Tumor and Stem Cell Biology

ESE3/EHF Controls Epithelial Cell Differentiation and Its Loss Leads to Prostate Tumors with Mesenchymal and Stem-like Features

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Abstract
Cancer stem cells (CSC) play a significant role in tumor progression, disease recurrence, and treatment failure. Here, we show that the endogenously expressed ETS transcription factor ESE3/EHF controls prostate epithelial cell differentiation and stem-like potential. We found that loss of ESE3/EHF induced epithelial-to-mesenchymal transition (EMT), stem-like features, and tumor-initiating and metastatic properties in prostate epithelial cells, and reexpression of ESE3/EHF inhibited the stem-like properties and tumorigenic potential of prostate cancer cells. Mechanistically, ESE3/EHF repressed the expression of key EMT and CSC genes, including TWIST1, ZEB2, BMI1, and POU5F1. Analysis of human tissue microarrays showed that reduced ESE3/EHF expression is an early event in tumorigenesis, frequently occurring independently of other ETS gene alterations. Additional analyses linked loss of ESE3/EHF expression to a distinct group of prostate tumors with distinctive molecular and biologic characteristics, including increased expression of EMT and CSC genes. Low ESE3/EHF expression was also associated with increased biochemical recurrence of prostate cancer and reduced overall survival after prostatectomy. Collectively, our findings define a key role for ESE3/EHF in the development of a subset of prostate tumors and highlight the clinical importance of identifying molecularly defined tumor subgroups. Cancer Res; 72(11); 1–12. ©2012 AACR.

Introduction
Prostate cancer is the most common cancer and a leading cause of cancer death in men in developed countries (1). Prostate cancer has a very heterogeneous clinical behavior ranging from indolent to aggressive forms that rapidly progress to metastatic and androgen-independent state (2). The factors that determine this heterogeneous clinical evolution are largely unknown (2, 3). Cancer cells with stem cell–like properties (CSC) within the primary tumors are considered major elements in tumor initiation and progression and a possible source of tumor heterogeneity (4–7). CSCs can derive from transformation of tissue/adult stem cells or from more differentiated cells that acquire stem-like properties (6, 7). Recent studies indicate that in the prostate epithelium cells, both in the basal and luminal cell compartments, are susceptible to malignant transformation and acquire tumor-initiating properties (8–11). However, our knowledge of the nature and factors governing the behavior of CSCs in prostate tumors is still limited (12, 13). Understanding the pathways controlling CSCs and defining the relationship between progenitor cells and the molecular characteristics of the ensuing tumors could be an important step toward development of more effective treatment strategies.

In this study, we investigated the role of the epithelial-specific ETS factor ESE3/EHF in these processes. ETS transcription factors are important elements in differentiation and developmental programs in many tissues and their expression is tightly regulated according to specific tissue- and time-dependent patterns (14, 15). Gene rearrangements leading to ectopic expression of ETS genes, such as ERG, are found in about 50% of prostate tumors (16–19). We reported recently that downregulation of ESE3/EHF is an additional relevant event in prostate tumorigenesis (20, 21). Here, we show that ESE3/EHF controls the balance between differentiation and self-renewal of prostate epithelial cells (PrEC) and that ESE3/EHF downregulation confers stem-like, tumor-initiating, and metastatic properties to the cells. Our data indicate that downregulation of ESE3/EHF characterizes a subset of prostate tumors with unique molecular, biologic, and clinical characteristics of aggressive disease. Overall, this study points...
Figure A: Bar graph showing cell viability (% of T0) over time (0-120 h). The graph compares PrECs, ESE3, and Ras-PrECs.

Figure B: Bar graph showing colony number over time (0-120 h). The graph compares PrECs, ESE3, and Ras-PrECs.

Figure C: Bar graph showing fold change in mRNA levels of PrECs, ESE3, and Ras-PrECs.

Figure D: Bar graph showing fold change in mRNA levels of PrECs, ESE3, and Ras-PrECs.

Figure E: Images showing PrECs and ESE3-PrECs under different conditions.

Figure F: Images showing PrECs and ESE3-PrECs with markers for E-cadherin, Vimentin, Twist1, and Tubulin.

Figure G: Images showing PrECs and ESE3-PrECs with markers for E-cadherin, Vimentin, Twist1, and Tubulin.

