Title

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TMEM2 is a SOX4 regulated gene that mediates metastatic migration and invasion in breast cancer

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Abstract

The developmental transcription factor SOX4 contributes to the metastatic spread of multiple solid cancer types, but its direct target genes that mediate cancer progression are not well defined. Using a systematic molecular and genomic approach, we identified the TMEM2 transmembrane protein gene as a direct transcriptional target of SOX4. TMEM2 was transcriptionally activated by SOX4 in breast cancer cells where, like SOX4, TMEM2 was found to mediate pro-invasive and pro-migratory effects. Similarly, TMEM2 was sufficient to promote metastatic colonization of breast cancer cells and its expression in primary breast tumors associated with a higher likelihood of metastatic relapse. Given earlier evidence that genetic inactivation of SOX4 or TMEM2 yield similar defects in cardiac developmental, our findings lead us to propose that TMEM2 may not only mediate the pathologic effects of SOX4 on cancer progression but also potentially its contributions to embryonic development.

Introduction

Breast cancer is the most common cancer in women and the second most common cancer in the United States (The National Cancer Institutes Surveillance, Epidemiology, and End Results database of 2015). Most breast cancer patients die from metastasis, the spread of cancer cells from a primary tumor to distal organs (1). In order to study the biology of cancer metastasis, we *in vivo* select for breast cancer cells with high metastatic capability (2). This system has been used to identify molecular factors and key regulatory pathways that mediate metastasis in various cancer types (3–8). In breast cancer, molecular analysis of independently derived *in vivo*-selected cell populations led to the identification of miR-335 as one of three endogenous human microRNAs (miRNAs) that suppress breast cancer metastasis (9). In that study, *SOX4* was identified a miR-335-regulated gene. Reduction of miR-335 levels in breast cancer cells led to increased expression of SOX4, resulting in metastatic progression as a result of increased cell invasion and migration. Subsequent to its discovery as a mediator of breast cancer metastasis, SOX4 expression has been shown to be also increased in other cancer types, including leukemia (10,11), glioblastoma (12,13),

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medulloblastoma (14,15), hepatocellular carcinoma (HCC) (16), and prostate cancer (17–19). Despite this evidence supporting the tumorigenic properties of SOX4, comparative analyses of the SOX4 transcriptional network revealed a high degree of variation between the target genes in different cancers, which suggest the tumor-specific and context-dependent mechanisms of target gene selection (20). Recent studies have implicated SOX4 as a regulator of TGF-β-induced epithelial-mesenchymal transition in breast cancer cells (21,22). However, the broader transcriptional targets of SOX4 in breast cancer are poorly characterized. SOX4 transcriptional networks have also been studied in other cancer types, and these studies have revealed that SOX4 targets are involved in various oncogenic pathways (10,11,18). In this study, we used an unbiased genomic approach to identify *TMEM2* as a metastasis-promoting gene that is transcriptionally regulated by SOX4 in breast cancer cells and mediates SOX4-driven metastatic progression in breast cancer. Furthermore, we find that TMEM2 expression in breast cancer positively correlates with reduced overall survival of patients. Our work implicates TMEM2 as a direct transcriptional target and a mediator of SOX4 in breast cancer progression.

Materials and Methods

Analysis of mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from cancer cells was extracted and purified using the mirVana (Applied Biosystems) or Total RNA Purification Kit (Norgen Biotek). mRNA expression quantification was performed as described previously (7). 1 μg of total RNA was reverse transcribed using the cDNA First-Strand Synthesis kit (Life Technologies) and diluted 1:5 with water. Real-Time PCR reaction mix consisted of 5 μl of the diluted cDNA, 2.5 μl of 1μM primer mix, 17.5 μl of water, and 25 μl of FAST SYBR Green Master mix (Life Technologies, 4385612). Each reaction was performed in quadruplicate and mRNA expression was quantified by qRT-PCR using an ABI Prism 7900HT Real-Time PCR System (Applied Biosystems). 18S or GAPDH was used as an endogenous control for normalization. Primers were listed in Supplementary Table S1.

Animal Studies

All animal work has conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. For lung colonization assays, 4×10^4 MDA-LM2 cells or 5×10^4 CN34-LM1A cells were resuspended with 100 μl of PBS and injected into the lateral tail vein of NOD-SCID gamma female mice (The Jackson Laboratory) age-matched between six and eight weeks. Lung colonization was monitored once every week using bioluminescence. For orthotopic lung metastasis assays, 5×10^5 MDA-LM2 cells were resuspended in a 1:1 mixture of PBS and growth-factor-reduced Matrigel (BD Biosciences), and bilaterally injected into the mammary fat pads of NOD-SCID gamma female mice in a total volume of 100 μl at day 0. Primary tumor growth rates were monitored from day 14 to day 21, and tumor volume was calculated based on the formula $\pi LW^2/6$ (L, tumor length; W, width). At day 21, primary tumors were resected, and orthopic lung metastatic progression was monitored using bioluminescence. At day 50, lungs were extracted and fixed in 4% paraformaldehyde in PBS

for over-night at 4°C, underwent serial washes with PBS, 50% ethanol, and 70% ethanol, for 30 min each at room temperature, and Haematoxylin-and-eosin-stained. Quantification of *in vivo* bioluminescence (the total photon flux in the region of interest) was done in Living Image software (L.P. Larson Corporation).

