Induction of Human Epithelial Stem/Progenitor Expansion by FOXM1

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Abstract

Stem cells are permanent residents of tissues and thought to be targets of cancer initiation. The frequent, and often early, upregulation of the FOXM1 transcription factor in the majority of human cancers suggests that it may participate in the initiation of human tumorigenesis. However, this hypothesis has not been tested. Herein, we show that targeting the ectopic expression of FOXM1 to the highly clonogenic cells of primary human keratinocytes with stem/progenitor cell properties, but not to differentiating cells, caused clonal expansion in vitro. We show, using a functional three-dimensional organotypic epithelial tissue regeneration system, that ectopic FOXM1 expression perturbed epithelial differentiation generating a hyperproliferative phenotype reminiscent of that seen in human epithelial hyperplasia. Furthermore, transcriptional expression analysis of a panel of 28 epithelial differentiation-specific genes reveals a role for FOXM1 in the suppression of epithelial differentiation. This study provides the first evidence that FOXM1 participates in an early oncogenic pathway that predisposes cells to tumorigenesis by expanding the stem/progenitor compartment and deregulating subsequent keratinocyte terminal differentiation. This finding reveals an important window of susceptibility to oncogenic signals in epithelial stem/progenitor cells prior to differentiation, and may provide a significant benefit to the design of cancer therapeutic interventions that target oncogenesis at its earliest incipient stage. Cancer Res 70(22): 9515-26. ©2010 AACR.

Introduction

FOXM1 directs a transcriptional network of genes that is necessary for cell cycle promotion and for the coordination of timely cell division and exit from the cell cycle (1, 2). Previous studies have shown its role as a major regulator of the G2-M phase of the cell cycle where it is required to ensure successful execution of the mitotic program and the maintenance of chromosome stability (3). In 2002, we provided the first evidence that directly linked FOXM1 with human cancer, demonstrating that FOXM1 was abundantly expressed in human basal cell carcinomas and further identified this gene as a downstream target of the oncogenic transcription factor GLI1 (4). FOXM1 has since been listed amid the most common differentially expressed genes in the majority of human cancers (1, 2). Our finding that transcriptional upregulation of FOXM1 precedes malignancy in a number of solid human cancer types including oral, esophageal, lung, breast, kidney, bladder, and uterine cancers suggests a role for FOXM1 in tumor initiation (5). We have also found that ectopic FOXM1 expression induces genomic instability in primary human oral and skin cells (5, 6), which supports a role for FOXM1 in both malignant progression and metastatic invasion found in a number of human cancer types (1, 2). The fact that FOXM1 is implicated throughout early and late stages of cancer progression suggests a fundamental role for FOXM1 in both tumor initiation and maintenance. FOXM1 activity is dependent on oncogenic signaling downstream of Ras (7) and cyclin D1 (2), the deregulation of which is often implicated in epithelial tumor initiation and promotion. As an important executor of oncogenic stimulation, the aberrant activation of FOXM1 in models of chemically induced carcinogenesis in mice is tumor-promoting (2, 8, 9), whereas its ablation has strong tumor-suppressive effects (10, 11). Although the tumorigenic role for FOXM1 has been attributed to aberrant cell cycle regulation, the mechanism of how its upregulation contributes to cancer initiation in human epithelial cells, if at all, remains unclear.

