INTRODUCTION

With a large proportion of oral squamous cell carcinomas (OSCC), tumors of the head and neck region are among the most common tumor diseases worldwide. The prognosis of patients with advanced tumor disease has only slightly improved in recent decades (Bray et al., 2018).

Tumor progression, including increasing tumor invasiveness and metastatic spread, is a complex process regulated by direct tumor cell changes and by interactions between the tumor cells and the tumor...
To detach from their surroundings, tumor cells must lose their cell polarity and acquire migratory abilities in an epithelial–mesenchymal transition (EMT) process (Stone et al., 2016). Tumor cells with EMT characteristics have been observed on the invasive front—a region rich in stromal transforming growth factor-β (TGF-β) and other cytokines (e.g., TNFα, EGF, IGF-1) that cooperate in EMT induction (Katz et al., 2013). The TGF-β signaling pathway is the major pathway known to promote tumor cell invasiveness and metastasis (Liao et al., 2019). Little is known about OSCC-specific biomarkers relevant for tumor progression. Their identification might allow more accurate patient’s overall estimated prognosis and foster novel targeted tumor therapy (Marcu et al., 2018).

To identify new genes involved in OSCC progression, we treated the human OSCC cell line UPCI-SCC-040 in vitro with TGF-β1 and performed transcriptome analysis. Compared with untreated controls, many DEGs were found that could play a crucial role in OSCC progression. To validate the impact of some selected DEGs on patient prognosis, we evaluated their corresponding protein levels by immunohistochemistry using a tissue microarray (TMA) generated from the tissue samples of 39 OSCC patients, and we correlated the expression profiles with disease-free survival (DFS) as the primary clinical endpoint. We identified stearoyl-CoA desaturase-1 and sclerostin as independent prognostic factors in OSCC.

2.1 | Cell culture

The human OSCC cell line UPCI-SCC-040 was obtained from DSMZ. Cells were cultured in MEM with Earle’s salts, 2.2 g/L NaHCO₃, stable glutamine, low-endotoxin medium (Merck) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom), 1% non-essential amino acids (NEAA) (PAN Biotech), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated in a humidified chamber with 5% CO₂ at 37°C.

2.2 | TGF-β1 treatment

Cells were treated with 5 or 10 ng/ml TGF-β1 (PeproTech) and seeded onto 6-well plates (Greiner Cellstar) at 200,000 cells/well in 2 ml of medium. After 24 hr, medium was removed, cells were washed with 1× Dulbecco’s phosphate-buffered saline (DPBS) (PAN Biotech), and fresh culture medium without FBS and penicillin/streptomycin was added. After 2 hr of starvation, control cells (no TGF-β1 treatment) were collected and frozen. Other cells were treated with TGF-β1 in culture medium without FBS and penicillin/streptomycin for 48 or 72 hr.

2.3 | Sandwich ELISA

Concentrations of MMP-9 in the supernatants were measured at dilution 1:100 using commercial DuoSet ELISA kits (R&D systems) according to the manufacturer’s instructions.

2.4 | Migration assay

UPCI-SCC-040 cells (5x10⁴) were seeded in 96-well plates, and when confluency was reached, a scratch was made using a toothpick. After detached cells were washed away, the wounded cells were incubated with or without TGF-β1, and images were acquired at immediately (0 hr) or after 15 hr. The distance between the two sides of the scratch was measured at multiple points along the scratch using the ImagePro plus 4.5 software (Media Cybernetics). The average length after 15 hr was subtracted from the average length at 0 hr to determine the length to which the cells migrated.

2.5 | Proliferation assay

Tumor cells (5x10⁴ cells) were seeded in 96-well plates, and after overnight incubation, the medium was replaced with 0.5% FCS-supplemented medium and incubated with or without TGF-β1 (10 ng/ml) for 48 hr. The CCK-8 solution (Sigma-Aldrich) was then added to the wells according to the manufacturer’s instructions, and after 2 hr, the absorbance at 450 nm with 540 nm reference was read.

