Receptor of Activated Protein C Promotes Metastasis and Correlates with Clinical Outcome in Lung Adenocarcinoma

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***Rationale*: Efficient metastasis requires survival and adaptation of tumor cells to stringent conditions imposed by the extracellular milieu. Identification of critical survival signaling pathways in tumor cells might unveil novel targets relevant in disease progression.**

***Objectives*: To investigate the contribution of activated protein C (APC) and its receptor (endothelial protein C receptor [EPCR]) in animal models of lung cancer metastasis and in patients with lung adenocarcinoma.**

***Methods*: Signaling pathway triggered by APC/EPCR and its relevance in apoptosis was studied *in vitro*. Functional significance was assessed by silencing and blocking antibodies in several *in vivo* models of lung cancer metastasis in athymic nude *Foxn1^{nu}* mice. We examined EPCR levels using a microarray dataset of 107 patients. Immunohistochemical analysis was performed in an independent cohort of 295 patients with lung adenocarcinoma.**

***Measurements and Main Results*: The effects of APC binding to EPCR rapidly triggered Akt and extracellular signal–regulated kinase signaling pathways, leading to attenuated *in vitro* apoptosis. *In vivo*, silencing of EPCR expression or blocking APC/EPCR interaction reduced infiltration in the target organ, resulting in impaired prometastatic activity. Moreover, overexpression of EPCR induced an increased metastatic activity to target organs. Analysis of clinical samples showed a robust association between high EPCR levels and poor prognosis, particularly in stage I patients.**

***Conclusions*: EPCR and its ligand APC promote cell survival that contributes to tumor cell endurance to stress favoring prometastatic activity of lung adenocarcinoma. EPCR/APC is a novel target of relevance in the clinical outcome of early-stage lung cancer.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Activated protein C binding to its receptor endothelial protein C receptor (EPCR) expressed in endothelium promotes cytoprotective, antiinflammatory, and antithrombotic properties. EPCR is also expressed in tumors, including the lung.

What This Study Adds to the Field

Activated protein C/EPCR promotes tumor cell survival, which results in increased metastatic activity in lung adenocarcinoma. EPCR might be a useful prognostic factor and a potential therapeutic target.

Keywords: survival; microenvironment; bone metastasis; prognosis; adrenal gland

Metastasis is a frequent and incurable complication of lung adenocarcinoma (ADC), the most frequent histological subtype of lung cancer (1–6). Tumor cells tend to disseminate locally in the thoracic cavity as well as in distant organs including the skeleton, adrenals, brain, pericardium, and liver (7). This multistep process often initiates soon after the tumor develops, but is highly inefficient, and the majority of tumor cells undergo apoptosis. Tumor cells leaving the primary site require survival abilities in the bloodstream to overcome sheer stress in the circulation. In the target organ, the progression of metastasis is based on complex interactions that trigger signaling pathways in the tumor and its neighboring milieu, and modulate tumor cell survival and organ colonization (8, 9). Secondary sites, such as bone, also impose stringent conditions (10), including low pH, high calcium, and mechanical stiffness of the extracellular matrix (10), which impair the development of micrometastasis (11, 12). Thus, survival signaling pathways activated in tumor cells might offer an advantage promoting metastasis development (13). These key determinants enabling cells to overcome stress conditions could be of great interest, but few have been identified.

Endothelial protein C receptor (EPCR) is a transmembrane receptor highly expressed in endothelial cells. The main natural ligands are protein C and activated protein C (APC). Protein C activation is a tightly regulated process that physiologically occurs inside the vascular system (14). APC binding to EPCR exerts a cytoprotective function on endothelial cells, which includes resistance to inflammation (15–17) and apoptosis (18).

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The complex APC–EPCR can activate protease-activated receptor 1 (PAR1), a transmembrane coreceptor coupled to guanosine triphosphate–binding proteins, which triggers intracellular signaling (19).

Although EPCR is expressed in a variety of human tumors, including breast, lung, glioblastoma, and erythroleukemia (20, 21), the effects of APC binding to EPCR remain ill defined (22, 23).

In this study, we addressed the involvement of APC/EPCR in critical functions of the metastatic process using lung ADC as a model. We found that the APC/EPCR axis conferred a significant survival advantage to lung ADC cells. This function was relevant in several *in vivo* models of lung cancer metastasis. Consistently, we found a strong association in stage I ADC patients between EPCR levels and patient survival. These findings reveal a novel role of EPCR in metastatic events of lung ADC and suggest its potential relevance as a novel prognostic marker. Some of the results have been reported previously in the form of an abstract (24).

METHODS

In Vivo Intracardiac Inoculation

In vivo intracardiac inoculation was performed using athymic nude-*Foxn1*^{nu} female mice (Harlan Iberica, Barcelona, Spain) according to the approved protocols of the Local Animal Committee, as previously detailed (25).

In Vivo EPCR-Blocking Experiments with F(ab)2 Antibody

An *in vivo* administration regimen was performed from the day before intracardiac inoculation of the cells, and every 2 days for 22 days with a highly human-specific $F(ab)_2$ RCR252 anti-EPCR antibody with no cross-reactivity with murine EPCR (Figure E2 in the online supplement) or IgG.

Flow Cytometry Experiments

All the flow cytometry experiments were performed in a FACSCalibur (BD Biosciences, Sparks, MD). The expression of EPCR on the surface of cells was assessed using anti-human EPCR RCR-252 monoclonal antibody. Apoptosis of staurosporine-stimulated endothelial cells was assessed by incubating them with fluorescently labeled annexin V and actinomycin D.

Transcriptomic Analysis of APC Effect and *In Silico* Experiments

Analysis in A549 cells was performed after incubation of these cells with 50 nM APC for 4 hours, and the RNA isolated was hybridized in human Gene 1.0 ST microarrays (Affymetrix, Santa Clara, CA).

