Periostin, a cell adhesion molecule, facilitates invasion in the tumor microenvironment and annotates a novel tumor invasive signature in esophageal cancer

Carmen Z. Michaylira1,2,4,*, Gabrielle S. Wong1,2,4,*, Charles G. Miller1,2,4, Christie M. Gutierrez1,2,4, Hiroshi Nakagawa1,2,4, Rachel Hammond4,5, Andres J. Klein-Szanto6, Ju-Seog Lee7, Sang Bae Kim7, Meenhard Herlyn9, J. Alan Diehl4,8, Phyllis Gimotty4,5, and Anil K. Rustgi1,2,3,4

1Division of Gastroenterology, University of Pennsylvania, Philadelphia, PA, 19104, USA
2Department of Medicine, University of Pennsylvania, Philadelphia, PA, 19104, USA
3Department of Genetics, University of Pennsylvania, Philadelphia, PA, 19104, USA
4Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, 19104, USA
5Division of Biostatistics, Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania, Philadelphia, PA, 19104, USA
6Division of Basic Science, Tumor Cell Biology, Fox Chase Cancer Center, Philadelphia, PA, 19104, USA
7Department of Systems Biology, MD Anderson Cancer Center, Houston, TX, 77030, USA
8Wistar Institute, Philadelphia, PA, 19104, USA
9Department of Cancer Biology, University of Pennsylvania, Philadelphia, PA, 19104, USA

Abstract

Human squamous cell cancers are the most common epithelially derived malignancies. One example is esophageal squamous cell carcinoma (ESCC), which is associated with a high mortality rate (1) that is related to a propensity for invasion and metastasis (2). Here we report that periostin, a highly expressed cell adhesion molecule, is a key component of a novel tumor invasive signature obtained from an organotypic culture model of engineered ESCC. This tumor invasive signature classifies with human ESCC microarrays, underscoring its utility in human cancer. Genetic modulation of periostin promotes tumor cell migration and invasion as revealed in gain of and loss of function experiments. Inhibition of EGFR signaling and restoration of wild-type p53 function were each found to attenuate periostin, suggesting interdependence of two common genetic alterations with periostin function. Collectively, our studies reveal periostin as an important mediator of ESCC tumor invasion and they indicate that organotypic (3D) culture can offer an important tool to discover novel biologic effectors in cancer.

Corresponding Author: Anil K. Rustgi, MD, T. Grier Miller Professor of Medicine & Genetics, Chief of Gastroenterology, 600 CRB, University of Pennsylvania, 415 Curie Blvd., Philadelphia, PA 19104, 215-898-0154, FAX: 215-573-5412, anil2@mail.med.upenn.edu.

Authorship note: Carmen Z. Michaylira and Gabrielle S. Wong have contributed equally to this work.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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Introduction

Development of ESCC involves a multistep, progressive process starting with increased esophageal epithelial cell proliferation leading to basal cell hyperplasia, dysplasia, carcinoma in situ and finally, advanced invasive carcinoma (3). Several genetic alterations, such as amplification of the epidermal growth factor receptor (EGFR), dysregulation of cyclin D1 and somatic mutations in the DNA binding domain of the tumor suppressor p53, are involved in initiation and progression of ESCC (4). While EGFR overexpression and p53 inactivation are key genetic alterations associated with ESCC, how these genetic alterations contribute to ESCC progression remains to be elucidated. Previously, we had addressed this by modeling EGFR overexpression and p53 missense mutation (R175H) in primary human esophageal epithelial cells (EPC2), which have been immortalized by hTERT overexpression (designated as EPC2-hTERT-EGFR-p53R175H cells). EPC2-hTERT-EGFR-p53R175H cells were grown in 3D organotypic culture, resulting in invasion of these cells into the underlying extracellular matrix (ECM) compared to EPC2-hTERT-EGFR or EPC2-hTERT-p53R175H cells, which did not invade (5). Combined expression of these genes also resulted in anchorage-independent growth and in tumor formation in xenograft models, which was not observed in control cells overexpressing either EGFR or mutant p53 alone (5).

Recent experimental results have provided mounting evidence that altered expression of cell adhesion molecules can contribute directly to tumor progression by modulating cell signaling. Therefore, we sought to identify genes involved in cell adhesion that were differentially expressed in invading tumor cells and could shed new insights into processes affecting tumor progression.

