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

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Running title: ESE3/EHF controls prostate cell fate

Keywords: ETS transcription factor, ESE3/EHF, epithelial to mesenchymal transition, EMT, cancer stem cells, basal cells, luminal cells, prostate cancer

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Abstract

Cancer stem cells (CSCs) play a significant role in tumour 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 tumour-initiating and metastatic properties in prostate epithelial cells, and re-expression 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 tumours with distinctive molecular and biological 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 tumour subgroups.
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 behaviour 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 (CSCs) within the primary tumours are considered major elements in tumour initiation and progression and a possible source of tumour heterogeneity (4-7). Cancer stem cells 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 compartment are susceptible to malignant transformation and acquire tumour initiating properties (8-11). However, our knowledge of the nature and factors governing the behaviour of CSCs in prostate tumours is still limited (12, 13). Understanding the pathways controlling CSCs and defining the relationship between progenitor cells and the molecular characteristics of the ensuing tumours 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, like ERG, are found in about 50% of prostate tumours (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 and that ESE3/EHF downregulation confers stem-like, tumour initiating and metastatic properties to the cells. Our data indicate that downregulation of ESE3/EHF characterize a subset of prostate tumours with unique molecular, biological and clinical characteristics of aggressive disease. Overall, this study points to an important tumour suppressor role of ESE3/EHF in prostate tumorigenesis and to the possibility to identify prostate tumour subtypes with different phenotypes.
Materials and Methods

Detailed methods are included in Supplementary Information

Patient samples

Tissue microarrays containing samples from two cohorts of patients with organ-confined prostate cancer treated with radical prostatectomy were included in the study. Histopathological and clinical are shown in Supplementary Figure 1. Tissue samples were collected with the approval of the Ethics Committees of the IRCCS Multimedica of the Regione Lombardia, Italy, and of the Insespital (Bern, CH) with patient’s written informed consent.

Cell lines, selection of cell clones and growth assays

Immortalized human prostate epithelial cells (PrECs) and RPWE-1 stably expressing ESE3-directed shRNAs 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’UTR) or control siRNA using Interferin (20). Cell proliferation, clonogenic, soft-agar, anoikis and wound-healing assays were performed as previously described (21-23). For in vitro sphere-forming single cell suspensions were plated in ultra-low attachment dishes (Corning Life Sciences) in serum-free Mammary Epithelial Basal Medium (MEBM, Cambrex) (24). Each experiment was carried out in triplicate and repeated at least three times.

Tumour 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 RT-PCR

Total RNA was extracted and quantitative real-time RT-PCR was performed using custom primers (see Supplementary Table 1 for primer sequences) and SYBR Green chemistry as previously described (21). For analysis of prostate tumours qRT–PCR was performed 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 FC > 0.58 and adjusted P value <0.01. Data are MIAME compliant and have been deposited in the Gene Expression Omnibus: GEO accession numbers GSE23197. Experimental procedure and analysis of the Kunderfranco microarray dataset from tumour samples (GEO accession number GSE14206) have been previously described (21). Details for the inclusion of tumours 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 short-hairpin RNAs (shRNA) (Figure 1A). Immortalized PrECs are non-transformed, AR 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 (Figure 1b). Interestingly, colony forming efficiency of ESE3kd-PrECs was similar to that of PrECs expressing oncogenic H-Ras (Figure 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 prostate epithelial cells and express both luminal and basal type cytokeratins (26). ESE3kd-RWPE-1 cells established with three different shRNAs (Figure 1C) formed numerous colonies in soft-agar, while shControl cells were unable to do so (Figure 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 inter-cellular contacts (Figure 1E), which are typical features of cells undergone epithelial-to-mesenchymal transition (EMT). These morphological changes were associated with altered expression of E-cadherin, vimentin and Twist1 along with marked cytoskeleton reorganization (Figure 1F-G). Consistent with the induction of EMT, ESE3kd-PrECs exhibited
increased motility (Figure 1H) and resistance to anoikis (Figure 1I). ESE3kd-RWPE-1 cells showed similar phenotypic changes toward EMT in terms of morphology (Supplementary Figure 2A) and cell motility (Supplementary Figure 2B). Furthermore, to rule out shRNA-induced off-target effects, we knocked-down ESE3/EHF transiently with two distinct small-interfering RNAs (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 Figure 3A-B).

