall and of its active form (non-phosphorylated Ser37/Thr41) in the respective PD/S-SCCs (Fig. 3C). Moreover, β-catenin transcriptional activity was analyzed by transducing tumor cells isolated from early and advanced SCCs (Supplementary Figs. S3A–S3C) with a β-catenin reporter construct (29). WD-SCCs and PD/S-SCCs were regenerated by engrafting transduced WD and PD cells into nude mice. We detected a subset of EGFP-expressing cells with β-catenin transcriptional activity at the base of the nest in WD-SCCs (Fig. 3D), where CD34⁺-CSCs reside (11, 15, 16). However, EGFP-expressing cells were absent from PD/S-SCCs (Fig. 3D). Therefore, although β-catenin activity plays an important role in SCC generation and growth in early SCCs, this pathway is switched off in advanced SCCs.

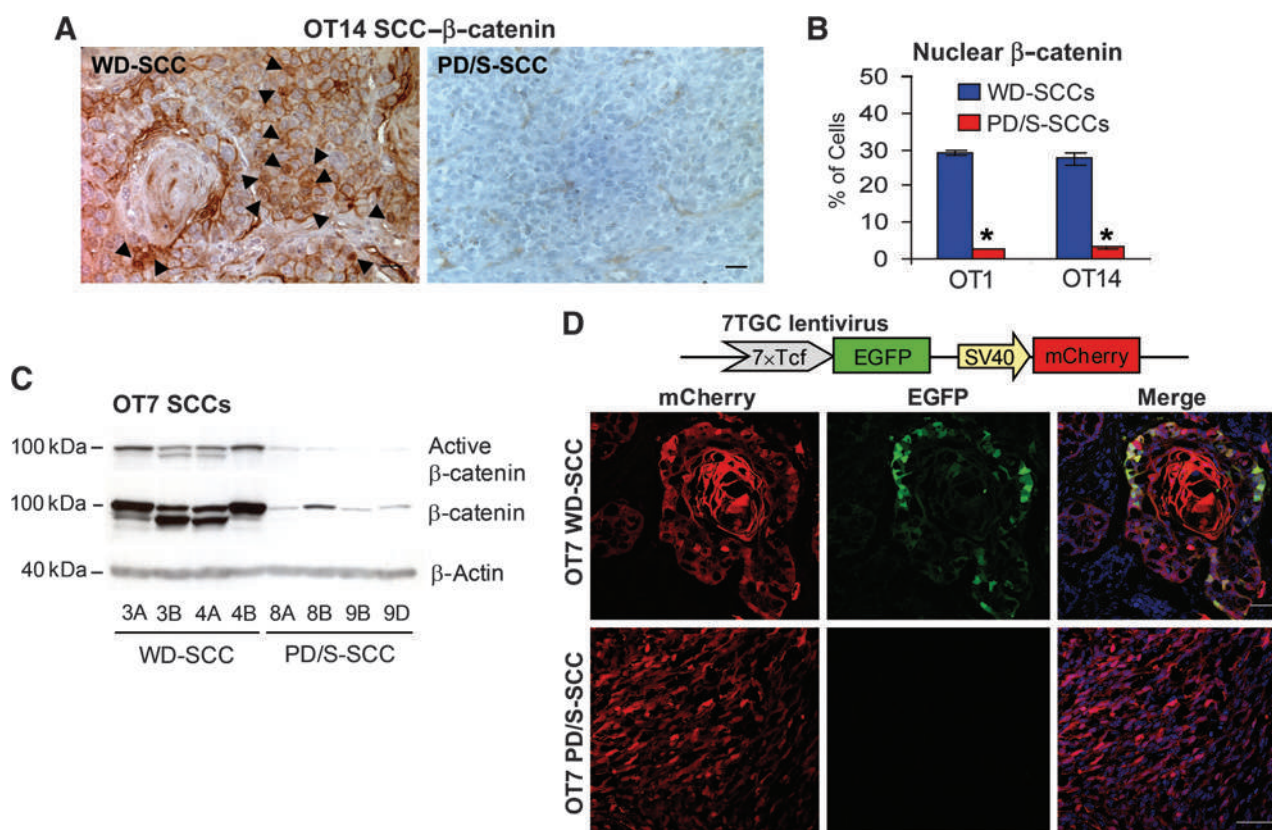
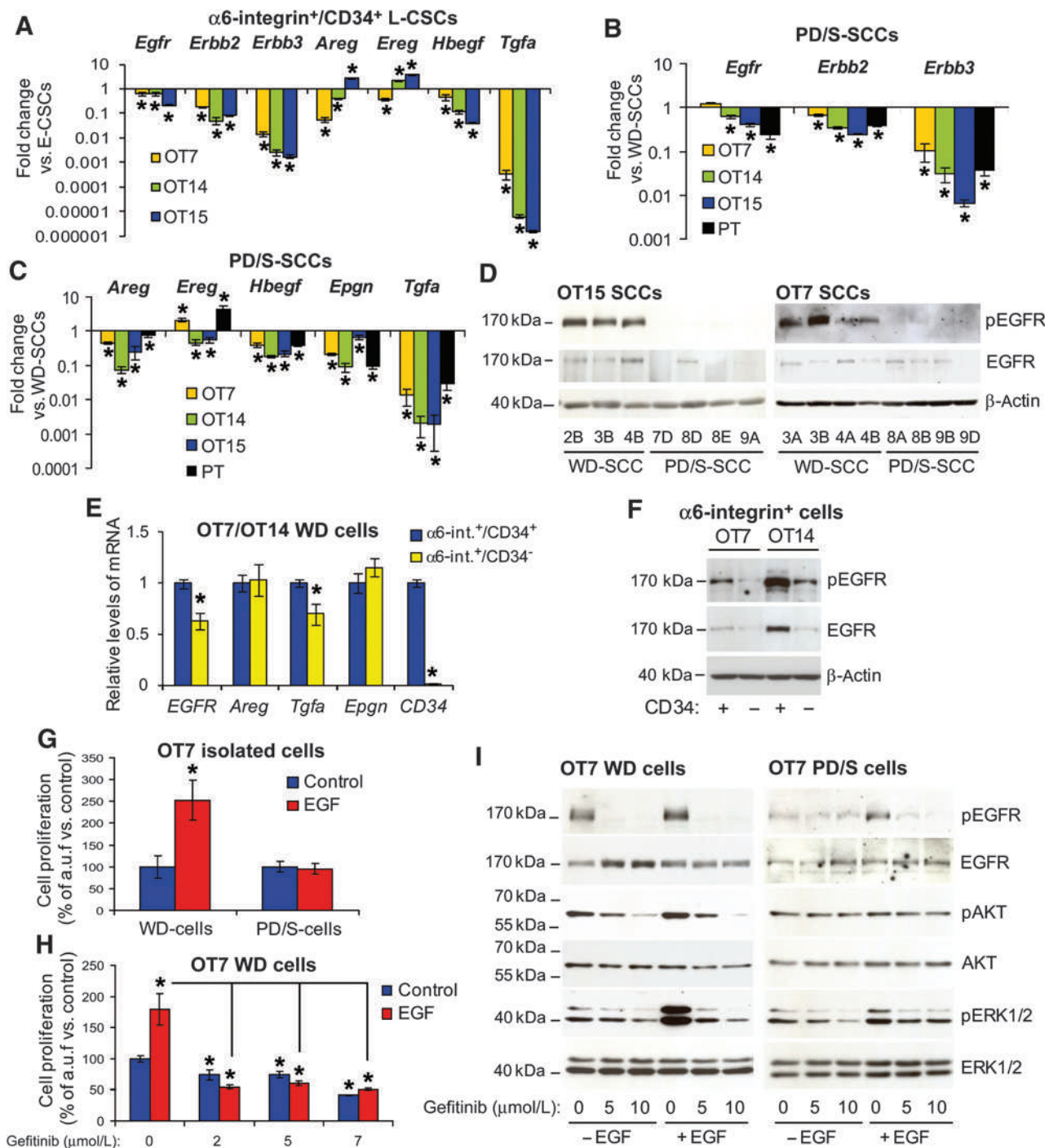
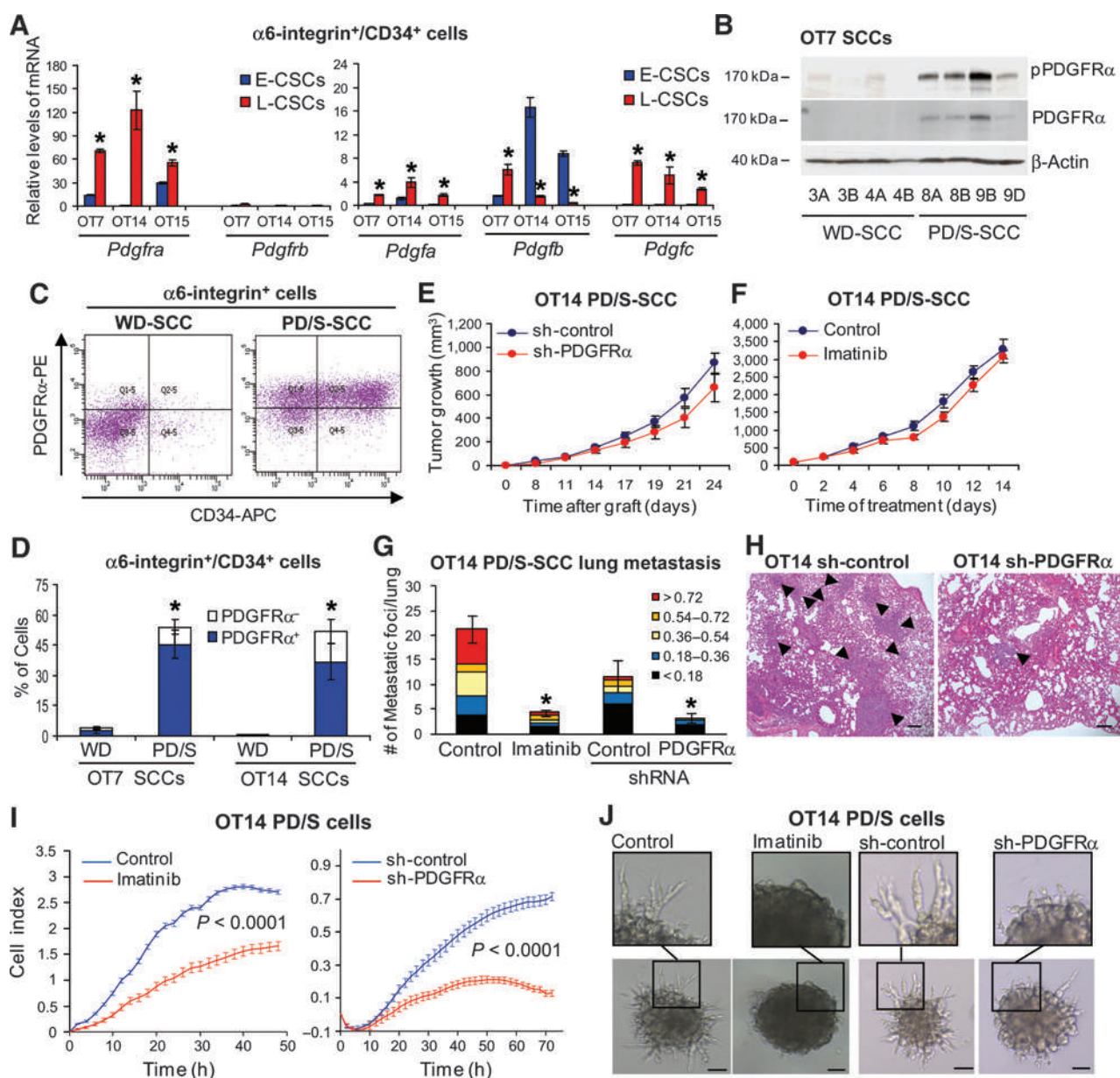