2.6 | Western Blot analysis (WB)

UPCI-SCC-040 cells lysates were prepared in RIPA buffer, and equal amounts of total protein (15 μg/lane) were loaded onto 12% SDS-PAGE and separated by electrophoresis. Proteins were transferred onto a nitrocellulose membrane and after blocking in AdvanBlock-Chemi (R-03726-E10, Advansta), and the membrane was incubated with anti-E-cadherin, anti-vimentin, or anti-β-actin primary antibodies (Abcam) diluted 1:1,000 for 1 hr, then washed three times in 1X TBS with 0.05% Tween-20 and incubated with HRP-conjugated secondary antibody or 1 hr. After additional three washes, the membrane was incubated with Western Bright ECL-HRP substrate (K-12045, Advansta), and the membrane was incubated with HRP-conjugated secondary antibody or 1 hr. The membrane was stripped and re-probed with the next antibody.

2.7 | Immunofluorescence (IF)

Tumor cells (2x10⁵ cells) were seeded on sterile round cover slips, placed in a 24-well plate, and treated with or without TGF-β1 (10 ng/ml) for 48 hr. The cells were then fixed with 300 μl of ice-cold methanol for 5 min. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min, and incubated in blocking solution (2% Normal Donkey Serum, 0.1% Triton X-100 in PBS) for 1 hr at room temperature. Then, cells were incubated for another 1 hr at room temperature with the primary antibodies (rabbit anti-human vimentin or E-cadherin, Abcam) diluted 1:500 in blocking solution. After three washes in PBS, cells were incubated with the secondary antibody (Alexa fluor®-555-conjugated donkey anti-rabbit IgG, Jackson Immuno-Research labs)
diluted 1:1,000 in Blocking Solution for 1 hr. Then, cells were washed three time with PBS for 5 min, incubated in DAPI (300 nM) at room temperature for 5 min, and washed again. Cover slips were mounted on slides with Fluoromount-G, and images were acquired by upright fluorescent trinocular microscope (Olympus BX-60) using the MS60 camera and the MShot Image Analysis System V1 (MSHOT).

### 2.8 | RNA isolation

RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA concentration was measured using a NanoDrop spectrophotometer. Samples containing 1 µg of total RNA were subjected to quality control and sequencing.

### 2.9 | RNA-seq library preparation and transcriptome analysis

RNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs). Libraries were pooled and sequenced on a HiSeq 4,000 sequencer (Illumina) to generate 50 base pair single-end reads.

### 2.10 | Raw read and quality check

Sequenced images were transformed with Illumina BaseCaller to BCL files, which were demultiplexed to fastq files with bcl2fastq. The sequencing quality was asserted using FastQC (Andrews, 2015).

### 2.11 | Mapping and read quantification

Sequences were aligned to the reference genome *Homo sapiens* using STAR aligner (Dobin et al., 2013). Subsequent read counting was performed using featureCounts (Liao et al., 2014). Read counts were analyzed in the R/Bioconductor environment using the DESeq2 R package (Love et al., 2014, p. 2). Candidate genes were filtered using an absolute log2 fold change >1 and FDR-corrected p-value <.05. Only the corresponding proteins of the most relevant genes (Figure 3b) with an absolute log2 fold change >3.55/<−3.55 and a p-value <.05 were considered for further evaluation. Gene annotation was performed using biomaRt R (Durinck et al., 2009). Data are available at GEO under ID GSE16324.

### 2.12 | Patients, tissue microarray (TMA) generation, and immunohistochemistry

Tissue samples of 39 OSCC patients treated primarily by surgery between 2003 and 2007 were used in TMA generation and immunohistochemical evaluation. The patient clinical characteristics can be found in Table 1. For evaluation of clinical baseline characteristics, stages T1 and T2, as well as T3 and T4, were combined into one group. Moreover, AJCC stages 1 and 2, as well as AJCC stages 3 and 4, were combined into one group. For nodal status analysis, patients were divided into positive and negative lymph node groups. DFS was the primary clinical endpoint, defined as the time interval from R0 tumor resection until local recurrence, LNM, or distant metastasis in months. Patients provided written informed consent before participating in the trial. The study was approved by the ethics committee of our institution (vote no. 07/06/09, updated in April 2018).
Tissue samples were obtained immediately after tumor resection, fixed in neutrally buffered 4% formalin, and embedded in paraffin. TMA was constructed, using the Arraymold TMA kit (60 cores with a diameter of 2 mm). Tissue samples of 26 primary OSCCs, 24 LNM, and 10 OM (internal controls) were used in TMA preparation (Figure S1). Immunohistochemical reactions (Table S1) were performed on 2-µm sections of the TMA using a fully automated slide stainer (Agilent Technologies).