Squamous epithelia are continuously maintained by permanent stem cell populations that are responsible for homeostasis (12–14). During wound repair, however, a different, slow-cycling population of cells (regenerative stem/progenitor cells) is responsible for tissue regeneration (15), and much like stem cells, it is also capable of repopulating all the lineages of...
the tissue in which it resides. Both populations, however, behave like clonogenic (Clonehi) populations of keratinocytes when expanded in vitro (12, 13). We therefore refer to these cells as stem/progenitor as there are currently no methods to isolate bona fide stem cells from human tissues (12–14). Although progenitor cells can self-renew very efficiently in culture, they inevitably exit this compartment as a consequence of either differentiation (16, 17), senescence (17–20), or both, and then resemble transit-amplifying cells (or paraclones; Clonehi) in that they quickly exit the cell cycle and terminally differentiate (18). The highly self-renewing cell forms colonies that have a high cloning efficiency on transfer or, in other words, have a high capacity for self-renewal and have been termed holoclines (18). The progressive reduction in self-renewal capacity leads to the formation of paraclones, which only form small or abortive colonies that quickly terminally differentiate. Numerous approaches have been used to separate Clonehi from Clonehi keratinocytes (17, 21–23) on the basis of cell surface expression signatures. This has facilitated the investigation into the response of such populations to various oncogenic stresses. In response to overexpression of mitogen-activated protein kinase (24) or constitutively active β-catenin (25), Clonehi keratinocytes retain their high clonogenic capacity when detached from integrin or expand in numbers, respectively. On the other hand, the overexpression of c-MYC depletes the Clonehi pool both in vitro (26) and in vivo (27). Furthermore, the Clonehi population can retain its clonogenic capacity by adenovirus E1A, which inactivates a variety of tumor suppressor pathways (28), but not by a single oncogene. In the in vivo setting, the RAS oncogene could induce neoplasia when targeted to the stem/progenitor-containing basal layer of the epidermis (29), although it can only produce regression-prone papillomas when its expression is driven by differentiation-specific promoters (29–31).

The upregulation of FOXM1 during tissue regeneration in response to injury (32) suggests that its physiologic activation represents a signal which is required for the expansion of regenerative stem/progenitor cells for the replenishment of the epithelium. FOXM1 is an early event in human epithelial neoplasia (5), and in line with the current “cancer stem cell” concept that malignancies are maintained by a subpopulation of cells that possess tumor initiation and self-renewal capacity, we hypothesized that the acquisition of aberrant FOXM1 gene expression in stem/progenitor cells may give rise to progeny cells inheriting excessive FOXM1 levels, as often found in many premalignant tissues (5) and malignant cancers (1, 2). To date, studies of FOXM1 as a cancer-causing gene have been performed in the context of transformed and/or immortalized cell lines, undoubtedly providing invaluable insight into the molecular mechanisms mediated by FOXM1. However, due to the aberrant molecular background already present in these cell line systems, the role of FOXM1 in the earliest stages of neoplasia could not be delineated and is not fully understood. Herein, we sought to investigate the role of FOXM1 (isoform B; FOXM1B) proto-oncogene, by examining its regulation and the effect of its overexpression, in normal primary human oral keratinocyte stem/progenitor cells as they represent the main targets of human malignancies.

Materials and Methods

Clinical samples

The use of human tissue was approved by the relevant Research Ethics Committees at each institution. The present study involved three oral SCC tumor tissue explants primary cultures for flow cytometry and gene expression analysis, and six independent normal oral mucosa tissues donated by healthy volunteers with informed patient consent and ethical approval.

Cell culture

Primary normal human oral keratinocytes were established from clinically normal oral buccal or gingival tissue and were maintained in RM+ medium (5) and were grown on mitomycin (10 μg/mL for 4 h) treated 3T3-feeder layers which were plated at a density of 1.8 × 10^5/cm^2 for 24 hours prior to keratinocyte seeding. All established cell lines and tumor explant culture procedures used in this study have been characterized and cultured as described previously (5, 33). Retroviral transduction cell synchronization and cell clonogenic assay protocols are described in the Supplemental Data file.

Figure 1. A, immunohistochemical staining of p75NTR (green) and FOXM1 (red) in normal human oral mucosa epithelium. B and C, magnified views of the tips of connective tissue papilla and deep-rete ridges, respectively (dotted boxes) in A are shown here where coexpression (yellow) of p75NTR and FOXM1 is detected (20×, 40×). D, absolute qPCR analysis of FOXM1B and p75NTR endogenous mRNA expression levels in p75NTR-sorted primary oral keratinocytes following 3 d after flow sorting. RS, random sorted populations. All values are representative of three independent experiments. Columns, mean of triplicate samples; bars, SEM (*, P ≤ 0.05; **, P ≤ 0.005; ***, P ≤ 0.001). E, endogenous FOXM1 and ΔNp63α protein expression patterns in p75NTR-sorted primary oral keratinocytes following 3 d (i) or 12 d (ii) in culture. β-Tubulin was blotted for protein loading control. F, clonogenicity assays for p75NTR-sorted primary oral keratinocytes. Primary human oral keratinocytes were FACSorted according to p75NTR levels (low or high) and were plated at equal densities in six-well plates (n = 6). Cells were then allowed to clonally expand for 12 d, after which they were either trypsinized to obtain cell number values, or were stained with rhodamine B for colony visualization and the quantification of colony growth. G, p75NTRhi cells were transduced with either (i, ii) pSIN-EGFP or (iii, iv) pSIN-EGFP-FOXM1B and individual keratinocyte colonies were examined under bright and fluorescence microscopy following 3T3-feeder removal. H, clonogenic assays performed on p75NTRhi and p75NTRlo oral keratinocytes following transduction with either pSIN-EGFP or pSIN-EGFP-FOXM1B. I, quantification of the average colony area of p75NTRlo and p75NTRhi oral keratinocytes transduced with either construct. Columns, mean obtained from n = 9 replicates performed in two individual primary human oral keratinocyte strains; bars, SEM. J, graphical representation of the colony size distribution according to surface area (mm^2) that emerged 15 d after transduction. A total of 200 to 500 colonies were counted (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Role for FOXM1 in Human Stem/Progenitor Cell Research.