Cell Culture

The MDA-MB-231 was derived from the pleural effusion of a breast cancer patient who developed distant metastasis years after primary tumor resection, and its metastatic derivatives LM2 cells were selected from the MDA-MB-231 cell population for their ability to metastasize to lungs by two rounds of *in vivo* selection (3). CN34 parental cells were obtained from the pleural fluid of another patient with metastatic breast cancer, and its metastatic derivatives LM1A cells were isolated from the lung metastatic nodules originated by the CN34 parental cells (9). These cell lines (MDA-parental, MDA-LM2, CN34-parental, and CN34-LM1A) were authenticated by RNA-seq profiling from GSE45162 (23). Breast cancer cell lines, HEK293T, and 293LTV cells were propagated *in vitro* with Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 2mM L-Glutamine, 1mM sodium pyruvate, and 2.5 μg/ml fungizone. Mycoplasma contamination was monitored periodically.

Chromatin immunoprecipitation and qPCR (ChIP-qPCR)

ChIP was performed using EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore, 17-10086) according to the manufacture's protocol. 2×10^7 LM2 cells were used per IP and the nuclear pellet was sonicated three times for 15 sec at amplitude 60 with 3 min lapse between each pulse (Branson sonifier). The lysate was incubated at 4°C for 4 hr with 5 μg of anti-SOX4 antibody (abcam, ab70598) or control IgG. Real-Time PCR reaction mix consisted of 1 μl of the ChIPed DNA, 2.5 μl of 1μM primer mix, 21.5 μl of water, and 25 μl of FAST SYBR Green Master mix (Life Technologies, 4385612). The optimized primers used for ChIP-qPCR were listed in Supplementary Table S1.

Clinical correlation analysis

Expression Project for Oncology (ExpO) Data—Expression levels of SOX4 and the putative target genes were analyzed in the total of 236 breast cancer samples obtained from the ExpO microarray database (GSE2109).

Gene expression-based Outcome for Breast cancer Online (GOBO) Data— Assessment of gene expression levels and association with overall survival outcome for SOX4 and TMEM2 in breast cancer subgroups (stage 0, I, II, III, IV) were done by GOBO http://co.bmc.lu.se/gobo/gobo.pl, (24).

Kaplan-Meier Plotter for Breast Cancer Data—Evaluation of TMEM2 in predicting survival of intrinsic subtypes (HER2 positive and luminal B) of breast cancer patients was done by http://kmplot.com/analysis/, (25).

Generation of over-expressing and knockdown cell lines

For SOX4 overexpression studies, human cDNA of SOX4 with N-terminal FLAG tag was cloned into pBabe-puro retroviral expression vector. 10 μg of DNA were transfected into H29 packaging cell line in a 10cm plate by Lipofectamine 2000 (ratio of μg DNA: μl Lipofectamine 2000 is 1:4). Virus-containing supernatant at 48 hr and 72 hr post-transfection were harvested, filtered through a 0.45 μm syringe filter, and added to breast cancer cells with polybrene (8 μg/ml). The cells were selected with puromycin (1 μg/ml) for 2–3 days. For doxycycline inducible SOX4 over-expression studies, FLAG-SOX4 was sub-cloned into pEN_Tmcs Entry vector (Addgene Plasmid #25751) then digested with BamHI and EcoRV to be ligated with pSLIK-Neo lentiviral vector (Addgene plasmid #25735) that was digested with AleI and XhoI. Lentivirus was generated by co-transfecting 2.5 μg of appropriate lentiviral plasmid with 7.5 μg of lentiviral packaging plasmids (5 μg of gag/pol and 2.5 μg of env) with Lipofectamine 2000 into 293LTV cells in a 10cm plate.

For TMEM2 overexpression studies, human cDNA of TMEM2 with C-terminal V5 tag was obtained from CCSB Lentiviral Expression Library (OHS6087) (26). The C-terminal frame shift mutation present in the ORFeome library was fixed and cloned into pLX304 lentiviral expression vector by Gateway cloning.

For knockdown studies, Sigma shRNA glycerol stocks were purchased: TRCN0000018216, TRCN0000018217, TRCN0000148114, TRCN0000147636, control shRNA (SHC002)

Luciferase reporter assay

The SOX4 bound regions of TMEM2 obtained from ChIP-qPCR were cloned into the pGL3-Promoter vector (Promega, E1761) that contains the Firefly luciferase gene downstream from a multiple cloning site into which the promoter of interest is inserted. The three putative SOX4 binding motifs in this construct were mutated by Multi Site-Directed mutagenesis kit (Agilent, 210515). These vectors (25 ng) were co-transfected with the Renilla luciferase vector (2.5 ng) into HEK293T cells either transiently overexpressing FLAG tagged SOX4 (22.5 ng) or control empty plasmid (22.5 ng). After 30 hr post transfection, the cells were processed with Dual-Luciferase Reporter Assay System (Promega, E1910) according to the manufacturer's protocol. Briefly, cells were lysed with 100 μl of Passive Lysis buffer for 20 min at room temperature. 30 μl of the lysate were used per Luciferase reaction. The Firefly and Renilla luciferase activities were measured in a luminometer (Perkin-Elmer EnVision plate reader) and transfection efficiency was normalized by the Renilla luciferase activity.