Figure H: Images showing PrECs and ESE3-PrECs with markers for E-cadherin, Vimentin, Twist1, and Tubulin.

Figure I: Graph showing % cell viability over time (1-3 days). The graph compares PrECs and ESE3-PrECs.

Figure J: Images showing PrECs and ESE3-PrECs under different conditions.

Figure K: Images showing PrECs and ESE3-PrECs with markers for H&E, No Ab, SV40LT, and Twist1.
to an important tumor suppressor role of ESE3/EHF in prostate tumorigenesis and to the possibility to identify prostate tumor subtypes with different phenotypes.

Materials and Methods
Detailed methods are included in Supplementary Information

Patient samples
Tissue microarrays (TMA) containing samples from 2 cohorts of patients with organ-confined prostate cancer treated with radical prostatectomy were included in the study. Histopathologic and clinical characteristics are shown in Supplementary Fig. S1. Tissue samples were collected with the approval of the Ethics Committees of the IRCCS Multimedica of the Regione Lombardia, Italy, and of the Inselpit (Bern, Switzerland) with patient’s written informed consent.

Cell lines, selection of cell clones, and growth assays
Immortalized human PrECs and RPWE-1 stably expressing ESE3-directed short hairpin RNAs (shRNA) and ESE3-expressing DU145 cells were established as previously described (21). For transient gene knockdown, cells were transfected with siRNAs directed to ESE3 [exon2 and 3' untranslated region (UTR)] or control siRNA using INTERFERin (20). Cell proliferation, clonogenic, soft agar, anoikis, and wound-healing assays were conducted as previously described (21–23). For in vitro sphere-forming assays, single-cell suspensions were plated in ultra-low attachment dishes (Corning Life Sciences) in serum-free mammary epithelial basal medium (MEBM, Cambrex; ref. 24). Each experiment was carried out in triplicate and repeated at least 3 times.

Tumor xenografts
Mice were purchased from the Harlan Laboratories. All protocols involving animals were conducted in conformity with the institutional guidelines for experiments on animals and in compliance with national and international policies. Study protocol was approved by the Swiss Veterinary Authority (No.5/2011).

Quantitative reverse transcriptase PCR
Total RNA was extracted and quantitative real-time reverse transcriptase PCR (qRT-PCR) was carried out using custom primers (see Supplementary Table S1 for primer sequences) and SYBR Green chemistry as previously described (21). For analysis of prostate tumors, quantitative RT-PCR (qRT-PCR) was carried out using Power SYBR Green One-step RNA-to-Ct system (Invitrogen).

Gene expression profiling
RNA from cell lines was amplified, labeled, and hybridized according to the two-color microarray-based gene expression analysis protocol (Agilent Technologies). Slides were scanned with the dual-laser scanner Agilent G2505B and analyzed as described (21). Differentially expressed genes were obtained by selecting probes with absolute log fold change > 0.58 and adjusted P value < 0.01. Data are Minimum Information about a Microarray Gene Experiment (MIAME)-compliant and have been deposited in the Gene Expression Omnibus (GEO) accession number GSE23197. Experimental procedure and analysis of the Kunderfranco microarray data set from tumor samples (GEO accession number GSE14206) have been previously described (21). Details for the inclusion of tumors in specific subgroups according to ETS expression patterns have been described (21).