Next, we assessed the effects of ESE3/EHF knockdown on \textit{in vivo} tumourigenicity and metastatic capability of immortalized prostate epithelial cells. Stable ESE3kd-PrECs and sh-control PrECs were implanted subcutaneously in SCID mice. After 25 days ESE3kd-PrECs generated large tumours containing dense masses of SV40 large T antigen positive cells, while only small nodules were seen with shControl cells (Figure 1J). To determine \textit{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 Figure 4). At 12 weeks metastatic lesions resembling adenocarcinomas and smaller peribronchiolar lesions were present in mice injected with ESE3kd-PrECs and not in control mice (Figure 1K). The metastatic lesions were positive for SV40 large T antigen and Twist1 confirming that they derived from colonization and expansion of ESE3kd-PrECs (Figure 1K).

\textbf{Loss of ESE3/EHF function confers stem-like and tumour-initiating properties}

EMT is a transdifferentiation program responsible for the acquisition of stemness and tumour-initiating capability by non-transformed cells (27, 28). Therefore, we investigated whether ESE3/EHF knockdown affected stem-like potential of prostate epithelial cells by testing their ability to propagate \textit{in vitro} as prostatospheres (PS) and form tumours \textit{in vivo} in immunodeficient mice. The \textit{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), while ESE3kd-PrECs formed more numerous (≥ 4-fold increase; P< 0.01) and larger (75% vs. 15% with diameter ≥100 μm) prostatospheres (PS) (Figure 2A). SFE of ESE3kd-PrECs was higher than PrECs also in limiting dilution assays under conditions in which each PS derived from individual stem/progenitor cells (P< 0.01; Supplementary Figure 5A). The clonal
origin of PS was further demonstrated by differential staining with fluorescent dyes. All the PS contained cells stained with a single dye, demonstrating that each PS derived from a single PS-forming cell (Supplementary Figure 5B). In addition to the number of PS-forming cells, ESE3/EHF knockdown increased their self-renewal capability. Primary PS-forming cells from ESE3<sup>kd</sup>-PrECs were propagated for up to 4 generations, while primary PS from shControl PrECs did not grow past the second generation (Figure 2B). ESE3/EHF knockdown in RPWE-1 cells led to a similar increase in SFE and self-renewal capability compared to shControl cells (Figure 2C-D). To rule out nonspecific effects in shRNA-knockdown cells, we performed transient ESE3/EHF knockdown using siRNAs. Interestingly, transient ESE3/EHF knockdown by two different siRNAs in PrECs and RWPE-1 cells increased SFE (Supplementary Figure 6A-D). 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 Figure 6E-F). Next, we determined whether these in vitro stem-like properties correlated with acquisition of in vivo tumour-initiating capability. To this end, primary PS were dissociated into single cell suspensions and implanted subcutaneously in SCID mice (Figure 2E). PS-forming cells derived from ESE3<sup>kd</sup>-PrECs and ESE3<sup>kd</sup>-RPWE-1 cells generated tumours that grew rapidly forming large masses, while shControl cells did not produce any tumour (Figure 2F). Furthermore, the tumours formed by ESE3<sup>kd</sup>-PrECs and ESE3<sup>kd</sup>-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.