siRNA-mediated mRNA knockdown

1×10^6 cells were seeded in a 10cm plate. Next day, siRNAs targeting either SOX4 (Thermo Scientific oligo ID ROSJN-00001, ROSJN-00003) or TMEM2 (Integrated DNA Technologies (IDT) siRNA identity HSC.RNAI.N013390.12.10, HSC.RNAI.N013390.12.2) and control siRNA (Thermo Scientific, siGENOME Control siRNA #1) were transfected with Lipofectamine reagent according to the manufacturer's protocol. 48 hr after transfection, the cells were collected and applied to either trans-well invasion assay or trans-well migration assay.

Statistical Data Analysis

Unless otherwise noted, all data are represented as mean ± standard error of the mean (s.e.m.) with p-values: ****, $P < 0.0001$; ***, $P < 0.0005$; **, $P < 0.001$; *, $P < 0.05$. One-tailed Student's t test was used and $P < 0.05$ was considered to be statistically significant. For KM plot analysis two-tailed log rank t test was used. Statistic analyses were conducted using PRISM version 6 for Mac (GraphPad software, La Jolla, California).

Transcriptomic profiling analysis

For poorly and highly metastatic cells, RNA-seq data from GSE45162 (23) were used in this study. For SOX4 knockdown microarray, 1 μg of total RNA was extracted using the MiRvana kit (Ambion) from two independent RNAi-mediated knockdown of SOX4 and two control cells. Each sample was labeled and hybridized on Illumina BeadChip array (GPL10558_HumanHT-12_V4) by the Genomics core facility at Rockefeller University. Each sample was median-normalized and the average of two control cells and the average of two SOX4 siRNAs was used to calculate the relative fold-change upon SOX4 knockdown by dividing a value from control cells over a value from SOX4 knockdown cells for each genes. The data from SOX4 knockdown microarray is deposited in Gene Expression Omnibus under accession number GSE79202. Heat maps depicting relative expression levels of genes from microarray data was created with matrix2png (27).

Trans-well invasion assay

The trans-well invasion assays were performed as described previously (9). Briefly, cells were incubated overnight in the culture media with 0.2 % FBS. The next day, the cells were trypsinized, re-suspended in the low serum media, and seeded at 5×10^4 for MDA or 1×10^5 for CN34 per well into the growth factor reduced matrigel invasion chambers (8 μm pore size, BD Biosciences). After 18–22 hr, the chambers were washed with PBS twice and the cells on the apical side of each inserts were scraped off. The invaded cells fixed with 4% paraformaldehyde were stained with DAPI using VectaShield (Vector Laboratories) and were counted in four fields per insert and then quantified with ImageJ (National Institutes of Health).

Trans-well migration assay

Trans-well migration assays were performed as described previously (9) with minor modifications: over-night serum-starved cells (1×10^5) were seeded per cell-culture insert made of Track-etched polyethylene terephthalate (PET) membranes with pores (3 μm pore size, BD Biosciences). After 12 hr, the migrated cells were processed as scribed above for the Trans-well invasion assays.

Western Blotting

Cells were lysed with ice-cold RIPA buffer with protease inhibitor cocktail (Roche) and sonicated. The cleared lysate by centrifugation was reduced and run on Bis-Tris NuPAGE gels in MOPS buffer (Life Technologies). Proteins were transferred to PVDF membrane. Following primary antibodies were used: 1:100 for custom-made rabbit polyclonal anti-

Sox4, Yenzyme, immunogen CGR SPA DHR GYA SLR; 1:1000 for rabbit polyclonal anti-Tmem2 (Abcam, ab98348); 1:4000 for rabbit polyclonal anti-Gapdh (Sigma, G9545).

Results

Systematic analysis of SOX4-regulated direct target genes in breast cancer

To identify potential SOX4 target genes that could mediate breast cancer metastasis, we employed RNA-sequencing and transcriptomic profiling approaches. Endogenous SOX4 expression becomes upregulated in highly lung-metastatic breast cancer sub-populations (MDA-LM2, CN34-LM1A) that were derived through *in vivo* selection from poorly metastatic parental cell lines (MDA-parental, CN34-parental, respectively) (9). Given this, and knowing that SOX4 is an established transcriptional activator (28–30), we searched for genes for which expression levels are increased in the metastatic cell sub-populations and were also reduced upon RNA interference-mediated SOX4 depletion. RNA-sequencing of MDA-parental/MDA-LM2 and CN34-parental/CN34-LM1A revealed 100 genes that were significantly upregulated in both highly metastatic sub-lines. To determine if any of these genes are transcriptional targets of SOX4, we performed transcriptomic profiling of MDA-LM2 cells transfected with two independent siRNAs targeting SOX4 as well as a control siRNA. Analysis of this data revealed that a total of 1356 genes were significantly downregulated upon SOX4 depletion in MDA-LM2 cells. Of the 1356 genes downregulated by SOX4 knockdown, 26 were also upregulated in the two highly metastatic cell lines based on the above mentioned RNA-sequencing analysis, resulting in a list of candidate SOX4-regulated genes in breast cancer cells (Fig. 1 and Supplementary Table S2–S4).