Results
ESE3/EHF downregulation promotes cell transformation and epithelial-to-mesenchymal transition
To establish a link between ESE3/EHF and cell transformation, we knocked down ESE3/EHF in immortalized human PrECs using shRNAs (Fig. 1A). Immortalized PrECs are non-transformed, androgen receptor-negative, and express basal cell markers (25). While shControl PrECs did not grow in soft agar, stable ESE3KD-PrECs formed numerous colonies, exhibiting properties of transformed cells (Fig. 1B). Interestingly, colony-forming efficiency of ESE3KD-PrECs was similar to that of PrEC-expressing oncogenic H-Ras (Fig. 1B). Notably, ESE3/EHF is silenced in H-Ras-PrECs (20). We assessed the effects of ESE3/EHF knockdown also in immortalized prostate epithelial RWPE-1 cells. These cells are defined as intermediate or transit-amplifying PrECs and express both luminal and basal-type cytokeratins (26). ESE3KD-RWPE-1 cells established with 3 different shRNAs (Fig. 1C) formed numerous colonies in soft agar, whereas shControl cells were unable to do so (Fig. 1D). Thus, ESE3/EHF knockdown promoted cell transformation in both cell lines. In addition, ESE3KD-PrECs showed elongated fibroblast-like appearance and formed scattered colonies with reduced intercellular contacts (Fig. 1E), which are typical features of cells undergoing epithelial-to-mesenchymal transition (EMT). These morphologic changes were
associated with altered expression of E-cadherin, vimentin, and Twist1 along with marked cytoskeleton reorganization (Fig. 1F and G). Consistent with the induction of EMT, ESE3kd-PrECs exhibited increased motility (Fig. 1H) and resistance to anoikis (Fig. 1I). ESE3kd-RWPE-1 cells showed similar phenotypic changes toward EMT in terms of morphology (Supplementary Fig. S2A) and cell motility (Supplementary Fig. S2B). Furthermore, to rule out shRNA-induced off-target effects, we knocked down ESE3/EHF transiently with 2 distinct siRNAs. Transient knockdown of ESE3/EHF resulted in changes in morphology and gene expression consistent with induction of EMT in PrECs and RWPE-1 cells (Supplementary Fig. S3A and S3B).

Next, we assessed the effects of ESE3/EHF knockdown on in vivo tumorigenicity and metastatic capability of immortalized PrECs. Stable ESE3kd-PrECs and shControl PrECs were implanted subcutaneously in severe combined immunodeficient (SCID) mice. After 25 days, ESE3kd-PrECs generated large tumors containing dense masses of SV40 large T antigen-positive cells, whereas only small nodules were seen with shControl cells (Fig. 1J). To determine in vivo metastatic capability, ESE3kd-PrECs and shControl cells were injected in the tail vein of SCID mice. After 8 weeks, cell infiltrates were detected in the lung of mice injected with ESE3kd-PrECs and not with shControl PrECs (Supplementary Fig. S4). At 12 weeks, metastatic lesions resembling adenocarcinomas and smaller peribronchiolar lesions were present in mice injected with ESE3kd-PrECs and not in control mice (Fig. 1K). The metastatic lesions were positive for SV40 large T antigen and Twist1 confirming that they were derived from colonization and expansion of ESE3kd-PrECs (Fig. 1K).

Loss of ESE3/EHF function confers stem-like and tumor-initiating properties

EMT is a transdifferentiation program responsible for the acquisition of stemness and tumor-initiating capability by nontransformed cells (27, 28). Therefore, we investigated whether ESE3/EHF knockdown affected stem-like potential of PrECs by testing their ability to propagate in vitro as prostatospheres and form tumors in vivo in immunodeficient mice. The in vitro sphere-forming assay has been widely used with primary cells and cancer cell lines to enrich for stem-like cells in heterogeneous cell populations and assess their self-renewal potential (24, 27, 29–31). shControl PrECs had low sphere-forming efficiency (SFE), whereas ESE3kd-PrECs formed more numerous (≥4-fold increase; $P < 0.01$) and larger (75% vs. 15% with diameter ≥100 μm) prostatospheres (Fig. 2A). SFE of ESE3kd-PrECs was higher than PrECs also in limiting dilution assays under conditions in which each prostatosphere derived from individual stem/progenitor cells ($P < 0.01$; Supplementary Fig. S5A). The clonal origin of prostatospheres was further shown by differential staining with fluorescent dyes. All the prostatospheres contained cells stained with a single dye, showing that each prostatosphere derived from a single prostatosphere-forming cell (Supplementary Fig. S5B). In addition to the number of prostatosphere-forming cells, ESE3/EHF knockdown increased their self-renewal capability. Primary prostatosphere-forming cells from ESE3kd-PrECs were propagated for up to 4 generations, whereas primary prostatospheres from shControl PrECs did not grow past the second generation (Fig. 2B). ESE3/EHF knockdown in RPWE-1 cells led to a similar increase in SFE and self-renewal capability compared with shControl cells (Fig. 2C and D). To rule out nonspecific effects in shRNA-knockdown cells, we conducted transient ESE3/EHF knockdown using siRNAs. Interestingly, transient ESE3/EHF knockdown by 2 different siRNAs in PrECs and RWPE-1 cells increased SFE (Supplementary Fig. S6A–S6D). Furthermore, co-transfection of the ESE3/EHF construct lacking 3’UTR rescued the effect of the 3’UTR siRNA on SFE, linking its action specifically to ESE3/EHF knockdown (Supplementary Fig. S6E and S6F). Next, we determined whether these in vitro stem-like properties correlated with acquisition of in vivo tumor-initiating capability. To this end, primary prostatospheres were dissociated into single-cell suspensions and implanted subcutaneously in SCID mice (Fig. 2E). Prostatosphere-forming cells derived from ESE3kd-PrECs and ESE3kd-RPWE-1 cells generated tumors that grew rapidly forming large masses, whereas shControl cells did not produce any tumor (Fig. 2F). Furthermore, the tumors formed by ESE3kd-PrECs and ESE3kd-RPWE-1 cells were highly undifferentiated with a high mitotic rate, moderate to severe nuclear atypia, and the presence of the macronucleoli and nuclear pleomorphism (Fig. 2G).