Figure 3. β-Catenin signaling is attenuated during late stages of SCC progression. A, β-catenin cell localization by IHC assays. Arrowheads, cells with β-catenin at nuclei. Scale bar, 30 μm. B, quantification of cells (mean ± SE; three samples per group) with β-catenin at nuclei in WD-SCCs and PD/S-SCCs of the indicated lineage. C, levels of β-catenin and its active form in WD-SCCs and PD/S-SCCs. β-Actin was used as a protein-loading control. Sample number identifies tumor passage and letters indicate independent tumor replicates. D, top, schema of the construct used to determine β-catenin transcriptional activity; bottom, representative images showing transduced cells (red) and cells with induced β-catenin signaling (green cells) in WD-SCCs and PD/S-SCCs. Scale bar, 30 μm.

**Figure 4.**

E-CSC-induced EGFR signaling is downregulated in CSCs of advanced SCCs. A–C, levels of the indicated mRNAs (mean $\pm$ SE) in L-CSCs relative to E-CSCs (A; three samples per group) and in PD/S-SCCs relative to WD-SCCs (B and C; 6–8 samples per group) in the indicated tumor lineages (OT) and spontaneous primary SCCs (PT; four samples per group). D, EGFR and its phosphorylated form in WD-SCCs and PD/S-SCCs. E, mean ($\pm$ SE) mRNA levels relative to *Gapdh* expression of the indicated genes and populations of WD cells (three samples per group). F, levels of EGFR and its phosphorylated form in the indicated populations of WD cells. G, cell proliferation upon EGF treatment, as measured by MTT. Means ($\pm$ SE) indicate arbitrary units of fluorescence (a.u.f.) in EGF-treated cells relative to cells growing without factor. H and I, effect of gefitinib on proliferation (H; mean $\pm$ SE of a.u.f.; two samples per group) of cells treated without or with EGF, and EGFR, AKT, and ERK1/2 phosphorylation (I). Note that the time of exposure was increased to detect EGFR and p-EGFR in PD/S cells. *, significant differences between groups (*t* test; *P* < 0.05). β -Actin was used as a protein-loading control in D and F.