We next asked if the expression levels of any of these 26 SOX4-regulated genes positively correlated with SOX4 expression in breast cancer. To do this, we examined gene expression levels in publicly available transcriptomic profiling datasets (Oncomine Cancer Microarray database (31) and Expression Project for Oncology microarray database (ExpO; GSE2109). This analysis showed that 8 out of these 26 candidate SOX4-regulated genes were overexpressed in multiple datasets comprised of non-cancerous and cancerous breast tissues when SOX4 was overexpressed (Fig. 2A). Of these 8 genes, we found a positive and significant correlation between MMP1, SEMA4B, and TMEM2 levels and SOX4 levels in the ExpO microarray database (Fig. 2B). In order to find SOX4 targets that would mediate metastatic progression of breast cancer, we next examined the transcript levels of SOX4 and these three genes in patients with high-risk primary breast tumors to evaluate the prognostic value of these putative targets. Of the three genes, only TMEM2 expression significantly correlates with reduced overall survival (OS) in high-risk grade 3 breast tumors as shown by the Kaplan-Meier Plotter software (Fig. 3A). Moreover, combined expression levels of SOX4 and TMEM2 performed as a superior prognostic factor relative to TMEM2 alone (Fig. 3B). To further investigate whether TMEM2 might be associated with any intrinsic subtype of breast cancer, we tested the prognostic values of either TMEM2 alone or TMEM2 with SOX4 in intrinsic subtypes of breast cancer. TMEM2 expression associated with poor prognosis in luminal B and HER2 positive subgroups. In HER2 positive subgroup, high expression of TMEM2 alone most significantly correlated with poor distant metastasis free survival (DMFS, Fig. 3C) and OS (Fig. 3D). In relapse free survival (RFS) of HER2

positive group and OS of the luminal B group, expression of TMEM2 alone did not show a statistically significant correlation, however, in both cases, the combination of TMEM2 with SOX4 significantly correlated with poorer outcomes compared to low expression of both genes (Fig. 3E–3H). These analyses reveal that TMEM2 is a SOX4-regulated gene which expression positively associates with reduced overall survival in breast cancer and suggest that TMEM2 may serve as prognostic factor for HER2 positive subtypes of breast cancer and potentially luminal B breast cancer. Importantly, using the Cancer Tissue Atlas we observed that TMEM2 is expressed at the protein level in breast cancer tissues as well as in almost every cancer type analyzed; and multiple cancer types including hepatocellular carcinomas and a few pancreatic, colorectal and gastric cancer samples showed strong positive staining for the antibody used against TMEM2 (Supplementary Fig. S1). Therefore, we sought to further investigate the role of SOX4 as a transcriptional activator of TMEM2.

SOX4 regulates TMEM2 transcription

Next, using quantitative real-time polymerase chain reaction (qRT-PCR), we validated the upregulation of SOX4 and TMEM2 transcript levels in highly metastatic MDA-LM2 cells relative to the poorly metastatic MDA-parental cells (Supplementary Fig. S2A and S2B). The relative expression levels of TMEM2 and SOX4 were also assessed by qRT-PCR in the CN34-parental cell line, an independent malignant breast cancer cell population, and its highly metastatic lung derivative, the CN34-LM1A cell line. (Supplementary Fig. S2C and S2D). In both cell lines, SOX4 and TMEM2 were upregulated in highly metastatic sub-lines relative to poorly metastatic parental cell lines. Depletion of SOX4 by two independent SOX4-targeting siRNAs in MDA-LM2 cells also significantly reduced TMEM2 transcript levels (Supplementary Fig. S2E and S2F). Additionally SOX4 depletion in CN34-LM1A cells by RNAi also significantly reduced TMEM2 transcript levels (Supplementary Fig. S2G and S2H). The efficiency of knockdown of SOX4 by siRNAs was measured by western blot (Supplementary Fig. S2I)

We next questioned if SOX4 overexpression is sufficient to increase TMEM2 transcript levels. MDA-parental cells over-expressing Flag-tagged SOX4 displayed increased TMEM2 transcript levels relative to control cells (Fig. 4A and 4B). To determine the temporal regulation of SOX4-mediated TMEM2 expression, we generated doxycycline inducible Flag-tagged SOX4-expressing MDA-parental and CN34-parental cell lines. In both cell lines, TMEM2 mRNA levels were increased at least at 24 hours after inducing SOX4 overexpression (Fig. 4C–4F). These findings suggest that TMEM2 transcript levels are regulated by SOX4 in breast cancer cells.