We expanded our analysis to assess the relationship between stem-like potential, tumorigenicity, and ESE3/EHF in human prostate cancer cells. We showed previously that ESE3/EHF expression was very low in PC3 and DU145 cells, intermediate in VCaP and 22Rv1, and high in LNCaP cells (20, 21). We found that SFE was higher in DU145 and PC3 cells, intermediate in 22Rv1 and VCaP cells, and lower in LNCaP cells (Fig. 3A), suggesting an inverse correlation with ESE3/EHF expression. To link directly stem-like potential to ESE3/EHF, we reexpressed the gene in DU145 cells (Fig. 3B). DU145 cells exhibited high SFE and self-renewal capability and prostatosphere cells derived form DU145 cells had high expression of various stem cell marker genes (Supplementary Fig. S7A and S7B). Reexpression of ESE3/EHF in DU145 cells reduced SFE significantly (Fig. 3C). ESE3/EHF-expressing DU145 cells exhibited also reduced clonogenic capability (Fig. 3D), migration (Fig. 3E), and proliferation (Fig. 3F) along with a senescent phenotype (Fig. 3G). Moreover, ESE3/EHF-expressing cells, unlike control DU145 cells, had reduced ability to form tumors when injected subcutaneously in nude mice (Fig. 3E). Thus, both in vitro and in vivo data indicate that ESE3/EHF has a relevant impact on stem-like and tumor-initiating capability of normal and transformed PrECs.

ESE3/EHF controls transcription of mesenchymal and CSC genes

To understand the mechanism by which ESE3/EHF controls PrECs differentiation and self-renewal, we conducted gene expression profiling (GEP) analysis in PrECs and ESE3kd-PrECs. ESE3/EHF knockdown induced broad transcriptional changes with 811 upregulated and 1,083 downregulated genes (adjusted $P < 0.01$) in ESE3kd-PrECs (Supplementary Table S2). Functional annotation analysis showed that EMT was the most...
predominant pathway deregulated in ESE3kd-PrECs, along with cell adhesion, cytoskeleton remodeling, and immune response (Fig. 4A). Consistently, among the most affected genes we found transcriptional regulators, such as TWIST1, ZEB2, NANOG, POU5F1, and epigenetic effectors, such as EZH2 and BMI1, which are known to be involved in EMT and cell stemness (27, 32–35). However, none of them, with the exception of EZH2 (21), had been previously linked to ESE3/EHF. The qRT-PCR confirmed the changes of expression of these and other genes linked to EMT (i.e., GNG11, VIM, CDH1, FOXA1, and TSPAN3) and cell stemness (i.e., COL1A1 and FBN1) in ESE3kd-PrECs (Fig. 4B). Conversely, many genes involved in EMT and cell stemness were repressed upon ESE3/EHF expression in DU145 prostate cancer cells (Fig. 4C). To relate these effects to ESE3/EHF, we searched for ETS-binding sites (EBS) in the promoters of the upregulated genes and conducted chromatin immunoprecipitation. ESE3/EHF was bound to the promoters of TWIST1, ZEB2, BMI1, POU5F1, and COL1A1 in