A disease progression study was performed with the expression and clinical data of one cohort (n = 107) (26).

Patient Samples

A cohort of 295 patients diagnosed with ADC at the M. D. Anderson Cancer Center was included in the clinical study. Tissue specimens were classified according to the 2004 World Health Organization classification system and further classified according to the revised International System for Staging Lung Cancer. Criteria for study inclusion were ADC and time to progression (TP) > 6 months.

Immunohistochemical Analysis

Sections were incubated with an anti-EPCR antibody overnight at 4°C and anti-mouse polyclonal EnVision complex (Dako, Glostrup, Denmark). The peroxidase activity was demonstrated by diaminobenzidine and sections were counterstained with hematoxylin, dehydrated, and mounted in DPX mounting media. The extension and the intensity of EPCR staining was performed by two observers and scored as percentage of positive cells and the intensity of staining, according to the H-score system (27, 28). Median values of the H-score were used as the cut-off to separate high and low EPCR patients.

Statistical Analysis

For each analysis, a study of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and a study of the homogeneity of variances (Levene test) were performed. Analysis of variance was performed for more than two groups with Tukey test for multiple comparisons. Student *t* test was used for comparison between two groups. Data were expressed as mean ± standard deviation. In nonparametric analysis, Kruskal-Wallis test was performed followed by the Mann-Whitney multiple comparison test. If, in addition, there was also heteroscedasticity, a median test was performed. The *P* value was adjusted, if there were multiple comparisons, using Bonferroni correction. Nonparametric tests were represented with the median ± interquartile range.

Kaplan-Meier curve significance was calculated by log-rank test. The univariate or multivariate study of relapse or death risk was performed with the Cox regression test. The variables analyzed included EPCR expression, age, sex, smoking status, tumoral size, and neoadjuvant and adjuvant therapy.

A detailed version of the METHODS is available in the online supplement.

RESULTS

APC–EPCR Axis Promotes Survival and Prevents Apoptosis in Lung ADC Cell Line

Biopsies from human lung ADC showed different expression levels of EPCR in the patients analyzed (Figure 1A). To assess the role of the APC/EPCR pathway, we performed analysis of EPCR in human lung ADC A549 cells. This cell line expressed high levels of EPCR (Figure 1B). Based on previous work, we investigated whether APC could activate prosurvival signaling pathways in A549 cells. Cells treated with growing concentrations of APC showed increased phosphorylation of Akt and extracellular signal–regulated kinases (ERK) (Figure E1a). Furthermore, a specific anti-human EPCR blocking antibody (RCR-252) (Figure E2) reduced APC-induced Akt and ERK phosphorylation at early time after stimulation (Figure 1C). A slight effect was observed by control IgG antibody probably due to nonspecific binding.

Next, we performed transcriptomic analysis for the identification of APC targets. Gene clustering analysis revealed that APC stimulation modified gene expression profiling of multiple genes. Functional gene enrichment analysis showed a significant alteration of apoptosis-related genes (Figure 1D and Table E1). Thus, we functionally tested the involvement of APC in apoptosis. APC reduced staurosporine-induced apoptosis in A549 cells, evaluated by annexin V staining and polyADP ribose polymerase cleavage, in a dose-dependent manner. Preincubation of cells with RCR-252 abolished the APC-mediated inhibition of staurosporine-induced apoptosis (Figures 1E, 1F, E1B, and E1C). Our *in vitro* results showed that APC interacting with EPCR conferred a prosurvival function.

Effects of Knockdown of EPCR Expression on Metastasis

We also examined EPCR expression after lentiviral transduction of two short hairpin (sh)RNAs targeting EPCR in A549 cell line that showed partial (shRNA1) or complete (shRNA2) abrogation of EPCR levels as compared with those in control cells (Figure 1G). Silenced cells (shEPCR1 and -2) showed a potential to form colonies in anchorage-independent conditions similar to that of the control cells (data not shown).

