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SOX2 in the pathogenesis of Head and Neck Squamous Cell  
Carcinoma

*Análise funcional do fator de transcrição SOX2 na patogênese de Carcinoma  
Espinoceleular de Cabeça e Pescoço*

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This investigation presented here was developed in the Molecular Genetics division of the German Cancer Research Center, beginning on the second year of the Ph.D. in the Oral Pathology department of the Federal University of Rio Grande do Sul, Brazil. The thesis advisor committee, as part of the Helmholtz International Graduate School for Cancer Research, was formed by: Prof. Dr. Peter Lichter (co-supervisor in Germany; DKFZ), Prof. Dr. Jochen Hess (DKFZ), Dr. Martina Seiffert (DKFZ), Prof. Dr. Kolja Freier (Heidelberg University Hospital) and Prof. Dr. Pantelis Varvaki Rados (UFRGS).

## Introdução

*A tese aqui apresentada foi desenvolvida no Centro de Pesquisa de Câncer Alemão (DKFZ) em programa conjunto com a Universidade Federal do Rio Grande do Sul. O comitê responsável pela correção e aprovação da tese dentro do DKFZ é composto pelos Professores Peter Lichter (DKFZ e Faculdade de Medicina da Universidade de Heidelberg), Jochen Hess (DKFZ e Faculdade de Medicina da Universidade de Heidelberg), Kolja Freier (Faculdade de Medicina da Universidade de Heidelberg) e Pantelis Varvaki Rados (Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul). Seguindo o padrão do DKFZ o presente formato é de tese e em inglês.*

*Este trabalho originará 3 artigos a serem publicados. O primeiro artigo consistirá no papel de SOX2 na migração celular e prognóstico de carcinoma espinocelular de cabeça e pescoço (CECP). Os resultados mostram que o silenciamento de SOX2 em células de CECP aumenta a migração celular e que as células que expressam SOX2 acumulam-se no centro das colônias celulares enquanto que as células negativas para SOX2 tendem a estar localizadas no “frente de invasão”. O perfil de transcrição após o silenciamento de SOX2 mostrou genes afetados relacionados ao movimento celular, sendo Vimentin o gene com maior expressão inversa a SOX2. Os achados in vitro serão publicados em conjunto com os resultados realizados a partir de estudo em pacientes do hospital universitário de Heidelberg, os quais mostram uma melhor resposta à terapia de CECP em pacientes com expressão de SOX2. Sugere-se que a pior resposta ao tratamento nos casos com baixa ou nenhuma expressão de SOX2 seja devida à maior capacidade migratória dessas células. A primeira autoria do artigo será dividida com a pesquisadora responsável pelos resultados em pacientes de CECP, Dra. Pilar Bayo.*

*O segundo artigo compreenderá os estudos funcionais com células de CECP, nos quais observou-se maior apoptose após o silenciamento de SOX2 e em resposta à doxorubicin. Para este artigo mais experimentos serão realizados com o objetivo de validar os resultados já encontrados e entender de que forma SOX2 afeta a apoptose.*

*O terceiro artigo terá como foco o papel de SOX2 na resposta imunológica em CECP, visto que a principal via celular afetada após o silenciamento de SOX2 foi a de apresentação de antígeno. Para este trabalho será investigada a regulação epigenética de Human Leucocyte Antigen classe I e SOX2 em células e amostras de pacientes de CECP.*

*Os desenvolvimento do segundo e terceiro artigo será realizado no Hospital Universitário de Heidelberg e no DKFZ em colaboração com a Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul.*

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## Abstract

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent and lethal human malignancies worldwide. To date there are no reliable biomarkers for prognosis or therapy decision making in HNSCC oncology. Recurrent copy number gain and overexpression of the transcription factor SOX2 has been shown in HNSCC; however, the impact of SOX2 expression on tumor relevant processes and clinical outcome remains largely elusive. Expression of SOX2 was assessed in 22 HNSCC cell lines with and without 3q gain. Mean expression level was significantly higher in cell lines with 3q gain as compared to cell lines without 3q gain ( $p = 0,0012$ ). We performed global gene expression profiling of control and SOX2 silenced HNSCC cell lines, specifically tongue squamous cell carcinoma, with SOX2 gene amplification. Results showed 749 affected after SOX2 knock down, considering the adjusted  $p$  value  $\leq 0,05$ . 456 genes were found to be down-regulated and 293 genes to be up-regulated after SOX2 knock down. The top up-regulated gene was vimentin and its inverse expression with SOX2 was confirmed by quantitative real time polymerase chain reaction. Indeed, immunofluorescence showed inverse expression of SOX2 and vimentin. Ingenuity pathway analysis of transcriptome data revealed an enrichment of differentially expressed genes with a functional annotation in cell mobility and cellular movement. In line with these findings, SOX2 silenced cells showed increased migration as compared to controls in two independent HNSCC cell lines, from tongue and hypopharynx, suggesting a higher risk for local recurrence and metastasis of tumors with low or no SOX2 expression. In addition, SOX2 negative cells were preferentially localized in the "invasion front" whereas SOX2 positive cells were seen in the center of the cell colonies, suggesting that silencing SOX2 could be a necessary step to initiate migration in HNSCC cells. On the other hand we also found that cell survival decreased after SOX2 knock down coherently with SOX2 role as an oncogene. Also, after apoptosis induction with doxorubicin, HNSCC SOX2 silenced cells had significantly more apoptosis than control. Interestingly, Antigen Presentation Pathway was the main affected pathway after the knock down of SOX2. The genes involved in this pathway that were found affected in our transcriptome data were mainly HLA class I, and they were all down-regulated after SOX2 knock down, showing a positive correlation with SOX2. This finding suggests a role of the transcription factor SOX2 in

the underlying immune response of HNSCC and could indicate a promising approach for new therapies development for SOX2 expressing tumors. In addition to the potential of SOX2 as prognostic biomarker, a better understanding of the underlying molecular mechanisms could help to establish new strategies for targeted therapy.



## Resumo

*Carcinoma espinocelular de cabeça e pescoço (CECP) é uma das malignidades mais prevalentes e mortais mundialmente. Até o momento não existem biomarcadores confiáveis para prognóstico e decisão de terapia na oncologia de CECP. Ganhos cromossômicos do fator de transcrição SOX2 foram demonstrados em CECP; entretanto, o impacto da expressão de SOX2 em processos relevantes tumorais e evolução clínica permanecem incompreendidos. Expressão de SOX2 foi analisada em 22 linhagens celulares de CECP, com e sem ganho em 3q. O nível médio de expressão de SOX2 foi significativamente maior nas linhagens com ganho em 3q quando comparada às linhagens sem ganho em 3q ( $p = 0,0012$ ). Neste trabalho, realizamos o perfil global de expressão genica das linhagens de CECP controle e após silenciamento de SOX2, especificamente carcinoma espinocelular de língua com amplificação de SOX2. Os resultados mostraram 749 genes afetados por SOX2 “knock down”, considerando o valor ajustado de  $p \leq 0,05$ . 456 genes foram encontrados negativamente regulados enquanto que 293 genes foram positivamente regulados após o “knock down” de SOX2. O principal gene positivamente regulado foi vimentin e sua expressão inversa com SOX2 foi confirmado usando “quantitative real time polymerase chain reaction”. De fato, a imunofluorescência indicou expressão inversa de SOX2 e vimentin. A análise da “transcriptome data” usando o software “Ingenuity pathway analysis” revelou genes diferentemente expressos envolvidos nas funções de mobilidade celular e movimento celular. Alinhados com esses achados, as células silenciadas para SOX2 apresentaram maior migração quando comparadas aos controles em duas linhagens independentes de CECP, uma de língua e outra de hipofaringe, sugerindo um maior risco para recidiva e metástase de tumores com baixa ou nenhuma expressão de SOX2. Complementando esses resultados, as células negativas para SOX2 localizaram-se preferencialmente no “frente de invasão”, enquanto que as células positivas para SOX2 estavam no centro das colônias celulares, sugerindo que o silenciamento de SOX2 pode ser necessário para o início da migração em células de CECP. Por outro lado, também encontramos uma diminuição na viabilidade celular após o “knock down” de SOX2, coerente com o papel de SOX2 como oncogene. De acordo, após indução de apoptose com doxorubicina, células de CECP silenciadas para SOX2 mostraram significativamente mais apoptose que as controles. Interessantemente, a via de apresentação de antígeno foi a mais afetada após o “knock down” de SOX2. Os genes envolvidos nessa via e afetados nos nossos resultados, principalmente genes HLA classe I, estava afetados negativamente, mostrando correlação positiva com expressão de SOX2. Estes achados sugerem um papel de SOX2 na resposta imunológica de CECP e podem indicar um enfoque promissor para o*

*desenvolvimento de novas terapias para tumores de CECP com expressão de SOX2. Além do potencial de SOX2 como biomarcador para prognóstico de CECP, uma maior compreensão dos mecanismo moleculares subjacentes pode ajudar no estabelecimento de novas estratégias de terapia direcionada.*

## Introduction

Head and neck cancer is the 6th most common solid malignant tumour worldwide, affecting approximately 600.000 people per year (1). Around 95% of cases are squamous cell carcinoma, that is a cancer originated from the epithelial cells of the mucosa lining the mouth, oropharynx, hypopharynx, larynx, nasopharynx and nasal/paranasal sinuses (2, 3). It is important to consider that HNSCC comprehend a heterogeneous group of tumors from different anatomical sites, which has an important impact on the cellular and clinical behaviour of these cancers (4). Despite advances in other types of cancer, the 5-year survival rate for head and neck squamous cell carcinoma (HNSCC) remains at 55% and did not improve in the last decades (1, 5). Delay in diagnosis, despite the direct visual access for clinical examination, at least of the oral cavity, may contribute to the bad prognosis (6).

Known risk factors for HNSCC are tobacco and alcohol consumption. Another risk factor, mainly for oropharyngeal SCC, is infection by high-risk types of human papilloma virus (HPV) (7, 8). Traditionally the most affected population of HNSCC was men, more than 40 years, smokers and/or heavy drinkers. In the last years a change in the incidence of HNSCC was seen, increasing the number of cases in young patients probably due to HPV infection (9, 10).

Currently, standard treatment of HNSCC patients includes surgery or radiochemotherapy for low stage tumors and multimodal therapy for advanced HNSCC. Some HNSCC patients are treated with antibody-based immunotherapy with a monoclonal anti-EGFR (epidermal growth factor receptor) antibody (cetuximab). The treatment with EGFR inhibitors is based on the fact that some tumors become independent from growth factors due to alterations in the EGFR pathway (11, 12).

Recently, next generation sequencing approaches unraveled the mutational landscape of HNSCC and demonstrated that individual risk factors drive carcinogenesis with different mutational profile. As observed by Agrawal et al. there are more mutations in tumors from smokers than tumors from non-smoker patients and that HPV-negative tumors have more mutations than HPV-positive counterparts. These differences most likely influence the biological and clinical behavior in response to therapy, since it is well established that patients with HPV-related

HNSCC have better overall and disease-specific survival (13-15). However, HPV status does not seem to influence the efficacy for treatment with EGFR inhibitors (16).

For the HPV negative tumors sub-classification is still preliminary and is mainly based in the TP53 mutation, frequently found in the HNSCC with high chromosome instability (17-19). However this is not enough considering that other alterations can also have an important impact on treatment response and patient survival. Therefore it is necessary to gain a more comprehensive view on the mutational landscape in HNSCC development and identify driver mutations that are operative in those cancers. Recent advances in whole genome sequencing technology allow the identification of thousands of somatic mutations in HNSCC. A better understanding of mutation profile of HNSCC can have an impact in diagnostic and therapy. Recently, four different signatures, meaning different combinations of mutations types, were found for HNSCC. Those signatures are probably associated with the age of the patients, the apobec family of cytidine deaminases, the habit of smoking and the exposure to ultraviolet light (20).

Stransky et al. revealed new mutated genes implicated in HNSCC analyzing whole exome sequencing data from 74 tumor-normal pairs. The authors found genes implicated in HNSCC, like TP53, CDKN2A, PTEN, PIK3CA and HRAS. They also found mutations in genes not previously related to HNSCC like NOTCH1, IRF6 and TP63, and key regulators of squamous differentiation (21).

Previously, gains of 3q21-29, 11q13 and the loss of 8p21-22 were associated with short survival in HNSCC and were identified as independent prognostic markers, suggested to be more accurate than only nodal status (22). However it is necessary to understand whether chromosome copy number changes and transcriptional activity of genes in those chromosome sub-regions are correlated. Janjetovic et al. investigated by comparative expressed sequence hybridization the relative mRNA expression in 28 oral SCC. The authors found increased expression of genes located at 3q26.3-qter and 11q12-q13.2 and decreased expression on 8p22-p23 among others (23). In a previous work of our group, recurrent amplification of 3q26 was found in oral squamous cell carcinoma and 11 out of 40 cases shared a minimal amplified region of 0.85 mega base pair (Mbp) with SOX2 as the only candidate gene (24). This finding makes SOX2 an attractive candidate gene for further analysis.

## SOX2

SOX2 is an oncogene located in the chromosomal region 3q26.3 and encodes a 317 amino acid long transcription factor that belongs to the SRY (sex determining region of Y)-related HMG (high mobility group) box gene family (SOX family). The SOX family of genes plays a key role in stem cell biology during embryonic development as well as adult tissue homeostasis (25). They share the HMG box domain, a highly conserved DNA binding domain of 80 amino acids.

HMG box genes have evolved from canonical HMG domain proteins, which share the ability to alter the DNA conformation, increasing protein accessibility and plasticity. That happens because of unique properties of the HMG domain proteins. Unlike other DNA-binding proteins, those proteins bind the DNA in the minor groove. Other common features of the HMG domain proteins are the ability to bind DNA irregular structures and to modulate DNA structure by bending. With that comes the widening of the minor groove and the compression of the major groove, facilitating the formation of enhanceosomes, meaning active complexes of transcription factors on gene enhancer sequences (26). HMG box proteins, opposite to the HMG domain proteins, have tissue-restricted expression and the HMG box domain is identical only in around 20% to the HMG domain. However, importantly, the amino acids that allow the DNA conformation are conserved (27). These unique characteristics of this group of DNA-binding proteins, including SOX2, may allow the function versatility observed for this transcription factor.

SOX2 exerts pleiotropic functions in the context of maintaining pluripotency, in reprogramming somatic cells and being critical for development and differentiation. Those paradoxical functions are probably consequences of interaction with other transcription factors and cofactors. SOX2 interacts with the transcription factor OCT4 for the stabilization of embryonic stem cells (ESCs) in their pluripotent state. SOX2 and OCT4 bind cooperatively the DNA at non-palindromic sequences for the transcription activation of key pluripotency factors (28-30). Actually, it was suggested that SOX2 primary function in ESC is to control OCT4 expression. The expression of those two transcription factors can be maintained permanently when both factors are expressed concurrently, and therefor the pluripotency as well (30).

Besides the role of SOX2 in pluripotency, SOX2 has a role in reprogramming somatic cells. In 2007, Yu et al. and Takahashi et al. showed that induction of pluripotent stem cells from mouse and human somatic cells, respectively, was possible to be achieved using SOX2 together with the transcription factors Oct3/4, Klf4, and c-Myc (31, 32).

Although SOX2 is frequently associated with a stemness phenotype, Adachi et al. demonstrated the function versatility of this transcription factor during embryogenesis. The authors compared by whole-genome expression analysis ESCs and trophoblast stem cells (TSCs), after SOX2 knockout induction for each cell type. Compared target genes by using transcriptome and chromatin immunoprecipitation analysis they observed that SOX2 regulates different set of genes in ESCs and TSCs (33).

SOX2 is important in determining cell fate. Small changes in SOX2 level can trigger differentiation in mouse embryonic stem cells (34, 35). This transcription factor is critical for the differentiation of tongue, esophagus, stomach, lung, retina and brain and in those tissues it remains expressed in adulthood (36-38). It also plays an important role in the development of sensory systems (36, 39). Okubo et al. studied the function of SOX2 during development of tongue in mice. The authors found SOX2 expression in the basal cells of the tongue epithelium. Specifically, in tongue taste bud placodes, fungiform papillae, and mature taste cells they observed high levels of SOX2, whereas in filiform papillae they found low levels of SOX2. They suggest that SOX2 regulates the differentiation of endodermal progenitor cells of the tongue into taste bud sensory cells versus keratinocytes and that this function of SOX2 occurs in a dose-dependent manner (36).

Since SOX2 tightly regulates so many critical physiological processes in development and in adult life, de-regulation of SOX2 can play an important role in the establishment and maintenance of human disorders, including cancer.

## SOX2 and Cancer

The function of SOX2 appears to be different in cancer when compared to its physiological functions in ES cells. Watanabe et al. compared profiles of SOX2 regulated genes in several SCC cell lines and ES cells and found SOX2 binding at different loci depending on the cellular origin (40). The role of SOX2 probably differs not only in a physiological versus cancer conditions, but also in a cancer specific manner.

Bass et al. described SOX2 as an oncogene in lung and esophageal SCC. The authors found that the tumors driven by SOX2 expressed pluripotency and squamous differentiation markers (41). In SCC SOX2 preferentially colocalizes with the squamous marker p63, and SOX2 overexpression in lung SCC cells induces p63 expression (40, 42).

There are conflicting results concerning the functional role of SOX2 in the cancer cell cycle. In lung SCC cells impairment of cell growth was observed after SOX2 knock down (43). In line with this data, SOX2 was shown to facilitate the G1/S transition in glioma, prostate and breast cancer (44-46). In contrast, overexpression of SOX2 causes cell cycle arrest through down-regulation of cyclin D1 and phosphorylated Rb in gastric cancer (47).

SOX2 anti-apoptotic properties were seen in different types of cancer. In lung carcinomas, SOX2 silencing caused not only apoptosis but also autophagy (48). In prostate cancer cell lines, SOX2 down-regulation increases apoptotic sensitivity to chemotherapy. Jia et al. found a tumor size reduction in silenced SOX2 cells xenograft mice compared to control xenograft mice. The authors also investigated the functional role of SOX2 by its overexpression and down-regulation in a human prostate cancer cell line. They found that SOX2 increased the apoptosis-resistant properties in those cells (49). Apoptosis induction after SOX2 silencing was also found in melanoma cells (50). Apostolou et al. suggested that in colon cancer stem cells the AP-1 complex might have an important role in maintaining cancer stem cells (CSCs) properties, as they found decreased SOX2 expression and increased apoptosis after AP-1 complex knockdown (51).

Concerning cell motility and metastasis, SOX2 expression in different types of cancer is frequently associated to cell migration and invasion characteristics, and it was also found to regulate epithelial-mesenchymal transition (EMT) (52-55). It is

worth noting that those associations with SOX2 are rather indirect, as those studies often use SOX2 as a marker for stemness, and only a few really investigate the functional role of SOX2.

Han et al. investigated the functional role of SOX2 in colorectal cancer cells and observed that SOX2 silencing not only reduced cell migration and invasion but also induced mesenchymal-epithelial transition (MET) (56). Also in glioma, cell migration and invasion was impaired by silencing of SOX2 and induced by ectopic SOX2 expression (46, 57).