Deriving a novel invasive tumor signature from a gene expression profile analysis of invading EPC2-hTERT-EGFR-p53R175H cells in 3D culture and human ESCC tumor microarrays, we have identified periostin to be the most significantly upregulated gene triggering tumor cell invasion. Periostin (POSTN) is a secreted 90 kDa protein that was identified originally as a bone adhesion molecule responsible for differentially recruiting and attaching osteoblasts to the periosteum (6). Periostin is a member of the fasciclin (FAS) family and has a N-terminal signal peptide sequence, a cysteine-rich domain, four internal homologous repeats and a hydrophilic C-terminal domain (6). These four internal repeat domains share structural homology with Fasciclin 1, an insect neuronal adhesion protein (6, 7) and big-h3, a TGF-β1 inducible gene (7, 8). The high degree of structural and sequence similarity with these cell adhesion molecules suggest that periostin has a role in cell adhesion and migration. Indeed, not only is its expression induced in human ESCC and mouse tumor xenografts, but more importantly, genetic modulation of periostin also affects tumor invasion in a direct fashion. The novel identification of periostin as an essential mediator of tumor invasion in the microenvironment has implications upon tumor detection and therapy.

Materials and Methods

Cell culture

Primary esophageal cells (EPC2) were established from surgical specimens of normal esophagus as described previously (9) and immortalized by overexpression of the catalytic
subunit of telomerase (hTERT) (EPC2-hTERT cells) (10). All cells were maintained in keratinocyte-SFM medium (KSFM) (Invitrogen) supplemented with 40 mg/ml BPE, 1.0 ng/ml EGF, 100 U/ml penicillin, and 100 mg/ml streptomycin (Full KSFM). Cells were grown at 37 °C in 5% CO₂.

Retroviral vectors and cell line generation

The PFB-neo or pBABE-zeo retroviral vectors were used to overexpress human EGFR in EPC2-hTERT cells. Additionally, p53<sup>R175H</sup> was subcloned into the pBABE-puro or the pBABE-zeo retroviral vectors. Periostin cDNA was used (Open Biosystems) and subcloned into the pFBneo retroviral vector. Two periostin short hairpin RNAs (shRNA#1 5′-CCCATGGAGAGCCAATTAT-3′ and shRNA#2 5′-CTCTGACATCATGACAACAAAT-3′) were used (Open Biosystems). Plasmids were transfected into Phoenix-Ampho packaging cells (gift of G. Nolan) using the Lipofectamine 2000 reagent (Invitrogen). Supernatants of the retroviruses encoding EGFR, mutant p53, periostin over-expression and shRNA vector constructs were collected 48 hrs. and 72 hrs. after transfection. Sub-confluent EPC-hTERT cells were infected with retrovirus supernatant in 4mg/ml polybrene (Sigma). 48 hours after infection, cells were selected in 300 μg/ml G418, 0.5 μg/ml puromycin, 2 mg/ml zeocin or 5 mg/ml blastocidin for 5 days. Overexpression of EGFR, mutant p53 and periostin, as well as periostin knock-down was confirmed by Western blot analysis.

Organotypic culture

Keratinocytes were grown in organotypic culture to recreate their microenvironment by supplying extracellular matrix components including collagen and laminin and fetal esophageal fibroblasts, its detailed procedures were described previously (5). Cells grown in organotypic culture were processed for histology by fixing in 10% formaldehyde and paraffin embedded for LCM studies by embedding in OCT medium followed by freezing in liquid nitrogen and then stored at −80 °C.

LCM and RNA isolation

Frozen organotypic cultures were sectioned to 8 μm onto membrane mounted metal frame slides (MMI, Switzerland) using a Microm HM 505E cryostat (Richard Allen Scientific). Sections were immediately fixed, stained and dehydrated before laser microdissection. Microdissection was performed with a Nikon Eclipse TE 2000-5 microscope with a UV laser (MMI, Switzerland). Microdissected cells were collected and 50 ul of extraction buffer (Arcturus) was added and incubated for 30 min at 42 °C, followed by centrifugation at 800 x g for 2 minutes. RNA was extracted using the Arcturus PicoPure RNA isolation kit following the manufacturer’s instructions. The integrity and concentration of the RNA was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies).