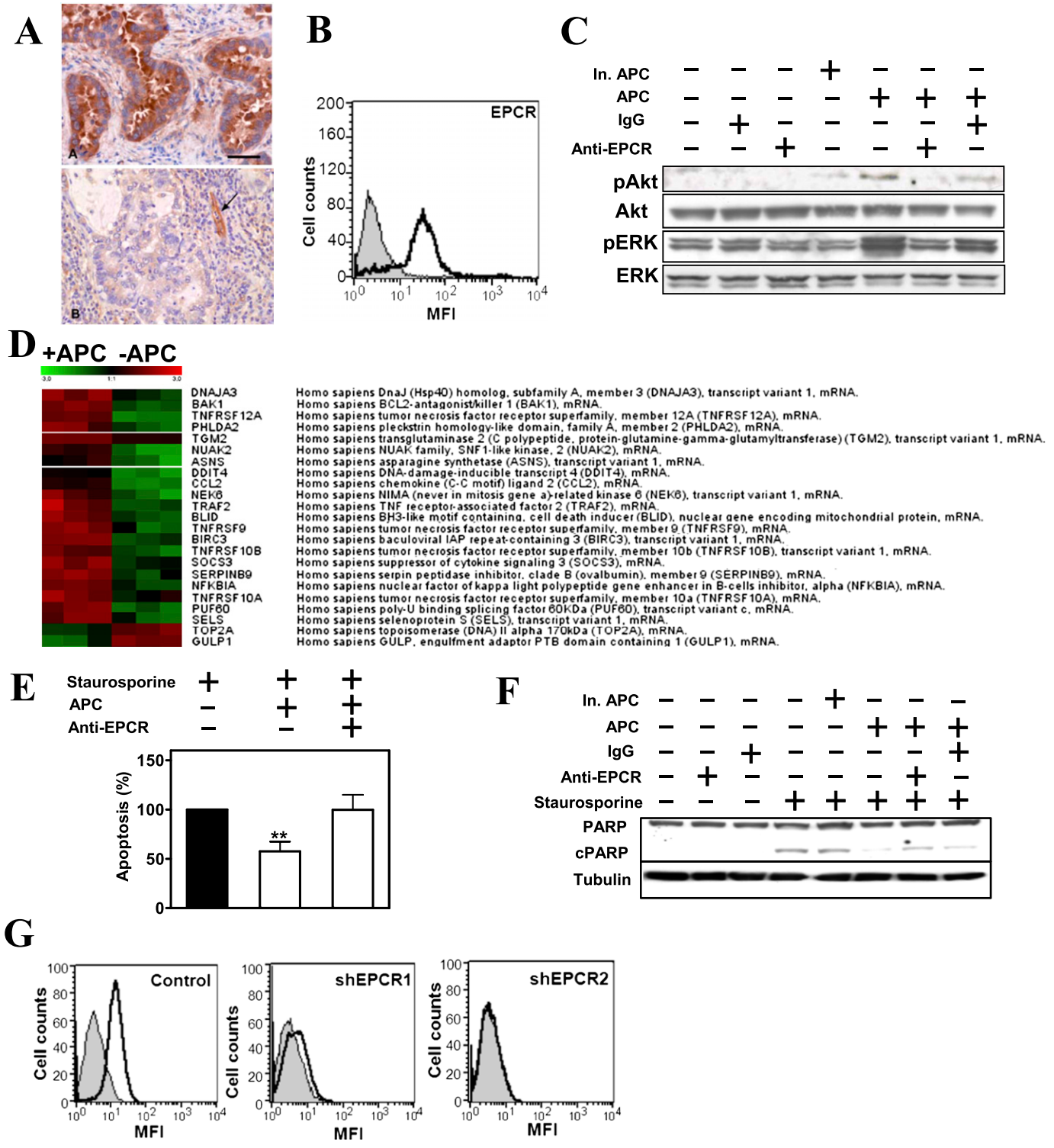


Figure 1. Endothelial protein C receptor (EPCR) mediates *in vitro* cell survival. (*A*) Representative immunohistochemical images of EPCR in human lung adenocarcinoma samples with high (*A*) and low (*B*) EPCR levels. *Arrow* indicates positive immunostaining of the endothelium. *Scale bar* = 50 μm. (*B*) Flow cytometry analysis of EPCR expression in A549 cells. The *gray histogram* represents the isotype control. (*C*) Western blot analysis of the phosphorylation status of ERK and Akt in A549 cell lysates after incubation. Cells were incubated for 15 minutes with activated protein C (APC) or inactivated APC (In. APC) and for 30 minutes with IgG or anti-EPCR antibody. (*D*) Hierarchical cluster diagram of differentially expressed genes in A549 cells after incubation with vehicle (−APC) or with 50 nM APC (+APC). *Horizontal rows*, single-gene probe sets; *vertical columns*, results from the single microarray hybridizations. Each box is the hybridization signal value of a gene probe set in the microarray assay. (*E*) Flow cytometry analysis of apoptosis was performed by annexin V binding. Anti-EPCR antibody abolished the APC-mediated inhibition of staurosporine-induced apoptosis, represented by mean ± SD derived from three independent experiments. (*F*) Western blot analysis of the effect of APC on PARP cleavage induced by staurosporine. Experiment was performed three times with similar results. (*G*) Flow cytometry analysis of EPCR protein expression in A549 cells transduced with scramble shRNA (control) or shRNA targeting EPCR (shEPCR). The *gray histogram* represents the isotype control. ERK = extracellular signal–regulated kinases; PARP = polyADP ribose polymerase.