Interestingly, Berezovsky et al. showed in glioblastoma tumors and in patient-derived mouse xenografts that SOX2 expression is not restricted to a stem like subpopulation of cells, and instead it is expressed in differentiated cancer cells in different molecular subtypes (58). Probably in the heterogeneity of the tumor SOX2 has a more complicated role in regulating cell behavior than the one seen in the subpopulation of cancer stem cells.

Overexpression of SOX2 was seen in HNSCC and in different types of cancer, like colorectal cancer, breast carcinomas, lung adenocarcinomas and SCC as well as esophageal, cervix uteri, skin and penis SCC (24, 59-63). SOX2 plays an important role as an oncogene in tumor development, however, the effects of SOX2 overexpression in cancer prognosis are not clear. There are contradictory results not only across the different cancer types but also within the same types of cancer (48, 64-66).

Loss of SOX2 expression was associated with poor prognosis (47, 67). In esophageal adenocarcinomas (EAC), Honing et al. investigated CSCs markers in 94 EAC patients and found that loss of SOX2 and loss of CD44 as markers for poor prognosis. Otsubo et al. analyzed 52 patients with advanced gastric cancers and found a significantly shorter survival in patients with tumors having SOX2 methylation compared to those without methylation (47).

Consistent with these findings, high SOX2 expression was associated with a better prognosis in lung and gastric carcinoma patients (64, 65, 68-70). Vural et al. investigated the presence of SOX group B (SOX1, SOX2 and SOX3) antibodies in serum samples from 90 small-cell lung carcinoma (SCLC) patients. SOX2 was present in 20 cases and overlapped significantly with SOX1, suggesting that SOX1 and SOX2 may have very similar or overlapping immunogenic epitopes. The authors



correlated the findings with clinical parameters and found that SOX group B antibodies correlate with better survival in SCLC (65). The mechanistically explanation of the role of SOX2 in a better outcome is still missing. The authors affirm that further studies are necessary to comprehend the underlying immune responses.

On the other side, SOX2 is known to be a stemness marker, found in CSCs which are frequently associated to a more aggressive phenotype in cancer cells and to cancer therapy resistance (48, 71). Indeed several publications refer to SOX2 as a poor prognostic marker in cancer.

For example, Rudin et al. found a correlation between SOX2 expression and advanced stage in a cohort of 110 SCLC tumor samples (72). In lung adenocarcinomas (LA), Xu et al. investigated self-renewal mechanisms using LA cell lines, 200 patients' specimens and xenografts. The authors found that SOX2 maintains self-renewal of lung adenocarcinoma cancer stem-like cells by forming a complex with OCT4 and  $\beta$  catenin, which binds at the NANOG promoter, dependent on the insulin-like growth factor I receptor (IGF-IR). Co-localization of OCT4 and  $\beta$  catenin with overexpressed IGF-IR was significantly associated with poor prognosis (73). Accordingly, in colon cancer, Neumann et al. showed association of SOX2 with lymph node and distant metastases, which was also associated to nuclear  $\beta$ -catenin (74). In prostate cancer, Jia et al. found an association between SOX2 overexpression and high Gleason score (49).

In summary, these data strongly suggest a context dependent regulation and function of SOX2 in tumor cells with obviously different consequences on treatment response and prognosis. Therefore it is necessary to better understand its role in a cancer type specific manner as well as to pursue in unraveling a more comprehensive overview on SOX2-dependent gene interaction networks in different tumor cell type. Functionally, SOX2 was implicated in proliferation, apoptosis and migration in several types of cancer; however, the role of SOX2 in HNSCC is less clear (56, 75-80).

## SOX2 and HNSCC

Again contradictory results have been published regarding the expression of SOX2 and clinical outcome of HNSCC patients. Comparisons between those results are limited since some of the publications focus in specific sites of HNSCC and stage of the tumors are highly variable (78, 79, 81). It is also possible that SOX2 has opposite roles depending on the HNSCC site and tumor stage.

On a cellular level, Chen et al. observed that SOX2, Nanog, and Oct3/4 were significantly increased in HNSCC spheroid derived cells (77). Sun et al. isolated and investigated the biological characteristics of CD133+ CD44+ subpopulation from tongue squamous cell carcinoma (TSCC) cell lines. Fewer cells from this subpopulation were needed to form secondary tumor in nude mice, as compared to the total cell population. The authors concluded that the CD133+ CD44+ TSCC cells have stem-like characteristics and they found higher expression of SOX2 and also higher expression of the anti-apoptotic protein BCL-2 in this subpopulation of cells (82).

In the human HNSCC cell line SCC-25, Schröck et al. investigated SOX2 function by SOX2 knock down and overexpression. Considering spontaneous apoptosis they found no difference between SOX2 knock down and control cells but they found less spontaneous apoptosis in SOX2 overexpressing cells compared to control counterparts. The authors also found that SOX2 induced the expression of the antiapoptotic protein BCL-2 and suggested that this is a mechanism by which SOX2 inhibits apoptosis, pointing SOX2 as a mediator of therapy resistance in human HNSCC. Indeed, after treatment with the apoptosis-inducing agents staurosporine and cisplatin, they found enhanced apoptosis in the SOX2 knock down cells and reduced apoptosis in SOX2 overexpressing cells (83). Cisplatin resistance mechanisms in the stem-like subpopulation of the HNSCC cell line HSC-3 was also in part influenced by SOX2 as shown by Bourguignon et al. (84). Lim et al. found in high SOX2 HNSCC-driven squamospheres, resistance not only to cisplatin, but also to 5-fluorouracil (FU), paclitaxel and doxorubicin, drugs that are also used in HNSCC therapy (85).

Those findings in the cellular level point to a possible role of SOX2 in determining worst prognosis. However, findings from Züllig et al. showed that up-regulation of SOX2 is indicative of less aggressive and invasion growth. The authors

observed that in a set of 120 T1/T2 oral SCC, upregulation of SOX2 correlated with lower incidence of lymph node metastasis (86). In hypopharyngeal SCC, SOX2 together with OCT4 was associated with better prognosis and with better differentiation, and OCT4 alone was an independent predictive factor for SCC of the hypopharynx (78).

In contrast, SOX2 nuclear expression in TSCC was associated with poor prognosis. Du et al. investigated by immunohistochemistry SOX2 expression in 82 patients with histologically node-negative (pN0) TSCC and correlated with clinic-pathological factors and disease prognosis. 62.2% patients had tumors with SOX2 expression and had a significant association with large tumor size. The authors found that SOX2 expression was an independent marker for poor prognosis in pN0 TSCC and suggested that SOX2 is involved in tumor progression (79).

Huang et al. assessed by immunohistochemistry the protein expression levels of ALDH1, CD44, OCT4 and SOX2 in 66 TSCC tissue samples. The authors found that SOX2 expression alone can be used as a marker for poor prognosis in TSCC (81). Chang et al. pointed out that the combination of SOX2 and other pluripotency markers with the connective tissue growth factor could be a better prognostic indicator than an individual marker in HNSCC (87).

In a cohort of 496 patients, that included 253 metastases and 135 recurrences, Schröck et al. associated SOX2 overexpression to clinico-pathological parameters of worse prognosis, however they did not find differences in disease-free or overall survival time. The authors detected SOX2 amplification in 21% of the primary HNSCC and found a concordance with the corresponding metastatic tissue in most of the cases. Interestingly, in this study SOX2 amplification was more frequent in primary SCC of the hypopharynx and oropharynx and less frequent in SCC of the larynx and oral cavity (83).

In 2010, our group published for the first time overexpression of SOX2 in OSCC (24). Altogether, the published data about SOX2 in HNSCC leads to the assumption that SOX2 has a critical role in development and progression of HNSCC. However, the role of SOX2 in HNSCC is not yet understood. The aim of the present study is to understand the role of SOX2 in pathogenesis and clinical outcome of HNSCC. Main goals are to understand how SOX2 influence tumor relevant processes that critically contribute to treatment response and clinical outcome of

HNSCC patients, and to predict promising drug targets for innovative strategies for more efficient and less toxic therapy.

## Previous results from our group

Since DNA copy number aberrations are involved in HNSCC development, Freier et al. screened HNSCC cell lines for genetic imbalances by chromosomal comparative genomic hybridization (cCGH). Frequent DNA copy number gains were detected on 3q26.3-qter in a set of 20 HNSCC cell lines. Below there is a table from Freier et al. showing the gains and losses of the HNSCC cell lines, named HNO (Hals-Nasen-Ohren; Neck, nose and ear) cell lines. The original table is modified to show in yellow the cell lines with 3q gain (Fig. 1). We could see 13 HNSCC cell lines with 3q gain (88).

**Table 1** Chromosomal regions of DNA copy number aberrations detected by chromosomal CGH in HNSCC cell lines

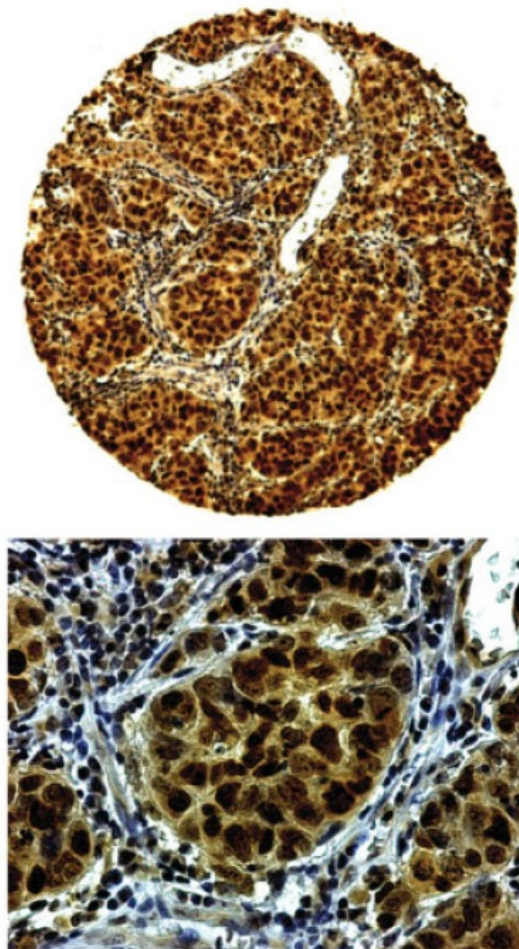
HNSCC cell line	Tumor site	Gains	Losses
HNO41	Hypopharynx	3q23-qter, 5p, 7p, 7q21-q22, 8q22.2-qter, 9q22-qter, 11q13	–
HNO89	Larynx	3q23-qter, 8q, 10q22, 11q13-q22, 12q14-q15, 13, 20q13.1	4q32.1-qter
HNO91	Oral cavity	3p13-p14, 20p, 20q	5p, 5cen-5q23
HNO97	Oral cavity	3p25-pter, 3q, 5p, 9q22-qter, 10p, 10q, 11cen-p14, 20p, 20q	18q
HNO124	Oral cavity	1p21-p31, 3q, 8q, 17q22-qter, 18p	8p, 11q22-qter, 18q22-qter
HNO147	Hypopharynx	8q23-qter, 11q12-13, 14q23-qter, 16q12-q21, 17p, 20cen-20q13.2, 22q13	7q31-qter, 13q31-q32, 18q
HNO150	Larynx	2p, 2q22-q32, 3p22-pter, 3q, 5p, 5q, 7q31-q33, 8q, 9q, 10p, 10q, 11cen-p14, 13, 14q13-q31, 20p, 20q	18q, 22
HNO160	Oral cavity	5p, 20p, 20q	3p, 4p, 4q
HNO180	Oropharynx	3q13.3-qter, 5q21-qter, 7cen-q31, 8p, 8q, 11q13-q21, 16q	4q23-qter, 7q31-qter, 11q23-qter, 13, 18p, 18q, 21
HNO199	Oral cavity	5p, 9p, 9q, 13q21-qter, 18p, 20p, 20q	3p, 8p, 15, 18q
HNO206	Oropharynx	5p, 11q13	7q32-qter, 11q23-qter, 18q21-qter
HNO210	Larynx	3q, 7p, 7q, 9p, 9q, 20p, 20q	3p, 4p, 4q, 21
HNO211	Oropharynx	7p, 8q, 11q13, 20p, 20q,	–
HNO212	Oral cavity	5p, 7cen-p13, 9p, 11p, 11q, 17p, 17q, 20p, 20q	8p, 18q21-qter
HNO222	Larynx	3q, 7p, 7q, 8q, 14	3p, 8p, 10p, 10cen-q22, 18q
HNO223	Oral cavity	3q22-qter, 8q, 9p, 9q, 11q13, 20p, 20q	–
HNO237	CUP	3q21-qter, 8q, 9p, 9q, 11q13, 20p	4q
HNO258	Oral cavity	1q41, 3q21-qter, 7p, 7cen-q21, 8q22-qter, 9cen-p13, 9q31-qter, 11q13, 15p, 15q	4p, 18q12-qter
HNO268	Oropharynx	3q, 5q14-q23, 18p, 20q, 22q13	4p, 4q, 6q24-qter, 8cen-p12, 8cen-q21, 10p, 10cen-q21, 18q21-q22
HNO306	Oral cavity	2cen-2p13, 2cen-2q21, 3q25-qter, 8q23-qter, 12p, 14q21-qter	18p, 18q

CUP, carcinoma of unknown primary.

Figure 1. Table from the publication of Freier et al. (88). HNSCC cell lines, named HNO, from different sites, chromosomal gains and losses are listed.

To delineate candidate genes inside critical chromosomal regions, Freier et al. applied array-CGH to 40 oral squamous cell carcinoma (OSCC) specimens using a microarray covering the whole human genome with an average resolution of 1 Mb. The authors found gene copy number gains in 3q26 in 11 of the 40 OSCC

specimens. Then they found high mRNA expression of SOX2 in 3q26.33 by quantitative real time polymerase chain reaction (RT-PCR). Tissue microarray (TMA) analyses done with fluorescence in situ hybridization (FISH) in a representative OSCC collection found gene copy number gain for SOX2 in 52% (115/223). Immunohistochemical analyses on TMA sections detected high expression of SOX2 in 18.1% (49/271). Below there is a figure extracted from the publication from Freier et al. showing the high SOX2 expression (Fig. 2) (24).



**Figure 2.** Figure from the publication of Freier et al.(24). SOX2 staining by immunohistochemistry in TMA sections. Top: X10; bottom: X 40.

## Objective

Understand the role of SOX2 in pathogenesis of HNSCC.

### Specific Objectives

- Understand how SOX2 influence tumor relevant processes
- Predict promising drug targets for innovative strategies for more efficient and less toxic therapy.

## Methods

### HNSCC cell lines and cell culture

HNSCC cell lines (HNO: Hals-Nase-Ohrenklinik) were provided by the Neurosurgery clinics of the Heidelberg University. Those cell lines were characterized by comparative genomic hybridization in previous work from our group. 11 of the 20 HNSCC cell lines have amplification in 3q26 (88). Cells were maintained at 37°C, 5% CO<sub>2</sub> in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biochrom), 1% penicillin and streptomycin.

### Short hairpin RNA, transfection and transduction

The following shRNAs (Sigma-Aldrich) were used:

#### SOX2 shRNAI

5'-CCGGTAGGACAGTTGCAAACGTGAACTCGAGTTCACGTTTGCAACTGCCTATTTTT-3'

#### SOX2 shRNAII

5'-CCGGCTGCCGAGAATCCATGTATATCTCGAGATATACATGGATTCTCGGCAGTTTTT-3'

#### SOX2 shRNAIII

5'-CCGGCGCTCATGAAGAAGGATAAAGTCTCGAGACTTATCCTTCTTCATGAGCGTTTTT-3'

#### Luciferase shRNA

5'-GATCCCCCTTACGCTGAGTACTTCGAttcaagagaTCGAAGTACTCAGCTAAGTTTTTGGAAA-3'

#### Non-target shRNA

5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTCTTTCATCTTGTTGTTTTT-3'

For stable SOX2 knock down of the HNO223 cell line, the shRNAs were cloned into the BglIII/HindIII-restriction sites of pSUPER- dL\_Zeo vector as described in Pscherer et al. (89), followed by transformation in *Escherichia coli*, isolation of plasmid DNA with a QIAprep Miniprep Kit (Qiagen) and Sanger sequencing. The vector pSUPER- dL\_Zeo was kindly provided by A. Pscherer (BioRN, Heidelberg,



Germany). Transfection was performed with a non-liposomal method (Effectene Transfection Reagent, Qiagen) with a control shRNA against luciferase and two different shRNAs (shRNAI and shRNAII) targeting SOX2. The cells that had the shRNA incorporated in the DNA were selected using Zeocin (2µg/ml) for the generation of stable cell lines. The transfection of HNO223 with luciferase control shRNA and two different SOX2 shRNAs was repeated, having two sets of stable cell lines (HNO223 1T and HNO223 2T).

The same transfection method used for HNO223 was applied to the cell line HNO41 without success. Interestingly, the control cells survived whereas the SOX2 knock down cells died or didn't have a significantly SOX2 knock down (data not shown).

To generate inducible shRNA-expressing lentiviral constructs in the HNO41 cell line, double-stranded oligonucleotides encoding the SOX2 shRNAI and shRNAIII were cloned into single vector inducible shRNA construct pLKO-Tet-On between the AgeI/EcoRI restriction sites. Briefly, reconstituted sense and antisense oligos were mixed with 10x Oligo Annealing Buffer (Invitrogen), incubated at 95°C for 4 minutes, the tube containing annealing mix was left in the turned off heat block for overnight. 10,000-fold diluted double-stranded oligos were cloned into pre-digested and purified pLKO-Tet-On vector using Roche rapid ligation kit under manufacture's instruction. Lentiviral particles were produced by cotransfection of HEK293T cells with the psPAX2 (Addgene 12260, Didier Trono; packaging plasmid), pMD2.G (Addgene 12259, Didier Trono; envelope plasmid) and respective inducible pLKO-Tet-On shRNA constructs. Transfections were carried out using TransIT-LT1 (Mirus Bio LLC), and viruses were harvested at 48h and 72 h after transfection and combined. Infection of HNSCC cells with virus at a multiplicity of infection of 2 was carried out in the presence of 8µg/ml of polybrene (Chemicon). The supernatant containing virus was removed after 24h, and splitting of cells was performed 3, 5 and 7 days after transduction. This inducible lentiviral system was previously described in Wiederschain et al. (90).

Reverse transcription and quantitative real time polymerase chain reaction (qRT-PCR)

RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer. Reverse transcription of mRNA into cDNA was done as following: 1µg RNA was adjusted to a total volume of 8µl with RNase free water and then 2µl 5x first strand buffer and 1µl DNaseI (10U/µl) were added to remove possible DNA contaminations. After incubation for 20min at room temperature (RT) 1µl EDTA (25mM), 1µl dNTP mix (10mM) and 1µl oligo dT(20) (300ng/ µl) primer were added. Samples were incubated in a thermo-cycler for 10min at 65°C and 10min at 25°C. Thereby DNase was inactivated, secondary structures within the RNA were removed and oligo dT primers were allowed to bind to the RNA. A master mix done with 2µl of DTT (0.1M), 2µl of 5x first strand buffer and 1µl of H<sub>2</sub>O was added and the samples were incubated for 2min at 42°C. For the cDNA synthesis 1µl SuperScript II reverse transcriptase (200U/µl) and 0.2µl T432 protein (2mg/ml) were added and the samples were incubated for 50min at 42°C. Samples were left for 10min at 95°C to stop the reaction, diluted to 100µl total volume with H<sub>2</sub>O and stored at -20°C.

