The transcription factor FOXM1 regulates the balance between proliferation and aberrant differentiation in head and neck squamous cell carcinoma

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Running title: FOXM1 limits aberrant differentiation in HNSCC

ABSTRACT

Sustained expression of FOXM1 is a hallmark of nearly all human cancers including squamous cell carcinomas of the head and neck (HNSCC). HNSCCs partially preserve the epithelial differentiation program, which recapitulates fetal and adult traits of the tissue of tumor origin but is deregulated by genetic alterations and tumor-supporting pathways. Using shRNA-induced knockdown, we demonstrate a minimal impact of FOXM1 on proliferation and migration of HNSCC cell lines under standard cell culture conditions. However, FOXM1 knockdown in three-dimensional (3D) culture and xenograft tumor models resulted in reduced proliferation, decreased invasion and a more differentiated-like phenotype, indicating a context dependent modulation of FOXM1 activity in HNSCC cells. By ectopic overexpression of FOXM1 in HNSCC cell lines, we demonstrate a reduced expression of cutaneous-type keratin K1 and involucrin as a marker of squamous differentiation, supporting the role of FOXM1 in modulation of aberrant differentiation in HNSCC. Thus, our data provide a strong rationale for targeting FOXM1 in HNSCC.

Keywords: FOXM1, head and neck cancer, invasion, squamous differentiation.

INTRODUCTION

Transcription factors of the Forkhead family are crucial regulators of embryonic development and many vital functions in adult tissues [1]. These proteins also play a pivotal role in the progression of cancers. The most important Forkhead Box (FOX) transcription factor involved in a multitude of pro-tumorigenic processes is FOXM1 [2,3]. The main gene regulatory activity of FOXM1 is attributed to cell cycle and mitotic control [4,5]. However, recent studies revealed additional activities of FOXM1 as a co-regulator of oncogenic pathways [3,6]. In addition, high *FOXM1* expression serves as a biomarker of poor outcome across a large set of cancer types [7]. This strongly supports the concept of FOXM1 as an actionable target for therapy [3,6] and encourages further exploration of FOXM1 function.

Squamous cell carcinomas of the head and neck (HNSCC) is a heterogeneous group of deadly cancers associated with loco-regional recurrences and distant metastases caused by the invasive and metastatic behavior of cancer cells [8]. The most striking feature of all SCCs is their ability to preserve a functional epithelial differentiation program [9], which recapitulates many specific traits of the tissue of origin of the tumor. However, in contrast to normal tissues, tight control over the fate and size of the proliferating and differentiating cell populations is lost in SCCs due to inactivation of critical regulatory mechanisms [9], thus causing aberrant epithelial differentiation and stratification featured in particular by altered and heterogeneous expression of keratins [10-12].

Despite a large body of evidence that FOXM1 has a role in HNSCC [13–16], its oncogenic functions in this type of cancer are not yet sufficiently understood. In that regard it is

noteworthy that FOXM1 is upregulated in premalignant oral lesions and persisting in primary HNSCCs and metastases [17]. Ectopic FOXM1 overexpression in oral keratinocytes induces a hyperproliferative phenotype and suppresses the expression of epithelial differentiation-specific genes [18] via a mechanism that includes differential regulation of DNA methylation [15]. Also, upon loss of p53 or overexpression of c-Myc, ectopic FOXM1 partially rescued primary human keratinocytes from terminal squamous differentiation, while promoting proliferation and genome instability [19]. In turn, differentiating normal human keratinocytes lose FOXM1 expression [20], thus suggesting that down-regulation of FOXM1 is part of the keratinocyte differentiation program. However, the effect of FOXM1 knockdown or overexpression on the differentiation state of HNSCC cells remains so far elusive. Given that FOXM1 is expressed in HNSCCs also early on during tumorigenesis [17,18], the demonstration of an effect on tumor growth and phenotype would support FOXM1 as a novel actionable target for adjuvant treatment of these cancers. In the present work, we thus sought to clarify the role of FOXM1 in HNSCC cell lines using cell culture and xenograft models.

Materials and methods

Cell culture

The human CAL27 cell line was purchased from ATCC (CRL-2095) and its authenticity was confirmed by STR profiling (Microsynth Gmbh, Balgach, Switzerland). Other cancer cell lines (LAU2083, LAU2088, and LOD148) and cancer associated fibroblasts (CAF142) were

established from tumor biopsies from patients undergoing surgery at the CHUV hospital (the procedures were all approved by the Cantonal Department of the Swiss Ethics Committees and the relevant informed consent forms were signed by the patients). All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. For a full list of the culture media used please see supplementary material, Supplementary materials and methods.