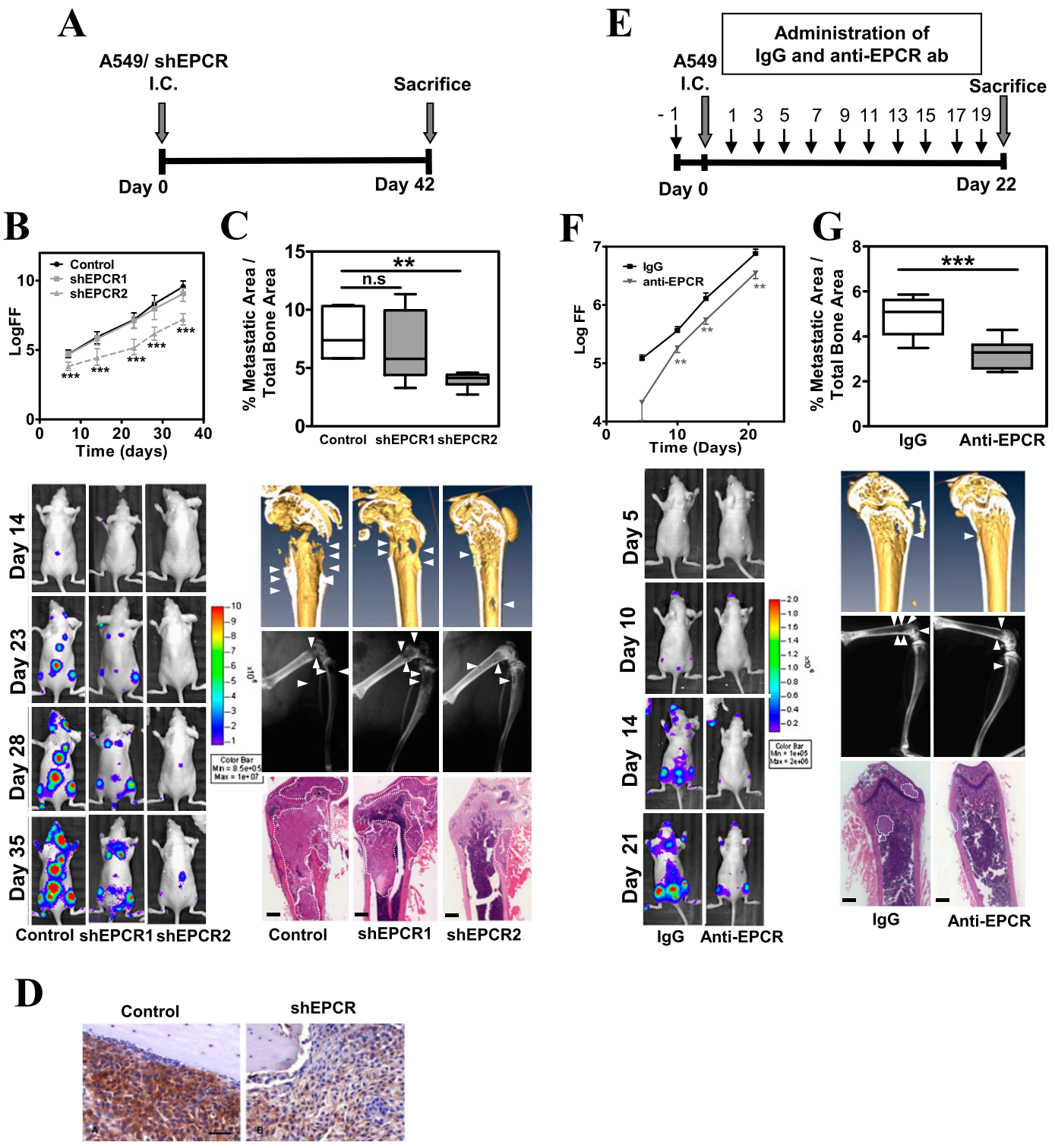


Figure 2. Endothelial protein C receptor (EPCR) mediates prometastatic activity to bone. (*A*) *In vivo* experimental regimen. (*B*) Prometastatic activity of EPCR after intracardiac inoculation with control, shEPCR1, and shEPCR2 A549 cells (n = 8). *Upper panel*: bioluminescence quantification in hindlimbs represented by the median and interquartile range. *Lower panel*: representative images. (*C*) *Upper panel*: quantification of X-ray image analysis of osteolytic lesions in hindlimbs on Day 35. Median and interquartile range are represented. *Lower panel*: representative micro–computed tomography scans (*top*), X-ray images (*middle*), and hematoxylin and eosin (H&E) sections (*bottom*). (*D*) Representative immunohistochemical images of EPCR metastatic lesions of control and shEPCR hindlimbs. (*E*) Experimental regimen for the anti-EPCR antibody. (*F*) Prometastatic activity of EPCR after intracardiac inoculation of cells into mice treated with anti-EPCR or IgG control (n = 10 per group). *Upper panel*: bioluminescence quantification in hindlimbs represented by the mean and standard deviation. *Bottom panel*: representative images. (*G*) *Upper panel*: quantification of osteolytic lesions in hindlimbs by X-ray imaging on Day 21. Median and interquartile range are represented. *Lower panel*: representative micro–computed tomography scans (*top*), X-ray images (*middle*), and H&E-stained sections (*bottom*). $^{**}P < 0.01$, $^{***}P < 0.001$. *Arrowhead* indicates the location of osteolytic lesions. Metastatic area is depicted by a punctate line. *Scale bar* = 500 μm.

Since tumor cell survival function is critical in metastasis progression, we examined to what extent EPCR could play a role in a mouse model of lung cancer metastasis with proclivity to form osseous metastasis (25). Intracardiac inoculation of shEPCR1 and -2 (Figure 2A) led to a significant decrease in tumor burden of hindlimbs, assessed by bioluminescence imaging at Day 35, in an EPCR level–dependent manner (Figure 2B). Similar results were obtained by X-ray image analysis. A dramatic decrease in bone metastatic area was observed in long bones derived from shEPCR2 mice, whereas this decrease was milder in mice inoculated with shEPCR1 cells, as compared with control mice (Figure 2C). Histological analysis, and micro–computed tomography scans revealed a similar decrease in bone colonization (Figure 2C). Analysis by immunostaining did not show differences in the number of activated caspase-3–positive cells in metastases (data not shown), suggesting that prosurvival effects mediated by EPCR could be more important in circulating tumor cells before reaching the target organ. The use of the lentivirally transduced shEPCR allowed permanent silencing of EPCR expression levels *in vivo* during the whole experimental period (Figure 2D). Thus, inhibition of EPCR expression levels decreased bone metastatic activity.

Effects of Anti-EPCR Blocking Antibodies on Metastasis

To substantiate the role of EPCR and to avoid spurious effects related to the complement system, we used the F(ab′)2 fractions of the specific anti-human EPCR blocking antibody RCR-252 according to the schedule outlined in Figure 2E. Twenty-two days after intracardiac inoculation of A549 cells, bioluminescence imaging and X-ray analysis showed a marked decreased tumor burden and osteolytic lesions in hindlimbs of anti-EPCR–treated animals as compared with controls (IgG-treated) (Figures 2F and 2G).

Effects of EPCR Overexpression in Other Cell Lines

Next, we intracardially inoculated a parental H727 lung cancer cell line, a non–EPCR-expressing cell line of non-ADC origin, which forms metastasis to the skeleton and to the adrenal glands. Consistent with previous findings, expression of EPCR was detected in highly metastatic subpopulations isolated from metastasis after intracardiac inoculation of parental H727 cells (Figure E3). These findings suggest that rare EPCR-expressing cells within the parental population are endowed with an advantageous function to metastasize.