We next investigated if *TMEM2* is a direct transcriptional target of SOX4 in breast cancer cells. To do this, we assessed SOX4 binding to the *TMEM2* promoter using chromatin Immunoprecipitation qPCR (ChIP-qPCR). We designed qPCR primers spanning from 4.8 kilobase (kb) pairs upstream of the *TMEM2* transcription start site (TSS) to 2.2 kb pairs downstream of the TSS. This experiment revealed that a genomic DNA region proximal to the *TMEM2* TSS and spanning base pairs 856-1178 upstream of the TSS, was highly enriched in SOX4-bound genomic DNA relative to that bound by IgG control (Fig. 5A). The functional consequence of SOX4 binding to this *TMEM2* promoter region was further

investigated using a luciferase promoter assay. To do this, we generated reporter constructs consisting of a luciferase reporter cloned downstream of the indicated *TMEM2* promoter region (Fig. 5B). SOX4 overexpression increased luciferase activity relative to control, consistent with a SOX4-dependent increase in transcription from the *TMEM2* promoter (Fig. 5C). The SOX transcription factor family is known to preferentially bind to the hexameric core sequence WWCAAW, where W indicates A or T (32–34). In order to refine the *TMEM2* promoter regions necessary for the observed SOX4-dependent increase in transcription, we searched for and identified three instances of the WWCAAW motif in the *TMEM2* promoter region. These core binding motifs were then mutated, either individually or combined, and these constructs were tested in luciferase promoter reporter assays. Upon SOX4 overexpression, cells transfected with a reporter containing the wild type TMEM2 promoter regions exhibited increased expression, while reporters containing mutations in the SOX4 binding motif did not exhibit enhancement of SOX4-dependent expression (Fig. 5C). The requirement for the regions of all three SOX4 binding sites suggests that cooperative multi-site binding by this transcription factor is required for transcriptional activation. Given that pre-mRNAs are transient intermediates and their steady-state levels reflect endogenous transcription prior to nuclear processing (35–39), we used qRT-PCR to analyze the levels of intron-containing TMEM2 pre-mRNA in control and SOX4 depleted cells. SOX4 knockdown significantly reduced TMEM2 pre-mRNA levels, consistent with a transcriptional role for SOX4 in *TMEM2* regulation (Fig. 5D and 5E). Collectively, these findings support a model where by SOX4 transcriptionally promotes *TMEM2* expression through direct interaction with the promoter region of *TMEM2*.

TMEM2 promotes invasion, migration, and breast cancer metastasis

Highly metastatic MDA-LM2 cells express higher levels of SOX4 relative to MDA-parental cells, and their ability to invade and metastasize to the lungs of mice is reduced upon SOX4 depletion (9). To determine if an increase in the expression of SOX4 is sufficient to enhance the invasive capacity of breast cancer cells, we performed a matrigel invasion assays in MDA-parental and CN34-parental breast cancer cell lines engineered to overexpress SOX4. SOX4 overexpression significantly increased the invasion capacity of breast cancer cells relative to control cells (Fig. 6A and 6B). Next, to investigate if the enhanced cancer cell invasion capacity observed upon SOX4 overexpression was mediated by TMEM2, we used shRNAs to knockdown TMEM2 levels in SOX4 overexpressing cells. Two independent shRNAs targeting TMEM2 were validated by western blot (Supplementary Fig. S3). Depletion of TMEM2 in SOX4 overexpressing cells significantly reduced the invasion capability of these cells (Fig. 6C). Furthermore, transient overexpression of SOX4 in CN34-parental cells increased their invasion capacity, an effect that was abrogated upon TMEM2 depletion (Fig. 6D–6F). This suggests that the increased invasion capacity conferred by SOX4 overexpression is dependent, at least in part, on its downstream target gene, *TMEM2*. Similarly, significantly greater invasion and migration capacity was observed in MDA-parental cells overexpressing TMEM2 relative to control cells (Fig. 6G and 6H). Consistent with this, a reduction in cell invasion capacity was observed upon TMEM2 depletion in MDA-LM2 cells (Fig. 6I and 6J). These findings are consistent with a model where the pro-invasive effects of SOX4 are mediated, at least in part, through transcriptional induction of *TMEM2*.

Next, we asked if TMEM2 can promote *in vivo* breast cancer metastatic colonization in a similar manner as SOX4. To do this, we assayed the metastatic lung colonization capacity of MDA-LM2 cells with stable TMEM2 knockdown or with TMEM2 overexpression. Depletion of TMEM2 in highly metastatic MDA-LM2 cells significantly reduced metastatic colonization (Fig. 7A and 7B). Consistently, knockdown of TMEM2 by an independent shRNA in CN34-LM1A cells also significantly decreased the ability of cells to colonize the lungs (Supplementary Fig. S4A and S4B). Conversely, TMEM2 overexpression significantly enhanced the metastatic capacity of MDA-LM2 cells (Fig. 7C and 7D). To study the effect of TMEM2 in primary tumor growth as well as in metastatic potential, we conducted orthotopic injection of MDA-LM2 cells with stable TMEM2 knockdown in mammary fat pads of the mice. In both *in vitro* proliferation assays (Fig. 7E) and *in vivo* primary tumor growth assays (Fig. 7F), depletion of TMEM2 did not have an effect on neither cell proliferation nor primary tumor growth relative to control. However, TMEM2 depletion caused decrease in lung metastasis development after primary tumor resection (Fig. 7G and 7H). Therefore, TMEM2 acts as a promoter of lung metastatic colonization in breast cancer cells and has a potential to mediate spontaneous breast cancer metastasis.