RNA amplification and microarray studies

The RNA obtained from the LCM studies was amplified using the Affymetrix GeneChip Expression 3′-Amplification Two-Cycle cDNA Synthesis Kit (Affymetrix) followed by the Affymetrix GeneChip Hybridization Wash and Stain kit (Affymetrix). The resulting cRNA (200 ng) was used as template for random-primed cDNA synthesis and a second round of in vitro transcription, which incorporated biotinylated CTP and UTP. The cRNA products were fragmented to 200 bp or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to Affymetrix U133Plus 2.0 oligonucleotide microarrays (Affymetrix). Microarrays were subsequently washed at low (6 x SSPE) and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-phycocerythrin. The fluorescence signal was amplified by addition of biotinylated anti-streptavidin, and an additional aliquot of streptavidin-
phycoerythrin stain. A confocal scanner was used to acquire the fluorescent signal after excitation (570 nm).

**qPCR**

LCM was repeated to isolate invading and non-invading EPC-hTERT-EGFR-p53\(^{R175H}\) cells grown in organotypic culture. Amplification and cDNA synthesis was performed using WT-Ovation RNA Amplification System (NuGen Technologies) according to manufacturer’s instructions. Real-Time PCR was performed and analyzed using ABI PRISM 7000 sequence detection system software (PE Applied Biosystems) and using Power SYBR Green PCR Master Mix (PE Applied Biosystems) for β-actin according to the manufacturer’s instructions. Taqman assays for periostin were run using Taqman Universal PCR Master Mix (PE Applied Biosystems) according to the manufacturer’s instructions.

**Tumor specimens**

Esophageal tumor tissue specimens and adjacent normal tissue were surgically procured from patients at the Okayama University Hospital (Drs. Shirakawa and Naomoto, Japan). All tumor specimens were pathologically diagnosed as esophageal squamous cell carcinomas (Grade III) and obtained from informed-consent patients in accordance with Institutional Review Board standards and guidelines.

Specimens were immediately snap-frozen for RNA and protein analyses. The human ESCC tissue microarray was subjected to immunohistochemistry analysis using a polyclonal anti-periostin antibody and scored for periostin expression as follows; Negative (0), Marginal considered negative (0.5), mild positive stain (1), moderate positive stain (2), and intense positive stain (3). Scores >0.5 are considered positive. Each case on the tissue microarray comprises of 2 cores and the mean scores of 2 cores were taken.

**Antibodies**

The following antibodies were used for immunoblotting: EGFR (NeoMarkers, Ab-12), p53 (Oncogene Research Products, Ab-6), phospho-EGFR (Cell Signaling, Tyr 1068), rabbit polyclonal periostin (Abcam, ab 14041), p21 (Oncogene Research Products), WAF1 (ab-1), β-actin (Sigma) was used as a loading control. For immunohistochemistry: periostin (Abcam, ab 14041).

**Western blotting**

To confirm protein expression, western blot analysis was performed as described previously (5). To confirm secreted periostin expression, conditioned media was collected and protein concentration was determined by Bio-Rad protein assay (Bio-Rad) and Western blot analysis was performed as described above.

**Migration and invasion assays**

Migration and invasion assays were performed as described previously (5). All experiments were performed at least three times in triplicate.

**Data Processing**

Affymetrix array cel files were processed in R using an algorithm that uses genes in the least variant set (LVS) to normalize the expression data (11). In the analysis of invasive and non-invasive cells, we then identified 6106 probe sets for subsequent statistical analysis that had a minimum expression level (expression greater than 100 in all samples) and that had significant variability among the 11 cell lines (standard deviation was greater than 150). The background correction, summarization and normalization of Affymetrix cel files were done.
in the same manner independently for each of the two tumor/normal studies. Gene Expression Omnibus (GEO) database accession number: GSE21293

**Statistical analysis**

All statistical analyses used natural log transformed expression data. Using the arrays of cells from the organotypic culture model, a multivariate regression model was fit for each gene that included two independent binary variables. The first variable indicated cells with both EGFR overexpression and P53 mutation only and the second indicated cells with EGFR overexpression, P53 mutation and invasion. The third group (the reference group) included cells with a non-invading phenotype that had either EGFR overexpression or P53 mutation. The p-values from the test that the regression model was significant (F-statistic, 2df) were adjusted using the Benjamini-Hochberg technique; probe sets with a False Discovery Rate (FDR) < 0.05 were selected for further investigation (12). Genes on this list were compared to annotated Gene Ontology biologic processes and a functional annotation cluster analysis was done using the online DAVID tool (http://niaid.abc.ncifcrf.gov/). Annotations were derived from the DAVID databases. Supervised hierarchical clustering used in the heat maps was based on Euclidean distance. The statistical analyses were done using SAS/STAT software version 9.2 of the SAS System for PC.