D

Relative mRNA copy number

E

(i) FOXM1

β-Tubulin

(ii) FOXM1

ΔNp63α

β-Tubulin

F

Relative cell number

Relative colony size (pixel area)

G

EGFP

EGFP-FOXM1B

H

EGFP

EGFP-FOXM1B

I

p75NTR

J

Total colony count (%)
Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was performed on BD FACSARia Cell-Sorter (BD Biosciences). Cells were washed once with cold PBS and resuspended in cold FACS buffer (PBS/5% fetal bovine serum, 1% penicillin/streptomycin) followed by direct immunofluorescence for 15 minutes on ice with FITC-conjugated anti-integrin β1 (10 μL antibody/10⁶ cells, CD29-FITC; Abcam), phycoerythrin conjugated anti-p75NTR (20 μL antibody/10⁶ cells, CD271-PE; BD Biosciences), or anti-CD44 (1 μL antibody/10⁶ cells, clone G44.26; BD Biosciences) in 100 μL FACS buffer. Cells were centrifuged twice (800 rpm, 3 min) with one PBS wash in-between and finally resuspended in cold FACS buffer containing 200 ng/mL mitomycin C (Sigma). Integrin β1, p75NTR, or CD44 high/low (hi/lo) fractions were sorted by selecting the highest/lowest 20% of stained cells, respectively. Random sorted controls were selected randomly from the total pool of DAPI-negative cells. Keratinocytes were then plated through the automated cell dispenser onto six-well plates at 50 to 1,000 cells/cm², containing preplated feeder layers, cells were incubated for 5 minutes in PBS/Versene (Life Technologies, Invitrogen; 1:1 ratio) to remove feeder cells, and cells were incubated for 5 minutes at room temperature. Incubation with primary antibody (in 10% goat serum (Sigma) in 0.025% Tween 20 (TBS) for 1 hour) and secondary antibody (which generally has very low proliferative index), this being in agreement with the finding that inactive proteins are sequestered in the cytoplasm and that upon mitogenic stimulation, FOXM1 protein translocates into the nucleus (7).

Organotypic culture on de-epidermalized dermis

Organotypic keratinocyte cultures were performed as described (22). Tissue processing was carried out by Dr. Wesley Harrison at the Centre for Cutaneous Research at Blizard Institute of Cell and Molecular Sciences, and tissue sections were cut at the Pathology Core Facility of Blizard Institute of Cell and Molecular Sciences. Organotypic culture on collagen matrix protocol is described in Supplementary Data file.

Immunoblotting

For protein extraction from keratinocytes growing on feeder layers, cells were incubated for 5 minutes in PBS/Versene (Life Technologies, Invitrogen; 1:1 ratio) to remove feeder cells prior to protein extraction. Protein extraction and separation on SDS-PAGE gels and immunoblotting was performed as previously described (5). All antibodies used are listed in the Supplementary Data file.