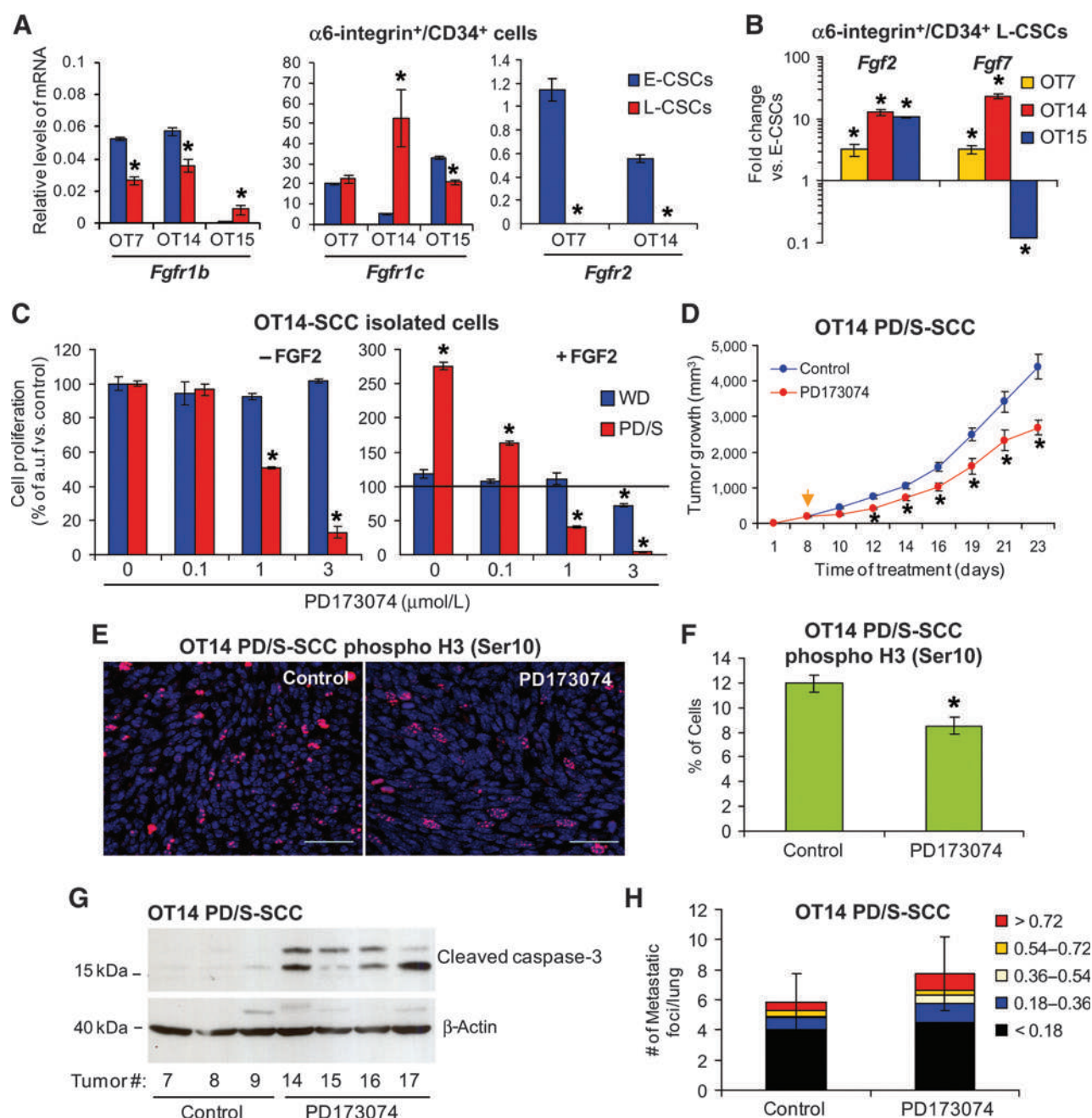
**Figure 5.**

Autocrine PDGFR α signaling induced in CSCs of advanced SCCs promotes metastasis. **A**, expression of indicated genes relative to *Gapdh* mRNA (mean $\pm$ SE) in E-CSCs and L-CSCs (two samples per group). **B**, PDGFR α protein and its phosphorylated form in WD-SCCs and PD/S-SCCs. β -Actin is shown as a protein-loading control. Sample number identifies the tumor passage and letters indicate independent tumor replicates. **C** and **D**, quantification of $\alpha 6$ -integrin⁺ cells expressing CD34 and PDGFR α in WD-SCCs and PD/S-SCCs by flow cytometry. **D**, percentage (mean $\pm$ SE) of $\alpha 6$ -integrin⁺/CD34⁺ cells with or without PDGFR α expression in the indicated tumors (5–6 samples per group). **E** and **F**, growth kinetics (mean $\pm$ SE of tumor size) of PDGFR α -expressing (sh-control) and PDGFR α -knocked-down (sh-PDGFR α) PD/S-SCCs (**E**; 8 tumors per group) and PD/S-SCCs treated with vehicle or imatinib (**F**; 10 mice per group). **G**, mean $\pm$ SE of metastatic foci per lung section (categorized by size, mm²) in mice injected with sh-control or sh-PDGFR α PD/S tumor cells (4 mice per group) and in control and imatinib-treated mice (10 mice per group). *, significant differences between the groups (*t* test; *P* < 0.05). **H**, metastatic lesions (indicated by black arrowheads) in the lungs of mice grafted with sh-control and sh-PDGFR α PD/S tumor cells. Scale bar, 200 μ m. **I**, comparison of the invasion capacity (indicated as cell index) in control and upon PDGFR α signaling inhibition in xCELLigence real-time cell analysis. **J**, effect of imatinib treatment and PDGFR α -knock-down on the formation of invasion structures in PD/S cells growing in Matrigel. Scale bar, 50 μ m.

EGFR signaling is downregulated in $\alpha 6$ -integrin⁺/CD34⁺-CSCs of advanced SCCs

We investigated the relevance of the receptor tyrosine kinase (RTK)-dependent pathways that were identified in the L-CSC signature to promote tumor progression. EGFR signaling is

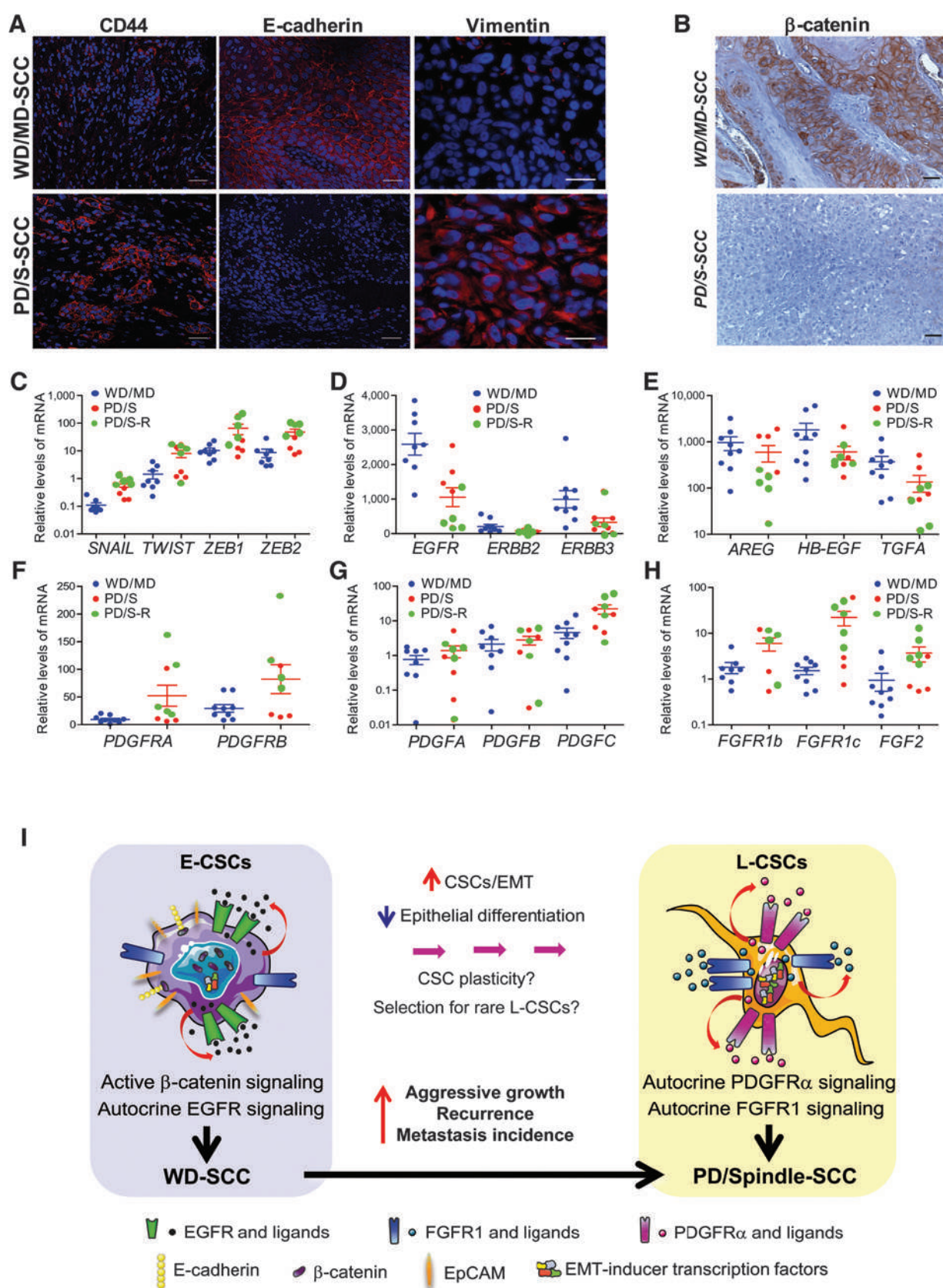
necessary to maintain the proliferation and survival of basal cell compartment in the early stages of SCCs (30, 31). Previous studies showed that *Egfr* expression is downregulated in advanced SCCs (22). However, whether this pathway regulates CSC proliferation and survival and is altered during progression remains to be