### Table 1: Patients’ clinical characteristics

| Sex     | Age | Localization of primary OSCC | pT   | pN   | pM   | Grading | AJCC Stage | Disease-free survival (months) |
|---------|-----|-------------------------------|------|------|------|---------|------------|-------------------------------|
| Male    | 62  | Floor of mouth                | pT3  | pN0  | pM0  | G2      | III       | 49                            |
| Female  | 62  | Tongue                        | pT4  | pN1  | pM0  | G2      | IV        | 11                            |
| Female  | 46  | Floor of mouth                | pT4  | pN2  | pM0  | G2      | IV        | 7                            |
| Male    | 48  | Floor of mouth                | pT4  | pN2  | pM0  | G2      | IV        | 18                            |
| Female  | 71  | Cheek mucosa                  | pT2  | pN0  | pM0  | G2      |  II       | 69                            |
| Female  | 50  | Gum                           | pT4  | pN0  | pM0  | G2      | IV        | 8                            |
| Female  | 55  | Floor of mouth                | pT2  | pN0  | pM0  | G2      |  II       | 36                            |
| Male    | 57  | Gum                           | pT4  | pN2  | pM0  | G2      | IV        | 30                            |
| Male    | 61  | Gum                           | pT4  | pN0  | pM0  | G1      | IV        | 47                            |
| Female  | 70  | Cheek mucosa                  | pT2  | pN0  | pM0  | G2      |  II       | 6                            |
| Male    | 46  | Tongue                        | pT4  | pN0  | pM0  | G2      | IV        | 73                            |
| Male    | 64  | Tongue                        | pT2  | pN2  | pM0  | G2      | IV        | 15                            |
| Female  | 66  | Tongue                        | pT4  | pN2  | pM1  | G2      | IV        | 8                            |
| Male    | 68  | Floor of mouth                | pT3  | pN0  | pM0  | G2      | III       | 24                            |
| Male    | 74  | Gum                           | pT4  | pN1  | pM0  | G2      | IV        | 11                            |
| Male    | 72  | Gum                           | pT4  | pN1  | pM0  | G2      | IV        | 27                            |
| Male    | 50  | Floor of mouth                | pT4  | pN1  | pM0  | G2      | IV        | 7                            |
| Female  | 82  | Gum                           | pT4  | pN0  | pM0  | G2      | IV        | 2                            |
| Male    | 38  | Floor of mouth                | pT4  | pN0  | pM0  | G3      | IV        | 163                           |
| Female  | 62  | Gum                           | pT4  | pN0  | pM0  | G2      | IV        | 3                            |
| Male    | 60  | Cheek mucosa                  | pT2  | pN2  | pM0  | G2      | IV        | 122                           |
| Male    | 72  | Gum                           | pT2  | pN2  | pM0  | G2      | IV        | 50                            |
| Female  | 65  | Tongue                        | pT2  | pN2  | pM0  | G1      | IV        | 179                           |
| Male    | 79  | Gum                           | pT1  | pN1  | pM0  | G2      | III       | 5                            |
| Male    | 72  | Floor of mouth                | pT4  | pN2  | pM0  | G2      | IV        | 15                            |
| Female  | 71  | Gum                           | pT4  | pN0  | pM0  | G1      | IV        | 8                            |
| Female  | 63  | Floor of mouth                | pTis | pN0  | pM0  | G1      |  I        | 27                            |
| Male    | 78  | Tongue                        | pT2  | pN0  | pM0  | G2      |  II       | 95                            |
| Male    | 61  | Oropharynx                    | pT2  | pN0  | pM0  | G2      |  II       | 30                            |
| Male    | 61  | Floor of mouth                | pT4  | pN2  | pM0  | G2      | IV        | 12                            |
| Male    | 51  | Gum                           | pT4  | pN1  | pM0  | G2      | IV        | 8                            |
| Male    | 50  | Gum                           | pT4  | pN2  | pM0  | G3      | IV        | 49                            |
| Female  | 79  | Gum                           | pT4  | pN0  | pM0  | G2      | IV        | 50                            |
| Male    | 67  | Palate                        | pT4  | pN1  | pM0  | G2      | IV        | 68                            |
| Male    | 40  | Palate                        | pT4  | pN1  | pM0  | G2      | IV        | 1                            |
| Female  | 62  | Gum                           | pT4  | pN0  | pM0  | G2      | IV        | 19                            |
| Male    | 32  | Tongue                        | pT2  | pN0  | pM0  | G2      |  II       | 8                            |
| Male    | 45  | Floor of mouth                | pT4  | pN2  | pM0  | G2      | IV        | 5                            |
to identify tissue cores, and the resulting TMA grids were verified manually and changed if necessary. Spot vector and background estimates were used to improve stain separation within the software using color deconvolution (Ruifrok & Johnston, 2001). Automated cell and cell membrane detection was used to identify cells across all TMA cores based on cellular and membranous staining. These procedures additionally estimate the full extent of each cell based on constrained expansion of the nucleus region and calculate up to 33 measurements of intensity and morphology, including nucleus area, circularity, staining intensity for hematoxylin and DAB, and nucleus/cell area ratio. Because not all of these measurements are expected to supply independent or useful information with respect to cell classification, a subset of 16 measurements was chosen empirically and supplemented for each cell by measuring the local density of cells and calculating a Gaussian-weighted sum of the corresponding measurements within neighboring cells using QuPath’s add smoothed features command. A two-way random trees classifier was trained within the software to distinguish OSCC cells from other cell or tissue types. Intensity thresholds were set to further subclassify tumor cells with negative, weak, moderate, or strongly positive staining based on mean DAB optical densities. An H-score was calculated for each tissue core by adding 3x% strongly stained tumor cells, 2x% moderately stained tumor cells, and 1x% weakly stained tumor cells (Goulding et al., 1995), yielding results in the range of 0 (all tumor cells negative) to 300 (all tumor cells strongly positive). The results of the semiautomated immunohistochemical evaluation were randomized and checked by a blinded pathologist trained to evaluate tissue sections.