Inducible shRNA knockdown

Inducible FOXM1 knockdown in CAL27, LAU2083 or LOD148 cells was achieved using the pTRIPZ system (Dharmacon, Lafayette, CO, USA). Cells were transduced with a lentivirus carrying a shRNA sequence targeting FOXM1 (shFOXM1, CloneId V3THS_396939, Dharmacon) or a Non-silencing shRNA Control (shCtr, RHS4743, Dharmacon) and further selected by 1.5 µg/ml puromycin (540222-25, VWR International GmbH, Dietikon, Switzerland). After transient activation of the tetracycline response element by its transactivator (rtTA3) with 2 µg/ml doxycycline (Dox, D9891, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) for two days, cells were sorted by FACS to enrich for those expressing a high level of the pTRIPZ reporter (TurboRFP[high]) and were further maintained without Dox. Prior to any *in vitro* experiment, cells were treated again for 5 days with 2 µg/ml Dox to induce FOXM1 down-regulation and were further maintained in Dox-containing medium throughout the course of the experiment.

Constitutive FOXM1 overexpression

Constitutive FOXM1 overexpression in CAL27, LAU2083, LAU2088 and LOD148 cells was achieved using the pLenti-Blast system. In brief, the lentiviral pLenti-PGK-Blast-FOXM1c ("FOXM1") and pLenti-PGK-Blast-mCherry ("mCherry") were generated by subcloning human *FOXM1c* (contains an N terminal FLAG epitope tag) and mCherry nucleotide sequences from pCW57.1-FOXM1c (Addgene plasmid # 68810; a gift from Adam Karpf) and LeGO-C2 vectors (Addgene plasmid # 27339; a gift from Boris Fehse) into pLenti-PGK-Blast-V5-LUC (w528-1) vector (Addgene plasmid # 19166; a gift from Eric Campeau and Paul Kaufman) by replacing V5-tagged firefly luciferase with the respective insert sequence. After transduction with either vector, cells were further selected by incubation with 5 µg/ml blasticidin (InvivoGen, Toulouse, France).

3D cell culture (organotypic culture, spheroids and tumorspheres)

Organotypic cell culture was performed as previously described [21] and is briefly outlined in the supplemental experimental procedures. After 8–16 days of culture, the organotypes were collected either in 4% PFA/PBS for histological staining or in TRI reagent (T9424, Sigma-Aldrich Chemie GmbH) for RT-qPCR analysis. As a simplified version of the organotypic culture, cells can be embedded inside the collagen instead of on top of it, in which case cells will grow as spheroids. In brief, 200,000 tumor cells were admixed into a regular collagen I solution at a final concentration of 2.8 mg/ml before seeding them in one well of a poly-HEMA coated 24-well plate. After 30 min at 37 °C, regular culture medium was added on top of the collagen and cells were kept in tissue culture plates for 9 days to allow the development of spheroids.

Spheroid-containing collagen gels were collected either in TRIzol for RNA analysis or with a PFA/Sucrose/OCT protocol for immunofluorescence staining. As a third alternative, cells were seeded in StemXVivo semi-solid medium (CCM012, R&D Systems Europe Ltd, Abingdon, UK) and cultured in poly-HEMA coated 24-well plates in order to grow as tumorspheres.

Submental implantation model

All mouse experiments were approved by the Ethics Committee for Animal Experimentation of Switzerland and performed following institutional guidelines. HNSCC cells were implanted in the neck of NMRI-nu immunodeficient mice (JANVIER LABS, Le Genest-Saint-Isle, France) as previously described [21]. 800,000 cells were resuspended in 30 µl HBSS (14175053, Gibco (Life Technologies Europe BV, Zug, Switzerland)), mixed with 20 µl BD Matrigel (356234, Becton Dickinson, Reinach, Switzerland) and subcutaneously injected along the submental midline of 8-week-old female NMRI-nu mice. Tumor volume was monitored using a Vernier caliper and calculated using the modified ellipsoid formula: Volume = $1/2 * (\text{Length} * (\text{Width})^2)$. Five days after implantation, FOXM1 down-regulation was initiated by providing 1 mg/ml Dox in drinking water containing 2% sucrose (35580.02, Serva (Witec AG, Sursee, Switzerland)). When tumors reached 150 mm^3 they were dissected in pieces that were either collected by snap freezing in liquid nitrogen for downstream RNA extraction or fixed in 10% formalin (HT501128, Sigma-Aldrich Chemie GmbH) for histological evaluation.

RNA-seq and Gene Set Enrichment Analysis (GSEA)

TruSeq stranded RNA library preparation and paired-end 100 bp read sequencing on an Illumina HiSeq 2500 machine were performed at the genomics facility of the University of Lausanne. The ENA study accession number is PRJEB29013. Transcripts were quantified using Salmon (v.0.8.2) [22]. Given the xenogeneic nature of the samples analyzed, the quantification was performed using an artificial hybrid transcriptome generated by merging *Homo sapiens* GRCh38 and *Mus musculus* GRCm38 cDNA sequences to efficiently distinguish reads from human or mouse origin. Downstream analysis was performed in R (https://www.R-project.org) and is described in the supplementary material, Supplementary materials and methods.