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 higher 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 (Figure 3A), suggesting an inverse correlation with ESE3/EHF expression. To link directly stem-like potential to ESE3/EHF, we re-expressed the gene in DU145 cells (Figure 3B). DU145 cells exhibited high SFE and self-renewal capability and PS cells derived form DU145 cells had high expression of various stem cell marker genes (Supplementary Figure 7A-B). Re-expression of ESE3/EHF in DU145 cells reduced SFE significantly (Figure 3C). ESE3/EHF-expressing DU145 cells exhibited also reduced clonogenic capability (Figure 3D) migration (Figure 3E) and proliferation (Figure 3F) along with a senescent phenotype (Figure 3G). Moreover, ESE3/EHF-expressing cells, unlike
control DU145 cells, had reduced ability to form tumours when injected subcutaneously in nude mice (Figure 3E). Thus, both in vitro and in vivo data indicate that ESE3/EHF has a relevant impact on stem-like and tumour-initiating capability of normal and transformed prostate epithelial cells.

**ESE3/EHF controls transcription of mesenchymal and cancer stem cell genes**

To understand the mechanism by which ESE3/EHF controls prostate epithelial cell differentiation and self-renewal, we performed GEP analysis in PrECs and ESE3kd-PrECs. ESE3/EHF knockdown induced broad transcriptional changes with 811 up-regulated and 1083 down-regulated genes (adjusted p-value <0.01) in ESE3kd-PrECs (Supplementary Table 2). Functional annotation analysis showed that EMT was the most predominant pathway deregulated in ESE3kd-PrECs, along with cell adhesion, cytoskeleton remodelling and immune response (Figure 4A). Consistently, among the most affected genes we found transcriptional regulators, like *TWIST1*, *ZEB2*, *NANOG*, *POU5F1*, and epigenetic effectors, like *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. 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*, *FBN1*) in ESE3kd-PrECs (Figure 4B). Conversely, many genes involved in EMT and cell stemness were repressed upon ESE3/EHF expression in DU145 prostate cancer cells (Figure 4C). To relate these effects to ESE3/EHF, we searched for ETS binding sites (EBS) in the promoters of the upregulated genes and performed ChIP. ESE3/EHF was bound to the promoters of *TWIST1*, *ZEB2*, *BMI1*, *POU5F1* and *COL1A1* in correspondence of the predicted EBS in PrECs (Figure 4D and Supplementary Table 3). 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 (Figure 4E). 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 prostate epithelial cells.

**ESE3/EHF loss is an early and ERG-independent event in prostate tumours**
To further define the relevance of the above findings at the clinical level, we assessed ESE3/EHF protein expression by immunohistochemistry in tissue microarray (TMA) containing samples of normal prostate, high-grade prostate intraepithelial neoplasia (HGPIN) and organ-confined prostate tumours. Clinical parameters and patient characteristics are reported in Supplementary Figure 1. ESE3/EHF was detected in normal prostate with staining prevalently nuclear and more abundant in basal than luminal cells (Figure 5A-B). Fifteen percent of tumours had normal levels of ESE3/EHF, while approximately 35% and 50% had, respectively, weak and negative ESE3/EHF staining. Forty percent of HGPINs were positive for ESE3/EHF, while 45% and 15% had weak or negative staining. Thus, a large fraction of HGPINs (~60%) and tumours (~80%) had reduced levels of ESE3/EHF. For 18 patients we had available both HGPINs and tumours (Figure 5C). In about half of these cases ESE3/EHF was reduced or absent in both HGPINs and tumours. In the remaining cases, normal levels of ESE3/EHF in HGPINs were associated with either normal or reduced expression in tumours. 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 (Figure 5A). About 15% of HGPINs (3/20) expressed ERG, consistent with previous reports (36, 37). Thirty eight percent of tumours were ERG positive and had either normal or reduced/absent expression of ESE3/EHF, confirming that deregulation of these two ETS factors could coexist in a subset of tumours (Figure 5A). On the other hand, about one third (35%) of tumours were ERG negative and did not express ESE3/EHF. These tumours would correspond to the subgroup of ERG fusion negative tumours with low ESE3/EHF expression (ESE3\textsuperscript{low} tumours), which we had identified previously by gene expression profiling (GEP) (21), confirming that a subset of prostate tumours have exclusive loss of ESE3/EHF in absence of ETS gene rearrangements.