Immunofluorescence and confocal microscopy

Frozen tissue sections were fixed in ice-cold methanol/acetone (1:1) for 10 minutes or in 4% formaldehyde (in PBS) for 20 minutes at room temperature and blocked by 10% goat serum (Sigma) in 0.025% Tween 20 (TBS) for 1 hour at room temperature. Incubation with primary antibody (in 1% bovine serum albumin/TBS) was performed for 2 hours at room temperature or overnight at 4°C. Sections were washed twice in TBS prior to incubation with secondary antibody for 1 hour at room temperature. Finally, sections were washed twice in TBS and mounted with Shandon Immu-Mount (Thermo Scientific) solution containing DAPI dilactate (final concentration at 300 ng/mL). Paraffin-embedded tissue sections were initially heated at 60°C for 8 minutes and were then deparaffinized in xylene (three times for 5 min) and dehydrated (95% ethanol, 90% ethanol, and 70% ethanol for 5 min each) prior to rehydration in water (5 min). Antigen unmasking was performed by incubation in sub-boiling temperature for 20 minutes in antigen retrieval solution (pH 9.0; DakoCytomation). The sections were washed twice in TBS and permeabilized with TBS 0.2% Triton X (Sigma) for 10 minutes at room temperature and were then processed as described above. Confocal and fluorescence microscopy were performed as described previously (22).

Real-time absolute quantitative reverse transcription-PCR

Absolute real-time absolute quantitative reverse transcription-PCR (qPCR) were performed according to previously established protocols involving standard curves for each gene (5). FOXM1 isoform-specific primers and reference genes (YAP1 and POL2A) have been previously described and validated (5). Absolute qPCR were performed using the LightCycler 480 qPCR system (Roche Diagnostics, Ltd.). Primer sequences are listed in Supplementary Table S1.

Results and Discussion

Of the three alternatively spliced isoforms, FOXM1B was found to be the key isoform involved in oncogenesis (1, 2). However, it remained unclear which isoform(s) was directly involved in the cell cycle. We addressed this question using both normal telomerase-immortalized human keratinocytes (N/TERT) and metastatic cervical carcinoma cells (HeLa), in which FOXM1B was the only isoform found to be differentially expressed in cycling cells upon release from growth arrest (Supplementary Fig. S1A and B; see Materials and Methods). Herein, we investigated the possibility that acquisition of aberrant FOXM1B expression in clonogenic stem/progenitor cells might be a fundamental oncogenic initiation mechanism which contributes to its widespread upregulation detected in most human cancers.

To test this hypothesis, we first investigated the expression profile of FOXM1 protein in human oral mucosa tissue. As the antibody for FOXM1 protein was unable to differentiate between isoforms, we henceforth use the term FOXM1 whenever isoform-specific information was not available. Second, we examined the cellular and molecular effects of its ectopic expression in primary human oral mucosa keratinocyte stem/progenitor cells identified and isolated using a well-established oral stem/progenitor cell marker p75NTR [a low-affinity nerve growth factor receptor (NGFR), also known as CD271, TNFRSF16, Gp80-LNGFR (ref. 23)]. In agreement, p75NTR expression was strictly basal, with regions of intense positivity at the tips of connective tissue papillae (Fig. 1A and B), whereas its expression was less intense in deep-rete ridges (Fig. 1C). In contrast, FOXM1 expression was found mainly in the more proliferative epibasal layers of the oral epithelium and occasionally detected in isolated basal cells (Fig. 1A and C). FOXM1 protein is mainly cytoplasmic within the normal mucosa (which generally has very low proliferative index), this being in agreement with the finding that inactive proteins are sequestered in the cytoplasm and that upon mitogenic stimulation, FOXM1 protein translocates into the nucleus (7).
We have further shown that in normal primary oral keratinocytes, FOXM1 proteins were predominantly found in the cytoplasm, whereas in the oral SCC cell line UK1 (33), both cytoplasmic and nuclear FOXM1 proteins were ubiquitously detected (Supplemental Fig. S1E), which is consistent with the FOXM1 protein expression pattern found in both premalignant dysplastic and oral SCC tumor tissues (5). In areas of intense p75NTR staining at the tips of connective tissue fibers, FOXM1 expression is also increased. This suggests a role for FOXM1 in the regulation of cell proliferation and differentiation in oral epithelial cells.