Histology, immunohistochemistry and immunofluorescence

Hematoxylin and eosin staining were performed on formalin fixed paraffin embedded (FFPE) tumors or organotypes using standard procedure. Immunohistochemistry staining was performed on FFPE samples, the details of antibodies are listed in supplementary material, Supplementary materials and methods. Involucrin immunofluorescence was performed on PFA-fixed spheroids. Sections were permeabilized for 10 min at room temperature (RT) with 0.1% Triton X-100 then blocked for 30 min in PBS 0.5% BSA containing 1% donkey serum before incubation for 2 h at RT with a 1/200 antibody dilution in 0.5% BSA/PBS. After incubating 1 h at RT with donkey anti-mouse-A488 (A-21202, Life Technologies Europe BV) antibody diluted 1/500 in 0.5%BSA/PBS, sections were mounted in ProLong Gold antifade reagent with DAPI (P-36931, Life Technologies Europe BV) and evaluated by fluorescence microscopy.

Statistics

The experimental results presented in the figures are generally representatives of at least three observations. Unless stated otherwise, the experimental data points in the figures represent the mean from triplicate experiments and the statistical significance of the data was determined using two-tailed Student's *t*-test.

For details of cell culture, 3D cell culture, RNA-seq and Gene Set Enrichment Analysis, chromatin immunoprecipitation, Alamar Blue assays, time-lapse cell tracking, double thymidine blocking, RNA isolation, RT-qPCR, image data analysis, immunohistochemistry and western blotting, please see supplementary material, Supplementary materials and methods.

RESULTS

Impact of FOXM1 knockdown on HNSCC cells in 2D and 3D cultures.

To validate well-known *in vitro* activities of FOXM1 such as regulation of cell proliferation [23], migration and cellular invasion [24], we generated stable doxycycline (Dox)-inducible shFOXM1- and shCtr-expressing CAL27, LAU2083 and LOD148 cell lines (Figure 1A). FOXM1 knockdown using a validated construct [14] induced only slight (CAL27 and LOD148) or no difference (LAU2083) in cell proliferation (Figure 1B). Correspondingly, there were fewer cells in S-phase and slightly more cells in G2-phase upon shRNA-induced FOXM1 knockdown (Figure 1C). Next, we monitored the expression of the cell cycle related genes

GTSE1 and *CCNB2* (a known FOXM1 target) as read-out for the transcriptional activity of FOXM1. *GTSE1* was identified amongst the top-genes that correlated with *FOXM1* expression in the TCGA HNSCC dataset (Spearman's Rho 0.784), and we hypothesized that its promoter is a potential direct target of FOXM1. Using qChIP, we confirmed FOXM1-binding to the *GTSE1* promoter region in CAL27 cells as well as to the *CCNB2* promoter (Figure 1D). However, *GTSE1* expression in FOXM1-depleted cells was only slightly lower than in control cells (Figure 1E), while the effect of FOXM1 downregulation on *CCNB2* expression was more pronounced. Thus, we were unable to clearly confirm that FOXM1 regulates proliferation of HNSCC cells and expression of cell cycle related genes *in vitro*.

There was also no major difference in respect to HGF-stimulated migration of control and FOXM1-depleted cells in a scratch wound healing assay (Figure 2A). Since HGF triggers the transcription of the *FOS* gene [25], an early-response transcription factor that stimulates expression of migration- and invasion-related genes such as *PLAU* (uPA) [26] and *PLAUR* (uPAR) [27], we looked at the expression of *FOS* and its target genes by RT-qPCR in HGF-stimulated cells. The transcription of *FOS* was strongly induced 1 h after HGF stimulation, although it did not show a significant difference between control and FOXM1-depleted cells (Figure 2B). Three hours after HGF stimulation the expression of *PLAUR* in shCtr was slightly upregulated when compared to shFOXM1 cells, demonstrating that HGF-induced FOS activity seems to decline upon FOXM1 knockdown to some extent. Surprisingly, neither *PLAU* transcript levels, nor the abundance of its secreted product (uPA) changed upon FOXM1 knockdown in CAL27 cells (not shown).

To further evaluate the effect of FOXM1 knockdown on invasion, we used 3D organotypic culture (OT-culture) and assessed whether cancer-associated fibroblasts (CAFs), known to secrete HGF [28], would impact the invasive phenotype of the organotypes' epithelial layer in a FOXM1-dependent manner. Indeed, mixing control cells with CAFs and seeding them on top of collagen I gel, resulted in a more invasive phenotype when compared to OT-cultures developed from shFOXM1 cells, as demonstrated by the quantification of budding or detaching cell groups (Figure 2C,D).