**Results**

Identification of periostin from tumor invasion signature derived from gene expression profile analysis on invading EPC2-hTERT-EGFR-p53\textsuperscript{R175H} cells and ESCC tumor microarrays

The molecular pathways and networks underlying tumor invasion remain largely to be elucidated. In order to characterize a gene expression profile underlying the invasive phenotype of invading EPC2-hTERT-EGFR-p53\textsuperscript{R175H} cells, we profiled mRNA obtained from laser capture microdissected EPC-hTERT cells overexpressing EGFR and with a p53 missense mutation (R175H) that was either invasive or non-invasive in the organotypic culture model, as well as non-invasive cells that either overexpressed EGFR alone or had p53 (R175H) mutation alone, using Affymetrix U133-2 plus microarrays.

Analysis of the gene expression microarray data revealed a tumor invasive signature corresponding to a probe set list (PSL) of 939 probesets, where there was significant differential expression of these probe sets in invasive versus non-invasive cells (Supplementary Table 1). A supervised hierarchical cluster analysis demonstrated that this tumor invasive signature could distinguish significant differences between cell lines in organotypic culture with and without an invasive phenotype as presented in a heat map (Figure 1A).

To determine whether this invasive signature derived from invading EPC2-hTERT-EGFR-p53\textsuperscript{R175H} cells grown in organotypic culture can be extended to human ESCC, we analyzed gene expression profiles from five paired human ESCCs. We found that overall gene expression patterns of invasive cells grown in organotypic culture are highly similar to those from ESCC tumors and this tumor invasive signature was able to discriminate esophageal tumors from adjacent normal tissue and classified all tumors and 80% of the normal samples correctly (Figure 1B). To validate this tumor invasive signature, we performed a secondary analysis on an independent cohort of five paired human ESCC tumors using this tumor invasive signature and observed that it was capable of classifying 80% of ESCC tumors and all adjacent normal tissues correctly (Figure 1C). Using the t-test statistic to rank the probesets in this tumor invasive signature, we identified periostin, a cell adhesion molecule, with the most significant difference in gene expression between invading transformed...
esophageal epithelial cells compared to non-invading normal or dysplastic epithelial cells (t-
test for two independent groups, p<0.0001). Strikingly, periostin was also found to be
significantly upregulated in both independent cohorts of human ESCC tumors compared to
adjacent normal tissue, the fold change in mean periostin expression was found to be 5.0 and
5.7 in the respective cohorts.

To explore the biological processes associated with invasion of ESCC, we tested for
enrichment of gene ontology (GO) terms within this tumor invasive signature. This tumor
invasive signature was found to be significantly enriched in nine biological processes (FDR,
p<0.01) (data not shown, Supplementary Table S1 and S2). To investigate the cellular
processes altered within the tumor microenvironment, we selected three biological processes
for further investigation and observed that probesets clustered in each biological process
(cell/biological adhesion, development-related and cellular component organization and
biogenesis) were able to classify invading transformed esophageal epithelial cells from non-
invading cells (Table 1 and Supplementary Figure S1A-C). Interestingly, periostin was
found to be the highest upregulated gene (6.54 fold) across the three biological processes
(Table 1), and was unique in this feature amongst all the genes in the datasets. In aggregate,
these results reveal a tumor invasive signature derived from the organotypic culture model
which is applies faithfully to human ESCC and highlights periostin as an important gene
required for facilitating tumor cell invasion.