**ESE3\textsuperscript{low} tumours are enriched of mesenchymal and cancer stem cell genes**

To determine whether tumours with exclusive loss of ESE3/EHF (ESE3\textsuperscript{low} tumours) had unique molecular features, we analyzed GEP data from three independent studies (21, 38, 39). ESE3\textsuperscript{low} tumours were identified based on the ETS gene expression patterns and represented about 25% of all tumours in each of three datasets (Figure 6A). We extracted ESE3\textsuperscript{low} specific gene signatures from each dataset applying differential gene expression analysis. The ESE3\textsuperscript{low}
signatures were very robust and contained many genes significantly up- and down-regulated in ESE3\textsuperscript{low} tumours compared to all other tumours (FDR<0.05; Supplementary Tables 4-6). Functional annotation analysis revealed that EMT was one of the most enriched pathways along with cytoskeleton remodeling, cell adhesion and chemotaxis in all three datasets (Figure 6B). Thus, the most affected pathways in ESE3\textsuperscript{low} tumours were very similar to those affected in ESE3\textsuperscript{kd}-PrECs.

To further probe the similarity between ESE3\textsuperscript{low} tumours and ESE3/EHF-knockdown cells, we performed Gene Set Enrichment Analysis (GSEA) using the list of genes deregulated in ESE3\textsuperscript{kd}-PrECs (Supplementary Table 2). Notably, GSEA showed significant enrichment of these genes in ESE3\textsuperscript{low} tumours compared to all other tumours in the three datasets (Figure 7A) confirming that ESE3\textsuperscript{low} tumours had unique features closely resembling those found in ESE3\textsuperscript{kd} prostate epithelial cells. Next, to explore the specific molecular features of ESE3\textsuperscript{low} tumours and their relation with ESE3\textsuperscript{kd} cells, we used previously published “core EMT” and “core CSC” gene sets to perform GSEA. The “core EMT” gene set included genes regulated by EMT-inducing signals, like 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 tumours (30). ESE3\textsuperscript{low} tumours from all three datasets showed significant enrichment of EMT and CSC genes compared to all other tumours (Figure 7A). Furthermore, applying unsupervised hierarchical clustering we found that tumours clustered in two main groups with ESE3\textsuperscript{low} tumours prevalently in the group with high expression of “core CSC” genes (Figure 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 ESE3\textsuperscript{low} tumours compared to all other tumours in all microarray datasets (Figure 7C) and when measured by QRT-PCR in available tumour samples (Figure 7D).

Collectively, these findings indicate that human prostate tumours exhibit distinctive patterns of expression of EMT and stemness genes and that ESE3\textsuperscript{low} tumours show preferential deregulation of genes involved in these processes compared to the other tumours. In addition, the close similarity of the transcriptional profiles of ESE3\textsuperscript{low} tumours and ESE3\textsuperscript{kd} prostate epithelial cells, suggests a direct and causal role of loss of ESE3/EHF expression in driving the transcriptional and biological features observed in these tumours.
The molecular features that we found associated with ESE3\textsuperscript{low} tumours (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 IHC in a cohort of prostate cancer patients treated with radical prostatectomy and with long-term clinical follow-up (Supplementary Fig.1, bottom panel). 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-value: 0.02 and 0.03, respectively; (Figure 7E and Supplementary Figure 8). Gleason score distribution was not significantly different between ESE3 expressing and non-expressing tumours (Supplementary Figure 8).

Discussion

The molecular pathways that control prostate epithelial cell 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 prostate epithelial cells acquired transformed, mesenchymal and stem-like properties along with \textit{in vivo} tumour-initiating and metastatic capability. Conversely, re-expression of ESE3/EHF reversed the transformed phenotype and reduced migration, stem-like and tumorigenic potential of prostate cancer cells. Our study provides also mechanistic insights on how ESE3/EHF controls these processes. Gene expression profiling studies in cell lines and tumour samples indicates 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, like \textit{TWIST1}, \textit{ZEB2}, \textit{POU5F1/OCT4}, \textit{NANOG}, \textit{BMI1} and \textit{EZH2}, were directly repressed by ESE3/EHF and upregulated upon ESE3/EHF knockdown in prostate epithelial cells. 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 suggest also a mechanism by which ectopically expressed ETS, like ERG, can disrupt cell differentiation
and induce cell transformation by functionally interfering with the normal transcriptional program established by the endogenous ESE3/EHF in prostate epithelial cells.