Thus, our data demonstrate that shRNA-induced FOXM1 knockdown in HNSCC cells did not significantly impact on their capacity to proliferate and migrate under conditions of 2D culture. However, FOXM1 depletion resulted in a significant inhibition of cellular invasion in organotypic co-cultures with fibroblasts.

FOXM1 knockdown reduces the number of proliferating cells in 3D OT-cultures.

Next, we reasoned that the conditions of OT-culture are more physiological and hence more adequate to address the effect of FOXM1 knockdown. In OT-cultures, both shCtr and shFOXM1 CAL27 cells plated on top of collagen I (this time without CAFs to allow RT-qPCR analysis of cancer cells only) formed multilayered structures that in part resembled squamous epithelium (Figure 3A). Both control and shFOXM1 organotypes demonstrated widespread expression of keratins K14 (a marker of basal keratinocytes) and K8 (a marker of fetal epithelial differentiation in oral mucosa [29] that is aberrantly expressed in HNSCC [10]). In contrast, a slightly stronger expression of involucrin (a marker of squamous differentiation) and cells

positive for keratin K4 (a marker of mucosal differentiation) were observed in shFOXM1 organotypes, while K18 (another marker of fetal epithelial differentiation) was substantially downregulated in FOXM1-depleted organotypes (Figure 3B). This phenotype was associated with upregulation of *IVL* transcripts and its upstream regulator *JUND* [30], as well as with a strong decrease in expression of the FOXM1 target genes *CCNF* [31] and *GTSE1* in shFOXM1 cells (Figure 3C). Although below the standard statistical significance threshold, a trend for upregulation of *IVL* upon FOXM1 knockdown could be confirmed in a large series of OT-cultures (Figure 3D). Importantly, *FOXM1* expression already decreased in shCtr cells upon transfer from 2D to OT-culture (Figure 3E), indicating that *FOXM1* down-regulation is intrinsically associated with the activation of a differentiation program triggered upon 3D conditions of OT-culture. In the 3D context and in contrast to the minimal effect of FOXM1 knockdown on proliferation observed in 2D culture, the association of FOXM1 with a proliferative state was confirmed by Ki-67 IHC staining of shCtr and shFOXM1 OT-cultures. Although at day 8 we observed a comparable number of Ki-67 positive cells mostly enriched at the basal layer (Figure 3F,G), at day 16, Ki-67 positive cells expanded throughout the control OT-culture, but not upon FOXM1 knockdown (Figure 3F,G). Thus, in OT-culture, the level of FOXM1 is decreased upon formation of a multilayered, epithelial-like tissue. Nonetheless, further reduction of FOXM1 expression by shRNA-induced knockdown results in a decreased amount of proliferating cells mostly trapped in the basal layer and facilitates progression towards a more differentiated-like phenotype.

FOXM1 knockdown limits tumor growth and affects tumor phenotype in a xenograft model.

To find out whether effects of inducible FOXM1 knockdown observed in OT-cultures could be reproduced *in vivo*, control and shFOXM1-expressing cells were tested in the submental mouse model of HNSCC. In this model, human HNSCC cells injected into the submental region of immunodeficient mice initiate tumors featured by a squamous differentiation and heterogeneous expression of epithelial differentiation markers such as involucrin, keratins 8, 18, 14 and 4 (supplementary material, Figure S1). In line with the reduced number of proliferating cells seen in OT-cultures, FOXM1 knockdown (induced from day 7 after implantation) significantly slowed down the growth kinetics of xenograft tumors (Figure 4A). In addition, FOXM1 knockdown also affected tumor invasion (Figure 4B). For each sagittal section through the tumor attached to the floor-of-the-mouth (FOM), we selected a series of regions of interest (ROI) and examined the orientation of the tumor border relative to the horizontal axis in these regions (left panel). Upon FOXM1 knockdown, the tumor borders were significantly less invasive, as demonstrated by the quantified reduction in their *dispersion factor* (a measure of the curvature of the border) (right panel). In addition, the ability of FOXM1-depleted cells to form large cohesive cords was significantly reduced.

Histological analysis by H&E staining revealed an accelerated squamous differentiation by FOXM1 knockdown, as evidenced by formation of keratin pearls involving large areas (Figure 4C,D). However, immunostaining-based quantification of slightly upregulated keratin 1 expression (a marker of terminal squamous differentiation) in shFOXM1 tumors did not reach statistical significance due to widespread expression of this marker in well-differentiated shCtr

tumors (Figure 4E,F). In shCtr tumors, multiple cells positive for FOXM1 staining were observed in the less differentiated areas (Figure 4G1) and sometimes located in a cell layer close to tumor stroma (Figure 4G2). Surprisingly, rare single cells (Figure 4G3) or patches of cells (Figure 4G4) showing nuclear expression of FOXM1 were detected in shFOXM1 tumors, indicating an incomplete knockdown in a fraction of cells.