**Periostin is expressed preferentially in invading EPC2-hTERT-EGFR-p53R175H cells both in
vitro and in vivo**

We next sought to validate whether the upregulation of periostin is concomitant with
invading transformed esophageal cells as well as establish the localization of periostin
expression in these cells. Consistent with the results of the microarray analysis, increased
periostin mRNA expression in invading EPC2-hTERT-EGFR-p53R175H cells was confirmed
by LCM isolation of invading and non-invading EPC2-hTERT-EGFR-p53R175H cells in the
organotypic culture model followed by RNA isolation, amplification and qPCR analysis
(Figure 2A). Furthermore, periostin protein accumulation in invading EPC2-hTERT-EGFR-
p53R175H cells grown in organotypic culture was observed by immunohistochemical
staining (Figure 2B, Right panel b), but not in control EPC2-hTERT-EGFR cells (Figure 2B,
Left panel a). Interestingly, periostin protein expression was also observed in the epithelial-
stromal interface in tumors formed by EPC2-hTERT-EGFR-p53R175H cells in a xenograft
tumor model (Figure 2C, white arrows). These results establish preferential expression of
periostin in invading EPC2-hTERT-EGFR-p53R175H cells both in vitro and in vivo.

**Loss of periostin leads to decreased migration and invasion in EPC2-hTERT-EGFR-
p53R175H cells**

Previous efforts involving other epithelial cancers had indicated that periostin promotes
invasion and anchorage-independent growth in several epithelial cancer cell lines and in
tumors such as head and neck cell carcinoma, oral, breast and ovarian cancers (13-16).
Elevated levels of periostin have been detected in sera of patients with breast, thymoma and
non-small cell lung cancer, suggesting periostin secretion during tumorigenesis (17-19). We
sought to investigate directly whether periostin had a functional role in facilitating tumor
cell migration and invasion in ESCC. An RNA interference approach with shRNA was used
to induce stable knockdown of periostin expression in EPC2-hTERT-EGFR-p53R175H cells
(Figure 3A). Knockdown of periostin in EPC2-hTERT-EGFR-p53R175H cells led to
significant decrease in cell migration and invasion compared to control shscrambled cells
(Figure 3B, C). Notably, reduced periostin expression in EPC2-hTERT-EGFR-p53R175H
cells also resulted in decreased invasion of these cells into the underlying matrix in
organotypic culture (Figure 3D).
**Periostin overexpression promotes increased migration and invasion in EPC2-hTERT-EGFR-p53R175H cells**

In parallel studies, periostin was retrovirally overexpressed in two independent cell lines (EPC2-hTERT-EGFR-p53R175H-1 and EPC2-hTERT-EGFR-p53R175H-2) (Figure 4A) and while overexpression of periostin showed no effect on proliferation (data not shown), we observed that these cells displayed increased migration and invasion (Figure 4B, C). Furthermore, when EPC2-hTERT-EGFR-p53R175H cells overexpressing periostin were grown in organotypic culture, increased invasion into the underlying matrix was also observed (Figure 4D). Collectively, these results demonstrate the direct functional role of periostin in promoting tumor cell migration and invasion, particularly within the context of the tumor microenvironment.

**Upregulation of periostin expression in primary ESCC tumors and in a cancer tissue microarray**

We next sought to determine if upregulation of periostin is also found in human ESCC by assessing periostin expression in primary ESCC (Grade III) tumors. Increased periostin mRNA expression was observed in ESCC tumors compared to their matched normal controls (Figure 5A). A marked increase in periostin protein levels was observed in all ESCC tumors studied compared with their matched normal mucosa controls (Figure 5B and Supplementary Figure S2). To determine the localization of periostin in human ESCC, further immunohistochemical analysis of periostin expression was performed in a tissue microarray containing 73 ESCC tumors and adjacent normal tissue and revealed periostin expression in invasive ESCC tumor cells (Figure 5C, Bottom panel c, arrowheads) and consistent accumulation in tumor stroma (Figure 5C, Bottom panel c, arrows) compared to normal esophageal tissue where periostin expression was detected primarily in and around blood vessels (Figure 5C, Left panel a, arrows). Periostin expression was also detected in high grade esophageal intraepithelial neoplasia (EIN), which was found in a restricted number of cases, particularly in the epithelial-stromal interface (Figure 5C, Right panel b, arrows). In addition, the stroma in all 73 ESCC cases were scored for periostin immunohistochemical staining intensity and it was shown to have higher levels of periostin staining compared to matched normal esophagus which showed weak to no periostin staining. Specifically, the ESCC scored either 2 or 3, meaning moderate or intense staining, whereas the normal esophagus was 0 or 0.5 (Figure 5C, panel d), which would suggest that induction of periostin could also arise in the stroma during ESCC progression.