**Figure 6.**

Autocrine FGFR1 signaling is upregulated in L-CSCs promoting tumor growth. A and B, mRNA quantification (mean $\pm$ SE) of the indicated genes in E-CSCs and L-CSCs (two samples per group) from three lineages. Results are represented as mRNA levels relative to *Gapdh* expression (A) and n-fold change in L-CSCs relative to E-CSCs for each lineage (B). C, mean $\pm$ SE of WD and PD/S cell proliferation after the indicated treatments, as measured by MTT. D, growth kinetics (mean $\pm$ SE of tumor size) of PD/S-SCCs treated with vehicle or PD173074 (10 mice per group). Arrow, the start of the treatment. E, immunodetection of phosphorylated histone H3 (Ser10) in control and PD173074 treated tumors. Scale bar, 30 μ m. F, percentage (mean $\pm$ SE) of proliferating cells, as determined in E. G, levels of cleaved caspase-3 in mock and PD173074-treated tumors. β -Actin was used as a protein-loading control. H, mean $\pm$ SE of metastatic foci per lung section (categorized by size; mm²) developed in mock- and PD173074-treated mice (10 mice per group). *, significant differences between groups (*t* test; *P* < 0.05).

determined. Thus, we compared the expression of *ErbB* receptors and ligands in E-CSCs and L-CSCs isolated from various OT-SCC lineages. The expression of *Egfr*, *ErbB2*, and *ErbB3* receptors, as well as of the *Tgfa*, *Areg*, and *Hbegf* ligands was reduced in L-CSCs from most tumor lineages (Fig. 4A), consistent with the *ErbB* and ligand

expression profiles observed in spontaneous and orthotopically derived PD/S-SCCs (Fig. 4B and C). These results, along with the substantially lower phosphorylated status of EGFR consistently observed in PD/S-SCCs (Fig. 4D), indicate that EGFR signaling is downregulated in advanced SCCs. To determine the role of EGFR



signaling in CSC proliferation, we analyzed tumor cells isolated from WD-SCCs and their PD/S-SCC offspring and maintained in culture. Primary WD cells grew as adherent cells and had a typical epithelial shape, whereas PD/S cells grew as spheres (Supplementary Fig. S3A). These primary cultures were enriched in $\alpha 6$ -integrin⁺/CD34⁺-CSCs (50% and 90% in WD and PD/S cells, respectively), exhibited a similar expression profile of EMT inducer factors and *ErbB* and ligands to their respective parental tumors, and isolated CD34⁺-CSCs (Supplementary Figs. S3B–S3E). In accordance with the genetic profile of their parental tumors, no *Ras* mutations were detected in OT14 WD and PD/S cells, whereas 34% of *Hras* alleles were mutated (Q61L) in OT7 cells. $\alpha 6$ -integrin⁺/CD34⁺ WD cells showed a higher level of expression of EGFR and *Tgfa* ligand than $\alpha 6$ -integrin⁺/CD34[−] WD cells, whereas *Areg* and *Epgn* expression were similar in both subpopulations of cells (Fig. 4E). Furthermore, EGFR was mainly activated in $\alpha 6$ -integrin⁺/CD34⁺ WD cells, as determined by the levels of phosphorylated EGFR (Fig. 4F). In contrast to PD/S cells, EGF treatment significantly increased the proliferation of WD cells (Fig. 4G) concomitantly with a stronger induction of EGFR, AKT, and ERK1/2 phosphorylation, which were inhibited in response to the EGFR inhibitor gefitinib (Fig. 4H and I and Supplementary Figs. S3F and S3G). In the absence of EGF, WD cells showed basal levels of pEGFR, which were inhibited along with the phosphorylation of downstream effectors and cell proliferation in response to gefitinib (Fig. 4H and I and Supplementary Fig. S3G). However, these effects were stronger in OT14 WD cells (*Ras* WT) than in OT7 WD cells, which present activating mutations in a subset of *Hras* alleles (Supplementary Fig. S3H). Together, these results indicate that autocrine and probably paracrine EGFR signaling promotes the growth of E-CSCs, and that this pathway is attenuated in L-CSCs.