2.14 | Statistics

The in vitro experiments are presented as means ± SEM (standard error of means) and analyzed with two-tailed unpaired student’s t test. The distribution patterns of biomarkers over different tissue types were compared based on the H-score values using the Mann–Whitney U test and are presented using box plots. Missing H-score values were imputed by multiple imputations. For further survival analysis, H-score values were divided into high expression and low expression groups based on the median value of each protein. The influence of each individual biomarker and of the clinical baseline characteristics on the DFS was investigated using CR analysis (Gill, 1982). The strength of the influence was described by the hazard ratio (with

**FIGURE 2** TGF-β1 enhances the EMT process and metastasis. UPCI-SCC-040 cells (5 x 10^4 or 2 x 10^5 cells) were incubated with or without TGF-β1 for 48 hr, and the expression of the EMT marker E-cadherin and vimentin was evaluated with (a, b) immunofluorescence (n = 7) and (c) Western blot analysis. Additional properties that support the effect of TGF-β1 on metastasis, such as (d, e) migration (n = 5,7), (f) secretion of MMP-9 (n = 5), and (g) proliferation (n = 9), were also assessed.
an additional 95% confidence interval). Analyses were first performed in a univariate manner, that is, separately for each factor. Significant factors were further combined in a multiple CR analysis model. The influence of factors significantly correlated with DFS was shown using Kaplan–Meier survival curves. Differences between survival curves were investigated by the log-rank test. To investigate the association between the expression of different marker proteins and the occurrence of LNM in OSCC patients, Spearman’s rank correlation was performed using the respective grouped H-score values (above or below the median) and the pN status (LNM present or not). All of the tests were performed at a significance level of $\alpha = 5\%$ using Wizard Pro statistical software (version 1.9.41, by Evan Miller, Chicago, IL, USA) and SPSS (version 24, IBM). Graphs were created with GraphPad Prism software (version 8, GraphPad Software).