**Induction of periostin is dependent upon EGFR signaling and p53 mutation**

It is believed, but not established, that periostin might enhance tumor cell invasion and metastasis through increased integrin signaling, augmenting cell survival through the PI3K/Akt pathway or fostering epithelial-to-mesenchymal transition (EMT)(16, 20, 21). Given that periostin was identified initially in invading EPC-hTERT-EGFR-p53R175H cells through functional genomics and bioinformatic analysis, we hypothesized that periostin expression is dependent upon both activated EGFR signaling and p53 mutation. Notably, periostin expression was upregulated in EPC2-hTERT-EGFR-p53R175H cells compared to control cells overexpressing EGFR alone (EPC2-hTERT-EGFR) or mutant p53 alone (EPC2-hTERT-p53R175H) (Figure 6A). In addition, periostin promoter reporter assays in EPC2-hTERT-EGFR-p53R175H cells displayed the highest promoter activity compared to control cells with either EGFR overexpression or p53 mutation alone (Supplementary Figure S3), suggesting that both genetic alterations may be required to activate periostin expression at a transcriptional level. This result was further corroborated when EPC2-hTERT-EGFR-p53R175H cells were stimulated with EGF and increased periostin protein expression was observed (Figure 6B). This induction of periostin upon EGFR stimulation led us to test whether inhibition of EGFR signaling and/or restoration of wild-type p53 function could
inhibit periostin by treating EPC2-hTERT-EGFR-p53R175H cells. We employed AG1478, an EGFR tyrosine kinase inhibitor, and 5-iminodaunorubicin, a small molecule compound which restores wild-type p53 signaling by inducing apoptosis and cell cycle arrest, as illustrated by induction of p21(22). Periostin protein expression was noted to be decreased when inhibited by AG1478 or 5-iminodaunorubicin or both (Figure 6B). Taken together, these data support the notion that periostin expression is modulated mechanistically by activated EGFR signaling and p53 mutation.

**Discussion**

Overall, our results reveal a novel tumor invasive signature derived from invading transformed cells compared to control noninvading normal and dysplastic cells in organotypic (3D) culture. This signature is one that annotates primary invasive human esophageal squamous cell cancer as distinctive from adjacent normal human esophageal mucosa in two independent cohorts of tumors, underscoring the fidelity and utility of this tumor invasive signature. It is conceivable that this molecular signature might be informative in the future for other squamous cell cancers arising in different tissues.

Gene ontology analysis (DAVID databases) of specific biological processes that are believed to be involved in the tumor microenvironment reveals the consistent and unique upregulation of periostin, suggesting its critical functional role. First, periostin was found to be upregulated in invading transformed cells and in primary esophageal squamous cell cancers based upon immunohistochemical and Western blot analysis. Second, periostin’s direct functional role is underscored by overexpression and knockdown experiments in which genetic manipulation of periostin dramatically influences the degree of tumor invasion in organotypic culture. Third, EGFR signaling and p53 mutation, both canonical genetic alterations in ESCC, appear to converge upon periostin based upon luciferase reporter gene assays as well as inhibition of EGFR signaling and restoration of wild type p53 function. In aggregate, these novel results underscore the utility of the organotypic culture model for the discovery of direct biological effectors of tumor invasion into the microenvironment, which has been largely elusive to date. Local tumor invasion in the mesenchymal stromal compartment is important given that it temporally precedes tumor dissemination in the lymphatic and blood vessels for tumor metastasis.

Various avenues of investigation have highlighted the important role of the microenvironment in enhancing the initial dissemination of malignant tumor cells. Dynamic interactions between the epithelium and mesenchymal stroma contribute to boosting the invasive phenotype of tumor cells by activating a variety of genes facilitating cell proliferation, de-differentiation, migration and invasion (23, 24). Significant changes such as loss of cell-cell contacts, disruptions in cell adhesion junctions and altered cell-extracellular matrix interactions within the tumor microenvironment converge to increase the ultimate metastatic potential of tumor cells (25). Therefore, identification of gene expression pattern changes during initial stages of tumor progression within the tumor microenvironment is crucial to understanding the causes of tumor invasion, and ultimately, tumor metastasis to distant organ sites. Thus, our results provide new platforms into the investigation of tumor invasion.