Figure 2. Loss of ESE3/EHF confers stem-like and tumor-initiating properties. A, in vitro SFE of PrECs and ESE3kd-PrECs determined after 7 to 10 days. Bottom, representative images of prostatospheres. B, prostatosphere self-renewal potential. Prostatospheres formed by PrECs and ESE3kd-PrECs were counted and replated in sphere-forming conditions. C, in vitro sphere-forming assay with control (sh-) and ESE3kd-RWPE-1 cells (sh-1). Bottom, representative images of prostatospheres. D, self-renewal potential of RWPE-1 and ESE3kd-RWPE-1 prostatospheres. E, experimental design to assess tumor-initiating potential of prostatosphere (PS)-forming cells. F, growth of subcutaneous tumor xenografts from disaggregated prostatospheres of ESE3kd-PrECs, ESE3kd-RWPE-1 (sh-1), and control cells in SCID mice. Left, average tumor size after cell implantation. Right, average tumor weight. G, representative images of tumor xenografts formed by ESE3kd-PrECs and ESE3kd-RWPE-1 stained with H&E. P values were determined using t test. **, P < 0.01. CTRL, control.
correspondence of the predicted EBS in PrECs (Fig. 4D–E and Supplementary Table S3). To further understand the consequences of ESE3/EHF occupancy, we evaluated activating (histone H3 acetylation, H3Ac) and repressive (histone H3 K9 methylation, H3K9me) chromatin marks on the promoter of these target genes. Chromatin immunoprecipitation showed low H3Ac and high H3K9me on the gene promoters, indicating that binding of ESE3/EHF was associated with transcriptional repression (Fig. 4F). Collectively, these data indicate that ESE3/EHF directly controls a network of genes involved in EMT and cell stemness maintaining them in a repressive state in PrECs.

ESE3/EHF loss is an early and ERG-independent event in prostate tumors

To further define the relevance of the above findings at the clinical level, we assessed ESE3/EHF protein expression by immunohistochemistry in TMA containing samples of normal prostate, high-grade prostate intraepithelial neoplasia (HGPIN), and organ-confined prostate tumors. Clinical parameters and patient characteristics are reported in Supplementary Fig. S1. ESE3/EHF was detected in normal prostate with staining prevalently nuclear and more abundant in basal than in luminal cells (Fig. 5A and B). Fifteen percent of tumors had normal levels of ESE3/EHF, whereas approximately 35% and 50% had, respectively, weak and negative ESE3/EHF staining. Forty percent of HGPINs were positive for ESE3/EHF, whereas 45% and 15% had weak or negative staining. Thus, a large fraction of HGPINs (1/60%) and tumors (1/24) had reduced levels of ESE3/EHF. For 18 patients, we had available both HGPINs and tumors (Fig. 5C). In about half of these cases, ESE3/EHF was reduced or absent in both HGPINs and tumors. In the remaining cases, normal levels of ESE3/EHF in HGPINs were associated with either normal or reduced expression in tumors. Thus, ESE3/EHF loss of expression could occur both at the level of HGPIN and during progression from HGPIN to cancer.

We assessed also expression of ERG in the same TMA. ERG was not expressed in any of the normal prostate samples (Fig. 5A). About 15% of HGPINs (3 of 20) expressed ERG, consistent with previous reports (36, 37). Thirty-eight percent of tumors...
Figure 4. ESE3/EHF controls expression of EMT and CSC genes. A, GeneGo Pathway Maps significantly enriched (based on $P$ value) among deregulated genes in ESE3$^{kd}$-PrECs. B, interleukin. B, expression of selected EMT and CSC genes in PrECs and ESE3$^{kd}$-PrECs determined by qRT-PCR. C, expression of selected EMT and CSC genes in control (pcDNA) and ESE3-expressing (pESE3) DU145 cells determined by qRT-PCR. VIM, vimentin. D, diagrams of the 5’ proximal promoter with mapping of novel identified EBS in the indicated gene promoters. TSS, transcription start site. E, binding of ESE3/EHF to the promoters of the indicated genes in PrECs determined by chromatin immunoprecipitation. Right, densitometric quantification of the PCR bands. F, histone H3 acetylation (AcH3) and lysine 9 methylation (H3K9me) on the promoters of the indicated genes in PrECs and densitometric quantification of the PCR bands (right). **, $P < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
were ERG-positive and had either normal or reduced/absent expression of ESE3/EHF, confirming that deregulation of these 2 ETS factors could coexist in a subset of tumors (Fig. 5A). On the other hand, about one third (35%) of tumors were ERG-negative and did not express ESE3/EHF. These tumors would correspond to the subgroup of ERG fusion–negative tumors with low ESE3/EHF expression (ESE3low tumors), which we had identified previously by GEP (21), confirming that a subset of prostate tumors have exclusive loss of ESE3/EHF in absence of ETS gene rearrangements.