3 | RESULTS

3.1 | Confirmation that TGF-$\beta$1 induces EMT to acquire metastatic features

To demonstrate that TGF-$\beta$1 promotes EMT in UPCI-SCC-040 cells, and therefore mimic in vitro the malignant transformation of OSCC, we examined several properties of the cells after treatment. First, we observed that TGF-$\beta$1 treatment reduced E-cadherin expression and elevated vimentin expression (Figure 2a–c), typical marker of EMT. Secondly, using the scratch assay, we demonstrated that UPCI-SCC-040 cells could migrate to longer distances after TGF-$\beta$1 treatment (Figure 2d–e). This increase in migration was accompanied by an elevated level of MMP-9 that was secreted from the cells (Figure 2f). This enzyme is associated with degradation of the ECM and can serve as another marker of EMT. Finally, we could show that the TGF-$\beta$1 treatment enhanced cell proliferation (Figure 2g). Collectively, these results confirm that TGF-$\beta$1 promotes EMT and enhances the metastatic potential of these cells.

3.2 | Sequence annotation and identification of differentially expressed genes (DEGs)

Gene counts from individual samples were normalized, and two distinct plots were created: (1) a principal component analysis (PCA) plot (Figure 3a); and (2) a dendrogram clustering plot (Figure 3b), based on simple hierarchical clustering. Both plots show similar patterns of the control sample grouping among themselves and separate from the treated samples, whereas the 48 hr and 72 hr TGF-$\beta$1 treatment samples cluster together and are not distinguishable based on their different time points.

For the 3 sample groups (controls, 48 hr and 72 hr of TGF-$\beta$1 treatment), differential expression analyses were run as pairwise tests, resulting in 3 distinct differential expression tests: (1) control versus 48 hr TGF-$\beta$1; (2) control versus 72 hr TGF-$\beta$1; and (3) 48 hr TGF-$\beta$1 versus 72 hr TGF-$\beta$1. For each differential expression test, differentially expressed genes were considered those with adjusted $p$-values less than or equal to .05 and an absolute log2 fold change greater than or equal to 1.

Test (1) (control versus 48 hr TGF-$\beta$1) resulted in 2,203 DEGs genes, test (2) (control versus 72 hr TGF-$\beta$1) in 3,036 DEGs genes, and test (3) (48 hr TGF-$\beta$1 versus 72 hr TGF-$\beta$1) in 26 DEGs genes. As such, this analysis confirmed the assumption from the clustering plots that tests (1) and (2) might result in more DEGs genes than test (3). To determine how many DEGs genes were overlapping between the tests, the online tool Venny (Oliveros, J.C. (2007–2015), an interactive tool for comparing lists with Venn’s diagrams. https://

3.3 | Figure 3 The principal component analysis (PCA) plot reduces the multi-dimensional gene data to 2-dimensional representations of the analyzed samples. Control samples group among themselves and separate from the other samples (48 hr TGF-$\beta$1 and 72 hr TGF-$\beta$1), while 48 hr TGF-$\beta$1 and 72 hr TGF-$\beta$1 samples cluster together and are not distinguishable based on their different time points (a). Cluster dendrogram plot. Control samples group among themselves and separate from the other samples (48 hr TGF-$\beta$1 and 72 hr TGF-$\beta$1), while 48 hr TGF-$\beta$1 and 72 hr TGF-$\beta$1 samples cluster together and are not distinguishable based on their different time points (b)
bioinfogp.cnbc.csic.es/tools/venny/index.html), was used by overlapping the ENSEMBL gene IDs from the DEGs genes, resulting in most DEGs genes (52.6%) overlapping between tests (1) and (2), while only a few overlapped between test (3) and the other tests, indicating that of the most DEGs genes occurred between the controls and TGF-β1, regardless of the different time points (Figure 1a).

To reduce the number of DEGs genes and focus only on the most DEGs ones, differential expression analysis was repeated using more stringent thresholds of adjusted p-values < .0001 and an absolute log2 fold change > 3.55. For this new threshold, only 9 genes were found to be DEGs, and their normalized expression levels were plotted in a heatmap (Figure 1b), showing the clear differences in expression levels between the controls and TGF-β1-treated samples regardless of their time points. Antibodies were available for only 6 of the 9 identified genes (SCD1, TNFSF9, SOST, ID3, HIST3H2BB, and MYOM3) so that only these genes could be examined in the further immunohistochemical evaluation.