Periostin has been shown also to have a role in bone, tooth and heart formation during development (26, 27) and is only re-expressed and upregulated in adult tissue after vascular, skeletal or bone injuries (28, 29). Periostin is similarly overexpressed in human cancers (14), (30). Our results highlight that a tumor invasive signature defines genetically engineered ESCC that is reproduced in human ESCC. Our studies also suggest that induction of periostin, a secreted protein with a long half-life (Supplementary Figure S4 and data not
shown), may alter the tumor microenvironment by accumulating in the stroma and facilitating invasion through matrix remodeling. This may be achieved through regulation of collagen I fibrillogenesis (31), serving as a bridge between tenasin C and the extracellular matrix, as well as through interaction with αVβ3 integrins. Furthermore, periostin may promote tumor cell survival in the matrix of the microenvironment by activating the Akt/PI3K pathway. Lack of periostin may lead to suppression of Notch1 signaling (32), conversely, it is conceivable that overexpression of periostin could activate Notch1 signaling in cancers. Indeed, we have evidence of activated Notch signaling in invasive tumor cells grown in organotypic culture (data not shown). Future translational and clinical investigations might seek to exploit periostin as an attractive therapeutic target, especially in a combinatorial fashion, for example, with inhibitors of receptor tyrosine kinases.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

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References

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Figure 1. Invasive signature from EPC2-hTERT-EGFR-p53<sup>R175H</sup> cells classifies with human ESCC

(A) Microarray analysis of LCM-extracted RNA from invading EPC2-hTERT-EGFR-p53<sup>R175H</sup> cells grown in organotypic culture (n=3) compared to non-invading EPC2-hTERT-EGFR-p53<sup>R175H</sup> (n=3) as well as non-invading EPC2-hTERT-EGFR-puro (n=3) and EPC2-hTERT-neop53<sup>R175H</sup> cells (n=2). Differentially expressed probesets were subjected to supervised hierarchical clustering and expression is based on a log<sub>2</sub> scale where red represents upregulation and green represents downregulation. Heatmap denotes probeset list (PSL) of 939 probesets representing unique tumor invasive signature characterizing invasive phenotype. Tumor invasive signature comprises differentially expressed probesets (p<0.0001).

(B and C) Heatmap representation of gene expressing profiles from two independent cohorts each comprising of five paired ESCC tumors classified using the tumor invasive signature from (A). Due to microarray platform difference (U133 v2.0 and U133A) used in 2 independent cohorts, only 648 probesets were shared in both platforms. All probesets are in the same order as seen in (A).
Figure 2. Periostin is expressed preferentially in invading EPC2-hTERT-EGFR-p53R175H cells.
(A) Fold change of periostin expression in invading versus non-invading EPC2-hTERT-EGFR-p53R175H cells isolated by LCM from organotypic culture and quantified by qPCR. (*) P-value < 0.05
(B) Immunohistochemistry analysis of periostin expression in control EPC2-hTERT-EGFR cells (Left panel a) and invading EPC2-hTERT-EGFR-p53R175H cells (Right panel b) grown in organotypic culture (OTC). Rabbit polyclonal periostin antibody recognizes exogenous and secreted periostin.
(C) Periostin expression by immunohistochemistry of tumor formed in vivo by EPC2-hTERT-EGFR-p53R175H cells. Periostin expression in tumor epithelial-stromal interface indicated (white arrows). (200x Magnification)
Figure 3. Periostin knockdown in EPC2-hTERT-EGFR-p53^{R175H} cells reduces migration and invasion

(A) Western blot confirming periostin (90 kDa) knock-down in EPC2-hTERT-EGFR-p53^{R175H} cells using two independent shRNA constructs.

(B) Representative fluorescent images obtained from bottom filter of a Boyden chamber migration assay show reduced migration in EPC2-hTERT-EGFR-p53^{R175H} cells expressing shRNA to periostin versus control (scrambled) shRNA to periostin. Experiments were performed in triplicate. (*) P<0.04 for shPOSTN#1 vs shscrambled, (*) P<0.001 for shPOSTN#2 vs shscrambled.

(C) Images and quantifications of the Matrigel invasion assay were acquired and processed as in (B). Experiments were performed in triplicate. (*) P < 0.02 for shPOSTN#1 vs shscrambled, (*) P<0.05 for shPOSTN#2.