Taken together, these findings indicate that loss of ESE3/EHF could drive prostate tumorigenesis. Analysis of multiple prostate cancer series by IHC 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 tumour subgroup with distinctive molecular and biological features. We found a striking similarity between the transcriptional program of ESE3/EHF-knockdown cells and ESE3^low^ tumours. Prostate epithelial cells 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 ESE3^low^ tumours compared to the bulk of prostate tumours in multiple microarray datasets. Furthermore, unsupervised hierarchical clustering showed that ESE3^low^ prostate tumours could cluster together based on CSC markers. This strong connection between ESE3^low^ tumours across multiple platforms and ESE3/EHF-knockdown cells, further indicate that these tumours share a common origin and may derive from loss of ESE3/EHF expression in prostate epithelial cells. Furthermore, both EMT and CSC features have been associated with aggressive disease and poor prognosis in many cancers (27, 28, 41) suggesting that ESE3^low^ tumours might have a more aggressive clinical behavior. In support of this hypothesis, we found that tumours with low ESE3/EHF expression had higher recurrence and reduced overall survival compared to ESE3/EHF expressing tumours in surgically treated patients. EMT and stem-cell features have been associated also to castration-resistance in prostate cancer (42). Thus, the subset of primary tumours 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 tumours.

Collectively, our data point to a key role of ESE3/EHF in the pathogenesis of a subset of prostate tumours. These findings have also broader implications as they indicate that distinct tumour 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 tumour biology, clinical behaviour and therapeutic response. This is consistent with the data generated here from the in vitro and in vivo studies and the specific molecular and biological characteristics associated
with the ESE$^{low}$ tumour subgroup. However, more studies are clearly required to fully address these issues and establish the relevance of the identified molecular markers and tumour sub-grouping at the clinical level.

**Disclosure of Potential Conflict of Interest**

No potential conflicts of interest were disclosed

**Acknowledgements**

The authors would like to thank Enrica Mira Cato and Erica Ortelli from the IRB/IOSI Mouse facility for their support with in vivo experiments.

**Grant Support**

This work was supported by grants from Oncosuisse (KFS-01913-08 and KFS-02573-02-2010), Swiss National Science Foundation (FNS-31003A-118113) and Ticino Foundation for Cancer Research to GMC. MMG and GC were supported by Compagnia di San Paolo, Torino, Italy.

**References**

FIGURE LEGENDS

Figure 1. Knockdown of ESE3/EHF in prostate epithelial cells leads to cell transformation and epithelial-to-mesenchymal transition. A. ESE3/EHF expression in stable ESE3<sup>kd</sup>-PrECs and control PrECs evaluated by QRT-PCR. B. Colony formation in soft-agar by control, ESE3<sup>kd</sup>-PrECs and H-Ras-transformed PrECs. C. ESE3/EHF expression in stable ESE3<sup>kd</sup>-RWPE-1 (sh-1, sh-2 and sh-4) and control (sh-) RWPE-1 cells evaluated by QRT-PCR. D. Colony formation in soft-agar by control and ESE3<sup>kd</sup>-RWPE-1 cells. E. Morphology changes in ESE3<sup>kd</sup>-PrECs evaluated by phase-contrast microscopy. F. E-cadherin (green), vimentin (red), β-actin (green), F-actin (green) and nuclei (blue) staining in PrECs and ESE3<sup>kd</sup>-PrECs. Top panels, 600X; lower panels, 1000X. G. Expression of ESE3, E-cadherin, vimentin and Twist-1 in PrECs and ESE3<sup>kd</sup>-PrECs evaluated by immunoblotting. H. Wound healing assay with ESE3<sup>kd</sup>-PrECs and control PrECs. Lower panel, percentage of wound width relative to time 0. I. Survival of PrECs and ESE3<sup>kd</sup>-PrECs cells in anoikis in polyhema-coated plates. J. Tumour-seeding ability of ESE3<sup>kd</sup>-PrECs and PrECs. Representative images of tissue sections stained for SV40 large T antigen and with H&E. K. Formation of lung metastasis upon tail vein injection of PrECs and ESE3<sup>kd</sup>-PrECs. Representative images of lung sections stained for SV40 large T antigen and Twist1 and H&E. P values were determined using t-test. *P<0.05; **, P<0.01.