3.3 | Biomarker expression in different tissue types

3.3.1 | Stearoyl-CoA desaturase-1 (SCD1 gene)

Cytoplasmic expression (Figure 4) of stearoyl-CoA desaturase-1 could be demonstrated immunohistochemically in normal oral mucosa (OM) [mean H-score 12.49 ± 13.16 SD], primary OSCCs [mean H-score 49.49 ± 14.81 SD], and lymph node metastases (LNMs) of patients [mean H-score 37.53 ± 13.88]. We noted significantly higher stearoyl-CoA desaturase-1 expression in primary OSCC than in OM (p < .01) and significantly higher expression in LNM than in OM (p = .02). Stearoyl-CoA desaturase-1 expression in the OSCCs was slightly increased compared with that of LNM, but the difference was not statistically significant (Figure 4).

3.3.2 | CD137L (TNFSF9 gene, 4-1BBL)

Both cytoplasmic and membranous CD137L expressions were detected by immunohistochemical staining in all tissue types (OM, OSCC, and LNM). In addition, strong membrane expression in the invasive front was observed in the cells adjacent to the TME (Figure 4). The H-score values between the individual tissue types were OM [mean H-score 47.14 ± 57.34 SD], primary OSCC [mean H-score 70.46 ± 32.60 SD] and LNM [mean H-score 38.91 ± 17.15]. These values showed no significant difference in expression at the protein level (all p-values > .05) (Figure 4).

3.3.3 | Sclerostin (SOST gene)

Cytoplasmic sclerostin expression was found in all tissue types (Figure 4). However, the overall expression was found at low levels,
with OM [mean H-score 4.41 ± 6.02 SD], OSCC [mean H-score 18.02 ± 12.28 SD], and LNM [mean H-score 11.27 ± 7.08 SD]. No significant differences in expression were noted among all of the tissue types (all p-values >.05) (Figure 4) at the protein level.

### 3.3.4 DNA-binding protein inhibitor ID-3 (ID3 gene)

We detected high cytoplasmic DNA-binding protein inhibitor ID-3 expression in all tissue types. In addition, the adjacent cells of the TME showed high expression of ID3 (Figure 4). Significantly increased expression was observed in the OSCCs [mean H-score 102.58 ± 26.77 SD] compared with the OM [mean H-score 52.43 ± 28.80 SD] (p = .03) and in the LNM [mean H-score 99.42 ± 19.49 SD] compared with the OM (p = .01). The difference between OSCC and LNM was not significant (Figure 4).

### 3.3.5 Histone H2B type 3-B (HIST3H2BB gene)

For histone H2B type 3-B, nuclear expression could be observed in OM [mean H-score 33.79 ± 32.16 SD], primary OSCC [mean H-score 33.77 ± 19.58 SD], and LNM [mean H-score 29.56 ± 10.41 SD] (Figure 4). No significant differences in the expression among all of the tissue types were observed (all p-values >.05) (Figure 4).

### 3.3.6 Myomesin 3 (MYOM3 gene)

The cytoplasmic myomesin 3 expression in OM [mean H-score 8.89 ± 14.82 SD], OSCC [mean H-score 4.58 ± 5.01 SD] and LNM [mean H-score 1.08 ± 0.78 SD] was at the lower detection limit, but the cells adjacent to the TME showed a higher degree of expression (Figure 4). We noted a slightly higher myomesin 3 expression in OSCC than in LNM (p = .04) (Figure 4).

### 3.4 Association between protein expression, disease-free survival (DFS) and LNM

H-score values of the individual biomarkers were divided into low expression and high expression groups based on the median value (indicated in the graphs, Figure 5). These group values were
the MYOM3 expression and the presence of LNM (Spearman’s correlation coefficient $\rho = -0.361$ and $p = .022$, respectively). No significant correlation between the rest of the marker proteins (Sclerostin, CD137L, ID3, and HIST3) and the occurrence of LNM was found ($p$-values $>.05$, respectively).

### DISCUSSION

Oral carcinogenesis and progression are complex processes involving a variety of phenotypic and genotypic changes (Santosh et al., 2016). OSCC differs from other tumors of the head and neck region (Anderson & Slotkin, 1975; Vossen et al., 2018).