Figure 2. Characterization of FOXM1B-induced hyperproliferation phenotype using three-dimensional organotypic epithelial tissue regeneration model system. H&E-stained organotypic cultures of human oral mucosa derived from primary human oral keratinocytes retrovirally transduced with either pSIN-EGFP (A) or pSIN-EGFP-FOXM1B (B). Adjacent sections of respective organotypic tissues were stained with FOXM1 (C and D), Ki67 (E and F; arrows and arrowheads indicate expression of Ki67 in basal and suprabasal cells, respectively; dotted line demarcates the basement membrane), cytokeratin 16 (KRT16; G and H), transglutaminase-1 (TGM1; I and J), cytokeratin 13 (KRT13; K and L), and filaggrin (FLG; M and N). Nuclear DNA was stained with DAPI (blue).
tissue papillae, FOXM1 expression was almost undetectable (Fig. 1B), suggesting an inverse expression pattern between p75NTR and FOXM1. To further investigate this expression pattern, we have FACS-isolated p75NTRhi (containing differentiating/nonclonogenic cells or paraclines) and p75NTRlo (containing both stem and early progenitor cells which are clonogenic or are able to form holoclines in vitro) from early passage (P1) primary normal human oral keratinocytes and performed gene expression (Fig. 1D and E) and clonogenicity experiments (Fig. 1F; Supplementary Fig. S2). Although integrin α1, an epidermal stem cell marker (16), was found to be coexpressed with p75NTR (Supplementary Fig. S2A), the integrin β1hi/p75NTRhi double-sorted cells showed only marginal advantage in clonogenic potential over single-sorted p75NTRlo cells (Supplementary Fig. S2C–F), indicating that cells expressing p75NTR alone represent the majority of clonogenic stem/progenitor cells. In agreement with the inverse expression pattern found in oral mucosa tissue above, p75NTRhi cells were found to express significantly lower levels of FOXM1B mRNA (Fig. 1D) and protein (Fig. 1E, i) compared with p75NTRlo or randomly sorted keratinocytes. Although both FOXM1B and FOXM1C are transcriptionally active (32), the reduced mRNA expression was detected only for FOXM1B isoform (Fig. 1D), but not isoform C (Supplementary Fig. S1C). Given the proliferation-specific role of FOXM1B, we reasoned that the lower FOXM1B level was due to the quiescent nature of epithelial stem/progenitor cells in vivo and in vitro (17, 34, 35), as well as to the slow-cycling status of p75NTRhi keratinocytes in vitro (36) and in vivo (23). Expression analysis of human epidermal keratinocyte stem cells in vitro revealed that the maintenance of their quiescent state is partly attributed to a negative regulator of c-MYC, LRIG1 (37). As there is evidence that FOXM1 is a downstream target of c-MYC (38), it is possible that LRIG1-induced suppression of c-MYC indirectly contributed to the observed repression of FOXM1 in oral stem/progenitor cells (p75NTRlo). The upregulation of FOXM1 protein in cells immediately above the basal cell layer (Fig. 1A–C) led us to hypothesize that p75NTRhi cells may give rise to progeny with high clonogenic potential when maintained in prolonged culture (Fig. 1F). Indeed, the role of FOXM1 in progenitor compartment expansion became apparent following prolonged culture (12 d) in which FOXM1 was significantly upregulated in p75NTRhi cells and conversely, its expression diminished in p75NTRlo cells (Fig. 1E, ii). In both cultures, at days 3 and 12 (Fig. 1E), p75NTRlo cells retained high levels of another epithelial stem cell marker ΔNp63x (20), indicating that FOXM1 is induced specifically during the expansion of p75NTRhi cells with high clonogenic potential. In contrast, FOXM1 expression was suppressed in the differentiating p75NTRlo cells. Indeed, FOXM1 and involucrin (IVL, a keratinocyte differentiation marker) expression were found to be inversely correlated in early and late passage p75NTRlo cells (Supplementary Fig. S3A) while another differentiation marker cytokeratin 4 (KRT4) was found to be suppressed in p75NTRlo cells compared with p75NTRhi cells (Supplementary Fig. S3B). These findings further illustrate that FOXM1 expression is upregulated during the expansion of clonogenic stem/progenitor cells prior to the onset of epithelial differentiation. In agreement, during embryonic development, the expression of FOXM1 coincides with the transient proliferation of neural precursors preceding terminal differentiation (39), whereas in adult epithelial tissues in mice, its expression is confined to the most proliferative layers (32).