Discussion

SOX4 belongs to the HMG box superfamily of DNA-binding proteins and contains a single HMG domain through which it binds DNA in a sequence-specific manner (34,40–43). SOX4 was discovered as a transcriptional activator in B and T lymphocytes (40,44) and early genetic studies revealed that SOX4 is required for normal cardiac development (44–46).

SOX4 has also been found to be over-expressed in various cancers (30,47,48) and amplified in lung cancer (49). The identification of miR-335 as a suppressor of breast cancer metastasis led to the identification of a cancer progression role for SOX4—as a driver of cancer cell invasion and migration. SOX4 was shown to be downstream of the metastasis suppressor miRNA miR-335. As a result of miR-335 silencing in metastatic breast cancer, SOX4 expression is enhanced (9). The enhancement of invasive and migratory phenotypes caused by SOX4 up-regulation increased the metastatic lung colonization capacity of breast cancer cells. Other studies have shown a role for SOX4 in promoting cancer progression in a number of additional cancer types, including prostate cancer (17–19), hepatocellular carcinoma (16), glioblastoma (12,13), and leukemia (10,11).

Using a genomic and molecular approach, we have identified TMEM2 as a direct transcriptional target of SOX4 in breast cancer cells. We find that SOX4 directly binds to the promoter region of *TMEM2* and transcriptionally activates it in a sequence-specific manner. Molecular studies reveal that SOX4 also regulates *TMEM2* pre-mRNA levels, consistent with SOX4 transcriptional activation of this gene. TMEM2 promotes breast cancer cell migration, invasion, and metastatic colonization, phenocopying SOX4-dependent effects. Moreover, we find that expression of *TMEM2*, a gene that has not been previously implicated in cancer progression, correlates with breast cancer clinical outcome.

TMEM2 is a poorly described type II transmembrane protein that is composed of a N-terminal transmembrane domain and a C-terminal extracellular portion containing a G8 and

a GG domain. To date, no biochemical functions have been identified for these domains (50,51). Recent studies have revealed a role for *tmem2* during zebrafish heart development (52,53). Of the two different zebrafish mutants of *tmem2* identified, one displayed a specific loss of cardiac looping at late developmental stages, while the other exhibited aberrant atrioventricular canal differentiation. Both mutations resulted in a shortened Tmem2 extracellular domain, suggesting the missing extracellular domain is important for Tmem2 function during cardiac development. This zebrafish Tmem2 mutant phenotype could be rescued by knockdown of Bmp4, suggesting that Tmem2 regulates zebrafish heart development through inhibition of Bmp4. Interestingly, the heart developmental phenotype observed in zebrafish Tmem2 mutants resembles the heart developmental phenotype, a common arterial trunk defect, that is observed in *Sox4* null mice (44–46). In the myocardium, Tmem2 promotes the migration of myocardial and endocardial cells. It is interesting that Tmem2 functions in heart development in part by regulating cell migration, which resembles the role of SOX4 in both mouse heart development and breast cancer metastasis. Because of these similarities, it would be interesting to investigate if TMEM2 regulates cell migration and invasion by engaging BMP signaling pathways.

While the regulatory relationship between SOX4 and TMEM2 was previously unknown, the regulation of TMEM2 by SOX4 in breast cancer cells, the similarity of the SOX4 and TMEM2 phenotypes in cancer, and the common phenotypes resulting from the genetic inactivation of these genes, suggest that SOX4 and TMEM2 may form a evolutionarily conserved pathway that could govern normal cardiovascular development as well as cancer progression. Further studies are needed to determine if SOX4 regulates TMEM2 expression in the developing heart and, if so, whether enforced TMEM2 expression could rescue the SOX4 loss-of-function phenotype.