Metastatic spread accounts for the majority of cancer-related deaths despite treatment (Nagafuchi et al., 1987). To invasively migrate and metastasize, tumor cells must lose their cell polarity and break down the intercellular connections that are mainly established by E-cadherin. E-cadherin downregulation has been associated with a transition to the mesenchymal differentiation program (Roche, 2018; Vleminckx et al., 1991). The EMT process is crucial to increasing invasiveness and metastasis (Tracz-Gaszewska & Dobrzyn, 2019).

In this study, we identified changes in gene expression by treating the human OSCC cell line UPCI-SCC-040 with TGF-$\beta_1$ in vitro, and then, we validated the most important genes in human OM, OSCC, and LNM tissue samples. Furthermore, we translated
SCD1 expression is significantly increased in various human tumor cells (Holder et al., 2013; Makar et al., 1975; Ran et al., 2018; Wang et al., 2016), and in this investigation, we also demonstrated a significant SCD1 increase in OSCC and LNM compared with healthy OM controls. SCD1 is an important component of fatty acids (FA) de novo biosynthesis, which catalyzes the conversion of saturated fatty acids (SFAs) into \( \Delta^9 \)-monounsaturated fatty acids (MUFAs), and higher MUFAs levels have been found to modulate tumorigenic pathways (Scott et al., 2012). It has been shown that SCD1 promotes migration and invasion through accumulation of MUFAs (Ma et al., 2017). Inhibition of de novo FA synthesis in Src-transformed NIH/3T3 mouse embryo fibroblasts suppressed the formation of invadopodia, which are the membrane structures needed in the process of cell migration. However, this effect was reduced by the addition of oleic acid, the main product of SCD1 activity (Angelucci et al., 2018). Blockade of SCD1 activity with A939572 suppressed the migration and invasiveness of HCC cells (Mauvoisin et al., 2013), whereas oleic acid restored the original ability to migrate (Wheelock & Johnson, 2003a). SCD1 has been previously proposed as a therapeutic target in the treatment of metastatic breast cancer (Abraham et al., 2018).

One mechanism in which SCD1 might be involved in inducing EMT is the dysregulation of the \( \beta \)-catenin pathway (Michiels et al., 2017), which is a component of the adherent junctions and interacts with E-cadherin (Wheelock & Johnson, 2003b). SCD1-dependent degradation of \( \beta \)-catenin occurs in a manner involving glycogen synthase kinase 3b (GSK3b) (Tracz-Gaszewska & Dobrzyn, 2019).

Little is known about SCD1 function during OSCC carcinogenesis and progression. Abraham et al. examined SCD1 expression immunohistochemically in 15 patient tissue samples from normal oral mucosa (NOM), 20 samples from oral epithelial dysplasia (OED), and 20 samples from OSCC, and they correlated it with the overall survival rates (OS) of the patients. The authors reported reduced OS rates with increased SCD1 expression in the OSCC tissue samples (Liao et al., 2014).

Similar to the study described above, we here identified SCD1 as an independent prognostic factor in OSCC. SCD1 showed the highest impact in transcriptome analysis and in immunohistochemical validation and was significantly correlated with the DFS of patients. However, contrary to Abraham et al., patients with high SCD1 expression in OSCC and LNM tissue samples showed longer DFS rates than those with low expression, indicating better prognosis in these patients.

Local jaw bone invasion is a crucial step during OSCC progression, and it correlates with increased rates of recurrence and lower OS (Cannonier et al., 2016). Evidence suggests that tumor cells can communicate with osteocytes to create a microenvironment that promotes bone destruction and invasion (Atkinson & Delgado-Calle, 2019).

The Wnt signaling pathway inhibitor sclerostin, secreted by osteocytes, plays a crucial role during osteoblast activation (Zhu et al., 2017). Sclerostin has been shown to promote the migration, invasion, and bone osteolysis of breast cancer cells (Zhu et al., 2017), and pharmacological inhibition could serve as an effective strategy against bone metastases (Hesse et al., 2019). In the present investigation, we found significant upregulation of the SOST gene after TGF-\( \beta \) treatment and a significant influence of sclerostin expression on DFS in the CR analysis. In an established model of Down syndrome-dependent bone deficiency, Tamplen and colleagues treated mice with an anti-sclerostin antibody and could show that this treatment led to an increase in mandibular bone mass (Tamplen et al., 2019).