Many epithelial malignancies, including oral squamous cell carcinoma, are thought to arise from premalignant epithelial progenitors (40). To understand why FOXM1 is abundantly expressed in epithelial premalignancies (5) and a majority of human cancers (1, 2), we investigated the possibility that FOXM1 may be an initiating step that confers ectopic proliferation in keratinocyte stem/progenitor cells leading to the expansion of potentially premalignant progenies. To test this, FACS-sorted p75NTRlo and p75NTRhi cells were each retrovirally transduced with either pSIN-MCS (empty vector), pSIN-EGFP control, or pSIN-EGFP-FOX1B. pSIN-MCS and pSIN-EGFP-transduced cells gave identical experimental results (data not shown). Individual p75NTRhi keratinocyte colonies expressing FOXM1B showed an altered cellular morphology in which cells exhibited higher nuclear to cytoplasmic ratios whereas colonies appeared more compact and relatively larger in size (Fig. 1G, compare i and iii). EGFP was homogeneously expressed throughout the colony (Fig. 1G, ii), whereas EGFP-FOXM1B protein levels were heterogeneous with increased expression towards the periphery of each colony (Fig. 1G, iv). To establish whether ectopic expression of FOXM1B confers any functional effects, clonogenic assays were performed on primary keratinocytes derived from all subsets (p75NTRlo/hi–EGFP–FOXM1B). The p75NTRlo cells showed limited growth capacity regardless of the construct with which they have been transduced, consistent with a population containing differentiating/nonclonogenic cells. However, upregulation of FOXM1B in p75NTRlo cells significantly enhanced clonal expansion over control p75NTRlo–EGFP cells (Fig. 1H–J). p75NTRlo–FOXM1B cells showed a ∼2.6-fold increase (compared with p75NTRlo–EGFP) in the percentage of large cell colonies representing self-renewing holoclines (stem/progenitor cell–derived, >10 mm2), and a concomitant reduction in the percentage of smaller/abortive colonies (ref. 18; differentiating/nonclonogenic cell–derived, <5 mm2; Fig. 1J). Hence, the elevation of FOXM1B expression in p75NTRlo, but not in p75NTRhi keratinocytes, increased the intrinsic clonogenic capacity of primary keratinocytes. This suggests that the onset of epithelial differentiation renders cells refractory to FOXM1B-induced proliferation. To gain proliferative advantage, we therefore hypothesized that ectopic FOXM1B expression promotes stem/progenitor cell expansion by perturbing the onset of terminal differentiation.

To address this, we performed three-dimensional organotypic epithelial regeneration model systems using EGFP or EGFP-FOXM1B–transduced primary human oral keratinocytes according to our previously established method (22). In support, EGFP-FOXM1B–transduced oral keratinocytes grown in the organotypic cultures showed a hyperproliferative phenotype by producing thicker cell layers compared with control cultures using EGFP-transduced cells (Fig. 2, compare
Figure 3. Transcriptional regulation of keratinocyte differentiation genes in primary human oral keratinocytes transduced with varying levels of FOXM1B. Points, mean from triplicate determinations of six independent primary oral keratinocyte strains; bars, SEM. Second-order polynomial regression analyses were performed to obtain the $R^2$ coefficient of determination values which indicates the degree of correlation between each gene with FOXM1B.
A/B and C/D). FOXM1 protein was detected at low levels in the basal and proliferative epithelial layers in the control EGFP organotypics (Fig. 2C). In contrast, FOXM1 proteins were abundantly expressed in both the basal and suprabasal layers of the FOXM1B organotypics (Fig. 2D). As expected, the immunoreactivity of the proliferation marker Ki67 was strictly confined to the basal proliferative layer of the control EGFP organotypics (Fig. 2E). In contrast, keratinocytes of the suprabasal layers of FOXM1B organotypics retained ectopic FOXM1B expression, which correlated with increased Ki67 immunoreactivity (Fig. 2F; Supplemental Fig. S4F and G) indicating that they remained proliferative despite their locations within the upper spinous layers which should otherwise host nonproliferative differentiated cells. Only a subset of FOXM1-expressing cells are Ki67-positive and this is consistent with the staining patterns of FOXM1 and Ki67 in oral mucosa tissue (Supplementary Fig. S4E) and basal cell carcinoma (4), suggesting the involvement of FOXM1 in processes other than cell proliferation alone. Single cell expression analysis of human epidermal stem cells has revealed that the ablation of EGFR (growth factor receptor) by LRIG1 (stem cell marker) is required for the maintenance of stem cell quiescence, although it has also been suggested that loss of Lrig1 might trigger stem cell expansion and epithelial hyperproliferation through EGFR activation (37). In agreement with this model, EGFR was found to be significantly upregulated in FOXM1B organotypics (Supplementary Fig. S4A and B) and this further confirms the role of FOXM1B in progenitor compartment expansion resulting in epithelial hyperproliferation. The expansion of the suprabasal layers suggests that FOXM1B perturbed the balance between stem/progenitor cell renewal and the commitment to terminal differentiation. We therefore hypothesized that...
FOXM1B-induced hyperproliferation might intrinsically affect epithelial differentiation.