While we have identified TMEM2 as one transcriptional target of SOX4, our findings do not exclude the possibility of additional SOX4 transcriptional targets that mediate the SOX4-dependent pro-invasive and pro-migratory phenotypes. Additionally, it will be interesting to investigate the transcriptional and genomic landscape of SOX4 binding during development in order to establish the breadth of its developmental transcriptional program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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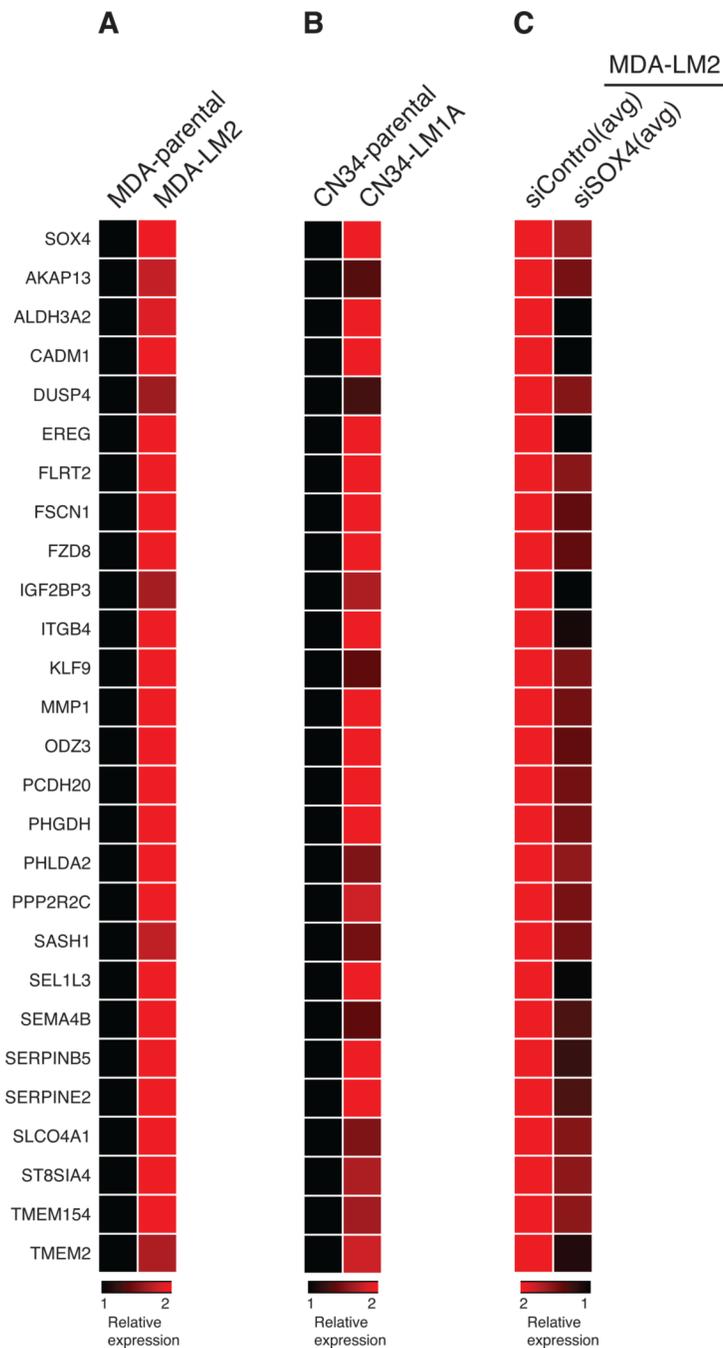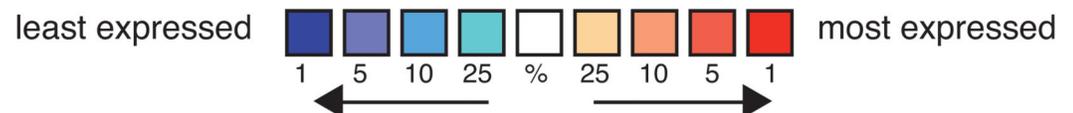
Figure 1. A systematic strategy for identification of SOX4 target genes that regulate breast cancer metastasis

Heat map depicting relative transcript levels of the 26 putative SOX4 target genes that were identified by the overlap of genes whose expression was upregulated in MDA-LM2 relative to MDA-parental cells (A, q < 0.05), upregulated in CN34-LM1A relative to CN34-parental cells (B, q < 0.05), and decreased upon SOX4 depletion using two independent siRNAs relative to control siRNA (C, P < 0.05).

A

Gene	Median Rank	P-value	Tissue Type								
			1	2	3	4	5	6	7	8	9
SOX4	2109.0	3.59E-11									
ST8SIA4	4981.0	2.18E-08									
TMEM2	5637.0	3.47E-07									
AKAP13	6635	1.46E-05									
SEMA4B	4805.0	2.63E-04									
MMP1	1199	1E-03									
PPP2R2C	3434.0	6E-03									
IGF2BP3	8523.0	1.6E-02									
PHLDA2	4463.0	4.9E-02									

least expressed — most expressed

1 5 10 25 % 25 10 5 1

1 Breast Adenocarcinoma Type: Ductal Breast Carcinoma

2 Intraductal Cribriform Breast Adenocarcinoma vs. Normal

3 Invasive Breast Carcinoma vs. Normal

4 Invasive Ductal and Lobular Carcinoma vs. Normal

5 Invasive Ductal Breast Carcinoma vs. Normal

6 Invasive Lobular Breast Carcinoma vs. Normal

7 Male Breast Carcinoma vs. Normal

8 Mixed Lobular and Ductal Breast Carcinoma vs. Normal

9 Mucinous Breast Carcinoma vs. Normal

B

correlation with SOX4	ExpO data (243 tumors)	
	Spearman R	P-value (two-tailed)
MMP1	0.2625	< 1E-04
SEMA4B	0.2331	2E-04
TMEM2	0.2126	9E-04