To substantiate this observation, we overexpressed EPCR in H727 cells (Figure 3A). Interestingly, H727 overexpressing EPCR (Figure 3B) showed an enhanced ability to form metastasis to the adrenal glands after intracardiac inoculation as compared with control injected mice as assessed by bioluminescence imaging (Figure 3C), and total weight of adrenal glands after necropsy (Figure 3D). Immunohistochemical analysis of adrenal glands revealed strong staining of EPCR in the metastases (Figure 3E). Immunostaining of activated caspase-3 cells revealed no differences between groups (data not shown), suggesting that EPCR-mediated effects might be occurring before tumor cells reach the adrenal glands. Moreover, overexpression of EPCR in H727 cells also increased the osseous prometastatic activity (Figures E4A–E4C). Furthermore, bone metastasis was significantly attenuated by RCR-252 in an *in vivo* model (Figures 3F and 3G).

Finally, forced expression of EPCR in a non–EPCR-expressing cell line of nonpulmonary origin (neuroblastoma) was again associated with an important increase in metastasis *in vivo* (Figure E5). Thus, EPCR expressing cells are endowed with an enhanced ability to form metastasis to different organs, including the skeleton and the adrenal glands. These findings suggest a common mechanism conserved in tumors of different origin.

EPCR Expression Promotes Early Events of Metastasis in the Target Organ

Early events of metastasis in the target organ entail several processes, including survival in circulation, the adherence to the target organ, and lastly, the survival and growth of tumor cells giving rise to micrometastases (29). To test whether the metastatic activity mediated by EPCR was due to changes in any of these early events, shEPCR1 and -2 or control A549 cells were intracardially inoculated (Figure 4A). Animals injected with shEPCRs showed a decrease in bioluminescence imaging as compared with control cells (Figure 4B). Consistent with previous findings, the number of isolated single-cell–derived colonies was also decreased in shEPCR-injected mice (Figure 4C). A similar experiment using F(ab′)2 RCR-252 was performed (Figure 4D). Although there were no detectable differences in bioluminescence between groups at Day 8 after inoculation (Figure 4E), the number of isolated single-cell–derived colonies in hindlimbs showed a trend to diminish in anti-EPCR–treated animals (Figure 4F). These effects were significant at longer times after Day 10 after inoculation, as shown in Figure 2F. Thus, the anti-ECPR blockade may require longer exposure to show significant effects as compared with the shEPCR silencing.

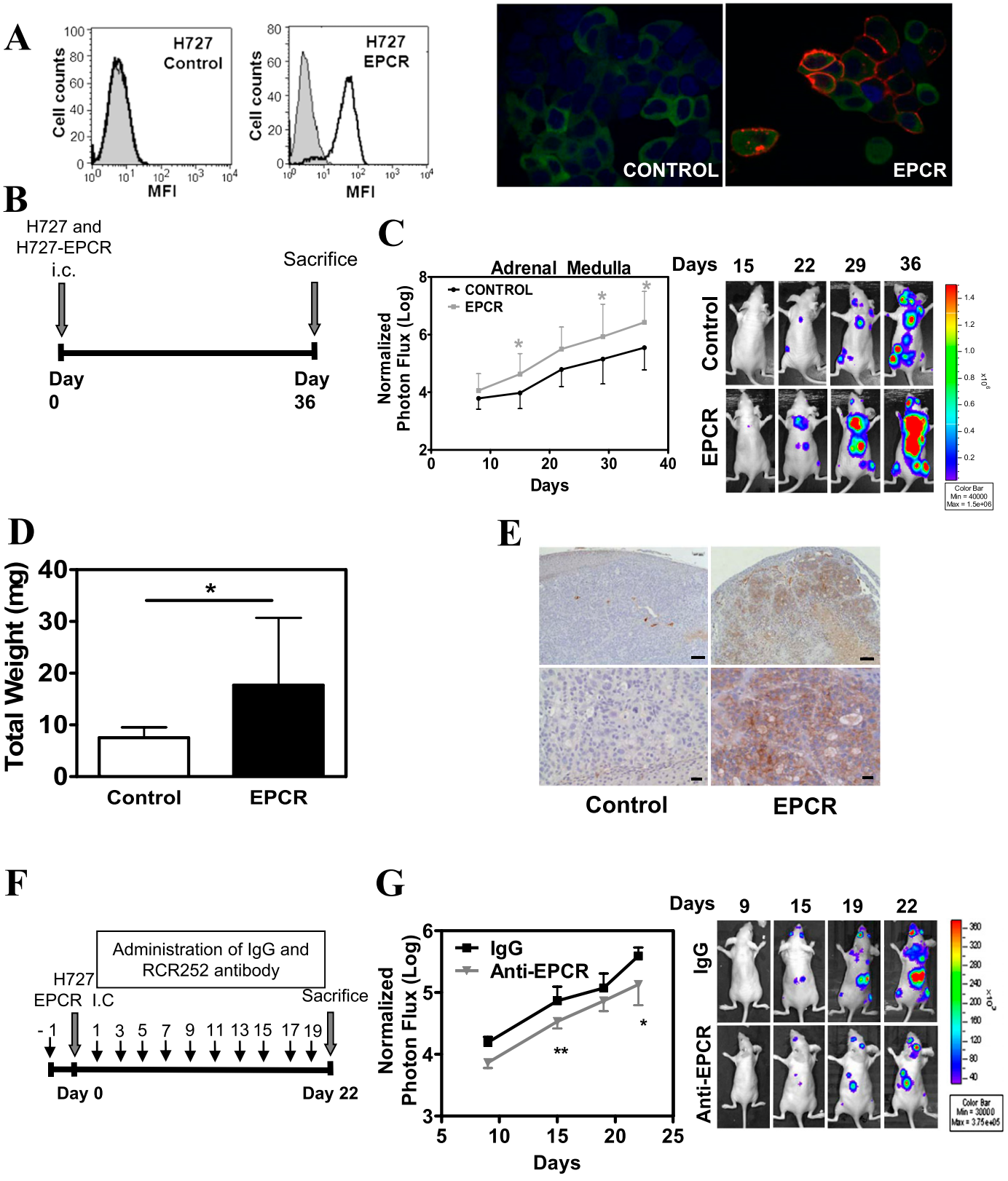
These results suggest that EPCR participates in early events of metastasis in the target organ.