To this end, we investigated the expression profile of cytokeratin 16 (KRT16), which is a marker of hyperproliferative conditions such as wound healing and its upregulation has been shown to delay differentiation (41), and transglutaminase-1 (TGM1), which is specifically expressed in the suprabasal layers of the oral epithelium (42). Both markers were significantly upregulated in FOXM1B organotypics (Fig. 2G–J), indicating a sustained keratinocyte hyperproliferation. Furthermore, cytokeratin 19 (KRT19), which is a marker for preneoplastic oral epithelia (43) and a target of Gli1 in hair follicle stem cells that is upregulated in basal cell carcinoma (44), was also upregulated in our FOXM1B-organotypics (Supplementary Fig. S4C and D). This expression pattern, along with the inappropriate mitotic activity of keratinocytes within the upper suprabasal layers, suggests that FOXM1B-expressing cells have acquired a defective terminal differentiation. Indeed, immunostaining with early and late keratinocyte differentiation markers cytokeratin 13 (KRT13; Fig. 2K and L) and filaggrin (FLG; Fig. 2M and N), respectively, showed complete lack of protein expression within FOXM1B-derived organotypics. Similar results were also obtained from organotypic cultures using collagen gels (data not shown).

To obtain a more comprehensive understanding of the differentiation characteristics of FOXM1B-expressing oral keratinocytes, we have measured mRNA levels using absolute qPCR on a panel of 21 classic cytokeratin genes (KRT1, 2E, 2P, 3, 4, 5, 6A, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 23) and 2 mesenchymal-specific genes (VIM and NES) in primary oral keratinocytes transduced with varying doses of FOXM1B in culture (Fig. 3). FOXM1B was found to dose-dependently transactivate a number of simple basal keratin genes including KRT7, 8, 18, 15, and 19. Keratins 15 and 19 are basal cell-specific markers in stratified epithelia including skin and oral mucosa (45–47). Expression of these keratins has also been associated with the clonogenic stem/progenitor cells of the bulge region in hair follicles that are responsible for maintenance and regeneration of the epidermis (48, 49). Keratins 7, 8, and 18 are simple or mixed epithelia–specific keratins that are normally not expressed in stratified epithelia but they are induced during carcinogenesis (47). Induction of simple epithelial–specific and stem cell–specific keratins indicates that ectopic FOXM1 expression activated a defective differentiation pathway—a common pathologic feature found in epithelial malignancies (50). High levels of FOXM1B expression (Fig. 3) were found to activate VIM, which may represent a signal for epithelial-mesenchymal transition (51). Furthermore, genes involved in keratinocyte differentiation, such as cornifin (SPRR1B; Fig. 4A), involucrin (IVL; Fig. 4B), and desmoglein 3 (DSG3; Fig. 4C), were found to be inversely correlated with FOXM1B mRNA expression across a large panel of human oral keratinocytes consisting of 14 primary normal human oral keratinocytes, 6 oral premalignant, and 19 oral squamous cell carcinoma cell lines. In agreement with expression patterns found in oral mucosa (Fig. 1A–C) and primary oral keratinocytes (Fig. 1D and E, i), p75NTR expression was also found to be inversely correlated with FOXM1B across this cell line panel (Fig. 4D), supporting a tumor suppressor role for p75NTR (52–54). Given that upregulation of p75NTR inhibits cell proliferation (54) and regulates epithelial differentiation (55), the inverse expression between FOXM1B and p75NTR indicates the deregulation of cell proliferation and differentiation pathways during malignant progression. A previously characterized FOXM1B target, CEP55 (5), as expected, correlated positively with FOXM1B expression (Fig. 4E). Collectively, these results indicate that the progressive upregulation of FOXM1B and downregulation of keratinocyte differentiation genes may be intricately associated not only during normal epithelial renewal, but also during malignant progression.