Quality control and quantification of purified RNA was done in the Nanodrop ND-100 spectrophotometer. The measurement was performed at 260nm, since nucleic acids have their maximum absorption at that wavelength. To estimate the contamination extent with organic solvents and proteins, measurements were done at 230nm and 280nm, respectively. Automatic calculation of RNA concentration was done on the basis of Beer-Lambert law:  $A = \Sigma \times c \times d$  ( $A$ = absorbance,  $c$ = RNA concentration,  $d$ = path length,  $\Sigma$ = extinction coefficient (40 for RNA)).

To determine relative amounts of specific transcripts, qRT-PCR was conducted on ABI Prism 7900RT Sequence Detection System using SYBR green as DNA intercalating dye. For each reaction 6µl of SYBR green, 100nM forward and reverse primer and 2µl of cDNA were used in a total volume of 14µl. A standard curve was measured in every run for every amplicon using Stratagene human reference RNA. Samples were initially denatured at 95°C for 15sec before the following steps: denaturation (40 cycles of 15sec and 95°C), primer annealing (10sec at 60°C) and elongation (60sec at 72°C). For the recording of a melting curve, three steps at 95°C for 15sec, 60°C for 15 sec and 95°C for 15sec were done.

Expression of SOX2, Vimentin (VIM), N-cadherin (CDH2) and Fibronectin 1 (FN1) was compared to the housekeeping genes (HKG) LamininB1, Actin and

PGK1 as reference. Primers were designed manually and ordered from Sigma Aldrich. from Sigma-Aldrich. The primers sequences are the following:

LamininB1: 5'-CTGGAAATGTTTGCATCGAAGA-3'/5'-GCCTCCCATTGGTGGATCC-3'

Actin: 5'-ATTGGCAATGAGCGGTTC-3'/5'-GGATGCCACAGGACTCCAT-3'

PGK1: 5'-AAGTGAAGCTCGGAAAGCTTCTAT-3'/5'- TGGGAAAAGATGCTTCTGGG-3'

SOX2: 5'-TTGCTGCCTCTTTAAGACTAGGA-3' /5'- CTGGGGCTCAAACCTTCTCTC- 3'

VIM: 5'- CTCTGGCACGTCTTGACCTT-3' / 5'- TCCTGGATTTCTCTTCGTG- 3'

CDH2: 5'- CACCGTGGTCAAACCAATCG-3'/5'- GGTGCTGAATTCCCTTGGCT -3

FN1: 5'-CTTTGGTGCAGCACAACCTTC-3' / 5'- TCCTCCTCGAGTCTGAACCA- 3'

### Western Blotting

For protein analysis of SOX2 expression in the HNSCC cell lines, total protein lysates were prepared using Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma Aldrich) plus protease inhibitor tablets (Roche). Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce). Equal amounts of protein samples were subjected to electrophoresis and blotted onto PVDF membrane (Millipore). Antibodies against SOX2 ( $\alpha$ -rabbit; 1:1000; Cell Signaling), alpha-tubulin ( $\alpha$ -mouse; 1:1000; Cell Signaling) as loading control and secondary antibodies (Rockland) were used. Imaging was done using the LiCOR Odyssey system.

### Gene expression profiling and data analysis

Gene expression profiling was performed using the Human GE 4x44K v2 Microarray according to manufacturer's instructions (Agilent, Santa Clara, CA). Briefly, total RNA was extracted from stable transfected cell clones with knockdown of SOX2 and the control from two different transfection experiments. The quality of the RNA was determined by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent). 1 $\mu$ g of RNA was used for cDNA synthesis, labelled in two different colours. After hybridization, slides were washed and scanned immediately. Data were extracted and pre-processed by Agilent Feature Extraction Software Version 10.7. Normalization and filtering was performed using R. Network analysis of the data was done by the Ingenuity Pathway Analysis (IPA) software

([www.ingenuity.com](http://www.ingenuity.com)). The log<sub>2</sub> fold change and the adjusted p value ( $p \leq 0,05$ ) were considered for the analysis.

## Survival assay

Apoptotic cell death was determined using the PE-AnnexinV Apoptosis Detection Kit (BD Bioscience), with 7-Amino-Actinomycin (7-AAD) (BD Bioscience), according to the manufacturer's guidelines. PE-AnnexinV and 7-AAD were used together to allow for identification of early apoptotic cells (7-AAD negative, PE Annexin V positive). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable for 7-AAD. Cells were harvested using trypsin (Sigma-Aldrich), stained with PE-AnnexinV and 7-AAD and kept for 15 min at RT in the dark. Apoptosis of cells was analysed by flow cytometry (Becton-Dickinson Canto II). Early and late apoptosis were visualized and quantified by constructing dot-plots using BD FACSDiva software.

## Cell cycle assay

HNO 223 SOX2 knock down and control cells were grown for 24h in a 6 well plate with DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin and then treated with 2 $\mu$ g/ml doxorubicin (Sigma) for 48 hours. Cells were harvested and stained with DAPI at time point 0h and 48h to see the difference in response to doxorubicin, regarding SOX2 expression. Cells were analyzed with UV laser in the Becton-Dickinson LSRII flow cytometer. Results were analyzed using the mathematical model Dean-Jett-Fox in the FlowJo software.

## Immunofluorescence

HNSCC cells were fixed with 4% paraformaldehyde (PFA) in PBS and washed with PBS. Permeabilization of cells was done in X-buffer (0.5% Triton X-100 in PBS) for 30min at RT. After washing three times with PBS cells were blocked with T-buffer (1% BSA/0.2% Tween20 in PBS) for 30min at RT. First antibody was diluted in T-buffer and incubated for 1h. Antibodies for SOX2 (anti-rabbit; 1:300; Cell Signaling) and vimentin (anti-mouse; 1:100; Progen) were used. Cells were washed three times in PBS. Second antibody was diluted in T-buffer and incubated for 1h in the dark.

Antibodies Cy3 (goat anti-rabbit; 1:100; Invitrogen) and Alexa 488 (goat anti-mouse; 1:100; Invitrogen) were used. Cells were washed three times in PBS and incubated for 5min in H33324 (3-(5-Fluoro-2-methoxyphenyl) propionic acid, 97%; Calbiochem, Merck) (1:1000). After washing three times in PBS cells were embedded with Mowiol 4-88 (Roth) on glass slides. Images were captured with a Nikon Eclipse Ti microscope at 20x magnification.

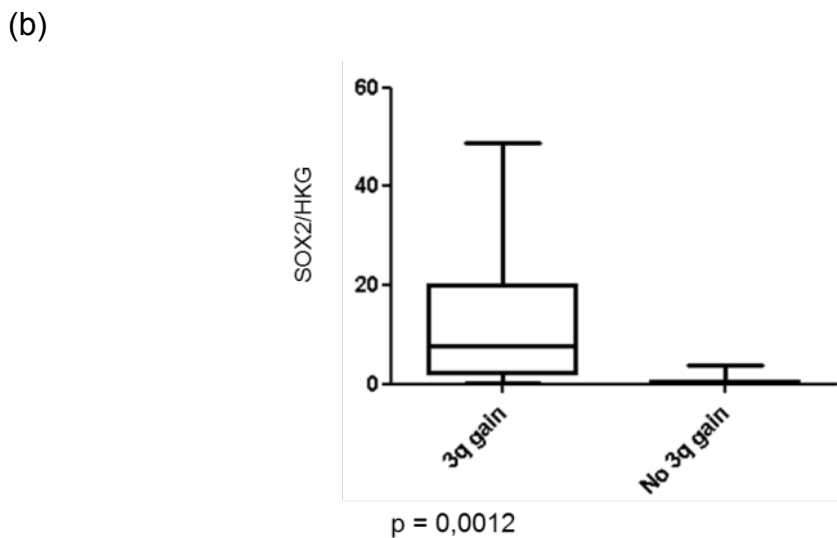
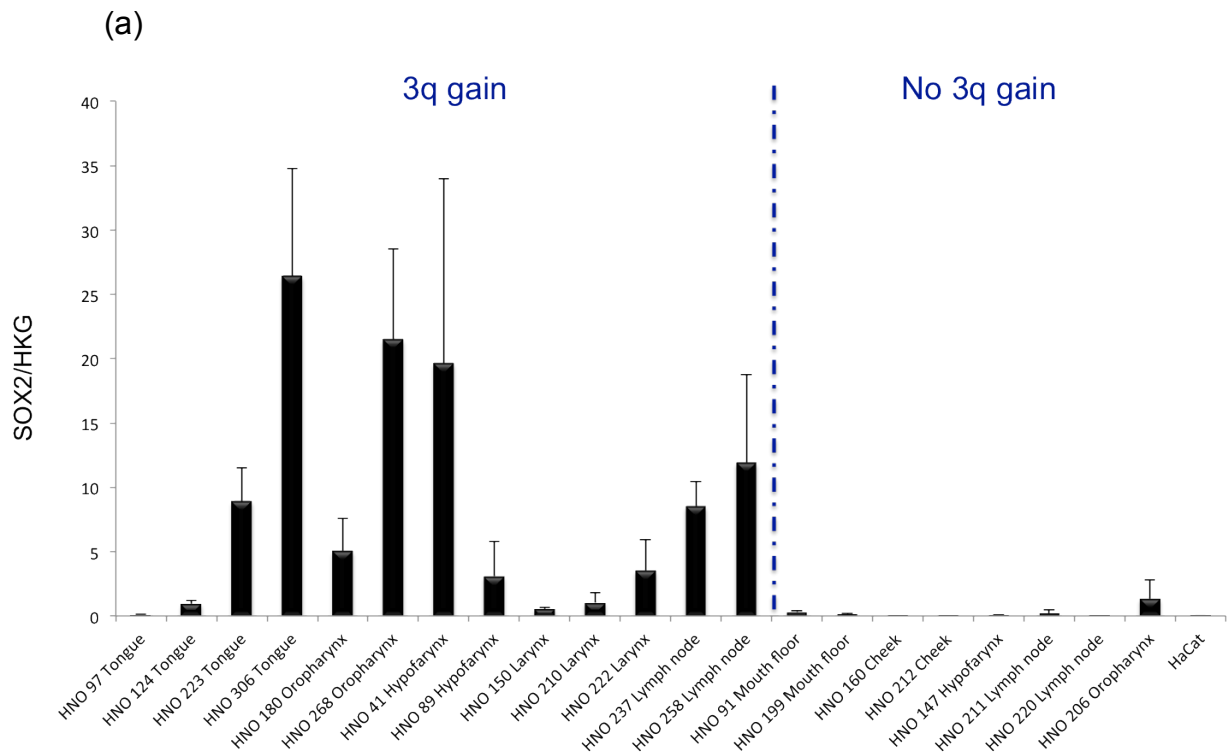
### Migration assay

Migration assay of control and SOX2 knock down HNSCC cell lines was performed using ibidi chambers accordingly to manufacturer. Briefly, cell suspension was prepared with 500.000 cells/ml and 70 $\mu$ l of suspension was applied into each well. Cells were incubated at 37°C and 5% CO<sub>2</sub>. After appropriate cell attachment (24h) the Culture-Insert was gently removed. Mitomycin C treatment (1h; 10 $\mu$ g/ml; Sigma-Aldrich) was used to stop proliferation. Images were taken at time point 0h, after 20-24h and after 48h, with 4x magnification. Images were captured with a Nikon Eclipse Ti microscope.

## Results

### SOX2 expression in HNSCC cell lines

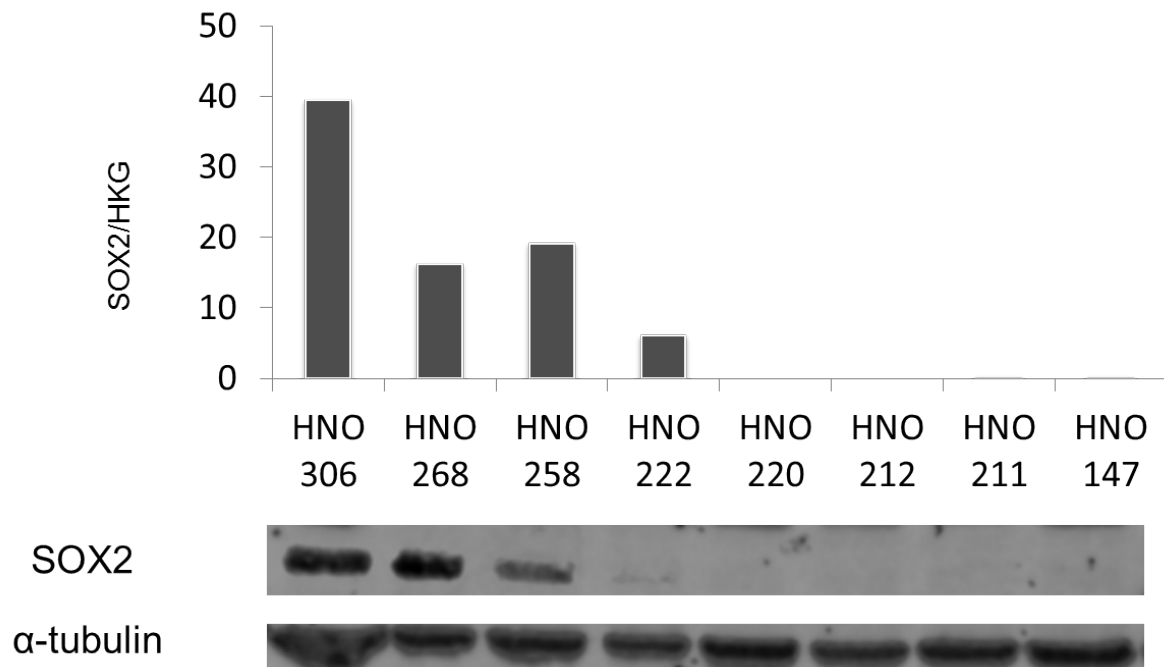
To investigate whether HNSCC cell lines with 3q gain display also higher SOX2 expression, we investigated SOX2 transcript levels in 22 HNSCC cell lines and in normal HaCat keratinocytes by quantitative RT-PCR analysis. While SOX2 transcription was not detectable in HaCat cells, we found a heterogeneous expression pattern in our pool of HNSCC cell lines ranging from high, moderate to low transcript levels (Fig. 3a). High SOX2 expression was more common in HNSCC cell lines with chromosome 3q gain and the mean expression level was significantly higher as compared to cell lines without 3q gain (Fig. 3b,  $p = 0,0012$ ). Moreover, western blot analysis with protein lysates from eight selected HNSCC cell lines confirmed that SOX2 transcript levels, as assessed by quantitative RT-PCR analysis in a second independent experiment correlated with protein expression (Fig. 4).



**Figure 3.** (a) Expression of SOX2 in HNSCC cell lines with or without 3q gain determined by quantitative RT-PCR showing variable levels of SOX2. (b) Distribution of cells with high and low SOX2 expression in the 3q or no 3q gain groups showing a correlation between 3q gain and high SOX2 expression and no 3q gain and low SOX2 expression. Expression of SOX2 was compared to the housekeeping genes (HKG) Laminin and PGK1. For each cell line at least three independent experiments



were performed using RNA from at least two different time points for SOX2 expression mean and standard deviation calculation.

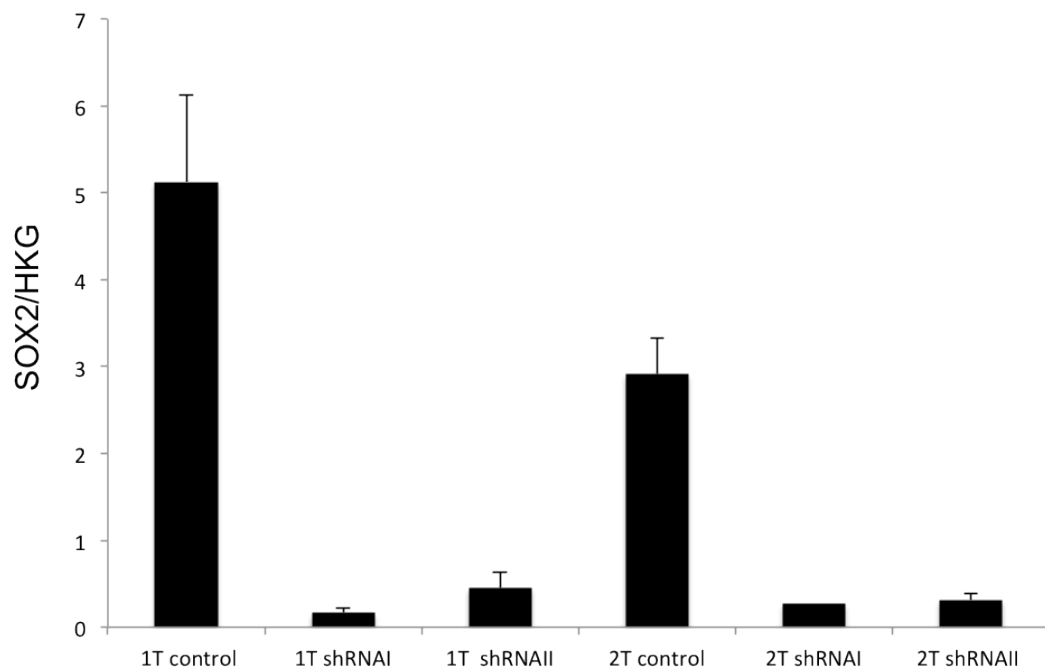


**Figure 4.** SOX2 mRNA level correlates with protein expression in four cell lines with high SOX2 and four cell lines with low SOX2 expression. mRNA level was determined by quantitative RT-PCR with the expression of SOX2 compared to housekeeping genes in an independent experiment from figure 3. SOX2 protein expression was determined by Western blot analysis. Detection of  $\alpha$ -tubulin serves to demonstrate equal quality and quantity of cell lysates.

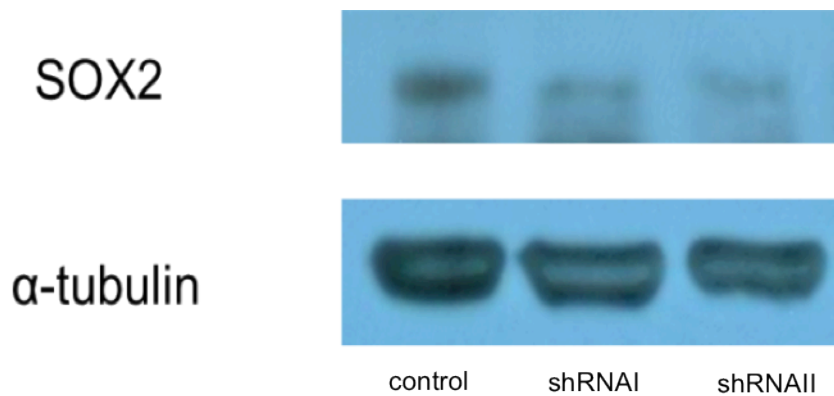
## SOX2 knock down in the HNO223 cell line

To unravel SOX2-dependent gene regulatory networks and affected signaling pathways, we established stable HNO223 cell lines with silenced SOX2 expression using two independent shRNAs. We selected HNO223 cells as model system due to high SOX2 expression and 3q gain. Total RNA was extracted from stably transfected cell lines of luciferase shRNA (control) and two different SOX2 knock down (shRNAI and shRNAII) from two independent transfection experiments (1T and 2T) (Fig. 5a). Moreover, efficient silencing of SOX2 expression in stable HNO223 SOX2 shRNA cell lines was confirmed on protein levels using Western immunoblot analysis (Fig. 5b)

(a)



(b)



**Figure 5.** (a) quantitative RT-PCR: SOX2 expression in HNO223 cells transfected with shRNA luciferase, and with two different shRNAs for SOX2, and the biological duplicates. Expression of SOX2 was compared to the HKG Laminin and PGK1. For each cell line at least three independent experiments were performed using RNA from at least two different time points for SOX2 expression mean and standard deviation calculation; (b) Western Blot: SOX2 protein in HNO 223

transfected with shRNA luciferase, and with two different shRNAs for SOX2,  $\alpha$ -tubulin was used as a loading control.