Clinical Relevance of EPCR in Lung ADC

Since EPCR participates in metastatic activity, we explored its clinical role in ADC. In an *in silico* analysis of microarray data of EPCR expression levels in the cohort from Nguyen and colleagues (26) (n = 107), we observed a longer metastasis-free period in patients with low EPCR levels ($P = 0.057$) (Figure 5A). The presence of other non–tumor-associated cells such as endothelium, macrophages, and normal bronchial epithelial cells, which also express EPCR, could preclude a rigorous evaluation of tumor EPCR. To circumvent this obstacle, we performed immunohistochemical analysis in a series of lung ADCs. The staining of tumors showed a variable degree of intensities. Clinical features are summarized in Table E2.

In ADC patients with high EPCR levels, a significantly shorter TP was observed as compared with that in those with low EPCR levels ($P = 0.021$) (Figure 5B). There was a significant association of high EPCR levels with progression-free survival adjusted by age, sex, smoking status, tumoral size, and treatment (Table 1). These findings indicate that EPCR constitutes an independent risk factor in disease progression in ADC.

Next, we stratified the cohort according to histopathological stage. Clinical features of stage I ADC (n = 180) are summarized in Table E3. In this subset, patients with high EPCR levels showed a shorter TP ($P = 0.0006$) (Figure 5C). Cox analysis revealed a statistically significant higher risk of progression independent of other variables (Table 1). Similarly, high EPCR levels in ADC also significantly decreased overall survival (Figure 5C and Table 1). However, the analysis of TP and survival in patients with stage II–IV disease revealed no differences (Table 1 and Figure E6A).



Finally, we analyzed the stage I ADC patients with high EPCR levels (n = 79), and we stratified them into those receiving adjuvant therapies and those untreated, ruling out those patients who had received neoadjuvant therapy. The analysis revealed a sharp increase in TP and survival in the subgroup of treated patients (Figure E6B). In contrast, patients with low EPCR did not benefit from treatment (Figure E6C). These findings suggest that ADC stratification according to EPCR levels could allow the identification of a subset of patients with high EPCR that could benefit from adjuvant treatment.

Figure 3. Effect of blocking endothelial protein C receptor (EPCR) antibody in bone metastasis formation in EPCR-overexpressing H727 cells. (*A*) EPCR protein expression analysis by flow cytometry of H727 control line (transduced with empty vector) and transduced with EPCR (H727 EPCR). The *gray histogram* represents the isotype control. *In vitro* confocal microscopy of the H727 control and H727 EPCR. EPCR staining, GFP, and nuclei are shown in *red*, *green*, and *blue*, respectively. The images were captured at ×630 magnification. (*B*) Schematic outline of the experiment. (*C*) Bioluminescence after intracardiac inoculation of athymic nude mice with H727 control (n = 7) and H727 EPCR (n = 6). *Left panel*: quantification of bioluminescence. *Right panel*: representative bioluminescence images. (*D*) Total weight of the adrenal gland of all mice. (*E*) Immunostaining of EPCR in histological sections of the adrenal gland from H727 control and EPCR representative animals. *Top images*: *scale bar* = 100 μm. *Bottom images*: *scale bar* = 20 μm. (*F*) Experimental regimen pretreating the animals with anti-EPCR or control antibody (50 mg/kg) before injection of H727 cells overexpressing EPCR. (*G*) Evaluation of the antimetastatic effects of treatment with control antibody (n = 7) or with an anti-EPCR antibody (n = 8). *Left panel*: quantification of bioluminescence of bone metastasis (median and interquartile range). *Right panel*: representative bioluminescence images. In all graphs, the mean and standard deviation are represented. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

In this study, we have characterized EPCR as a key factor of bad prognosis in lung ADC. EPCR promotes cell survival, which contributes to a robust prometastatic activity. These findings unveil a novel function of EPCR in the context of lung cancer and suggest that EPCR could be a critical factor in human lung ADC.

EPCR as Factor of Bad Prognosis in Stage I ADC

The analysis of clinical cohort of lung ADC patients showed the prognostic value of EPCR in stage I patients where high EPCR levels were associated with 2.4 to 3.8 fold increase in mortality and disease progression, respectively. This analysis was independent of other risk factors such as age, sex, tobacco, tumoral size, and treatment. In contrast, in patients with more advanced disease, EPCR did not show any influence on survival and disease progression. Other factors inherent to tumor biology or its microenvironment may display a more prominent role in advanced stages.

Of note, stage I ADC patients with high EPCR who received adjuvant treatment displayed a longer survival and time to progression than nontreated patients. Thus, the stratification of patients according to EPCR levels of stage I ADC could change the treatment strategy. According to our findings, it could be recommended to treat those patients with high EPCR levels in stage I ADC, but a prospective randomized study for treatment is necessary to confirm these results.