(D) H & E staining of organotypic culture comparing shRNA to periostin versus scrambled shRNA showing decreased invasion of EPC2-hTERT-EGFR-p53^{R175H} cells expressing shRNA to periostin. Bar graphs represent fold change in invasion +/- SEM, P=0.015 (200x magnification)
Figure 4. Periostin overexpression in EPC2-hTERT-EGFR-p53R175H cells promotes increased migration and invasion in vitro and in organotypic culture

(A) Western blot confirming periostin (90 kDal) overexpression in two independent EPC2-hTERT-EGFR-p53R175H cell lines. pFB neo was used as an empty control vector.

(B) Representative fluorescent images obtained from bottom filter of a Boyden chamber migration assay show enhanced migration in EPC2-hTERT-EGFR-p53R175H cells that overexpress periostin versus control EPC2-hTERT-EGFR-p53R175H-neo cells. Bar graphs represent fold changes +/- SEM (*) P<0.01 (Student t-test, EPC2-hTERT-EGFR-p53R175H periostin overexpressing cells vs. control cells). Note that P < 0.05 is statistically significant. Experiments were done in triplicate.

(C) Images and quantifications of the Matrigel invasion assay were acquired and processed as in (B). (*) P<0.04

(D) H & E staining of organotypic cultures comparing EPC2-hTERT-EGFR-p53R175H periostin overexpressing cells to empty vector control cells reveal increased invasion of EPC2-hTERT-EGFR-p53R175H periostin overexpressing cells. Bar graphs represent fold change in invasion +/- SEM, (*) P< 0.03 (EPC2-hTERT-EGFR-p53R175H periostin overexpressing cells vs empty control vector cells). (200x magnification)
Figure 5. Periostin is overexpressed in primary ESCC tumors and is associated with ESCC tumor progression

(A) Relative periostin mRNA expression measured by real-time PCR in 5 primary ESCC tumor specimens with paired adjacent non-invasive esophageal tissue. (*) P-value<0.05.

(B) Western blot analysis of periostin expression in 5 primary ESCC tumor specimens with paired adjacent non-invasive esophageal tissue. Expression of β-actin was used as an internal loading control.

(C) Immunohistological analysis of a tissue microarray comprising of 73 human ESCC cases with normal esophageal tissue. Representative photomicrographs of periostin expression in normal esophageal tissue, high grade esophageal intraepithelial neoplasia (EIN) and ESCC. Periostin expression was observed around blood vessels (Left panel a, arrows). Periostin staining observed in EIN epithelial-stroma interface (Right panel b, arrows) and accumulation in tumor stroma (Bottom panel c, arrows) and tumor cells (Bottom panel c, arrowheads). (400x magnification). Stroma in tissue microarray was scored for periostin staining intensity and average score of 2 cores from each case were taken. ESCC cases scored higher levels of periostin expression (n=73, Mean= 1.44, s.d +/- = 0.97) compared to matched normal controls (n=69, Mean= 0.44, s.d +/- = 0.68) (panel d). Closed circles represent outliers. Student’s t-test, ESCC cases vs matched normal esophagus controls. (**) p-value < 0.01
Figure 6. Periostin expression is dependent upon EGFR signaling and mutant p53 activation

(A) Western blot analysis of periostin (90kDa) in EPC2-hTERT-EGFR-p53\textsuperscript{R175H}, EPC2-hTERT-puro-neo, EPC2-hTERT-EGFR-neo and EPC2-hTERT-neo-p53\textsuperscript{R175H} cells.

(B) Western blot analysis of periostin expression in EPC2-hTERT-EGFR-p53\textsuperscript{R175H} cell lysates after 24h treatment with EGF (10ng/\(\mu\)l), EGFR inhibitor AG1478 (1 \(\mu\)M) and 5-iminodaunorubicin (3 \(\mu\)M). Immunoblotting for total EGFR and phosphorylated EGFR to confirm inhibition of EGFR as well as p21 to indicate restoration of wildtype p53 signaling. \(\beta\)-actin was used as a loading control. Densitometry ratios of periostin/\(\beta\)-actin were calculated and recorded.
Table 1
Genes with at least two-fold higher expression in invasive EPC2.hTERT.EGFR.p53R175H cell cell lines compared to non-invasive EPC2.hTERT.EGFR.p53R175H/EPC2.hTERT.EGFR.puro/EPC2.hTERT.ueo.p53R175H cell lines by GO Biological Processes

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### Cellular Component Organization and Biogenesis

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*Multivariate logistic regression models were used to test each probeset and adjusted for false discovery rate (p<0.01)