CSC-induced autocrine PDGFR α signaling promotes metastasis in advanced SCCs

PDGFR is not expressed in normal keratinocytes (32) and has not previously been associated with skin SCC CSCs. However, in accordance with our array data, *Pdgfra* expression was strongly induced in L-CSCs (Fig. 5A), correlating with the consistently observed stronger expression of this receptor in their respective PD/S-SCCs and in primary PD/S-SCCs (Supplementary Fig. S4A). Although expression of *Pdgfrb* was also enhanced in PD/S-SCCs (Supplementary Fig. S4A), $\alpha 6$ -integrin⁺/CD34⁺-CSCs and isolated WD and PD/S cells showed a low level of expression of this receptor (Fig. 5A and Supplementary Fig. S4C), suggesting that PDGFR β is essentially associated with stromal cells. Most of the PD/S-SCC $\alpha 6$ -integrin⁺/CD34⁺ cells expressed PDGFR α , in contrast to the sparse $\alpha 6$ -integrin⁺/CD34⁺ cell population exhibiting low levels of PDGFR α expression in WD-SCCs (Fig. 5C and D; Supplementary Figs. S4B and S4D). Furthermore, strong PDGFR α phosphorylation (Fig. 5B) and significant induction of *Pdgfa* and *Pdgfc* ligand expression were detected in PD/S-SCCs of different lineages, and specifically in their respective L-CSCs (Fig. 5A and

Supplementary Fig. S4E), suggesting that CSCs of advanced SCCs may induce autocrine activation of PDGFR α signaling. These events were directly related with tumor progression, as no changes in *Pdgfra*, *Pdgfrb*, or *Pdgfc* expression were detected in WD-SCCs that never progress to PD/S-SCCs after serial engraftments (Supplementary Fig. S4F; OT9). As further evidence of autocrine activation of PDGFR α , we observed that PD/S cells growing in the absence of growth factors exhibited PDGFR α phosphorylation, which was blocked in response to the imatinib PDGFR inhibitor (Supplementary Fig. S4G). Despite PDGFR α phosphorylation was further induced in response to PDGFR ligands (Supplementary Fig. S4G), there were no significant changes in PD/S cell proliferation upon PDGF or imatinib treatment (Supplementary Figs. S4H and S4I), and PD/S cells with PDGFR α knockdown expression had a similar proliferation rate to PDGFR α -expressing control cells (Supplementary Figs. S5A and S5B). Furthermore, similar tumor growth was observed when PDGFR α knockdown cells and their respective control cells were engrafted in nude mice (Fig. 5E and Supplementary Figs. S5C and S5D), and when orthotopically implanted PD/S-SCCs were treated daily with imatinib, compared with the control treatment (Fig. 5F). Nevertheless, we observed a significant reduction in the number and size of lung metastases developed in imatinib-treated mice and in those carrying PDGFR α knockdown tumors (Fig. 5G and H and Supplementary Fig. S5E). Infrequent metastases developed from PDGFR α -interfered tumors showed a high receptor expression, indicating that these lesions may have arisen from tumor cells that eluded the *Pdgfra* interference (Supplementary Fig. S5F). Reduced metastasis observed after PDGFR α inhibition was not associated with a reduction of tumor angiogenesis because, although significantly fewer CD31⁺ vessels were observed in imatinib-treated PD/S-SCCs (Supplementary Fig. S5G), as previously reported (33, 34), the frequency of CD31⁺ vessels was unaltered in PDGFR α -interfered tumors (Supplementary Fig. S5G). These results demonstrate that PDGFR α signaling plays an important role promoting metastasis development, but not L-CSC proliferation. Reduction of metastasis mediated by PDGFR α signaling inhibition was not due to a decrease in the frequency of tumor-initiating cells or to an attenuation of the EMT program (Supplementary Figs. S5H–S5J). However, both knocking down of PDGFR α expression and imatinib treatment significantly reduced the invasion capability of PD/S cells (Fig. 5I and J). These results indicate that autocrine PDGFR α signaling promotes CSC motility and invasion, consequently favoring CSC dissemination and metastasis.

Autocrine FGFR signaling promotes CSC proliferation and survival in advanced SCCs

Our results showed that *Fgfr2* was underexpressed in L-CSCs compared with E-CSCs (Figs. 2B and 6A), indicating that FGFR signaling is altered in CSCs of advanced SCCs. FGFR1 and FGFR2 are expressed in normal keratinocytes and play an important role in the epidermal homeostasis (35). However, the role of

Figure 7.

Advanced human skin SCCs with induced EMT recapitulate the changes in signaling pathways described in mouse PD/S-SCCs. A, representative images showing an expanded CD44⁺ cell population, lower E-cadherin, and higher vimentin expression levels in PD/S-SCCs compared with WD/MD-SCCs. Scale bar, 30 μ m. B, β -catenin expression and cell localization in WD/MD-SCC and PD/S-SCCs. Scale bar, 20 μ m. C–H, quantification of the indicated mRNAs in a set of human WD/MD-SCCs and PD/S-SCCs. Mean ($\pm$ SE) mRNA levels of these genes relative to *GAPDH* mRNA are shown (individual data and mean $\pm$ SE). Green dots, recurrent PD/S-SCCs. I, schema describing the identified changes in CSC features and regulatory mechanisms at different stages of mouse SCC progression.