## Global gene expression analysis with HNO223 control and SOX2 knock down cell lines

For experimental and biological replicates, all six transfected samples were used for the microarray experiment, always comparing one control with one SOX2 knock down RNA (shRNAI and shRNAII) (Fig. 5a). Raw data were normalized by quantile method using Limma package in R statistical environment. Next, two-color approach in Limma was used to identify differentially expressed genes according to the adjusted p value  $\leq 0,05$ . This analysis unraveled 749 genes to be de-regulated upon SOX2 silencing. The data will be uploaded in the GEO online platform. Of those, 456 genes were down-regulated (Table 1) and 293 genes were up-regulated (Table 2) in HNO223-SOX2 knock down cells as compared to HNO223-control cells. Analysis of annotated functions using Ingenuity pathway analysis (IPA) revealed a significant enrichment of differentially expressed genes implicated in cellular processes of cellular movement, cellular development, cellular growth and proliferation, cell death and survival, and cell cycle (Table 3).

The 20 main affected genes according to the Log<sub>2</sub> Fold Change (Log<sub>2</sub>FC) are listed below (Table 1 and 2). Information of gene location and type were extracted from IPA.

**Table 1.** 20 top down-regulated genes after SOX2 silencing as assessed by IPA.

Gene	Log2FC	Adj.p-value	Location	Type(s)
<b>MMP13</b>	-5,098	9,81E-03	Extracellular Space	Peptidase
<b>UBD</b>	-4,534	1,91E-02	Nucleus	Other
<b>MMP1</b>	-4,134	4,53E-02	Extracellular Space	Peptidase
<b>ATOH8</b>	-4,089	1,04E-02	Other	Other
<b>PIGR</b>	-3,940	5,12E-03	Plasma Membrane	Transporter
<b>CP</b>	-3,937	5,12E-03	Extracellular Space	Enzyme
<b>RARRES1</b>	-3,892	5,12E-03	Plasma Membrane	Other
<b>PTGIS</b>	-3,842	8,47E-03	Cytoplasm	Enzyme
<b>LUM</b>	-3,586	1,76E-02	Extracellular Space	Other
<b>IDO1</b>	-3,457	5,12E-03	Cytoplasm	Enzyme
<b>CFB</b>	-3,451	1,02E-02	Extracellular Space	Peptidase
<b>SNCAIP</b>	-3,396	9,81E-03	Cytoplasm	transcription regulator
<b>GCNT3</b>	-3,393	3,57E-02	Plasma Membrane	Enzyme
<b>SOX2</b>	-3,329	1,66E-02	Nucleus	transcription regulator
<b>ST6GALNAC1</b>	-3,295	1,59E-02	Cytoplasm	Enzyme
<b>S100A8</b>	-3,285	3,71E-02	Cytoplasm	Other
<b>AKR1B10</b>	-3,272	1,76E-02	Cytoplasm	Enzyme
<b>CYP2B6</b>	-3,238	1,76E-02	Cytoplasm	Enzyme
<b>PGLYRP4</b>	-3,216	3,71E-02	Plasma Membrane	transmembrane receptor
<b>SERPINB3</b>	-3,203	2,92E-02	Cytoplasm	Other

**Table 2.** 20 top up-regulated genes after SOX2 knock down as assessed by IPA.

Gene	Log2FC	Adj.p-value	Location	Type(s)
<b>VIM</b>	3,745	9,81E-03	Cytoplasm	Other
<b>SH2D5</b>	3,429	2,21E-02	Other	Other
<b>KRT34</b>	3,120	1,24E-02	Cytoplasm	Other
<b>IL1R2</b>	2,884	1,02E-02	Plasma Membrane	transmembrane receptor
<b>HMGA2</b>	2,723	1,66E-02	Nucleus	Enzyme
<b>NLRP3</b>	2,715	2,56E-02	Cytoplasm	Other
<b>EMP1</b>	2,673	3,36E-02	Plasma Membrane	Other
<b>NRG1</b>	2,661	2,08E-02	Other	growth factor
<b>NCF2</b>	2,562	1,02E-02	Cytoplasm	Enzyme
<b>NAV3</b>	2,511	2,02E-02	Nucleus	Other
<b>CSF2</b>	2,501	9,81E-03	Extracellular Space	Cytokine
<b>SPRR2C</b>	2,475	1,66E-02	Cytoplasm	Other
<b>APLN</b>	2,474	1,66E-02	Extracellular Space	Other
<b>IL36G</b>	2,468	3,36E-02	Extracellular Space	Cytokine
<b>ADAMTS6</b>	2,465	1,66E-02	Extracellular Space	Peptidase
<b>COL13A1</b>	2,447	3,05E-02	Plasma Membrane	Other
<b>KRTAP2-3</b>	2,373	4,05E-02	Other	Other
<b>PTX3</b>	2,275	4,14E-02	Extracellular Space	Other
<b>FN1</b>	2,262	3,05E-02	Extracellular Space	Enzyme
<b>MFNG</b>	2,252	3,79E-02	Cytoplasm	Enzyme

**Table 3.** The molecular and cellular functions affected by SOX2 as assessed by IPA.

Function name	Adj. p-value	Number of Molecules
Cellular Movement	2,62E-12 - 1,51E-02	118
Cellular Development	4,17E-10 - 1,51E-02	141
Cellular Growth and Proliferation	4,17E-10 - 1,49E-02	165
Cell Death and Survival	3,33E-07 - 1,51E-02	162
Cell Cycle	1,87E-06 - 9,31E-03	64

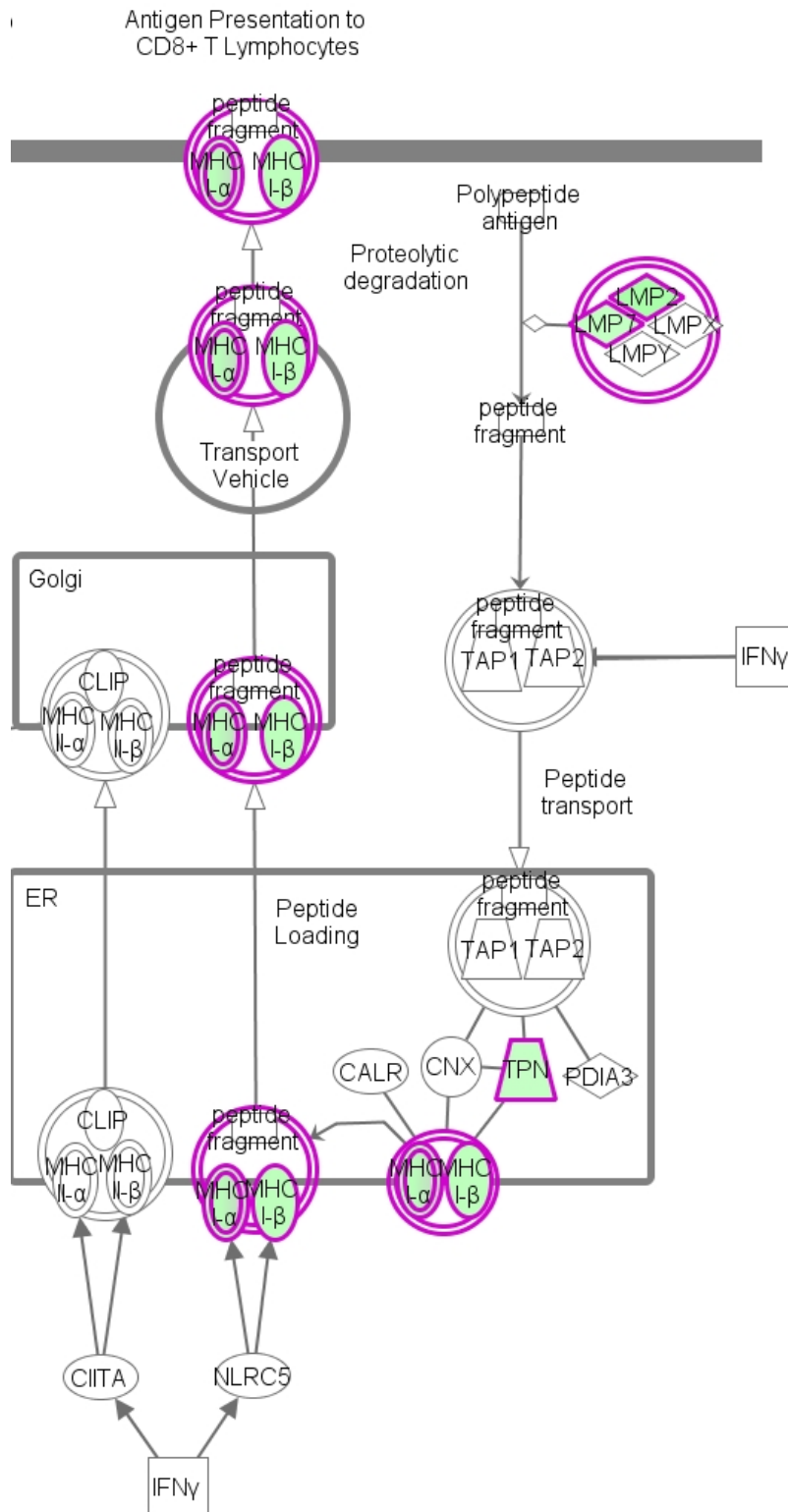


## Antigen presentation pathway is the top canonical pathway affected upon SOX2 silencing

Concerning canonical pathways affected by SOX2 silencing, we identified an enrichment of connected differentially expressed genes implicated in regulatory mechanisms of the immune system, including Antigen Presentation Pathway and Communication between Innate and Adaptive Immune Cells (Table 4). Accordingly, several genes are known to regulate MHC-I-mediated antigen presentation to the CD8<sup>+</sup> T lymphocytes (Fig. 6). Moreover, direct and indirect interactions have been reported in the literature for SOX2-regulated genes, which form part of the top affected pathway, Antigen Presentation Pathway (Fig. 7). The genes present in our data set that are part of the Antigen Presentation Pathway are down-regulated after SOX2 knock down (Table 5). In summary, these data suggest a role of SOX2-dependent gene regulatory networks in immune surveillance and tumor-stroma interaction.

**Table 4.** The top canonical pathways affected by SOX2 silencing as assessed by IPA.

Pathway name	Adj. p-value
Antigen Presentation Pathway	1,25E-05
Prostanoid Biosynthesis	2,86E-04
Communication between Innate and Adaptative Immune Cells	4,53E-04
Type I Diabetes Mellitus Signaling	1,2E-03
UDP-N-acetyl-D-glucosamine Biosynthesis II	1,21E-03



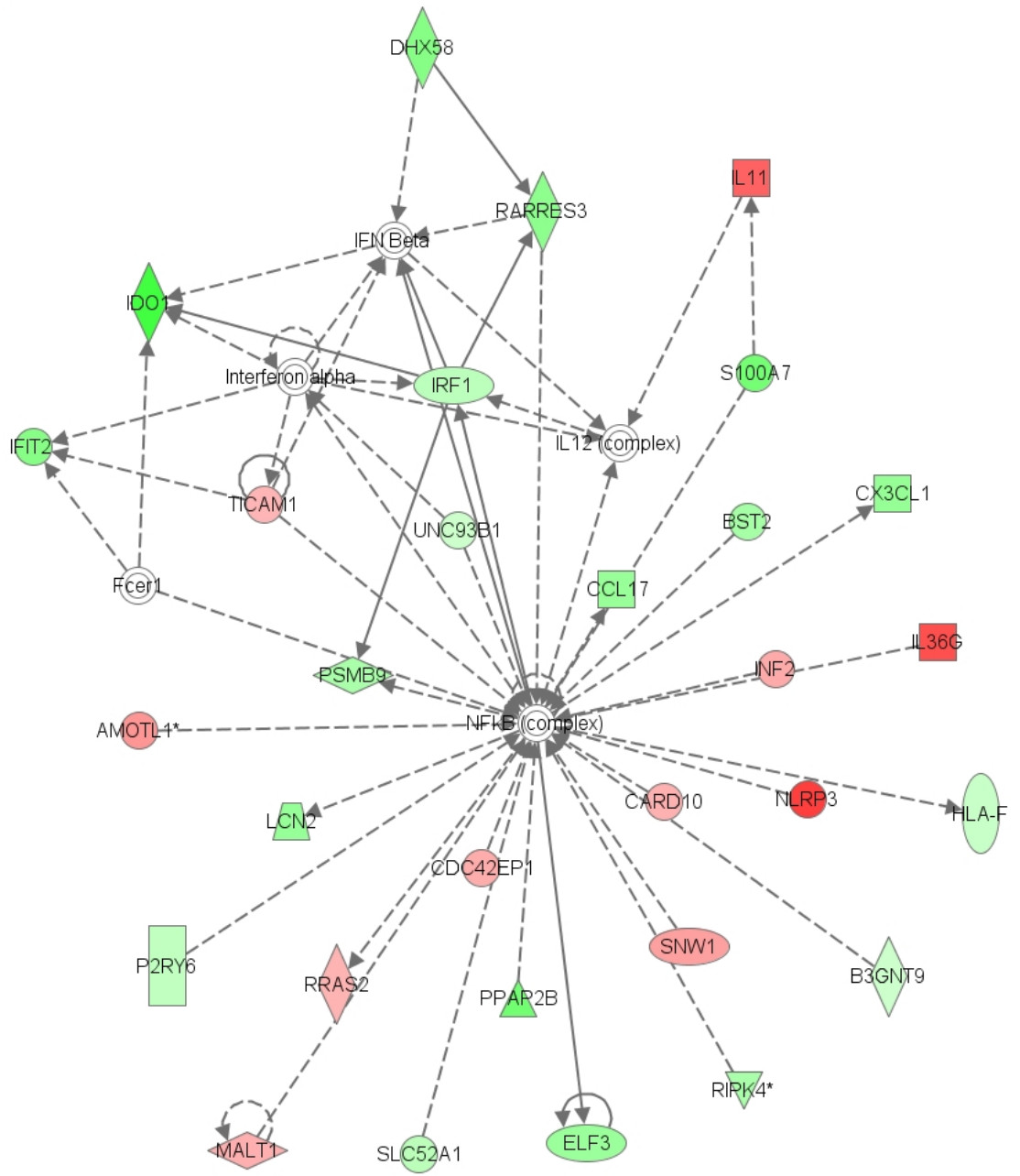
**Figure 6.** SOX2 affects MHC-I mediated antigen presentation pathway. In color are the genes positive affected after SOX2 silencing. The figure is part of a

figure generated by IPA for the Antigen Presentation Pathway. The rest of the figure was showing genes no affected by SOX2 in our data set.

**Table 5.** List of genes affected in the antigen presentation pathway after SOX2 silencing as assessed by IPA.

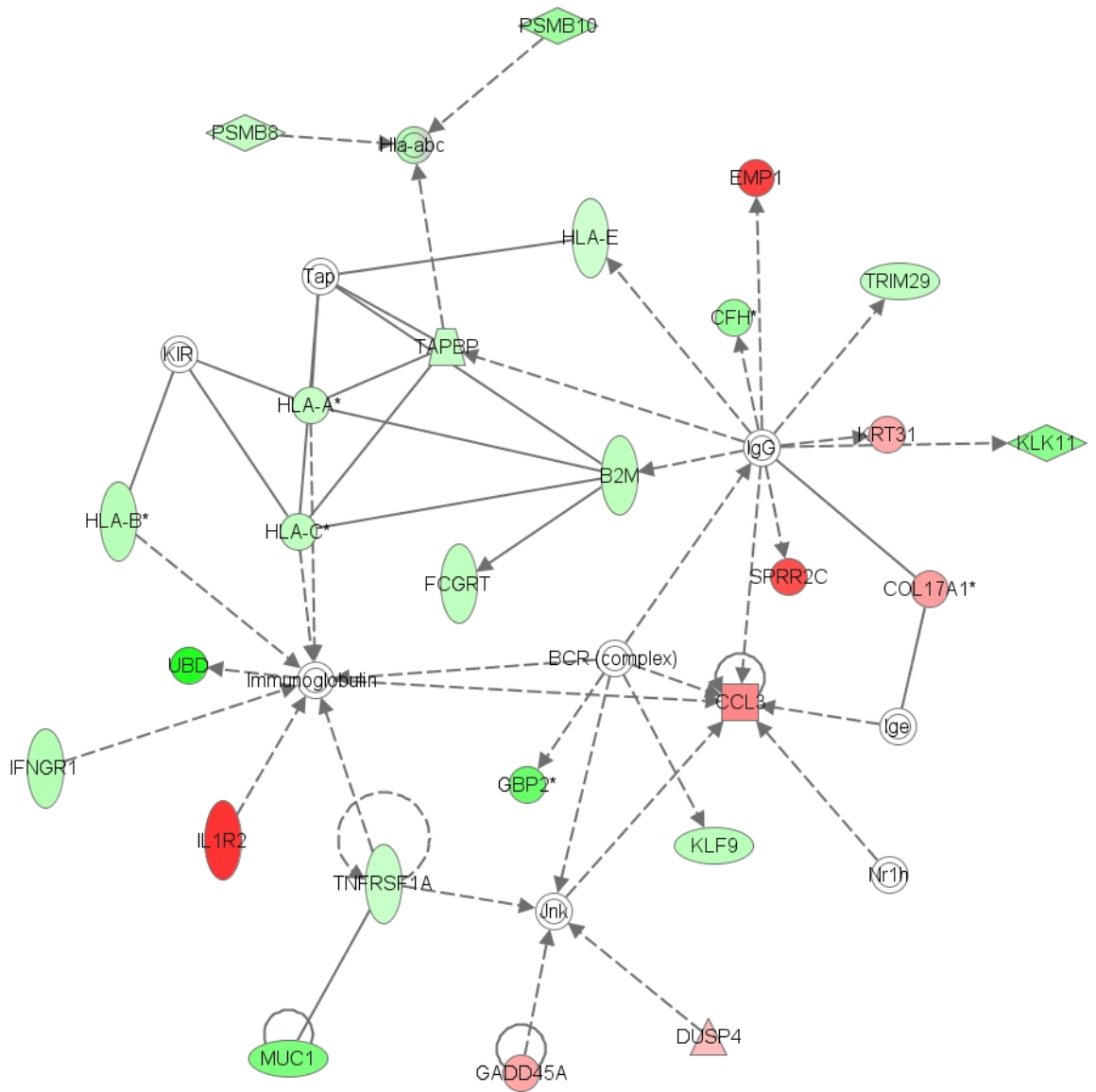
Gene	Log2FC	Adj.p-value	Location	Type(s)
<b>B2M</b>	-1,185	5,21E-02	Plasma Membrane	transmembrane receptor
<b>HLA-A</b>	-1,009	4,81E-02	Plasma Membrane	Other
<b>HLA-B</b>	-1,281	3,57E-02	Plasma Membrane	transmembrane receptor
<b>HLA-C</b>	-1,168	4,47E-02	Plasma Membrane	Other
<b>HLA-E</b>	-0,886	5,40E-02	Plasma Membrane	transmembrane receptor
<b>HLA-F</b>	-0,998	4,96E-02	Plasma Membrane	transmembrane receptor
<b>PSMB8</b>	-1,079	3,43E-02	Cytoplasm	Peptidase
<b>PSMB9</b>	-1,540	4,43E-02	Cytoplasm	Peptidase
<b>TAPBP</b>	-1,029	4,20E-02	Cytoplasm	Transporter