To analyze how FOXM1 knockdown globally affects the cancer cell transcriptome, 11 tumor samples were subjected to transcriptome sequencing (RNA-seq). As xenograft tumors include both human and mouse cells, reads were aligned using an artificially created human-mouse hybrid reference genome to ensure efficient and separate mapping of both human and mouse RNA sequencing reads. Owing to the different degrees of histologically evident squamous differentiation in shCtr samples and, respectively, to the varying level of FOXM1 expression and significant variations in FOXM1 knockdown efficiency in shFOXM1 samples, paired group analysis was not possible. Therefore, we used DEseq2 [32] to perform a correlative analysis of genes that were positively or negatively associated with *FOXM1* expression and used the resulting Wald statistics value (supplementary material, Table S1) as a metric to define a ranked list of genes for downstream gene set enrichment analysis (GSEA). Figure 5A (upper panels) illustrates positive and negative correlations with the level of *FOXM1* expression showing *GTSE1* and *IVL* genes as examples. The inverse correlations between *FOXM1* and differentiation-related genes such as *IVL* and *KRT1* were further confirmed by RT-qPCR in a larger number of RNA samples extracted from shCtr and shFOXM1 CAL27 xenograft tumors (Figure 5B). Notably, our results are in agreement with RNA-seq data from the TCGA HNSCC

dataset (Figure 5A, lower panels). In general, the top of the ranked list of genes was enriched in proliferation gene sets (e.g., G2-M cell cycle and EGFR signaling), while genes at the bottom of the ranked list were enriched in differentiation-related gene sets (Figure 5C, supplementary material, Table S2). Furthermore, clustering analysis using a differentiation-related subset of genes including keratin-coding genes revealed differential expression in our RNA-seq samples (supplementary material, Figure S2) and in the TCGA-HNSCC dataset (supplementary material, Figure S3), which can likely be attributed to deregulated mechanisms of squamous-like differentiation. Interestingly, in the TCGA dataset FOXM1 expression tends to be associated with cancers originating at the base of tongue and tonsils including HPV-related cases. In addition, FOXM1 clustered with simple epithelial-type keratins (K7, K8, K18, and K19) and basal keratinocyte keratin K15, suggesting a possible association of FOXM1 with traits of the fetal differentiation program.Altogether, by analyzing the effects of FOXM1 knockdown in HNSCC xenograft model we concluded that FOXM1 plays a key role in the tightly regulated balance of cancer cell proliferation versus differentiation.

Ectopic FOXM1 reduces expression of differentiation markers in HNSCC cell lines.

Finally, we provide complementary evidence that ectopic FOXM1 overexpression is accompanied by a less differentiated phenotype in a series of four head and neck cancer cell lines (Figure 6). Following immunoblotting in 2D cultures, we confirmed the downregulation of involucrin upon FOXM1 overexpression in LOD148, LAU2083 and LAU2088 cells, whereas keratin 1 was downregulated in LAU2083 and LAU2088 cells (Figure 6A). To further validate

the effect of ectopic FOXM1 on squamous-like differentiation, cells were cultured in 3D, either using collagen embedding or in semi-solid StemXVivo medium. LAU2083 and LAU2088 cells did not grow as spheroids, however CAL27 and LOD148 grew as spheroids (in collagen I gel) or as aggregates of cells (in StemXVivo medium) and confirmed the downregulation of *IVL* expression following FOXM1 overexpression (Figure 6B). Finally, immunostaining confirmed involucrin reduction upon FOXM1 overexpression in CAL27 collagen-embedded spheroids, as demonstrated by the reduced area of involucrin-positive cells within the spheroids (Figure 6C).

DISCUSSION

In this study, we demonstrated that the cell cycle promoting function of FOXM1 is context dependent in HNSCC and only evident under conditions of 3D culture and in tumor xenografts. Although mechanisms of FOXM1 action in the regulation of cell cycle in cancer cells are well explored [4], its role in controlling their cell fate decisions is largely unknown, in particular in SCCs which are characterized by complex patterns of deregulated differentiation states. Our work provides novel insight into the function of FOXM1 in such tumors demonstrating its supporting role in counterbalancing an aberrant squamous differentiation of HNSCC.

Mechanisms that govern normal differentiation towards stratified mucosal epithelia are impaired in HNSCC due to genetic and epigenetic alterations. An aberrant squamous differentiation program is evident in HNSCC by intra- and inter-tumor heterogeneity in morphological appearance paralleled by variable expression of differentiation-specific proteins

such as keratins [10,11]. In this respect, acquisition of the traits of fetal tissues (such as expression of simple epithelial-type keratins 8, 18 and 19) and developmental plasticity could be beneficial for growth and dissemination of cancer cells, as it may confer a survival advantage in a distinct microenvironment and upon anti-cancer treatment. Therefore, unraveling most critical pathways that trigger and control differentiation programs in HNSCC is urgently needed to discover actionable targets and to redirect cancer cell phenotype towards a less aggressive state. Accordingly, targeted induction of terminal differentiation may reduce tumor invasion as has been demonstrated by ectopic overexpression of S100A16, a EF-hand calcium binding protein of the S100 protein family, in HNSCC cell lines [33].