Our findings were consistent with the microarray analysis of another cohort such as the cohort of Nguyen and coworkers. However, the results of this study should be cautiously interpreted because it is possible that other tumor-associated cells, such as the tumor vasculature present in bulk biopsies, could influence EPCR

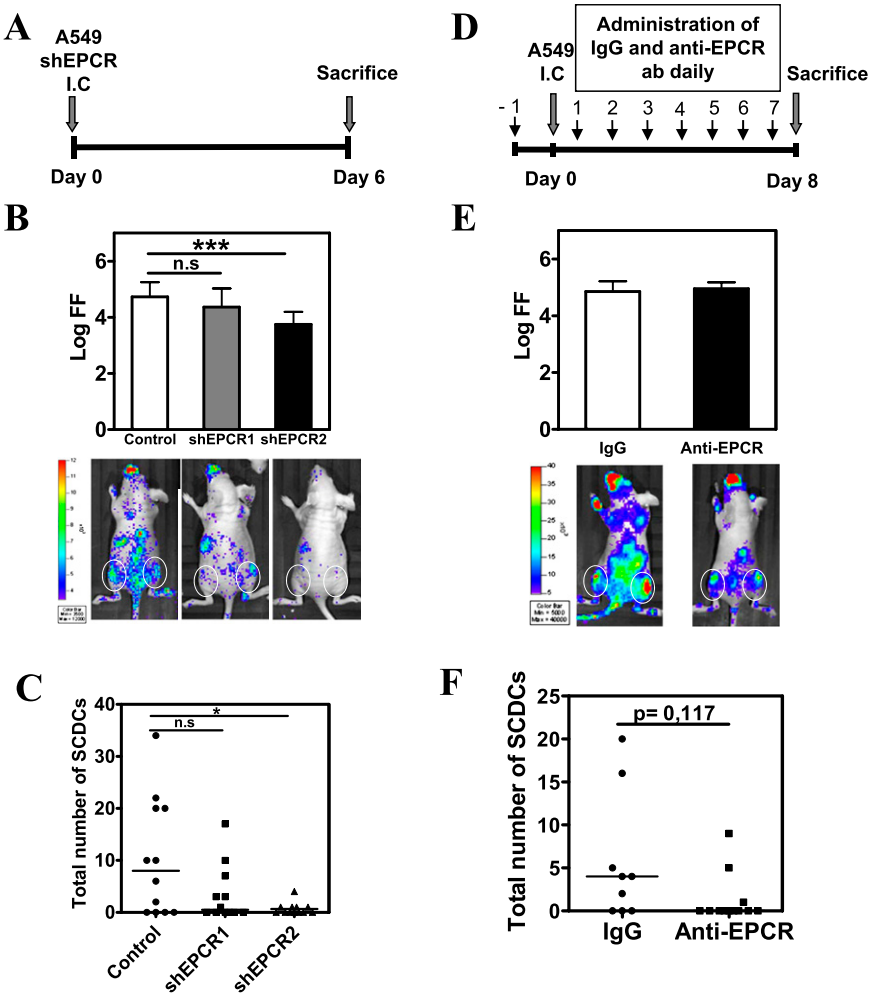


Figure 4. Prometastatic effects in the target organ mediated by endothelial protein C receptor (EPCR). (*A*) Experimental regimen of animals injected with control or shEPCR1 and shEPCR2 A549 cells. (*B*) *Upper panel*: quantification of bioluminescence in hindlimbs 5 days after intracardiac inoculation (n = 6 per group) represented by the mean and standard deviation. *Lower panel*: representative images. (*C*) Total number of single-cell–derived colonies (SCDCs) from bone metastasis of control and shEPCR-treated animals. The graph represents the median with a horizontal line. (*D*) Experimental regimen using anti-EPCR antibody and IgG (control) before and after intracardiac inoculation of A549 cells. (*E*) *Upper panel*: quantification of bioluminescence in hindlimbs 8 days after intracardiac inoculation (n = 6 per group) represented by the mean and standard deviation. *Lower panel*: representative images. (*F*) Total number of SCDCs from bone metastasis of control and anti-EPCR-treated groups. *$P < 0.05$, ***$P < 0.001$.