(a)



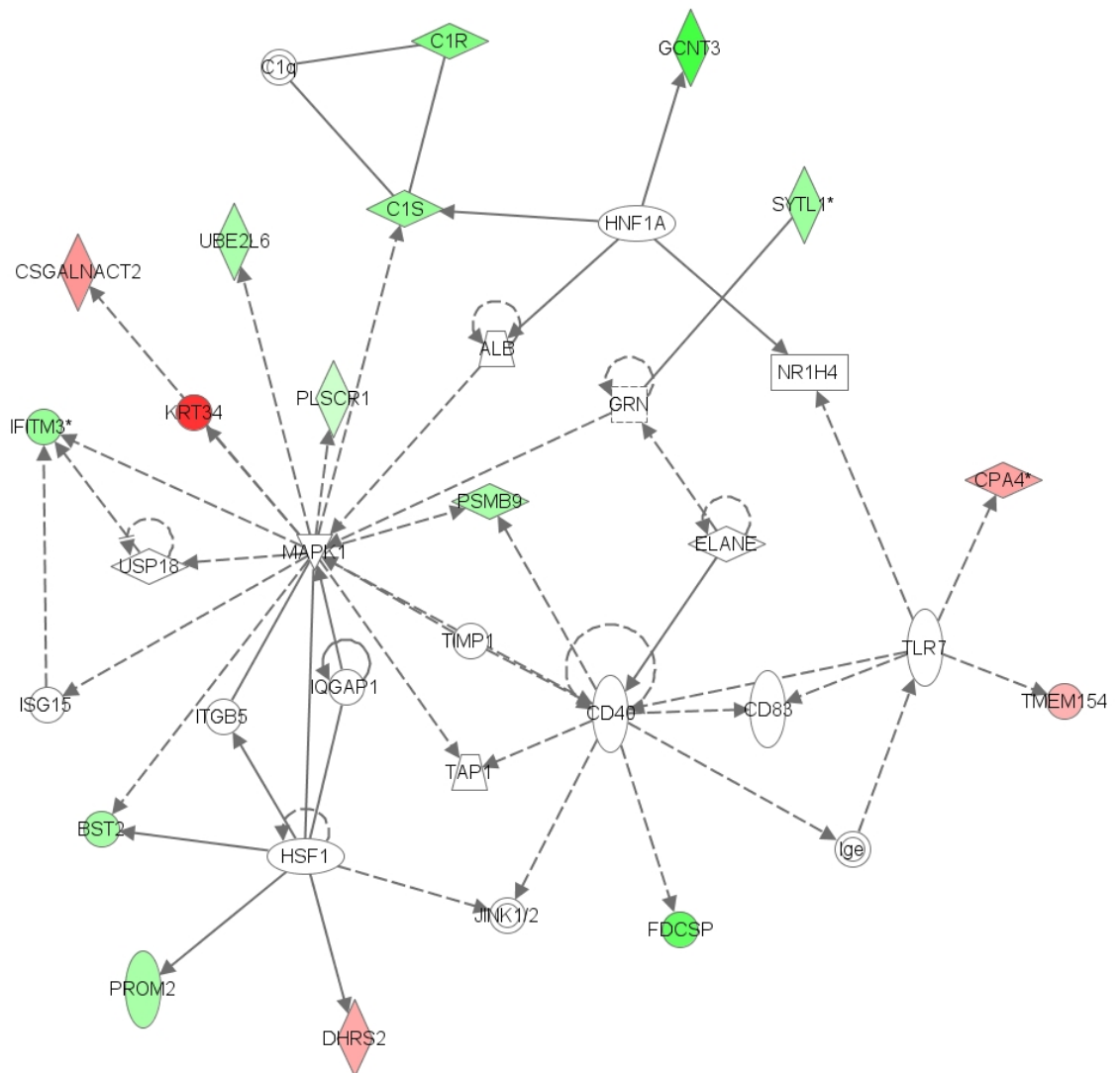
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(b)



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(c)



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**Figure 7.** Networks related to the antigen presentation pathway assessed by IPA. (a) HLA-A, HLA-B, HLA-C, HLA-E, PSMB8 and TAPBP network gene interaction; (b) HLA-F network gene interaction (c) PSMB9 network gene interaction. Red: up-regulated genes. Green: down-regulated genes. Genes without color are possible connections suggested by IPA and are not present in the HNO223 expression data.

→ Acts on

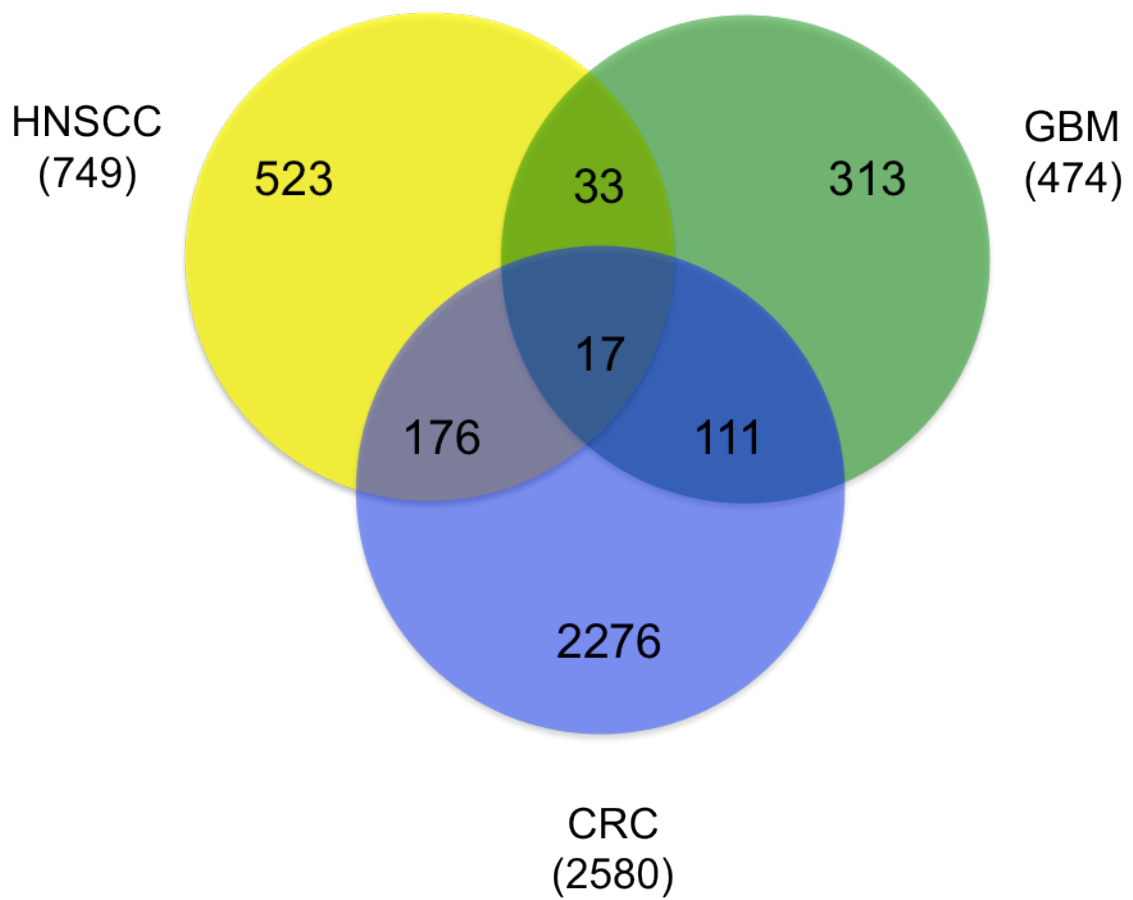
- -> Indirect regulation

— Binding only

### Common SOX2-regulated genes in HNSCC and other types of cancer

Since more than 700 genes were found to be affected we aimed to investigate which genes affected by SOX2 were playing an important role in cellular mechanisms related to cancer. To define a common set of genes affected by SOX2 independent of the cancer type and site, we compared our data set with published data from glioblastoma (GBM) (76) and colorectal cancer (CRC) (61) that used similar methods to ours to develop gene expression data of SOX2 knock down. We found 17 genes in common between the 3 types of cancer (Fig. 8 and Table 6). In this gene set, 9 were positively affected by SOX2 and 8 were negative affected by SOX2 expression. 12 genes have been described to be related to cancer, the most frequent one was Interferon Regulatory Factor-1 (IRF-1) (Table 7), which was also the only transcription factor found in common between the three cancer types analyzed here (Table 6).





**Figure 8.** Genes affected by SOX2 in HNSCC HNO223 cell line compared GBM and CRC cell lines (61, 76). The graph shows the genes found in common between HNSCC and GBM, GBM and CRC, HNSCC and CRC and in the middle the genes in common between HNSCC, GBM and CRC.

**Table 6.** List of common genes affected by SOX2 in HNSCC, GBM and CRC cell lines.

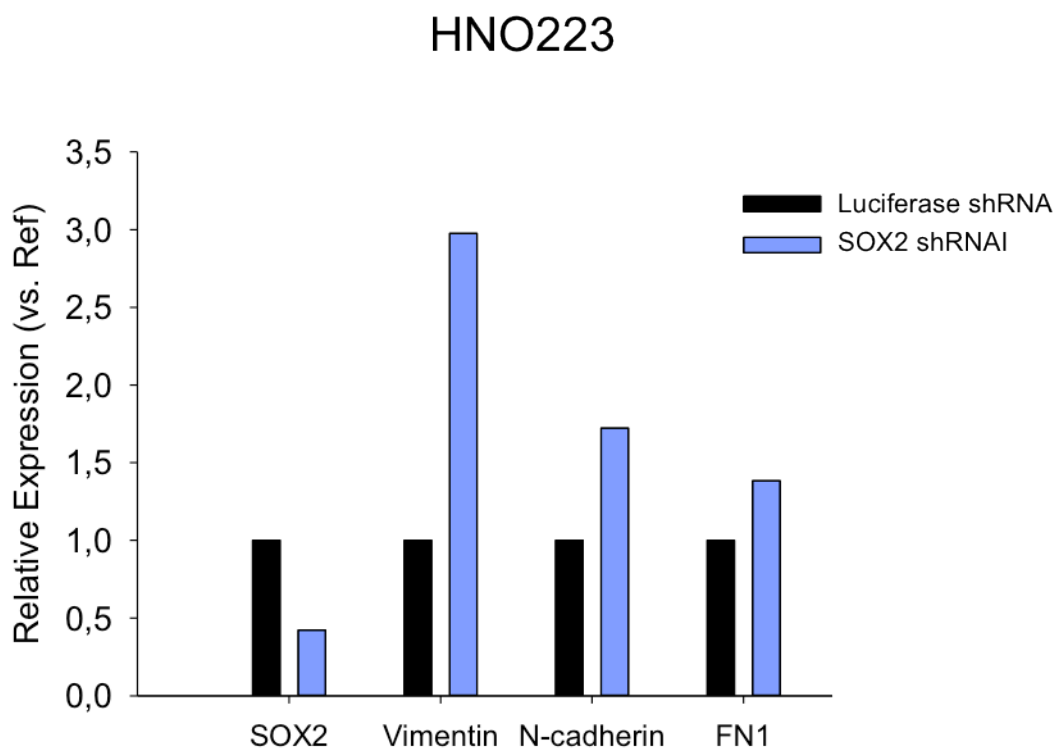
Gene	Log2FC	Adj.p-value	Location	Type(s)
<b>AKR1B10</b>	-3,187	1,19E-02	Cytoplasm	enzyme
<b>CFH</b>	-1,832	2,60E-02	Extracellular Space	other
<b>DHRS2</b>	1,213	4,55E-02	Nucleus	enzyme
<b>GADD45A</b>	1,252	2,59E-02	Nucleus	Other
<b>GBP2</b>	-2,741	1,03E-02	Nucleus	Other
<b>GLUL</b>	-1,670	4,78E-02	Cytoplasm	Enzyme
<b>IFIT2</b>	-2,188	3,36E-02	Cytoplasm	Other
<b>IL23A</b>	1,773	2,48E-02	Extracellular Space	Cytokine
<b>IRF1</b>	-1,228	2,99E-02	Nucleus	transcription regulator
<b>MFNG</b>	1,482	3,36E-02	Cytoplasm	Enzyme
<b>PTHLH</b>	1,584	2,21E-02	Extracellular Space	Other
<b>S100A2</b>	1,210	3,36E-02	Nucleus	Other
<b>SCRN2</b>	-1,072	4,77E-02	Extracellular Space	other
<b>SOX2</b>	-2,147	1,66E-02	Nucleus	transcription regulator
<b>STC2</b>	1,832	1,66E-02	Extracellular Space	other
<b>TNFAIP2</b>	-2,422	2,02E-02	Extracellular Space	other
<b>TRIM36</b>	1,247	2,60E-02	Cytoplasm	other

**Table 7.** List of genes affected by SOX2 between HNSCC, GBM and CRC cell lines and known to be related to cancer as defined using IPA.

Gene	Type of cancer	p-Value
<b>CFH,GBP2,IRF1</b>	prostatic intraepithelial neoplasia	1,15E-04
<b>GADD45A</b>	arrest in cell cycle progression of ovarian cancer cells	1,04E-03
<b>CFH,S100A2</b>	serous ovarian carcinoma process	8,50E-03
<b>AKR1B10,CFH,GBP2,GLUL,IRF1,PTHLH, S100A2,SCRN2,SOX2,TNFAIP2,TRIM36</b>	malignant neoplasm of abdomen	9,69E-03
<b>CFH,GLUL,IRF1,PTHLH,S100A2,SOX2</b>	breast or ovarian cancer	1,01E-02
<b>IRF1,PTHLH</b>	triple-negative breast cancer	1,03E-02
<b>SOX2</b>	ductal carcinoma in situ	1,75E-02
<b>CFH,GBP2,IRF1,S100A2,TNFAIP2</b>	pelvic cancer	2,10E-02
<b>GLUL,IRF1,PTHLH,S100A2,SOX2</b>	breast cancer	2,10E-02
<b>S100A2</b>	mucinous ovarian cancer	2,37E-02
<b>AKR1B10,IRF1</b>	non-small cell lung cancer	2,83E-02
<b>CFH,GBP2,IRF1,S100A2</b>	genital tumor	2,87E-02
<b>S100A2</b>	clear-cell ovarian carcinoma	2,88E-02
<b>SOX2</b>	trophoblastic tumor	3,08E-02
<b>CFH,GBP2,IRF1</b>	prostate cancer	3,81E-02
<b>GADD45A</b>	peripheral T-cell lymphoma	3,98E-02
<b>AKR1B10,IRF1,SOX2</b>	carcinoma in lung	4,05E-02
<b>IRF1</b>	esophageal squamous cell cancer	4,18E-02
<b>IRF1,SOX2</b>	gastric cancer	4,68E-02

## Confirmation of differentially expressed genes in HNO223-control and HNO223-shSOX2 cell lines

Since cellular movement was the main molecular and cellular function affected by SOX2, we selected three candidate genes, Vimentin (VIM), Fibronectin-1 (FN1) and N-cadherin (CDH2), with known function in cell mobility that were up-regulated after SOX2 silencing (Table 2). Induced expression of all three candidate genes was confirmed in the HNO223-shSOX2 cell line as compared to control by qRT-PCR (Fig. 9).

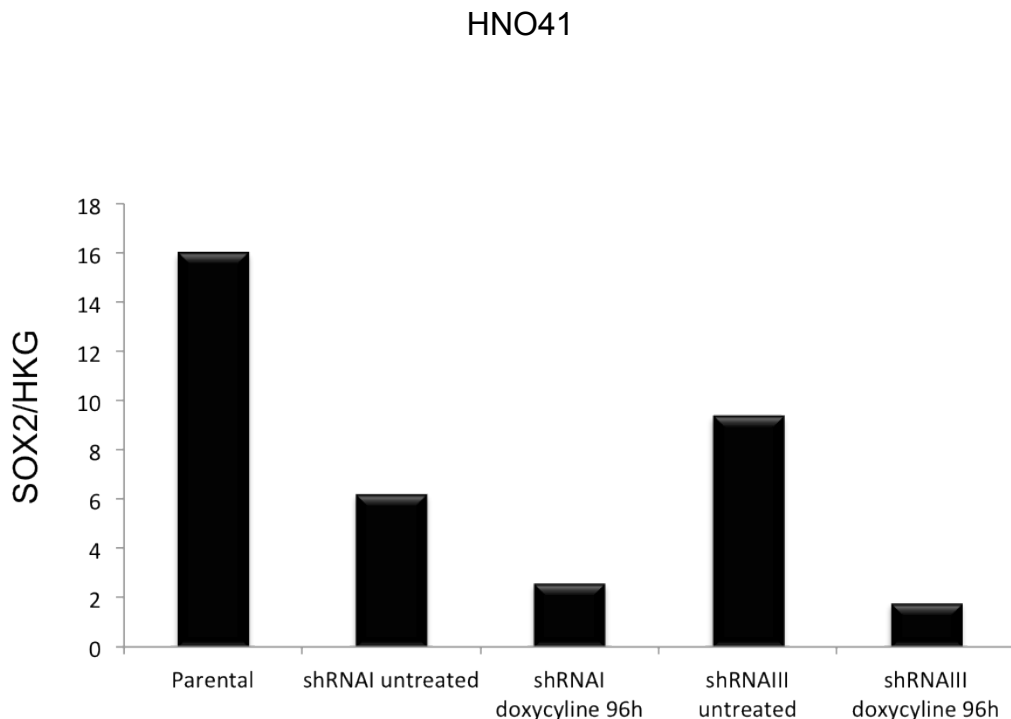


**Figure 9.** Induced transcription of VIM, CDH2 and FN1 in the HNO223-shSOX2 cell line. SOX2, VIM, CDH2 and FN1 mRNA was measured with quantitative RT-PCR. Expression of genes was compared to the HKG LamininB1. Relative

transcript levels were calculated related to HNO223-control, which were set to 1.  
Data were produced and kindly provided by Pilar Bayo.

## Conditional silencing of SOX2 in the HNO41 cell line

To further confirm SOX2-dependent regulation of newly identified candidate genes in an independent cellular model system, we generated two stable HNO41 cell lines with two different shRNA for TET-ON doxycycline inducible SOX2 knock down (shRNAI and shRNAIII) with an inducible SOX2 silencing based on the pLKO-Tet-on vector system and two independent shRNAs. The HSCC cell line HNO41 was selected due to 3q amplification and high SOX2 expression (Fig. 3). We achieved a 60-80% reduction in SOX2 transcript levels in HNO41 SOX2 knock down cells after 2-4 days of doxycycline treatment as compared to untreated controls (Fig. 10). However, it is worth mentioning that we also observed a decrease in SOX2 transcript levels in untreated HNO41-shRNAI and HNO41-shRNAIII in relation to parental HNO41 cells, that could be due to the stress of the method itself (Fig. 10).