The loss of a malignant phenotype upon the induction of differentiation has been exploited in the past in particular in hematological malignancies and coined differentiation therapy [34]. The most pertinent example is the treatment of acute promyelocytic leukemia (APL) with all-trans retinoic acid (ATRA) which results in a high rate of complete remissions [35]. Differentiation strategies to treat solid cancers are more difficult to realize because of their genetic heterogeneity and dependence on a complex combination of oncogenic pathways [34]. In HNSCC cells, retinoids are effective as anti-proliferative agents [36] that inhibit growth of tumor xenografts [37]. Not surprisingly, the proliferation-inhibitory effect of ATRA and bexarotene (a pan-RXR agonist) is associated with FOXM1 downregulation [38]. How FOXM1 regulates the tightly coordinated balance between proliferation and differentiation of HNSCC cells and whether this mode of action depends on direct or indirect interactions with other transcriptional

regulators of squamous differentiation such as p63 [39] or the ETS2/ELK3-AP1-SOX2-KLF5 network [40] remains to be elucidated.

It is intriguing to speculate that the retained ability to trigger a fetal differentiation program of oral mucosa and eventually acquire a reversible differentiation state may be advantageous to SCCs serving as an alternative to epithelial-to-mesenchymal transition (EMT) for survival. While proliferating cells are more sensitive to genotoxic drugs, differentiation may rescue a fraction of non-dividing, committed cancer stem cells (CSCs) from the treatment, especially if the process can be halted before the cells reach a state of permanent differentiation and reverse back to a proliferative state. In support of this idea, it has been reported that tumor cells re-growing after chemotherapy seem to originate from layers of cells directly surrounding hypoxic well-differentiated areas, which due to a scarce vascularization are insufficiently accessible for cytotoxic drugs [41]. Also, survival of residual cancer cells, which remain after surgery in the tumor surrounding area, may depend on their aptitude to switch reversibly into a dormant state via incomplete squamous differentiation and resume proliferation upon favorable conditions. In this respect, targeting of FOXM1 using specific inhibitors, such as RCM-1 [42], combined with a standard-of-care concurrent chemo-radiotherapy may potentially give an advantageous therapeutic effect.

In summary, FOXM1 being widely expressed in human cancers and in particular HNSCCs emerges as an interesting actionable target. Before that, preclinical investigations will have to address the potential and mechanisms of differentiation-promoting and invasion-inhibiting therapy using FOXM1 inhibitors in HNSCCs.

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Author contributions statement

Conceptualization, CS and GVT. Methodology, VR, AHF, VM, JPR, JH, GVT. Software, VR. Formal analysis, VR, AHF, JPR. Investigation, VR, AHF, VM, JPR, JS, MTT, SFL, ZT, MM. Resources, YM, JH, GVT, CS. Writing – original draft, VR, GVT, CS. Writing – review and editing, VR, MTT, JH, GVT, CS. Supervision, JPR, CS, GVT. Funding acquisition, CS, JH, YM, GVT.

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FIGURE LEGENDS:

Figure 1. shRNA-induced FOXM1 knockdown has minimal effect in 2D cell culture. (A) Inducible FOXM1 protein down-regulation in the presence of doxycycline (Dox) was confirmed by immunoblotting in three HNSCC cell lines. (B) The effect of FOXM1 knockdown on cell growth in all cell lines was very limited as measured by an Alamar Blue assay. Mean± SEM. (C) The cell cycle profile of FOXM1-depleted CAL27 cells following double thymidine block was assessed at the indicated time points. The increased number of cells in G2-phase was the only relevant difference observed in FOXM1-depleted cells. Each point represents an independent measure. (D) FOXM1 binding to *GTSE1* and *CCNB2* promoters was confirmed by qChIP. Efficient binding was observed independently on the presence of Dox or HGF. (E) RT-qPCR analysis confirmed the sustained down-regulation of *FOXM1* throughout cell cycle. While a substantial reduction in *CCNB2* mRNA was observed, *GTSE1* mRNA expression was only slightly reduced in FOXM1-depleted cells. Each point represents one measure from three independent experiments.