ESE3low tumors are enriched of mesenchymal and CSC genes

To determine whether tumors with exclusive loss of ESE3/EHF (ESE3low tumors) had unique molecular features, we analyzed GEP data from 3 independent studies (21, 38, 39). ESE3low tumors were identified on the basis of the ETS gene expression patterns and represented about 25% of all tumors in each of 3 data sets (Fig. 6A). We extracted ESE3low-specific gene signatures from each data set applying differential gene expression analysis. The ESE3low signatures were very robust and contained many genes significantly up- and downregulated in ESE3low tumors compared with all other tumors [false discovery rate (FDR) < 0.05; Supplementary Tables S4–S6]. Functional annotation analysis revealed that EMT was one of the most enriched pathways along with cytoskeleton remodeling, cell adhesion, and chemotaxis in all 3 data sets (Fig. 6B). Thus, the most affected pathways in ESE3low tumors were very similar to those affected in ESE3kd-PrECs.

To further probe the similarity between ESE3low tumors and ESE3/EHF-knockdown cells, we conducted gene set enrichment analysis (GSEA) using the list of genes deregulated in ESE3kd-PrECs (Supplementary Table S2). Notably, GSEA showed significant enrichment of these genes in ESE3low tumors compared with all other tumors in the 3 data sets (Fig. 7A), confirming that ESE3low tumors had unique features closely resembling those found in ESE3kd-PrECs. Next, to explore the specific molecular features of ESE3low tumors and their relation with ESE3kd cells, we used previously published core EMT and core CSC gene sets to conduct GSEAs. The core EMT gene set included genes regulated by EMT-inducing signals, such as Snail, Twist, TGF-β1 and E-cadherin, in human mammary epithelial cells (40). The core CSC gene set included genes deregulated in stem cell–enriched populations derived from immortalized mammary epithelial cells, breast cancer cell lines, and breast tumors (30). ESE3low tumors from all 3 data sets showed significant enrichment of EMT and CSC genes compared...
with all other tumors (Fig. 7A). Furthermore, applying unsupervised hierarchical clustering, we found that tumors clustered in 2 main groups with ESE3low tumors prevalently in the group with high expression of core CSC genes (Fig. 7B). These findings were confirmed at the single gene level by looking at CDH1, whose reduced expression is related to both EMT and cell stemness (27, 30) and was among the genes modulated by ESE3/EHF in different cell models. Consistent with the overall deregulation of EMT and CSC genes, expression of CDH1 was significantly reduced in ESE3low tumors compared with all other tumors in all microarray data sets (Fig. 7C) and when measured by qRT-PCR in available tumor samples (Fig. 7D). Collectively, these findings indicate that human prostate tumors exhibit distinctive patterns of expression of EMT and stemness genes and that ESE3low tumors show preferential deregulation of genes involved in these processes compared with the other tumors. In addition, the close similarity of the transcriptional profiles of ESE3low tumors and ESE3kd-PrECs suggests a direct and causal role of loss of ESE3/EHF expression in driving the transcriptional and biologic features observed in these tumors.

The molecular features that we found associated with ESE3low tumors (e.g., enrichment of EMT and CSC) suggested that loss of ESE3/EHF expression could have a negative impact on the disease, increasing the risk of progression and disease recurrence. To address this point, we evaluated ESE3/EHF level by immunohistochemistry in a cohort of patients with prostate cancer treated with radical prostatectomy and with long-term clinical follow-up (Supplementary Fig. S1, bottom). When patients were divided according to ESE3/EHF level, we found a statistically significant association of low ESE3/EHF expression with increased biochemical recurrence and reduced overall survival [log-rank (Mantel–Cox) \( P = 0.02 \) and 0.03, respectively; Fig. 7E; Supplementary Fig. S8]. Gleason score distribution was not significantly different between ESE3-expressing and -nonexpressing tumors (Supplementary Fig. S8).