**Figure 10.** Conditional SOX2 silencing in HNO41-shRNAI and HNO41-shRNAIII cell lines. SOX2 transcript levels were assessed by quantitative RT-PCR in parental HNO41 cells and stable HNO41 cell lines transduced with two independent shRNAs

for SOX2. Cells were treated with doxycycline for 96 hours to induce SOX2 silencing (TET-ON inducible system). Expression of SOX2 was compared to the HKG Laminin and PGK1.

## Impact of SOX2 silencing on apoptosis

Several reports provided experimental evidence that SOX2 plays a critical role in the regulation of tumor cell survival and its silencing induces apoptosis in distinct cancer cells, including HNSCC cells (49, 83). Indeed, conditional silencing of SOX2 in HNO41 cells increased the percentage total of dead and apoptotic cells (Fig. 11). Next, we addressed the question, whether silencing of SOX2 also influences induction of apoptosis by chemotherapeutic drugs. As expected, treatment with doxorubicin for 48 hours resulted in a strong induction of apoptosis in parental HNO223 and HNO223-control cell lines, which was even higher in HNO223-shRNAI SOX2 knock down cells (Fig. 12).

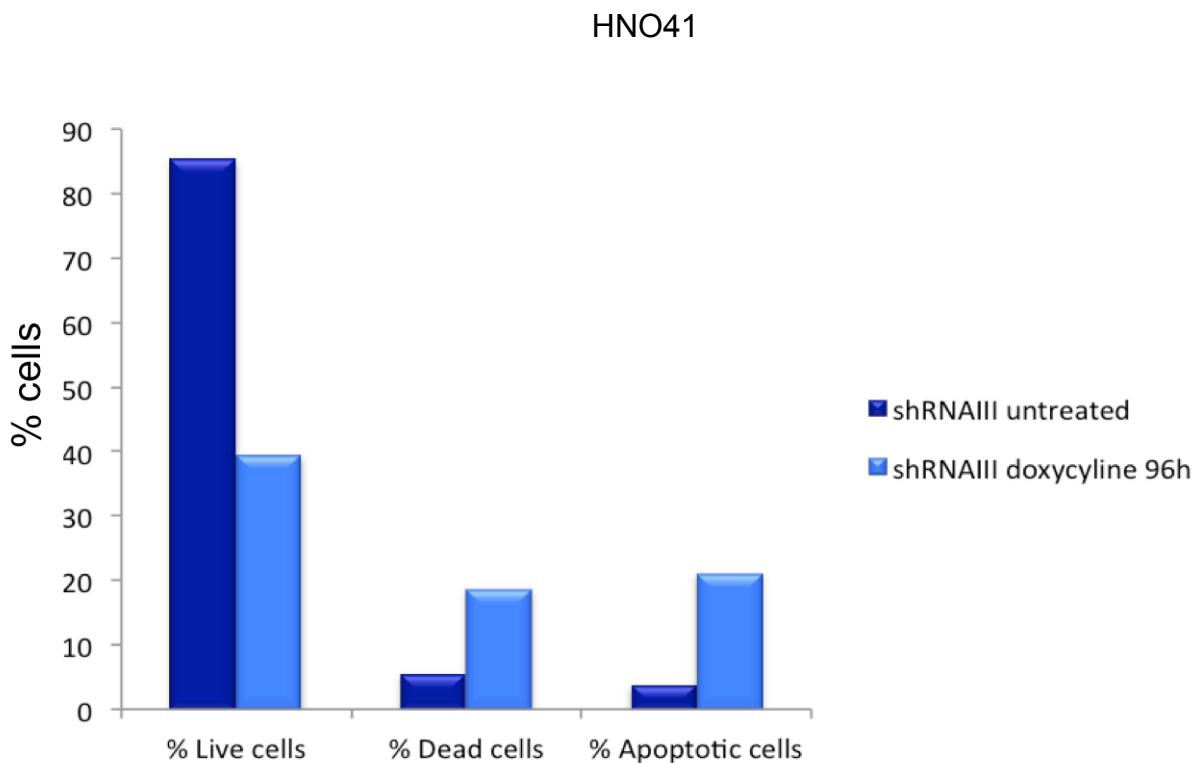
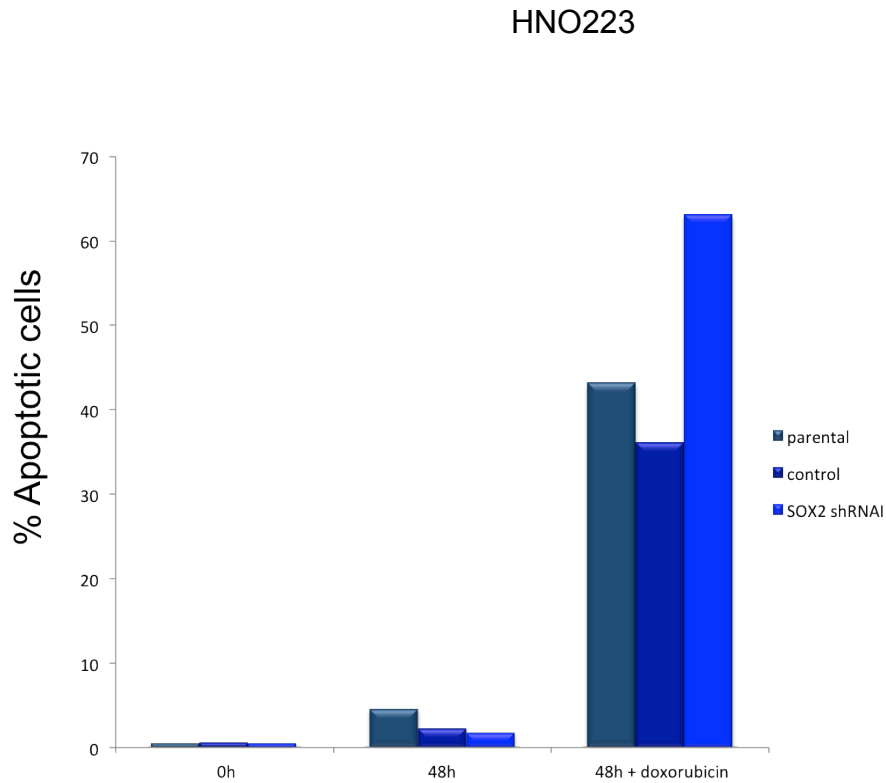


Figure 11. Conditional silencing of SOX2 in HNO41 decrease the percentage total of living cells by increasing of dead and apoptotic cells. The relative amount of dead and apoptotic cells was analyzed according to 7AAD and annexin V staining measured by FACS. Both 7AAD and annexin V staining



indicate late apoptotic cells whereas annexin V single positive cells are early apoptotic cells. 7AAD single positive cells are dead cells, likely necrotic.

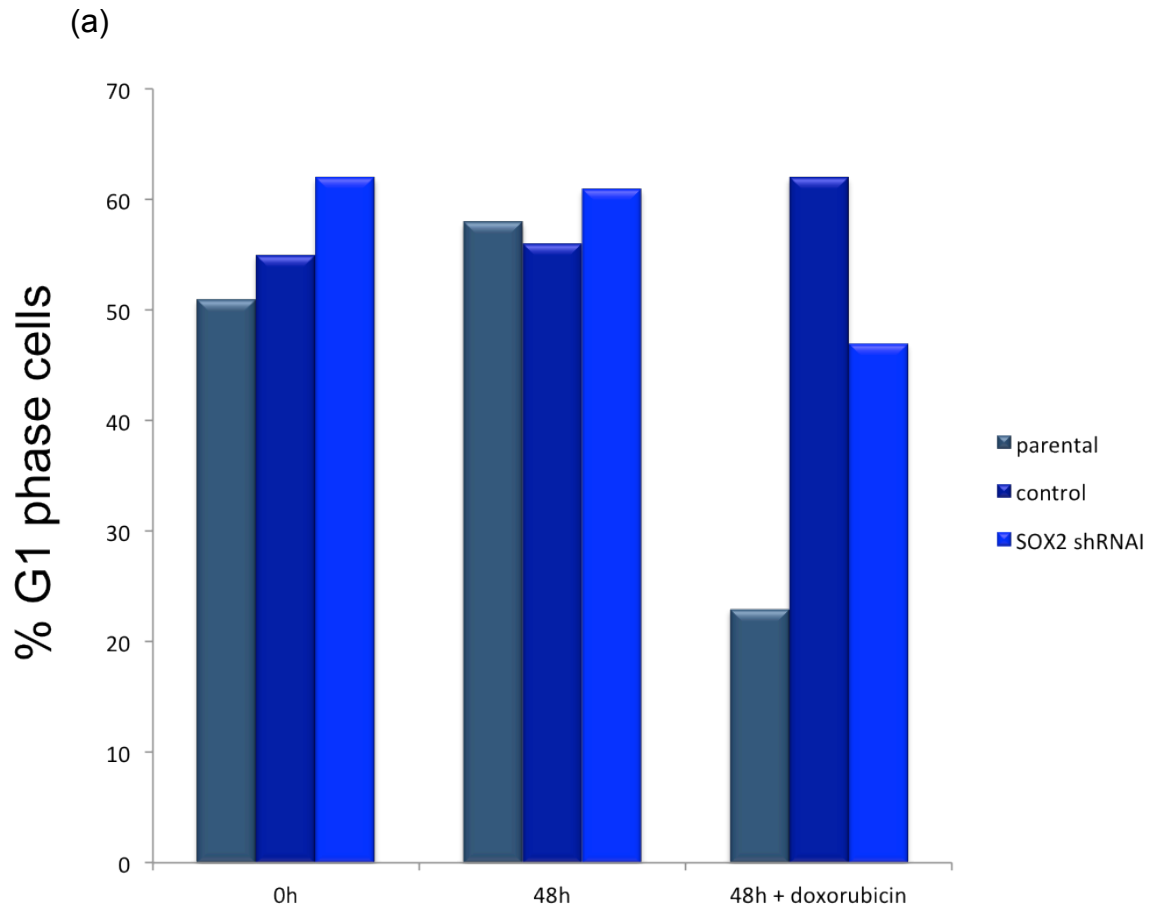


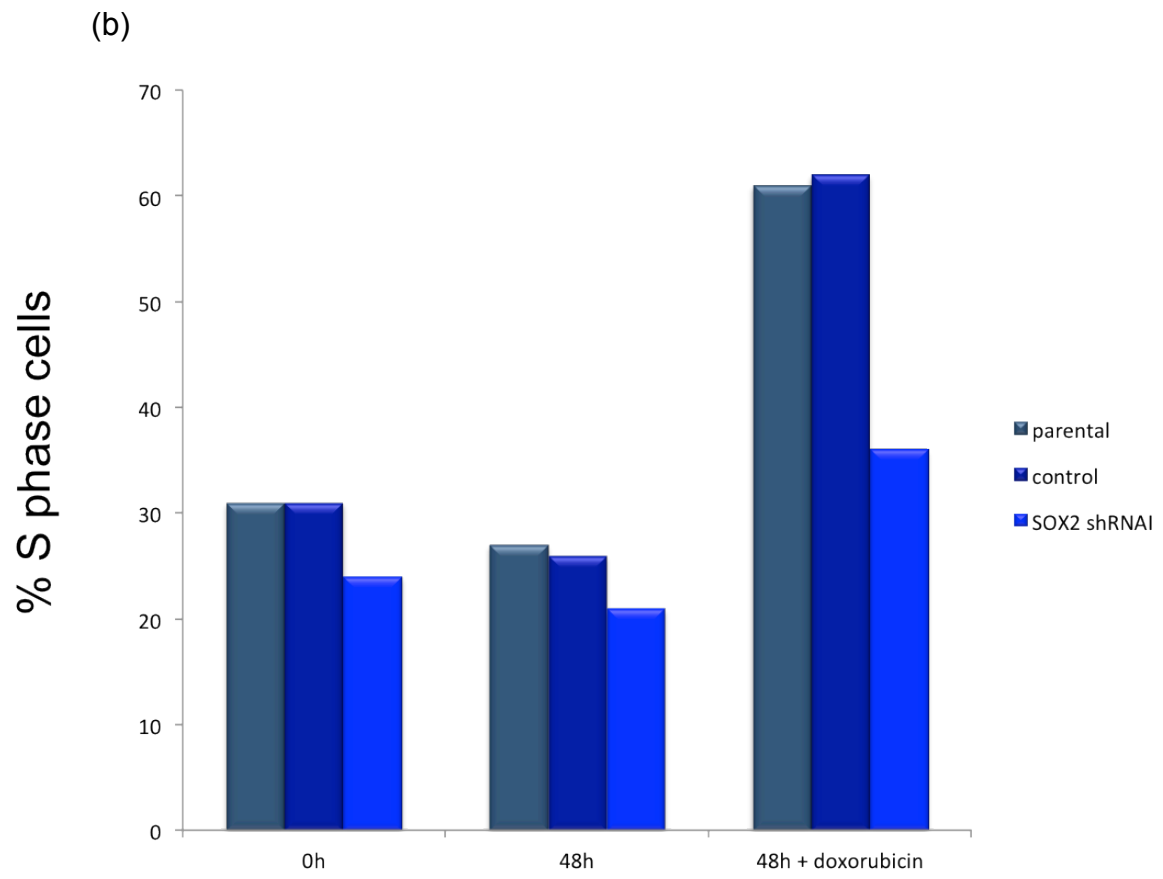
**Figure 12.** Enhanced apoptosis of HNO223 cells with silenced SOX2 expression after doxorubicin treatment. Apoptotic cells were considered to be the population sub-G1 phase. Cell DNA was stained by DAPI, measured with UV light in FACS and analyzed for cell cycle with the flowJo software, using the Dean-Jett-Fox mathematical model. Cells were measured at time point 0 hour, 48 hour without treatment and 48 hour with doxorubicin treatment for apoptosis induction.

## Impact of SOX2 silencing on cell cycle progression

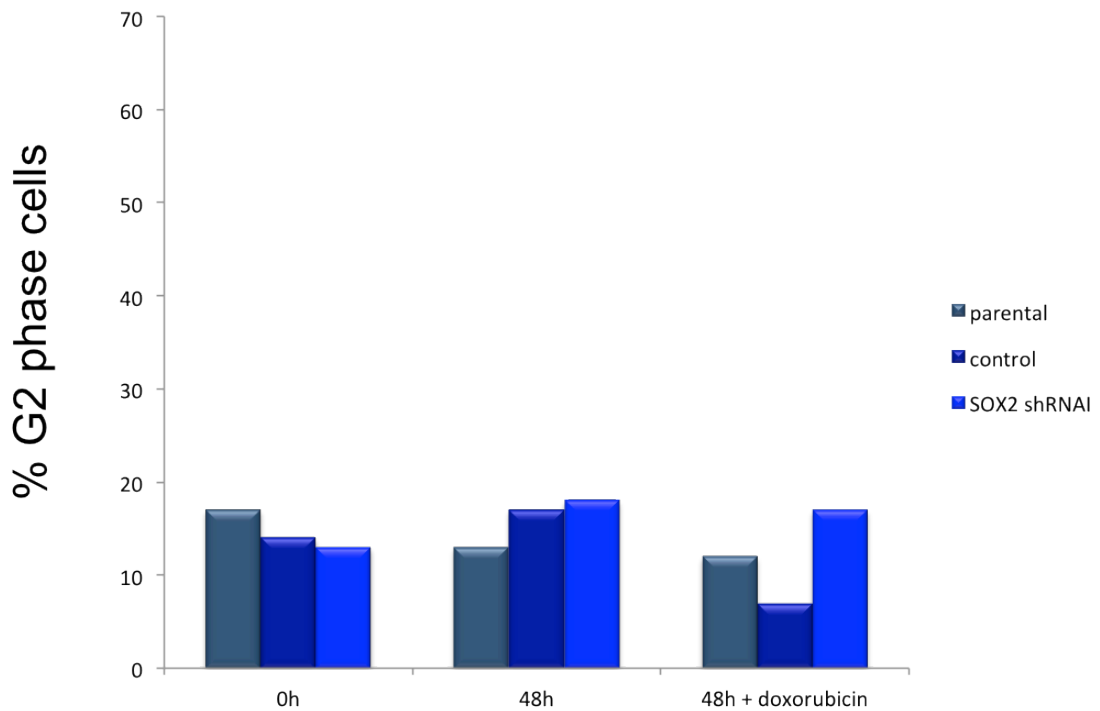
Since it was reported for other types of cancer that SOX2 affects the cell cycle we aimed to understand the effect in cell cycle after SOX2 knock down. HNO223 cells treated for 48 hours with doxorubicin accumulated less SOX2 knock down cells in S phase when compared to control or parental cell line (Fig. 13).

HNO223





(c)



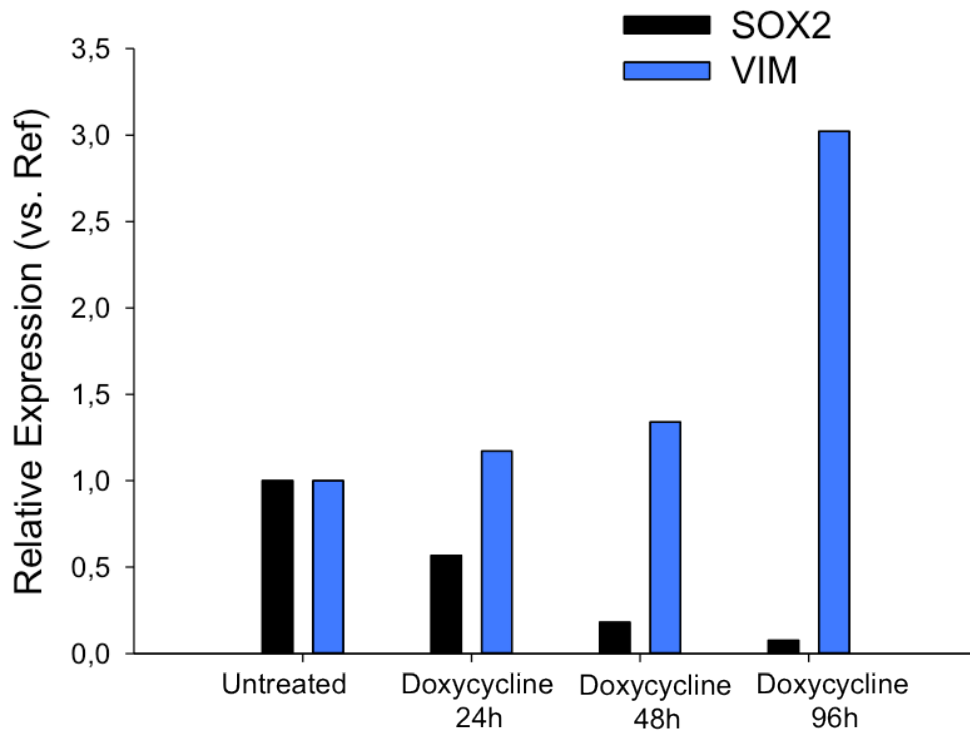
**Figure 13.** SOX2 knock down in HNO223 decreases the % of S phase cells after apoptosis induction. (a) G1 phase cells; (b) S phase cells; (c) G2 phase cells. Cell DNA was stained by DAPI, measured with UV light in FACS and analyzed for cell cycle with the flow jo software, using the Dean-Jett-Fox mathematical model. Cells were measured at time point 0 hour, 48 hour without treatment and 48 hour with doxorubicin treatment for apoptosis induction.