Contrary to the results of the presented studies, in which sclerostin expression was associated with a tumor-promoting effect, patients with high sclerostin expression showed a longer DFS than those with low sclerostin expression in the current investigation. However, sclerostin appears to be an interesting target during locally bone invasive growing OSCC and should be considered in more in-depth studies.

Interactions between CD137 and its ligand CD137L are mainly involved in cytotoxic T-cell activation (Wang et al., 2008) which can trigger the immune response against solid tumors (Glorieux & Huang, 2019). Moreover, this anti-cancer effect is based on the release of large amounts of IFN-\( \gamma \) (Vinay & Kwon, 2014). Little is known about CD137/CD137L expression in different types of tumor cells (Glorieux & Huang, 2019). It has been shown that CD137L is associated with patient prognosis and CD8\(^{+}\) T-cell infiltration levels in pancreatic cancer, and it likely contributes to modulating the M1 polarization of tumor associated macrophages (Wu et al., 2020). Data about CD137/CD137L involvement during head and neck squamous cell carcinoma (HNSCC) progression have been rare. Lucido and colleagues investigated the role of CD137/CD137L in HPV\(^{+}\) HNSCC in a mouse model (Lucido et al., 2014). The authors showed that amplification of this stimulation pathway synergized with cisplatin and radiotherapy to improve tumor clearance. In addition, tumor clearance was further potentiated by local tumor cell expression of CD137L (Lucido et al., 2014). Srivastava et al. investigated the additional benefit of the CD137 agonist urelumb in a cetuximab-based therapy for head and neck cancers (HNC) (Srivastava et al., 2017). Urelumb improved cetuximab-activated NK cell survival, dendritic cell maturation, and tumor antigen cross-presentation. The authors concluded that combined immunotherapy using cetuximab and a CD137 agonist has an additional benefit during the treatment of HNC (Srivastava et al., 2017).

In the present study, we describe strong CD137L expression in OSCC and the surrounding TME for the first time. The univariate CR analysis indicated a significant influence of CD137L on DFS; this influence did not reach statistical significance in the multiple analysis model. However, we believe that the CD137/CD137L axis is crucially involved in OSCC progression and could possibly be a biological tool for immunomodulation that requires further investigation.

In this study, we used DFS as the primary clinical endpoint for determining patient prognosis. A growing debate exists on the choice
of the correct endpoints in clinical trials in oncology. Thus far, only a modest correlation between disease-free survival and overall survival has been demonstrated (Love et al., 2014). Since overall survival is influenced by many other tumor-independent factors and is also difficult to properly record, we believe that DFS is the better clinical endpoint for capturing the changing tumor biology and the processes of invasion and metastasis. As stated above, our data again suggest the diversity of head and neck tumors and particularly OSCC, rendering further in-depth investigations necessary.

In conclusion, we identified changes in differentially expressed genes during OSCC progression in vitro and associated the most relevant ones with DFS as a primary clinical endpoint. Stearoyl-CoA desaturase-1 and sclerostin acted as independent prognostic factors in OSCC and thus could be of interest as new candidates for the development of targeted tumor therapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Patients gave written informed consent before participating the trial. The study was conducted in accordance with the Ethical Standards (Declaration of Helsinki) approved by the local ethics committee of the University Medical Center Goettingen (vote no. 07/06/09, updated in April 2018).

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

Boris Schminke: Data curation; Formal analysis; Investigation; Writing-review & editing. Orr Shomroni: Data curation. Gabriella Salinas: Data curation. Felix Bremmer: Formal analysis; Methodology. Philipp Kauffman: Formal analysis; Writing-review & editing. Henning Schliephake: Formal analysis; Writing-review & editing. Felix Oyelami: Data curation; Formal analysis. Michal Rahat: Formal analysis; Writing-review & editing. Philipp Brockmeyer: Conceptualization; Data curation; Formal analysis; Investigation; Supervision; Writing-original draft; Writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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