FGFR in regulating CSC proliferation and survival during SCC progression was unexplored. Analysis of the expression of different members of the FGFR family and variants (IIIb and IIIc) showed that *Fgfr1-IIIc* (*Fgfr1c*) was the FGFR most prominently expressed in E-CSCs and L-CSCs (Fig. 6A). Upregulation of *Fgfr1c* was not consistently found in L-CSCs of different lineages (Fig. 6A), although a strong FGFR1 expression was observed in spontaneous and orthotopically derived PD/S-SCCs relative to their respective WD-SCCs, but not in those WD-SCCs that never progressed (Supplementary Figs. S6A, S6C–S6E). These results indicate that CD34⁺-CSCs express high levels of FGFR1c and that the stronger expression of this receptor in advanced SCCs is due to the CSC expansion in these tumors. Interestingly, most L-CSCs from different lineages strongly induced the expression of *Fgf2* and *Fgf7* (Fig. 6B), correlating with their significant induction in PD/S-SCCs relative to WD-SCCs (Supplementary Fig. S6B). These results suggest that autocrine regulation of FGFR is induced in CSCs of advanced tumors.

Next, we evaluated the relevance of FGFR signaling to CSC proliferation and survival. $\alpha 6$ -integrin⁺/CD34⁺ population showed stronger expression of *Fgfr1c* than the $\alpha 6$ -integrin⁺/CD34[−] population in WD cells (Supplementary Fig. S6F), but the proliferation of these cells was unaltered in response to FGF2 or to the FGFR1 inhibitor PD173074 (36; Fig. 6C and Supplementary Fig. S6G). However, the response to FGF2 was significantly enhanced upon gefitinib-mediated EGFR inhibition (Supplementary Fig. S6G), indicating that the strong induction of the EGFR pathway attenuates FGFR signaling in WD cells. In contrast, the proliferation of PD/S cells was significantly induced upon FGF2 treatment (Fig. 6C, +FGF2), concomitantly with the induction of FGFR, AKT, and ERK1/2 phosphorylation, which were blocked in the presence of PD173074 similarly in *Ras* mutated or wild-type genetic backgrounds (Fig. 6C and Supplementary Fig. S6H). Furthermore, in the absence of growth factors, PD/S cell proliferation was significantly reduced in response to PD173074 (Fig. 6C; −FGF2). These results indicate that autocrine activation of FGFR1 signaling promotes CD34⁺-CSC proliferation in advanced SCCs. To analyze the effect of *in vivo* FGFR1 inhibition, mice carrying PD/S-SCCs were treated with PD173074. Tumor growth was significantly reduced in response to FGFR inhibition (Fig. 6D), coinciding with diminished tumor cell proliferation and induced apoptosis, as determined by phosphohistone H3 (Ser10) and cleaved caspase-3 levels, respectively (Fig. 6E–G). Despite the lesser tumor growth, no significant changes in the number and size of metastatic lesions were observed in PD173074-treated compared with mock-treated mice (Fig. 6H). These results indicate that autocrine FGFR1 signaling plays an important role promoting CSC survival and proliferation in PD/S-SCCs, although its inhibition does not impair the metastatic capability of these cells.

Advanced human skin SCCs recapitulate the molecular alterations described in mouse PD/S-SCCs

To determine the clinical relevance of our findings, we examined whether the molecular alterations described in mouse PD/S-SCCs were also associated with progression in human skin SCCs. The histopathologic grade of patient samples frequently showed intratumor regional heterogeneity, and they were classified as WD/MD-SCCs or PD/S-SCCs according to their main stage of progression and the percentage that this region represented in the overall sample (Supplementary Table S3). PD-SCCs and spindle

SCCs exhibited more tumor cells with CD44 expression, which identifies CSCs in human skin SCCs (37, 38), than WD/MD-SCCs (Fig. 7A). In addition, the EMT program was induced (Fig. 7A and C) mainly in PD-SCCs with spindle tumor cell regions and a history of recurrence after surgical resection (Supplementary Table S3). Consistent with the downregulation of β -catenin in advanced mouse SCCs, the expression of β -catenin was reduced in PD/S-SCCs compared with low-grade SCCs (Fig. 7B). Similarly, the level of expression of *EGFR*, *ERBB2*, *ERBB3*, and ligands was markedly lower in recurrent PD/S-SCCs (Fig. 7D and E), indicating that advanced human SCCs downregulate EGFR signaling. In contrast, the expression of *PDGFRA*, *PDGFRB*, *PDGFC*, *FGFR1b*, *FGFR1c*, and *FGF2* (Fig. 7F–H) was increased in most of the EMT-induced and advanced tumors. These results suggest that the signaling pathways controlling CSC proliferation and survival in advanced mouse skin SCCs may also operate in advanced human SCCs.

The L-CSC signature described here in mouse skin SCCs may identify progression and poor prognosis in other human SCCs. Indeed, this L-CSC signature identified a subtype of lung SCCs that exhibit poorly differentiated features and are associated with poor prognosis (Supplementary Fig. S7, left; refs. 39, 40). A similar association was found when this L-CSC signature was compared with a large subset of head and neck SCCs (Supplementary Fig. S7, bottom right; ref. 41). This suggests that CSC features in advanced skin SCCs may be commonly associated with SCC progression and poor prognosis in humans.