## Impact of SOX2 silencing on cellular movement

### **SOX2 expression inversely correlates with VIM in TSCC and HSCC cell lines**

To further confirm a causal link between SOX2 silencing and upregulation of Vimentin expression, we analyzed VIM transcript levels in HNO41-shRNA for SOX2 with or without doxycycline treatment. In line with previous findings in HNO223 cells, conditional silencing of SOX2 in HNO41 cells revealed a 3-fold increase in Vimentin expression 96 hours after doxycycline treatment (Fig. 14).

## HNO41 SOX2 shRNAI



**Figure 14.** Induced VIM transcript levels upon conditional SOX2 silencing in the HNO41-shRNAI cells. SOX2 and VIM transcript levels were measured with quantitative RT-PCR. Expression of genes was compared to the HKG, LamininB1. Transcript levels of untreated HNO41-shRNAI cells was set to 1 and relative expression values were calculated for each gene. Data kindly provided by Pilar Bayo.

### **Impact of conditional SOX2 silencing on cellular morphology**

In order to investigate the impact of conditional SOX2 silencing on the tumor cell physiology under normal growth conditions HNO41-shRNAI and HNO41-shRNA non-target (Nt) cells were cultured for 10 days with or without doxycycline. Concerning cell morphology, no obvious difference was observed between both cell lines. However, doxycycline treated HNO41-shRNAI cells acquired a bigger cell size and presented cellular protrusions, which was not visible for HNO41-NT cells growing under the same culture conditions (Fig. 15).



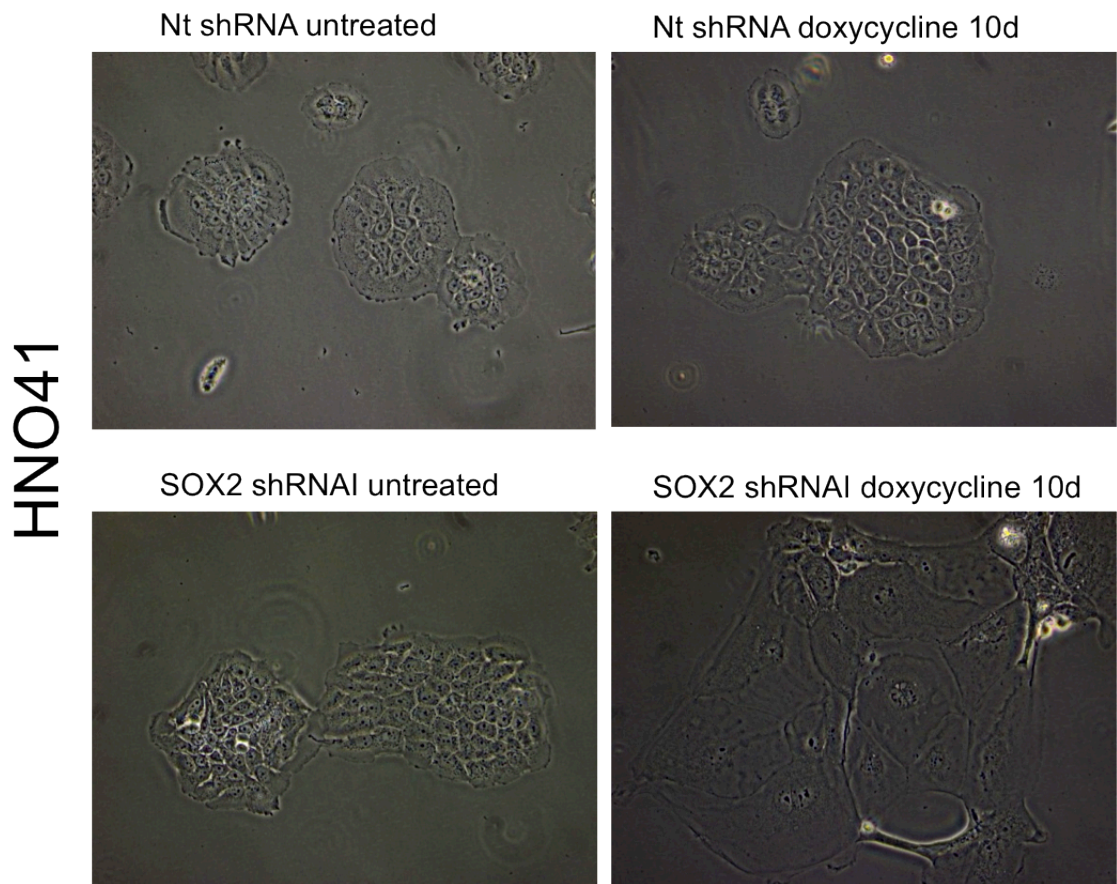
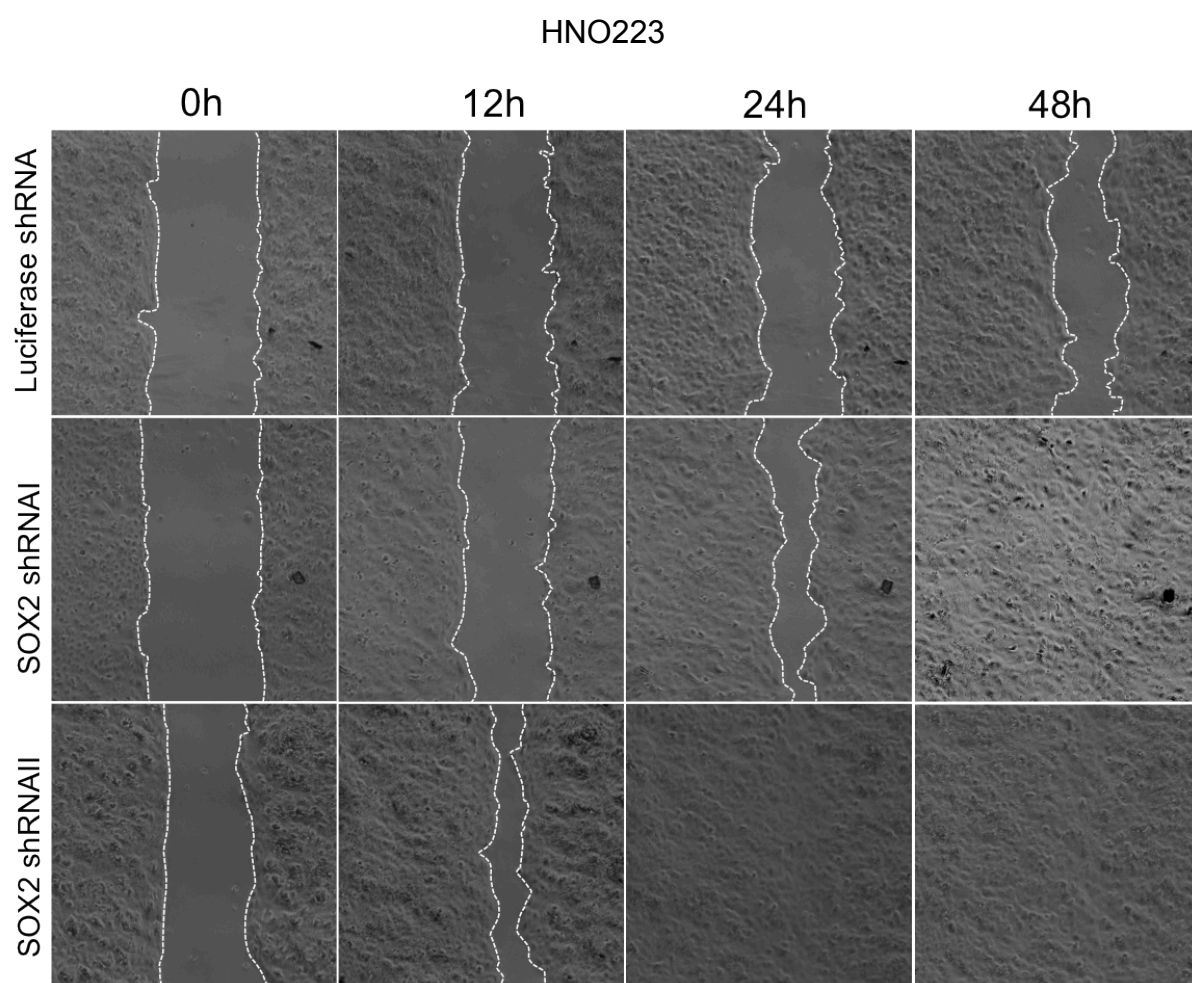


Figure 15. Conditional silencing of SOX2 effects cellular morphology. Upper: normal epithelial morphology of HNO41-Nt cells culture with or without doxycycline; lower: mesenchymal-like morphology of HNO41-shRNAI cells cultured with doxycycline for 10 days doxycycline. Data kindly provided by Pilar Bayo.

### Impact of SOX2 silencing on tumor cell motility

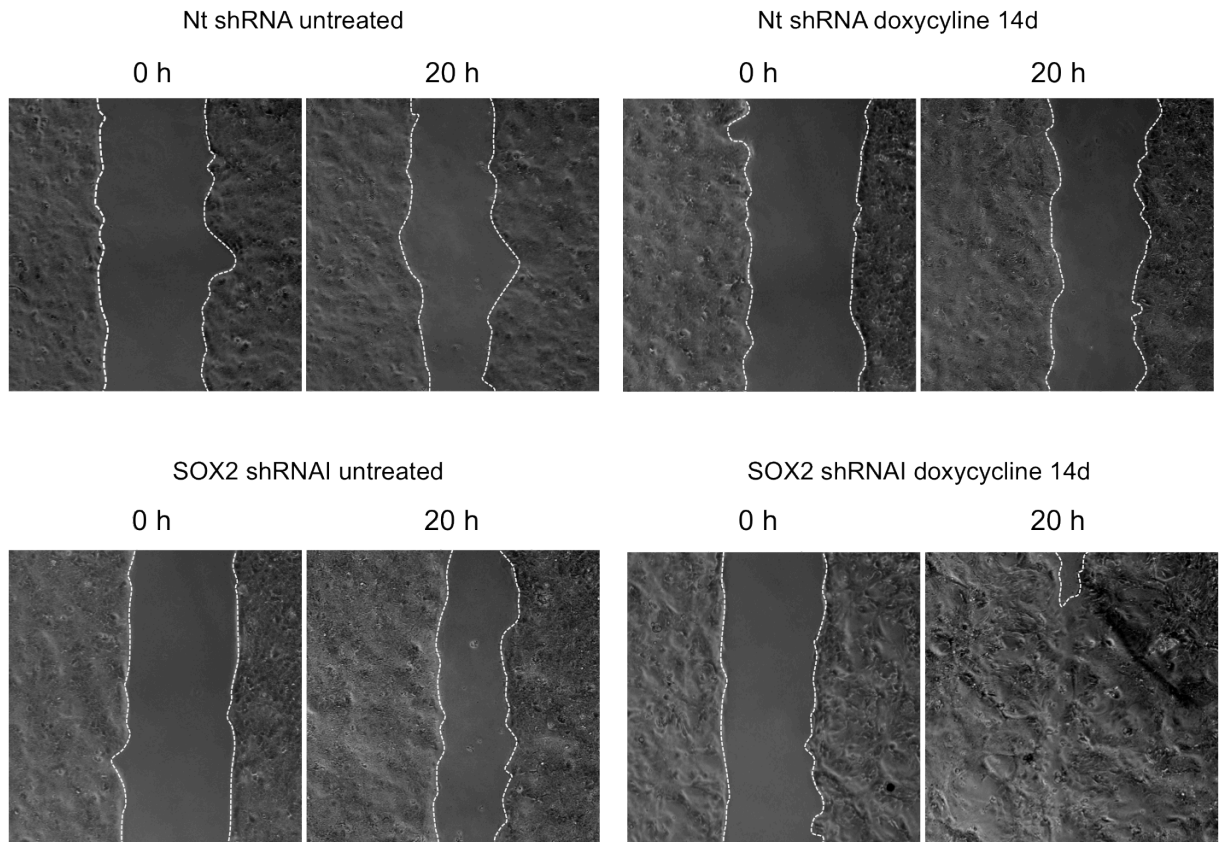
Since we confirmed an inverse regulation of Vimentin and SOX2, and observed changes in cellular morphology upon conditional SOX2 silencing we decided to monitor the migratory potential using an in vitro wound-healing scratch assay. While HNO41-shRNAI cells under doxycycline culture conditions closed the gap within 20 hours, a clear gap was still visible for HNO41-Nt controls (Fig. 16). These data demonstrate a severe increase in cellular migration after conditional SOX2 silencing. Indeed, an accelerated increase in cell migration was also evident for HNO223-shSOX2 cell lines as compared to HNO223-control (Fig. 16).

(a)



(b)

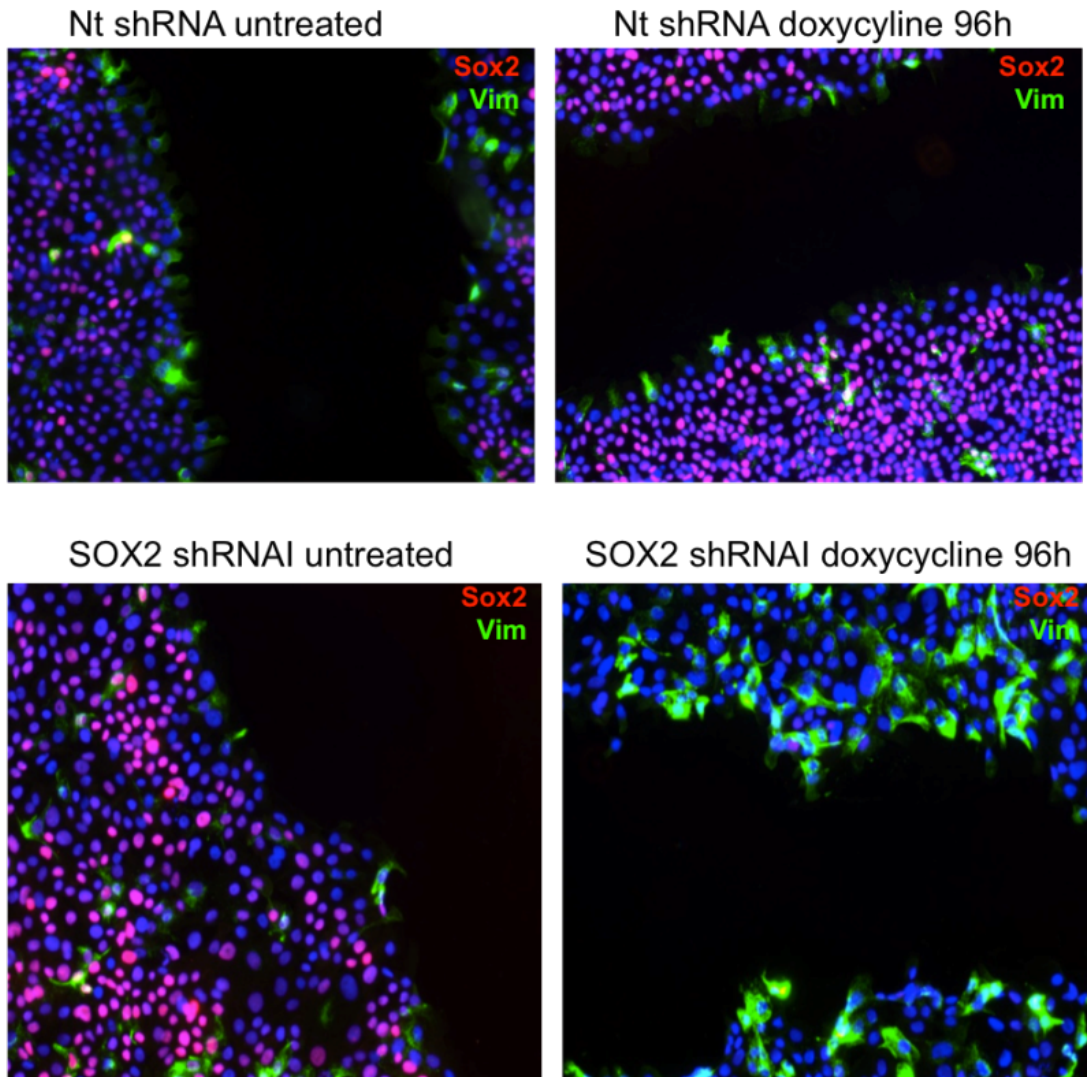
## HNO41



**Figure 16.** Enhanced migration of SOX2 silenced HNSCC cell lines in an in vitro wound healing scratch assay. Representative bright field pictures demonstrate migration of HNO223-control and HNO223-shRNAI SOX2 knock down cell lines (a) and HNO41-Nt and HNO41-shRNAI cell lines (b), which was monitored at indicated time point after scratching. Data kindly provided by Pilar Bayo.

In order to proof the hypothesis whether accelerated migration after SOX2 silencing is related to increased Vimentin expression, we conducted co-immunofluorescence staining of HNO41-Nt and HNO41-shRNAI cells after scratching. Tumor cells at the migratory front were preferably SOX2 negative and some showed a positive staining for Vimentin. Under doxycycline culture conditions, we confirmed loss of SOX2 expression accompanied by a strong increase in Vimentin staining in tumor cells of the migratory border (Fig. 17

HNO41



**Figure 17.** Conditional SOX2 silencing induces the amount of Vimentin-positive tumor cells at the migratory border. Representative picture of immunofluorescence staining for SOX2 (red), Vimentin (green) and nuclear staining (blue) of HNO41-Nt and HNO41-shRNAI cells untreated and under doxycycline culture conditions and following in vitro scratching. Data kindly provided by Pilar Bayo.

## Discussion

We aimed to explore the functional and mechanistic role of SOX2 for HNSCC, since results from Freier et al. pointed SOX2 as an interesting candidate gene for HNSCC (24). Freier et al. also screened a set of 20 HNSCC cell lines from different sites for genetic imbalances using chromosomal comparative genomic hybridization (cCGH). The authors found frequent DNA copy number gains on 3q26.3-qter, 5p, 7p11-p13, 8q23-qter, 9p11-p13, 9q31-qter, 11q13 and 20q13.1, and copy number losses on 3p, 4p, 4q32.1-qter, 8p11-p12 and 18q22. Consequently, to explore the role of SOX2 in HNSCC cell lines, we had cytogenetic characterized HNSCC cell lines with 3q gain and no 3q gain. At that point, the functional role of SOX2 in cell proliferation, apoptosis and migration was reported for different types of cancer but not for HNSCC (49, 56, 61, 76).

More recently, Schröck et al. showed a functional role of SOX2 in one HNSCC cell line. They described the already reported function, for other types of cancer, of improving cancer cells survival, which we also found here (83). Interestingly, our finding of higher cell migration after SOX2 silencing describes a so far unknown function for SOX2 in cancer cells. The genes found affected by SOX2 in our results were, some of them, already reported (61, 91). Nevertheless possible interesting directions pointed by our transcriptome data, like the SOX2 effect in the Antigen Presentation Pathway, were not found suggested or investigated in the literature.