Figure 2. shRNA-induced knockdown of FOXM1 impairs invasion in organotypic co-culture with CAFs. (A) Cell motility in presence of HGF was assessed in three cell lines by cellular tracking. The relative speed (distance covered per frame recorded) was independent on FOXM1 knockdown. Each data point represents one tracked cell. (B) RT-qPCR analysis of *FOXM1*, *FOS* and *PLAUR* expression following FOXM1 knockdown and stimulation with HGF (50 ng/ml). While FOXM1 was efficiently downregulated, the reduction in *FOS* and *PLAUR*

transcription was only suggestive of a trend and did not reach statistical significance (Student's *t*-test). Data represent three independent biological replicates. (C) H&E staining of CAL27 organotypic co-cultures with CAFs demonstrating an inhibition of budding as well as detached cellular invasion following FOXM1 knockdown. Scale bar, 100 μm. (D) Quantification of the invasion patterns observed in panel (C) showing a reduction following FOXM1 knockdown. Each point represents one analyzed culture (Student's *t*-test).

Figure 3. shRNA-induced FOXM1 knockdown decreases the number of proliferating cells and facilitates progression towards a more differentiated-like phenotype. (A) H&E staining of CAL27 organotypic cultures demonstrating a squamous-like phenotype after 16 days of culture. Scale bar, 100 μm. (B) Representative pictures of an immunostaining for a series of indicated differentiation markers in CAL27 organotypic cultures at day 16. Scale bar, 100 μm. (C) RT-qPCR analysis of organotypes showing a gradual decrease in *FOXM1* and its targets *CCNF* and *GTSE1* upon FOXM1 knockdown while differentiation-related genes such as *JUND* and *IVL* were upregulated both by FOXM1 knockdown and increased time of culture. Data shown are from one experiment. (D) RT-qPCR analysis of *FOXM1* and *IVL* expression levels in a large series of CAL27 shCtr or shFOXM1 organotypic cultures showing a trend for *IVL* upregulation following FOXM1 knockdown. Each dot represents one organotype. (E) FOXM1 expression was reduced by culturing cells in 3D organotypes as demonstrated by the reduction of *FOXM1* mRNA measured by RT-qPCR. Each data point represents one sample under one

condition or the other. (F) Computer-assisted quantification of the number of Ki-67-positive cells detected by immunohistochemistry in shCtr and FOXM1-depleted organotypes. Each data point represents one analyzed field of the organotype. (G) Examples for the Ki-67 immunohistochemistry data quantified in panel (F). Ki-67-positive cells were detected in the basal layer for both conditions at day 8. In the control, cycling cells were present throughout the organotype at day 16, while they were restricted to the basal layer upon FOXM1 down-regulation. Scale bar, 100 µm.

Figure 4. shRNA-induced FOXM1 knockdown delays tumor growth and promotes a squamous-like phenotype *in vivo*. (A) Tumor growth upon submental implantation of CAL27 cells in nude mice. FOXM1 knockdown resulted in a significant delay in tumor growth (Student's *t*-test) up to day 35. At this point, the sacrifice of four mice with bigger tumors in the shCtr group (ethical endpoint) resulted in the loss of significant difference between groups at day 42. (B) Based on histological evaluation of a series of hotspots randomly selected in xenograft tumors stained by H&E (left panel), the degree of cellular invasion was quantified using Fiji's directionality analysis tool and inferring the curvature of the tumor border with the dispersion index (right panel). Tumor borders from FOXM1-depleted tumors showed less aggressive invasion patterns (Student's *t*-test). Data points represent the mean dispersion value in each animal. (C) Tumor-bearing FOM were stained by H&E to examine the extent of squamous differentiation. One example H&E staining is shown for each condition. (D) Boxplot showing the

mean differentiated area in tumors grown from shCtr or shFOXM1 cells. The extent of differentiation in tumors increased significantly upon FOXM1 knockdown (Student's *t*-test). Each point represents one tumor. (E) Quantification of keratin 1 immunohistochemistry staining performed on shCtr and shFOXM1 tumor confirming a trend for keratin 1 upregulation upon FOXM1 knockdown. Each point represents one tumor. (F) One example of keratin 1 staining for each group. Scale bar, 100 μm. (G) FOXM1 immunohistochemistry was performed on shCtr and FOXM1-depleted tumors. Scale bar, 50 μm.

Figure 5. Differentiation markers are inversely correlated with FOXM1 *in vivo*. (A) Xenograft tumors analyzed by RNA-seq showed that *FOXM1* expression (measured by logarithmic read counts) was positively correlated with *GTSE1* expression, while it was inversely correlated with *IVL* expression (top). These correlations were validated in the TCGA HNSCC dataset (bottom). (B) RT-qPCR analysis confirming the inverse correlation of *FOXM1* expression with the differentiation markers *IVL* (top) and *KRT1* (bottom) in a larger group of xenograft tumors. (C) GSEA plots showing that a significant cluster of genes positively correlated with *FOXM1* expression (based on RNA-seq analysis) was listed in the Kobayashi EGFR signaling gene set (left), while another significant cluster of genes negatively correlated with *FOXM1* was listed in Rickman's well-differentiated tumor gene set (right).