Discussion

Signaling pathways controlling the CSC function at early stages of skin SCC progression have been previously described (11, 15, 16). However, it was not known whether CSC regulatory mechanisms change during disease progression, correlating with the enhanced recurrence and metastasis of high-grade SCCs (1), which could influence the selection of the most efficient therapy. In the study reported here, we generated lineages of SCC progression and showed that a high percentage of WD-SCCs exhibiting epithelial features progressed to PD and mesenchymal SCCs with enhanced metastasis capability, recapitulating the skin SCC progression reported in other mouse models (42). In contrast, previous reports suggested that spindle and EMT-like skin SCCs might arise by a route other than WD-SCCs after DMBA/TPA treatment, relying on a different cell of origin and/or lower requirement for inflammatory stimuli (22). In this regard, K14-HPV16 mice, which show chronic inflammation in the skin (43), mainly developed WD-SCCs spontaneously or after DMBA/TPA treatment. Although inflammatory signaling might favor WD-SCC development, our results show that once these tumors are generated they can progress to PD/S-SCCs after serial engraftments in nude mice, which show a proficient inflammatory response (44). However, it remains unclear whether some mutated cell populations may evolve more rapidly or efficiently to PD/S-SCCs than do others.

WD- to PD/S-SCC transition was associated with a robust induction of the EMT program and an expansion of $\alpha 6$ -integrin⁺/CD34⁺-CSCs and tumor-initiating capability, which were similarly observed in primary PD/S-SCCs spontaneously developed in K14-HPV16 mice, as well in other mouse models of advanced SCCs (12, 16). Cell hierarchy could be altered in advanced tumors, as $\alpha 6$ -integrin⁺/

CD34⁺ and α 6-integrin⁺/CD34⁺ of PD/S-SCC showed a similar tumor-initiating capability and impaired epithelial differentiation after initial engraftments in immunodeficient mice. However, previous studies showed that the tumor-initiating capability of CD34⁺ was significantly reduced after multiple serial engraftments, and only CD34⁺ cells have long-term self-renewal capability and are long-term tumor propagation cells (12).

Comparison of gene expression profiles between α 6-integrin⁺/CD34⁺-CSCs of WD- and PD/S-SCCs defined a CSC signature associated with highly malignant SCCs, which is characterized by the strong induction of stemness, the EMT program, cell proliferation, motility and metastasis, and reduced epidermal cell differentiation and cell adhesion-regulatory genes. Accordingly, tumor-initiating cells of PD/S-SCCs lose the ability to generate cells exhibiting epithelial traits. Therefore, CSC expansion may be the consequence of a sustained self-renewal and the inhibition of differentiation.

Furthermore, we found that the mechanisms that regulate CSC proliferation, survival, and dissemination change at different stages of progression. Indeed, β -catenin signaling, which plays a key role in regulating CD34⁺-CSC features in early SCCs (11), is downregulated in advanced SCCs. Moreover, EGFR signaling, which is strongly upregulated in E-CSCs to drive their proliferation, is similarly upregulated in L-CSCs, which instead induce autocrine FGFR1 signaling to promote tumor growth. Thus, although inhibition of EGFR signaling leads to WD-SCC regression and cancer cell differentiation (45), this treatment may be ineffective in advanced SCCs, which downregulated EGFR signaling. Although previous studies showed that the effectiveness of FGFR1 inhibitors is compromised in the presence of *Ras* mutations in other tumor types (46), FGFR1-dependent downstream signaling was similarly inhibited in PD/S cell with or without *Ras* mutations after PD173074 treatment, suggesting that the relevance of *Ras*-activating mutations promoting proliferation may be reduced in advanced SCCs, as previously reported in EMT-like skin SCCs (22) and in lung and pancreatic cancer cells with induced EMT (47).

In addition, autocrine PDGFR α signaling is strongly induced in L-CSCs. In this regard, the strong induction of EMT in L-CSCs may promote the activation of both FGFR1 and PDGFR α signaling, as reported in other tumor types (48, 49). So, breast CSCs generated from cell lines that spontaneously induced EMT, activated PDGFR/PLC γ /PKC α signaling, which promoted cell proliferation (48). However, inhibition of PDGFR α signaling in SCCs did not block tumor growth but strongly reduced the invasion capability of PD/S cells and metastasis in advanced SCCs. In contrast, FGFR1 inhibition reduced tumor growth without blocking metastasis. These results suggest that FGFR1 and PDGFR α signaling may act exclusively to promote tumor growth and metastasis, respectively.

The WD-to-PD/S-SCC transition may be induced by genetic, epigenetic, and/or, microenvironment alterations, which may modify the differentiation ability, frequency, and regulatory mechanisms of CSCs, promoting the generation and selection of a subset of tumor-initiating cells with strong growth advantages. Given the intratumor cell and clonal heterogeneity reported in some tumors (50), SCC progression may result from a selection and/or activation of rare cells with L-CSC features (α 6-integrin⁺/EpCAM⁺/CD34⁺/PDGFR α ⁺) residing in WD-SCCs. However, these cells were practically undetectable in WD-SCCs, as most of α 6-integrin⁺/CD34⁺ cells conserved EpCAM expression and

exhibited low levels of PDGFR α . Thus, our results indicate that the CSC features and mechanisms that control tumor-initiating cell proliferation and dissemination change in later stages of SCC progression, promoting aggressive growth and enhanced metastasis (Fig. 7I).

Interestingly, advanced and recurrent human skin SCCs with an expanded CD44⁺-CSC population and enhanced EMT, downregulated EGFR and β -catenin expression, and induced PDGFR α / β and FGFR1 expression, thereby recapitulating the alterations detected in advanced mouse SCCs. These results suggest that the use of specific inhibitors of these pathways may be a possible therapy to block relapse and metastasis in recurrent and advanced human SCCs, in which classic chemotherapy is of limited clinical benefit (4). Thus, identifying the regulatory mechanisms controlling CSCs at specific stages of progression may guide the choice of the most suitable therapy for selectively targeting the signaling pathways that regulate this subset of cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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