Initially we analyzed SOX2 expression in different HNSCC cell lines and found a close correlation between 3q amplification status and transcript levels, which was also found by Bass et al. for lung and esophageal SCC (41). These data are also in line with previous findings by Freier et al. in primary OSCC tumors (24). Variability in SOX2 expression was also reported for other tumor entities like glioma, gastric, breast and lung cancer (47, 62, 92, 93).

However, two tongue (TSCC) and two laryngeal SCC (LSCC) cell lines had low SOX2 expression despite the presence of 3q gain. Accordingly, genomic gain does not always resemble SOX2 overexpression during neoplastic transformation or malignant progression, and a combined analysis of amplification and protein expression is required in future clinical studies.

For the HNO150 cell line derived from a LSCC patient, we found hypermethylation of the proximal SOX2 promoter, which could explain silencing of SOX2

expression by an epigenetic mechanism (data not shown). Interestingly, Otsubo et al, found hyper-methylation of the SOX2 promoter to be associated with shorter survival time in advanced gastric cancer (47). Whether gene promoter hyper-methylation also contributes to low SOX2 expression in HNSCC, especially in those tumors with genomic 3q gain, or whether other modes of epigenetic or genetic alterations are involved will be interesting to investigate in the future.

However, for the other three cell lines with 3q gain but no elevated SOX2 expression no hyper-methylation was found at the proximal SOX2 promoter. A detailed analysis of the promoter methylation status with genomic DNA of the original tumor material will be necessary to exclude the possibility that the silencing by hyper-methylation is an artifact of in vitro culture conditions. Moreover, it will be interesting to study other modes of epigenetic silencing, such as regulation by microRNAs (94) as well as post-translational modifications of SOX2 regulation (95).

SOX2 function in normal and cancer cells critically depends on physical interaction with other transcription factors. In embryonic stem cells, SOX2 interacts with OCT4 to activate genes that maintain pluripotency, whereas in SCC cells, Watanabe et al. showed that SOX2 preferentially interacts with p63, jointly coordinating the activation of the oncogene ETV-4 (29, 40). As we see that it is important to understand which are the main interaction partners for SOX2 we describe here the transcriptome after silencing SOX2 in HNSCC cells, which was not previously reported in the literature. The functional annotation of differentially expressed genes after SOX2 silencing in HNO223 cells revealed alterations in several molecular mechanisms and signaling pathways related to cancer. Cell motility and cell movement were two of the main cellular functions affected by SOX2 silencing, and the Antigen Presentation Pathway was the main affected pathway positive related to SOX2. However, the consequence of these SOX2-related alterations on neoplastic transformation and even more important on response to established treatment options remains to be addressed in future experimental and clinical studies. As an example, SOX2 silencing revealed a higher doxorubicin induced apoptosis, suggesting an important role of SOX2 in the survival of HNSCC cells after treatment with chemotherapeutic drugs. This finding is in line with previous reports for HNSCC and other tumor cell lines (48, 83, 96, 97). Concerning tumor cell proliferation, silencing of SOX2 expression decreased the number of HNSCC cells in

S phase, which is similar to data provided for prostate cancer cells (49). In our transcriptome data, we found more than 23 genes involved in cell apoptosis regulation and 15 genes in cell proliferation regulation (data not shown). The specific function of each of these genes needs to be further explored for any conclusion.

Interestingly, the most up-regulated gene after SOX2 silencing in HNO223 cells encodes for Vimentin, a well-established marker for mesenchymal cells. Induced Vimentin expression after SOX2 silencing was confirmed on transcript and protein level in two independent HNSCC cell lines. An inverse correlation between SOX2 and Vimentin expression was reported in colorectal carcinoma cells (61), and Deng and colleagues found in breast cancer cells that overexpression of miR378 revealed reduced Vimentin expression accompanied by an up-regulation of SOX2. Vice versa ectopic Vimentin expression inhibited SOX2, suggesting the existence of a negative feedback loop in epithelial tumor cells. The clinical relevance of their finding was further supported by an inverse regulation of SOX2 and Vimentin in specimens of breast cancer patient (91).

In addition to Vimentin, other mesenchymal markers, N-cadherin and Fibronectin, were up-regulated after SOX2 silencing in HNSCC cells. This finding together with the fact that cellular movement was one of the main biological function, which was affected by SOX2 silencing led us to assume that SOX2 plays an important role in the regulation of cell migration and invasion. Indeed, silencing of SOX2 revealed a significant increase in cell migration and invasion. In line with our data, Chang et al. found that the connective tissue growth factor (CTGF) induces MET accompanied by induction of SOX2 via c-Jun (98). They suggest a model in which SOX2 acts as a potent oncogene, which induces a stem cell-like phenotype with epithelial characteristics that support tumor growth, but inhibits a mesenchymal-like phenotype, which is necessary for invasiveness and treatment failure after surgery. A role of SOX2 in MET is also well established in generation of iPS from fibroblast, indicating that it is an important function for SOX2 (99).

However, our findings are in contrast to several previous reports where SOX2 was associated with a mesenchymal and cancer stem-like phenotype (100-102). As an example, Han and colleagues reported that SOX2 knockdown decreases Vimentin but increases E-cadherin expression and suggested an involvement of SOX2 in epithelial-to-mesenchymal transition of colorectal cancer cells (56).



Moreover, Chen and colleagues demonstrated that spheroid cultures derived from HNSCC cell lines exhibit high expression of SOX2 and Vimentin, which was associated with higher invasiveness (77). In addition, several reports found an association between SOX2 expression and higher cell migratory ability (46, 98, 103, 104).

It is important to consider that those conflicting results, from both clinical data and in vitro models, could be due to differences in patient background, treatment approaches and methods for analysis, but could also be reflecting the dynamic and versatile function of SOX2. As in our results we saw SOX2 expression in the middle of the cell colonies and no SOX2 expression on the border cells, we could also speculate that SOX2, probably under epigenetic regulation could be silenced in the invasion front and again expressed once the metastasis favorable organ is achieved. The plasticity of the EMT and MET processes was seen to be possible by shifts in epigenetic regulation (94, 105). It is likely that SOX2 makes an important part in those dynamic and reversible processes.

Although the molecular mechanistic of how SOX2 silencing promotes cell migration and invasion remains largely elusive, our data suggest a crucial role of Vimentin. Indeed, silencing of Vimentin in HNSCC cell lines with reduced SOX2 expression partially reverted accelerated migration (data not shown). Vimentin is known to be related to cell migration and invasion in cancer, however the mechanisms are not completely clear. Interestingly, Vuoriluoto et al. found Vimentin expression and cell migration to be induced by ectopic expression of oncogenic H-Ras-V12G and Slug in pre-malignant breast epithelial cells (106). Havel et al., with the aim to answer the question why metastatic lung cancer cells expressing Vimentin are more motile and invasive, proposed a model whereby Vimentin promotes FAK stabilization through VAV2-mediated Rac1 activation (107).

Notably, in our results of differentially expressed genes, the parathyroid hormone-related protein (PTHrP) appeared to be correlated inversely with SOX2. PTHrP was found highly expressed at the invasive front of epidermal SCC (108). This is in line with our results of higher cell migration after SOX2 knock down and SOX2 negative cells at the “invasion front”.

Based on our findings, we speculated that HNSCC patients with low SOX2 expression in primary tumors could be at high risk for therapy failure upon local

treatment modalities, such as surgery and radiation. In fact, expression analysis in two independent and retrospective HNSCC patient cohorts demonstrated that low SOX2 protein levels served as unfavorable risk factor for poor progression and overall survival (data not shown).

In line with this data, lack of SOX2 expression in gastric and esophageal cancer, was associated with poor prognosis, while expression of SOX2 was associated with a better prognosis in other tumor entities (47, 48, 64, 65, 67, 68, 71). These conflicting results could also be due to the therapeutic approach, supporting our hypothesis that SOX2 expressing tumors would benefit more from local therapy whereas tumors lacking SOX2 would fit better in a system therapy approach. However, more studies controlling stage, site and type of therapy are necessary to conclude on that.

A possible explanation for the survival benefit of patients with tumors that express SOX2 is that SOX2 being an oncogene, tumors with SOX2 gain and overexpression require less other mutations and genomic aberrations. Considering that SOX2 would be enough to drives carcinogenesis this situation is similar to the HPV-related oropharyngeal tumors, which also present less genomic aberrations and somatic mutations (109).

To observe which genes are affected by SOX2 despite the tissue of origin of the tumor, we compared our HNSCC transcriptome data with from CRC and GBM. Combinatorial data analysis revealed more differentially expressed genes to be in common between HNSCC and CRC as compared to GBM transcriptome data, probably due to the similar histological background between HNSCC and CRC. Of the 17 genes in common, interferon regulatory factor 1 (IRF1) is the only transcription factor, therefore likely to influence the expression of other genes, and it is functionally related to the immune response against cancer (110). IRF1 is also known to regulate antigen presentation, by binding to the interferon stimulated response element in most HLA genes. Interestingly, the Antigen Presentation Pathway was the main affected pathway after SOX2 silencing, suggesting an important role of SOX2 in immune surveillance in the pathogenesis of HNSCC. Indeed, several genes implicated in antigen processing and presentation, such as beta-2-microglobulin (light chain of HLA-B), HLA class I, A, B, C, E and F, proteasome (prosome, macropain) subunits beta type 8 and 9, and TAP binding protein (tapasin) are co-regulated with

SOX2 in HNSCC cell lines. Consequently, another explanation for the poor clinical outcome of HNSCC tumors with low SOX2 expression could be the lack of an anti-tumor immune response. However, more extensive experimental and clinical approaches will be necessary to further confirm this assumption on the role of SOX2 in regulating gene regulatory networks that influence the immune response in the cancer microenvironment. Notably, Liu et al. found that cooperation of SOX2 with the microenvironment-activated STAT-3, which was previously related to influence the abnormal antigen presentation in cancer (111), is necessary for the carcinogenesis driven by SOX2 (112), pointing to the importance of SOX2 and microenvironment interaction for tumorigenesis.

Immunotherapy has been suggested as a promising approach to overcome resistance against chemotherapeutic drugs, which is well known characteristic of cancer cell with stem-like and tumor-initiating capacity (113, 114). Inoda et al., observed in a side population of colon cancer cells resistance to chemotherapy and expression of SOX2, OCT-4, LGR5, and ALDH1A1. For these resistant tumor cell subpopulation, cytotoxic T lymphocytes-based immunotherapy turned out to be a promising approach (115). In this context, it is worth noting that the CSC marker ALDH1A1 is also down-regulated after SOX2 silencing in HNSCC cell lines (data not shown). However, Busse et al. found down-regulation or loss of HLA-I in sphere cells of 8 of 10 human solid tumor cell lines (116). The fact that the Antigen Presentation Pathway was the main affected one may indicate a mechanism between stem-ness and antigen presentation that could be worth to pursue for targeting therapy.

Identification of novel drug targets and development of strategies for individualized therapy involving the SOX2 gene regulatory network is an attractive new goal after unraveling SOX2 as a prognostic biomarker for better survival in HNSCC. According to clinical results from our group, HNSCC patients with medium to high SOX2 expression respond better to standard treatment options, while the subgroup with low SOX2 expression are at high risk for treatment failure (data not shown). Based on our data derived from in vitro cell culture experiments, we hypothesize that the difference in clinical outcome resembles the more aggressive and invasive growth behavior of HNSCC tumor cells with low SOX2 expression. Accordingly, HNSCC patients with low SOX2 expression might benefit from a multimodal treatment protocol, including systemic chemotherapy, and urgently need

a more intensive follow-up monitoring. In contrast, patients with medium to high SOX2 expression should benefit from a local treatment with surgery and radiation alone or in combination, but are most likely resistant against chemotherapy. However, due to the co-expression of genes implicated in antigen processing and presentation, immunotherapy represents an attractive alternative for HNSCC patients with high SOX2 expression.

Accordingly, Schmidt et al. tested HLA-A\*0201-restricted SOX2-derived peptides for the activation of glioma-reactive CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) and found effective lysis of glioma cells in vitro. The authors found a raise in specific CTLs against the peptide TLMKKDKYTL and suggest that this antigen is a promising target for T-cell-based immunotherapy of glioma (93). Moreover, Dhodapkar et al., demonstrated in non-small cell lung carcinoma (NSCLC) an association between SOX2-specific T cells and better response to immunotherapy. In this study, patients were treated with antibodies against the programmed death 1 (PD-1) receptors and 24 patients were evaluable for a clinical response. A significant immune response against SOX2 was found in patients that responded to therapy compared to the ones that did not. No disease regression was found in patients lacking SOX2-specific T cells after the immune checkpoint blockade (117). Finally, Ikegame et al. found that crosslinking of HLA class I by C3B3 mediated a promising mechanism to overcome chemotherapy resistance in the cancer stem cell-like population as they observed decrease in  $\beta$ -catenin, SOX2, OCT3/4 and Nanog expression (118).

The role of SOX2 in neoplastic transformation, malignant progression and clinical outcome appears rather complex and context dependent. As a potent oncogene SOX2 promotes cancer development and is a well-established marker for cancer stem cell-like and tumor initiating cells (77). However, an increasing body of clinical and experimental studies demonstrate the critical involvement of SOX2-related gene regulatory networks in cellular and molecular processes, such as Antigen Presentation Pathway and tumor cell motility, with unexpected consequences on the clinical outcome of cancer patients. In order to gain a more comprehensive view on the underlying molecular mechanism extended analysis, including combinatorial analysis of SOX2-related alterations in the epigenome, transcriptome (coding and non-coding genes), and proteome, will be required. Furthermore, the expression analysis of SOX2 and newly identified target genes in

tumor samples of well-controlled and prospective patient cohorts with HNSCC but also other tumor entities should be initiated to further proof the concept of their clinical relevance as prognostic biomarkers.

The increasing understanding of the complex interactions in cancer is making clear that rather than isolated processes, there are genetic and epigenetic mechanisms and an intricate signaling coming from the microenvironment that determines this dynamic changes in the cell phenotype and cell function (105, 119, 120). Transcription factors like SOX2 may be involved in this complex regulation. The “functional versatility of transcription factors”, interestingly pointed out by Adachi et al. in embryogenesis, is likely to happen also in cancer, where they can also be recursively working in diverse manners within gene network structures.

Further investigation is necessary to clarify those SOX2 dependent mechanisms in HNSCC, leading to a use of SOX2 as a stratification marker for diagnosis and therapy decision, as well as for development of new therapeutic approaches. To achieve improvement in prognosis and therapy it is not only necessary to better understand the molecular basis of this pathology, but also to establish reliable biomarkers for prognostic, patient stratification and therapy decision-making.

## Conclusion

We showed here a novel role of SOX2 as an inhibitor of cell migration. In addition we found an enrichment of differentially expressed genes in SOX2 silenced HNSCC cells that are coherent with this cell function. These findings are of value for understanding the SOX2 role in metastasis process and prognosis, considering that SOX2 was related to better prognosis in different types of cancer, including HNSCC, but the mechanisms to explain that are not understood. This information can help the therapy decision-making process in the clinics.

Also, our results reinforce the ones already reported for HNSCC and other types of cancer that relates SOX2 as a tumor-promoting gene by maintaining cell survival. These paradoxical findings for SOX2, meaning silencing SOX2 resulted in more cell apoptosis and more cell migration, support the complex role of this gene in cancer.

Finally, we indicate here an effect of SOX2 in genes related to the cancer underlying immune response. This finding needs to be further investigated in future work. Interestingly, the interaction of transcription factors and cancer immune response is poorly explored in the literature and we suggest that this interaction could be an interesting focus for new therapies development.

## Conclusão

*Demonstramos aqui um novo papel para SOX2 como inibidor de migração celular em CECP. Coerentemente, encontramos genes envolvidos nessa função celular sendo afetados pelo silenciamento de SOX2 em células de CECP. Esses achados são importantes para o entendimento do papel de SOX2 no processo de metástase e prognóstico, considerando que SOX2 foi relacionado a um melhor prognóstico em diferentes tipos de câncer, inclusive CECP. Contudo, os possíveis mecanismos permanecem não entendidos. Essa informação pode ajudar o processo de decisão de terapia na clínica.*

*Ademais, nossos resultados reforçam os reportados para CECP e outros tipos de câncer, os quais apontam SOX2 como gene promotor de tumor por meio da manutenção da viabilidade celular. Esses achados paradoxais com relação a SOX2, ou seja, silenciar SOX2 causou mais apoptose celular e mais migração celular, suportam o papel complexo de esse gene no câncer.*

*Finalmente, nós indicamos aqui um efeito de SOX2 em genes relacionados à subjacente resposta imunológica em câncer. Esse resultado necessita uma investigação mais profunda em pesquisas futuras. Interessantemente, a interação de fatores de transcrição e resposta imunológica em câncer é pouco explorada na literatura. Sugerimos aqui que essa interação pode ser um interessante foco de estudo para o desenvolvimento de novas terapias.*

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## Considerações Finais

O presente trabalho, seguindo a motivação de encontrar novas perspectivas de diagnóstico e tratamento de Carcinoma Espinocelular de Cabeça e Pescoço (CECP), bem como ajustar medidas já existentes de forma individualizada para os pacientes, estabelece resultados a partir de um sistema *in vitro*. Sistema esse que teve como base linhagens celulares de CECP. Duas dessas linhagens, com ganho em 3q e expressão de SOX2 foram modificadas geneticamente para o silenciamento de SOX2.

SOX2, o qual é considerado um oncogene, vem sendo apontado como um marcador de prognóstico em diferentes tipos de câncer. Contudo, os resultados são controversos. Existe um consenso sobre o importante papel de SOX2 no desenvolvimento de tumor, mas não no significado da expressão de SOX2 para prognóstico ou decisão de terapia.

Baseando-se na discussão dos nossos resultados *in vitro* com resultados do mesmo grupo ainda não publicados, os quais apontam expressão de SOX2 como um marcador de prognóstico favorável, buscamos propor um mecanismo pelo qual SOX2 indica uma maior sobrevida para os pacientes de CECP tratados com terapia padrão para CECP. Sugerimos que o aumento da migração celular após o silenciamento de SOX2 indica que os pacientes sem expressão de SOX2 respondem pior à terapia pelo fato de as células do tumor adquirirem maior capacidade migratória, conseqüentemente de metástase. Um ponto crítico nessa discussão é que comparando o sistema *in vitro* com pacientes, a situação mais aproximada seria a daqueles pacientes que inicialmente expressavam SOX2 e essa expressão foi perdida ao longo do tempo, por meio de mecanismos que permanecem não entendidos.

Futuros trabalhos são necessários para entender essa situação. O estudo da regulação de SOX2 e de vias celulares reguladas por SOX2 permitirá avançar no entendimento do mecanismo envolvendo SOX2 durante a resposta à terapia. De igual importância é entender de que forma SOX2 está atuando no resposta imunológica de CECP, já que nossos resultados, ainda preliminares para maiores conclusões, indicam uma interação de SOX2 com o processo de apresentação de antígeno. Finalmente, pensamos ser imprescindível, após o alcance de um entendimento maior dos mecanismos de SOX2 em CECP, delinear estudos que busquem alternativas novas e individualmente adequadas para o tratamento de CECP.