Our finding that a defined genetic hit, such as FOXM1 upregulation, induces the sustained expansion of normal...
stem/progenitor cells led us to question whether putative cancer stem cells express different levels of FOXM1 compared with the rest of the tumor cell population. To test this, we investigated the endogenous expression levels of FOXM1 using absolute qPCR in cancer stem cells which were FACS-isolated from one premalignant oral dysplastic cell line (POE9n), two established oral SCC cell lines (5PT and CA1; ref. 33), and three primary explants of keratinocytes from independent oral SCC tumors (T1–T3; Fig. 4F), using an established oral cancer stem cell marker CD44 (56). We have shown that CD44hi cells were more clonogenic than CD44lo cells in oral SCC cell lines (5PT and CA1; Supplemental Figs. S1F and G). Interestingly, we have found that CD44hi and CD44lo cancer cells retain similar FOXM1 expressions, which suggests that FOXM1 expression is uniformly expressed in tumor cells regardless of their lineage hierarchy within the tumor subpopulations. The homogenous FOXM1 expression found in tumors is also consistent with our previous report that FOXM1 protein was abundantly expressed throughout the tumor mass of oral SCCs in vivo (5). In this study, we have shown that contrary to its heterogeneous expression pattern found in normal oral mucosal tissue, which retains an intact program of epithelial differentiation, FOXM1 is uniformly expressed in the whole population of tumor-derived keratinocytes. This suggests that the perturbation of epithelial differentiation during malignancy may further perpetuate FOXM1 expression in tumor cells. This is in agreement with our current hypothesis that a tumor may arise from a common premalignant progenitor which has acquired high levels of FOXM1 and later giving rise to progeny malignant cells (regardless of their “stemness”) with a hallmark of elevated FOXM1 expression. Furthermore, malignant cells are characterized by multiple genetic hits, many of which (e.g., RAS, loss of p53 function, c-MYC, p16/Rb pathway inactivation, and EGFR) are known to lead to constitutive upregulation of FOXM1 regardless of cancer stem cell status.

In summary, this study showed the first evidence that FOXM1B has a role in the proliferation of normal epithelial stem/progenitor cell. The aberrant upregulation of FOXM1B in these cells may perturb the balance between stem/progenitor expansion and commitment to terminal differentiation. These findings are consistent with the general consensus regarding epithelial carcinogenesis whereby a premalignant progenitor, possibly one with aberrant activation of FOXM1B, undergoes excessive rounds of clonal expansion within the epithelium. We have now shown that to achieve a protumorigenic “hit,” FOXM1B upregulation ought to be acquired during a “permissive window” during stem/progenitor cell expansion prior to their commitment to differentiation (Fig. 5). Although unable to revert the differentiated phenotype of committed keratinocytes, FOXM1B induced the expansion of the stem/progenitor compartment, which may explain why oncogenic stimulation upstream of FOXM1, such as RAS, is most efficient when targeted to the immature stem/progenitor epithelial compartment (30, 31, 57). Although large-scale whole-genome sequencing analysis across multiple human cancer types (58) did not find any DNA mutations within the FOXM1 gene (59), we have previously shown that environmental factors such as nicotine (5) and UV light (6) could directly stabilize and activate endogenous FOXM1B in primary human oral and epidermal cells, respectively, whereas others have shown that various oncogenic pathways lead to the activation of FOXM1B (1, 2). This study provides a novel role for FOXM1B in the initiation of human epithelial neoplasia, while our previous finding that ectopic FOXM1B expression contributed to genomic instability (5, 6) might also explain why this gene is implicated in both malignant progression and metastatic invasion (1, 2).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Role for FOXM1 in Human Stem/Progenitor Cell


Correction: Induction of Human Epithelial Stem/Progenitor Expansion by FOXM1

In this article (Cancer Res 2010;70:9515–26), which was published in the November 15, 2010 issue of Cancer Research (1), there is a typographical error in the name of the second author. The correct name is Daniela Elena Costea.

Reference


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Induction of Human Epithelial Stem/Progenitor Expansion by FOXM1

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