Figure 2. Loss of ESE3/EHF confers stem-like and tumour-initiating properties. A. In vitro sphere-forming efficiency (SFE) of PrECs and ESE3<sup>kd</sup>-PrECs determined after 7-10 days. Lower panels, representative images of prostatospheres. B. Prostatosphere self-renewal potential. Prostatospheres formed by PrECs and ESE3<sup>kd</sup>-PrECs were counted and replated in sphere-forming conditions. C. In vitro sphere-forming assay with control (sh-) and ESE3<sup>kd</sup>-RWPE-1 cells (sh-1). Lower panels, representative images of prostatospheres. D. Self-renewal potential of
RWPE-1 and ESE3\textsuperscript{kd}-RWPE-1 prostatospheres. E. Experimental design to assess tumour-initiating potential of prostatosphere-forming cells. F. Growth of subcutaneous tumour xenografts from disaggregated prostatospheres of ESE3\textsuperscript{kd}-PrECs, ESE3\textsuperscript{kd}-RWPE-1 (sh-1) and control cells in SCID mice. \textit{Left panels}, average tumour size after cell implantation. \textit{Right panels}, average tumour weight. G. Representative images of tumour xenografts formed by ESE3\textsuperscript{kd}-PrECs and ESE3\textsuperscript{kd}-RWPE-1 stained with H&E. P values were determined using t-test. *P<0.05; **, P<0.01.

**Figure 3. Re-expression of ESE3/EHF in prostate cancer cells reverts transformed and stem-like phenotype.** A. Prostato-sphere forming efficiency of human prostate cancer cell lines. B. ESE3 expression in control (pcDNA) and stable ESE3-expressing (pESE3) DU145 cells determined by immunoblotting. C. In vitro sphere-forming assay. D. Colony formation in adherent conditions. E. Wound healing assay. \textit{Lower panel}, percentage of wound width. F. Cell proliferation. G. β-galactosidase staining in control and ESE3-expressing DU145 cells. H. Growth of subcutaneous tumour xenografts of control and ESE3-expressing DU145 cells in nude mice. \textit{Right panel}, representative images of mouse injected with control and ESE3-expressing DU145 cells on right and left flank, respectively. P values were determined using t-test. *P<0.05; **, P<0.01.

**Figure 4. ESE3/EHF controls expression of EMT and cancer stem cell genes.** A. Gene Go Pathway Maps significantly enriched (based on P value) among deregulated genes in ESE3\textsuperscript{kd}-PrECs. B. Expression of selected EMT and cancer stem cell genes in PrECs and ESE3\textsuperscript{kd}-PrECs determined by QRT-PCR. C. Expression of selected EMT and cancer stem cell genes in control (pcDNA) and ESE3-expressing (pESE3) DU145 cells determined by QRT-PCR. D. Diagrams of the 5’ proximal promoter with mapping of novel identified ETS binding sites in the indicated gene promoters. E. Binding of ESE3/EHF to the promoters of the indicated genes in PrECs.
determined by chromatin immunoprecipitation. **Right panel**, 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 panel). *P<0.05; **, P<0.01.