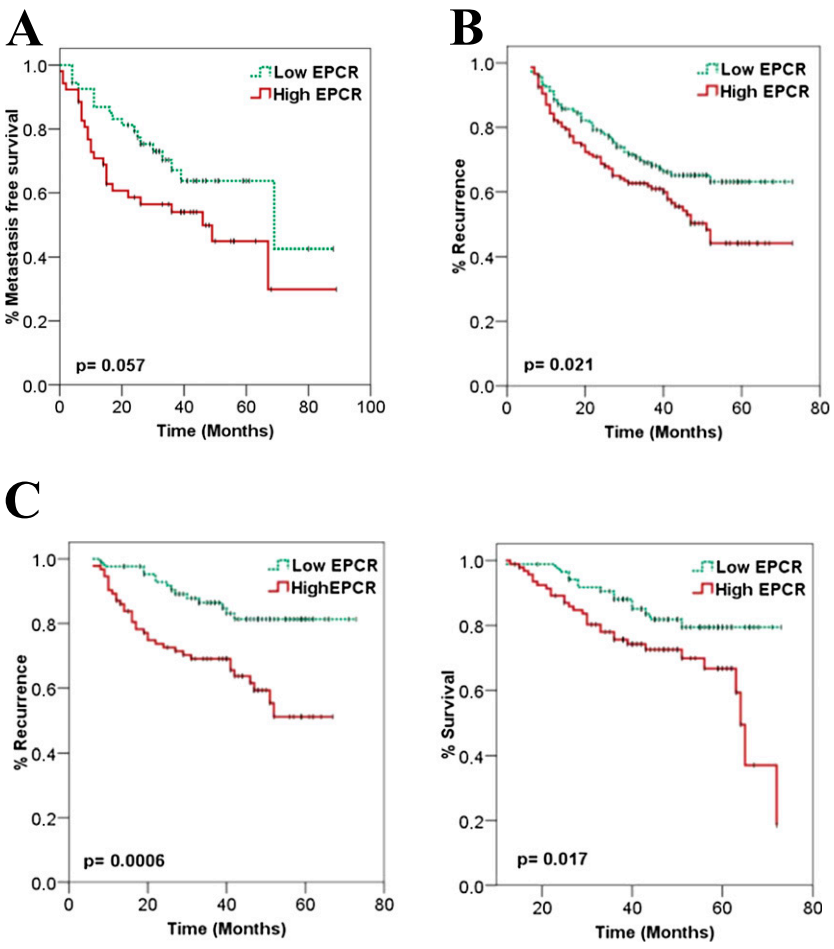


Figure 5. Clinical relevance of endothelial protein C receptor (EPCR) levels. (*A*) Metastasis-free time in a cohort of transcriptomic data of lung adenocarcinomas (ADCs) by Nguyen and colleagues (n = 107). (*B*) Kaplan-Meier progression-free survival curve for a cohort of 295 patients with lung ADC. EPCR expression was determined by immunohistochemistry. (*C*) Kaplan-Meier curve for a subgroup of 180 patients diagnosed with stage I lung ADC. (*D*) Schematic model summarizing that APC binding to EPCR in tumor cells triggers pro-survival signals through Akt and ERK phosphorylation. Cells in lung ADC tumors with high EPCR are endowed with enhanced tolerance to stress through the metastatic cascade. APC = activated protein C; ERK = extracellular signal–regulated kinases.

levels. In addition, the sample size of this cohort did not allow to determine clearly the role of EPCR in stage I patients.

Prometastatic Activity of EPCR

The clinical relevance of EPCR in stage I ADC patients suggest that EPCR could influence progression altering treatment resistance and metastasis progression. Since treated high-EPCR stage I tumors displayed a clinical benefit, EPCR did not confer chemo- or radioresistance. Thus, progressive disease in high-EPCR patients might be influenced by tumor metastatic activity, which could be the main factor associated with the adverse clinical course. EPCR could influence early stages of metastatic dissemination by conferring high survival or other properties to circulating cells, according to our microarray data.

In addition to this advantage in early metastatic events in the primary tumor, our animal experiments using different cell lines are consistent with clinical findings and suggest a direct effect of APC/EPCR axis in metastasis. The changes in prometastatic activity modulated by EPCR levels observed in our animal models suggest that cell survival in the circulation might account for such effect, although this point requires a rigorous demonstration in

TABLE 1. UNIVARIATE AND MULTIVARIATE COX REGRESSION ANALYSIS OF PROGRESSION-FREE AND OVERALL SURVIVAL

	Crude HR (95% CI)	Adjusted HR (95% CI)*
Progression-free survival		
All ADC (n = 295)	1.54 (1.06–2.23)†	1.77 (1.19–2.63)‡
Stage I (n = 180)	2.81 (1.51–5.24)‡	3.83 (1.90–7.71)§
Stage II–IV (n = 115)	1.03 (0.64–1.67)	1.07 (0.64–1.81)
Overall survival		
All ADC (n = 295)	1.34 (0.88–2.03)	1.42 (0.90–2.22)
Stage I (n = 180)	2.1 (1.13–3.9)†	2.38 (1.19–4.76)†
Stage II–IV (n = 115)	0.89 (0.49–1.6)	0.94 (0.50–1.77)

Definition of abbreviations: ADC = adenocarcinoma; CI = confidence interval; HR = hazard ratio.

*Adjusted by age, sex, smoking status, tumoral size, and treatment (adjuvant and neoadjuvant).

† $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.001$.

appropriate models. We cannot discard the possibility that other cellular functions modulated by EPCR (shown in our transcriptomic analysis) such as changes in cell adhesion might also participate in these early metastatic events in the target organ. Thus, EPCR could represent a "metastasis progression" gene, since it also endows cells with increase prometastatic activity in the target organ (30). The inhibition of metastasis by shRNA silencing was unlikely due to an off-target effect because the inhibition of EPCR with a monoclonal anti-EPCR antibody had similar effects reducing bone metastatic activity. The fact that A549 cells display a rapid, selective, and preferential tropism to form overt bone metastasis prevented the influence of EPCR in other target organs of metastasis in this model. Moreover, overexpression of EPCR in H727 led to high metastatic activity to adrenal glands. Thus, these results highly suggest that EPCR confers advantageous functions independent of the target organ. These experimental findings could explain the relevance of this protein in disease progression of patients with lung cancer.

It is also possible that APC/EPCR might be important in metastasis to target organs other than bones and adrenal glands, although this statement needs to be addressed in appropriate models. Moreover, the replication of these findings in other tumor cell lines (such as neuroblastoma) further implies that the axis APC/EPCR could be significant in other tumors.

Mechanism Mediated by EPCR in ADC

Our findings are consistent with the previously characterized role of APC in endothelial cells conferring an antiapoptotic and cytoprotective response in these cells (19). But our findings are also consistent with other cellular properties induced by the APC/EPCR axis, which have been found critical in metastasis and have not been dissected in this study, including heterotypic cell–cell adhesion (8) or cell–matrix interactions (31). These functions were partially affected upon APC/EPCR binding in our microarray analysis (data not shown). The fact that the antibody prevents the binding of EPCR with APC suggests that these effects are mediated by ligand–receptor binding. Circulating protein C could be activated in the local microenvironment of the target organ. In this regard in our model system, murine APC could bind human EPCR in tumor cells (data not shown), initiating the signaling response.

In apparent contrast to our findings, in a model of melanoma, APC decreased lung metastasis by the ability to prevent tumor transmigration through the endothelial cells, by the EPCR/PAR1/S1P1 axis (32, 33). This difference is explained by the fact that the melanoma cell line did not expressed EPCR, and therefore APC effects were exclusively mediated by the EPCR expressed in endothelial cells. In our model, by contrast, tumor cells did express EPCR on their surface.

In summary, the present study revealed an unexpected role of APC/EPCR signaling pathway in ADC eliciting enhanced cell survival. This functional advantage associates with increased tumor cell dissemination in early stages of lung ADC and endows cells with enhanced metastatic activity (Figure 5D). These findings underscore a novel role of EPCR in lung ADC as an independent indicator of bad prognosis.

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