**Discussion**

The molecular pathways that control PrEC differentiation and self-renewal and their involvement in prostate tumorigenesis are poorly understood. In this study, we show that ESE3/EHF has a relevant role in these processes. Upon ESE3/EHF knockdown, immortalized human PrECs acquired transformed, mesenchymal, and stem-like properties along with in vivo tumor-initiating and metastatic capability. Conversely, reexpression of ESE3/EHF reversed the transformed phenotype and reduced migration, stem-like, and tumorspecfic potential of prostate cancer cells. Our study also provides mechanistic insights on how ESE3/EHF controls these processes. GEP studies in cell lines and tumor samples indicate that loss of ESE3/EHF leads to a complex reprogramming of the cell transcriptome and that ESE3/EHF acts as key node controlling expression of a large network of genes. Specifically, ESE3/EHF keeps under control many genes that would drive cells to dedifferentiation and transformation. Key regulators of EMT and cell stemness, such as *TWIST1*, ZEB2, *POUSF1/OCT4*, *NANOG*, *BMI1*, and *EZH2*, were directly repressed by ESE3/EHF and upregulated upon ESE3/EHF knockdown in PrECs. These results are consistent with the known function of ETS transcription factors as regulators of tissue-specific developmental and differentiation programs (14, 15) and with the ability of ESE3/EHF to activate and repress genes depending on the promoter context (14, 15). Furthermore, these data also suggest a mechanism by which ectopically expressed ETS, such as ERG, can disrupt cell differentiation and induce cell transformation by functionally interfering with the normal transcriptional program established by the endogenous ESE3/EHF in PrECs.
Taken together, these findings indicate that loss of ESE3/EHF could drive prostate tumorigenesis. Analysis of multiple prostate cancer series by immunohistochemistry and microarrays shows that loss of ESE3/EHF expression is an early and frequent event, occurring independently of ERG deregulation. Moreover, loss of ESE3/EHF expression in the absence of ETS gene rearrangements defines a tumor subgroup with distinctive molecular and biologic features. We found a striking
similarity between the transcriptional program of ESE3/EHF-knockdown cells and ESE3low tumors. PrECs in which ESE3/EHF was downregulated showed preferential deregulation of EMT and CSC genes. Consistently, we found a significant enrichment of EMT and CSC genes in ESE3low tumors compared with the bulk of prostate tumors in multiple microarray data sets. Furthermore, unsupervised hierarchical clustering showed that ESE3low prostate tumors could cluster together on the basis of CSC markers. This strong connection between ESE3low tumors across multiple platforms and ESE3/EHF-knockdown cells further indicates that these tumors share a common origin and may derive from loss of ESE3/EHF expression in PrECs. Furthermore, both EMT and CSC features have been associated with aggressive disease and poor prognosis in many cancers (27, 28, 41), suggesting that ESE3low tumors might have a more aggressive clinical behavior. In support of this hypothesis, we found that tumors with low ESE3/EHF expression had higher recurrence and reduced overall survival than ESE3/EHF expressing tumors in surgically treated patients. EMT and stem cell features have been also associated to castration resistance in prostate cancer (42). Thus, the subset of primary tumors characterized by low ESE3 expression might be intrinsically less sensitive to androgen-deprivation therapy and more prone to progression to a castration-resistant state. If this hypothesis is confirmed, these findings would suggest the need for changes in management and treatment strategies for this subset of low ESE3-expressing prostate tumors.

Collectively, our data point to a key role of ESE3/EHF in the pathogenesis of a subset of prostate tumors. These findings have also broader implications as they indicate that distinct tumor subtypes could be defined on the basis of the expression patterns of specific molecular markers (e.g., ETS, EMT, CSC genes) and could reflect intrinsic differences in tumor biology, clinical behavior, and therapeutic response. This is consistent with the data generated here from the in vitro and in vivo studies and the specific molecular and biologic characteristics associated with the ESE3low tumor subgroup. However, more studies are clearly required to fully address these issues and establish the relevance of the identified molecular markers and tumor subgrouping at the clinical level.

Disclosure of Potential Conflict of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Made some experiments, including immunohistochemical experiments and evaluated immunohistochemical slides: M. Sarti

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