**Figure 5. Loss of ESE3/EHF expression is an early and ERG-independent event in prostate tumorigenesis.** A. Distribution of ESE3/EHF and ERG level by immunohistochemistry in normal prostate (N), high-grade PIN (HGPIN) and prostate cancer (PCA) tissue samples. B. Representative images of normal prostate and prostate tumours. C. ESE3/EHF and ERG expression in HGPIN and prostate tumours taken from the same patients.

**Figure 6. ESE3low tumours share common features.** A. Percentage of ESE3low tumours from three independent prostate cancer microarray datasets evaluated. B. Gene Go Pathway Maps most enriched (based on P value) among genes selectively deregulated (FDR≤0.05) in ESE3low tumours from the three prostate cancer microarray datasets.

**Figure 7. ESE3low tumours are enriched of EMT features and CSC gene features.** A. GSEA using genes upregulated in ESE3kd-PrECs (top panels), “core EMT gene signature” (middle panels) and “core cancer stem cell gene signature” (lower panels) comparing ESE3low tumours to all other tumours in three microarray datasets. B. Unsupervised hierarchical clustering of gene expression data from the indicated prostate cancer microarray datasets using the “core cancer stem cell gene signature”. Tumour subgroups are indicated as: ESE3low (blue), ERGhigh (red), ESE1high (green) and NoETS (yellow). C. E-cadherin (CDH1) expression in the ESE3low and other tumours in microarray datasets. D. QRT-PCR evaluation of E-cadherin (CDH1) expression
in ESE3low (n=11) and other (n=13) prostate tumours. E. Kaplan-Meyer analysis of biochemical recurrence-free survival and overall survival of prostate cancer patients subdivided according to ESE3/EHF expression status. Log rank test (Mantel-Cox): p=0.02 and 0.03, respectively.
Figure 1

A

Cost of change

PrECs  ESE3KD-PrECs  Ras-PrECs

B

Cost of change

PrECs  ESE3KD-PrECs  Ras-PrECs

C

Final change

RWPE-1  sh-  sh-1  sh-2  sh-4

D

Final change

RWPE-1  sh-  sh-1  sh-2  sh-4

E

PrECs  ESE3KD-PrECs

F

PrECs  ESE3KD-PrECs

G

PrECs  ESE3KD-PrECs

H

PrECs  ESE3KD-PrECs

I

% cell viability

PrECs  ESE3KD-PrECs

J

PrECs  ESE3KD-PrECs

K

H&E  noAb  SV40LT  TWIST1

PrECs  ESE3KD-PrECs
Figure 2

A

B

C

D

E

F

G

Primary PS

PS dissociation to single cell suspension

Subcutaneous injection in SCID mice

ESE3KD-PrECs

RWPE-1

ESE3KD-PrECs

RWPE-1

ESE3KD-PrECs

RWPE-1

ESE3KD-PrECs

RWPE-1

ESE3KD-PrECs

RWPE-1

400X
Figure 3

(A) PS forming efficiency (%)

(B) Western blot of DU145 pcDNA and pESE3

(C) SFE (%)

(D) Number of colonies

(E) DU145 pcDNA and pESE3

(F) Number of cells

(G) pcDNA and pESE3

(H) VOLUME (mm³)
Figure 6

A

B

Kunderfranco

Epithelial-to-mesenchymal transition (EMT)
Cytoskeleton remodeling
Immune response
Chemokines and adhesion
Chemotaxis

Wallace

Epithelial-to-mesenchymal transition (EMT)
Chemokines and adhesion
ECM remodeling
TGF-beta-dependent induction of EMT via SMADs
Cytoskeleton remodeling

Glinsky

Chemokines and adhesion
Cytoskeleton remodeling_TGF, WNT
Cytoskeleton remodeling
Epithelial-to-mesenchymal transition (EMT)
Induction of EMT TGF-beta-dependent
ESE3/EHF Controls Epithelial Cell Differentiation and Its Loss Leads to Prostate Tumors with Mesenchymal and Stem-like Features

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Cancer Res Published OnlineFirst April 13, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-0212

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