Figure 6. Overexpression of FOXM1 impairs differentiation in spheroid cultures of HNSCC cells. (A) Immunoblotting analysis of differentiation markers upon ectopic FOXM1 overexpression in a series of HNSCC cell lines. (B) RT-qPCR analysis of *FOXM1* (left panels) and *IVL* (right panels) mRNA expression in 3D spheroids (in collagen I gel) and tumorsphere cultures (in StemXVivo medium) upon FOXM1 overexpression. Each point represents one culture. (C) (left panel) Immunostaining analysis of control (mCherry) or FOXM1-overexpressing CAL27 spheroids confirming the reduction of involucrin-positive cells within spheroids following FOXM1 overexpression. Scale bar, 200 μm. (right panel) Quantification data. Each point represents one spheroid. FOXM1: FOXM1 overexpression. mCh: mCherry control vector.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. Differentiation markers in xenograft tumors.

Figure S2. Heatmap clustering of differentiation markers in RNA-seq data from xenograft tumors.

Figure S3. Heatmap clustering of differentiation markers in the TCGA HNSCC dataset.

Table S1. List of genes and their associated correlation factor with *FOXM1* as calculated by DEseq2.

Table S2. GSEA report for gene sets correlating with *FOXM1* expression.

A

Speed (Relative Units)

CAL27   LAU2083   LOD148

shCtr shFOXM1   shCtr shFOXM1   shCtr shFOXM1

B

□ shCtr
■ shFOXM1

FOXM1 $P = 10^{-5}$

Fold-change (versus shCtr)

FOS $P = 0.43$

Fold-change (versus shCtr at t0)

HGF Stimulation (1 h)

PLAUR $P = 0.07$

Fold-change (versus shCtr at t0)

0 h   1 h   3 h

HGF Stimulation

C

CAL27-shCtr + CAF142   CAL27-shFOXM1 + CAF142

detached   budding

D

budding   detached

Number of invading structures per field

p=0.047   p=0.053

shCtr shFOXM1   shCtr shFOXM1

A

shCtr | shFOXM1

day 8 | day 16 | day 8 | day 16

B

Keratin 18 | Keratin 8 | Keratin 14 | Keratin 4 | Involucrin

shCtr

shFOXM1

C

Organotypic culture

- ☐ shCtr day 8
- ☐ shFOXM1 day 8
- ☐ shCtr day 16
- ☐ shFOXM1 day16

Fold-change

FOXM1 CCNF GTSE1 JUND IVL

D

FOXM1

Fold-change (versus shCtr)

shCtr   shFOXM1

IVL

p=0.1513

shCtr   shFOXM1

E

2D | 3D

FOXM1 Fold-change (versus shCtr in 2D)

p=0.0035

p=0.0162

shCtr   shFOXM1   shCtr   shFOXM1

F

total Ki-67 positive cells per frame

● shCtr
■ sh-FOXM1

p=0.359

p=0.004

Day 8   Day 16

G

Day 8 | Day 16 | Day 8 | Day 16

Ki-67

shCtr

Ki-67

shFOXM1

A

Tumor volume (mm³) mean +/- SEM

CAL27 shCtr
CAL27 shFOXM1

Dox

Days post cell implantation

n=13
n=17
n=19
n=19 * *
* n=19
n=19
n=20
n=20
n=19
n=19

B

m751 m800

shCtr shFOXM1

Dispersion

p=0.01

shCtr shFOXM1

C

shCtr shFOXM1

Tongue Tongue

2 mm 2 mm

Tumor Tumor

m754 m803

200 µm 200 µm

D

Differentiated area (%)

p=0.01

shCtr shFOXM1

E

K1 positive area (%)

p=0.34

shCtr shFOXM1

F

shCtr shFOXM1

m754 m803

Keratin 1

G

CAL27 shCtr CAL27 shFOXM1

1 m317 2 m328 3 m324 4 m335

FOXM1

#

A

CAL27 · LOD148 · LAU2083 · LAU2088

mCh / FOXM1 (for each cell line)

kDa

130 — / 100 — — FOXM1

130 — / 100 — — Involucrin

70 — / 55 — — Keratin 1

40 — / 35 — — GAPDH

B

CAL27 — FOXM1 fold-change (versus mCh control cells): Collagen, StemXVivo (mCh, FOXM1)

CAL27 — IVL fold-change (versus mCh control cells): Collagen, StemXVivo (mCh, FOXM1)

LOD148 — FOXM1 fold-change (versus mCh control cells): Collagen, StemXVivo (mCh, FOXM1)

LOD148 — IVL fold-change (versus mCh control cells): Collagen, StemXVivo (mCh, FOXM1)

C

CAL27-mCherry · CAL27-FOXM1

DAPI
Involucrin

p=0.05

Involucrin area cells (%)

mCh · FOXM1