Figure 2. Clinical relevance of putative SOX4 target genes in breast tumors

(A) mRNA expression levels of SOX4 and its target genes for which positive correlation is observed in breast carcinoma and normal tissue. The values were obtained from Oncomine Cancer Microarray database (www.oncomine.org). The color of each box represent the expression level for each gene as depicted at the bottom of the table. The median rank for each gene across each of the analyses and their p-value are shown in the table. (B) The expression correlation of SOX4 with its target genes MMP1, SEMA4B, and TMEM2 as analyzed using the ExpO breast cancer database (www.intgen.org), which consists of a total of 243 tumors from 4 patients in stage 0, 38 patients in stage I, 129 patients in stage II, 67

patients in stage III, and 5 patients in stage IV. Bonferroni correction was used to adjust confidence intervals (P < 0.02).
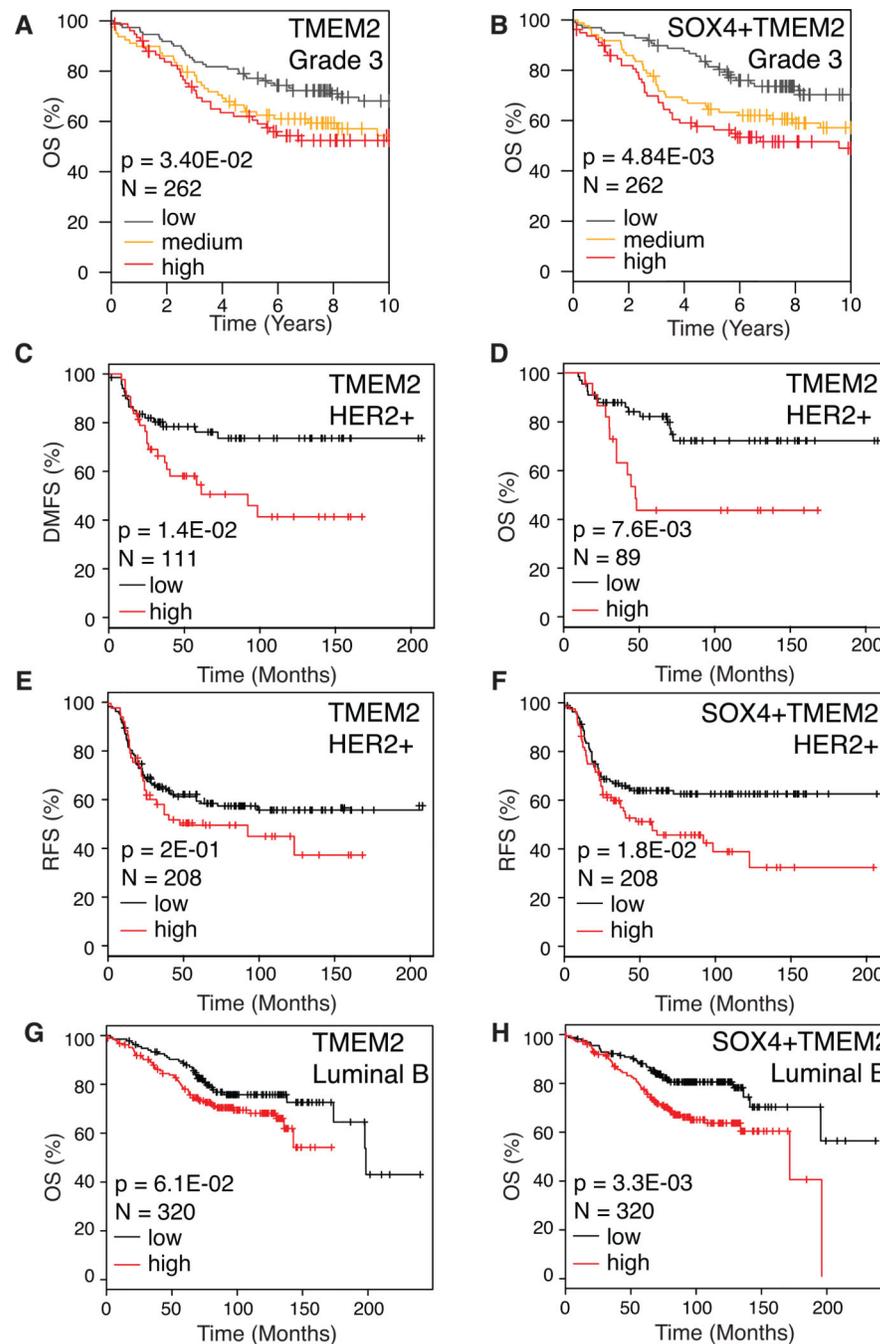
Figure 3. Prognostic value of TMEM2 on breast cancer survival

(A–B) Kaplan-Meier Overall Survival (OS) curves depicting the segregation of patients diagnosed with grade 3 tumors (n=262) expressing different levels of TMEM2 (A), and both SOX4 and TMEM2 (B). Data was obtained from Gene expression-based Outcome for Breast cancer Online (GOBO). Red colored line represents patients with high gene expression, yellow colored line with intermediate and grey color represents patients with low gene expression. TMEM2_low (log2 expression –2.531 to –0.243), medium (log2 expression –0.24 to 0.245), high (log2 expression 0.25 to 3.357), SOX4 + TMEM2_low (log2

expression −3.111 to −0.2), medium (log2 expression −0.199 to 0.215), high (log2 expression 0.22 to 2.196). (C–H) Kaplan-Meier plots for DMFS (C), OS (D), RFS (E) for TMEM2, and both SOX4 and TMEM2 (F) in HER2+ subtype are shown. (G–H) Kaplan-Meier OS plots for TMEM2 (G) and both SOX4 and TMEM2 (H) in luminal B subtype are shown.
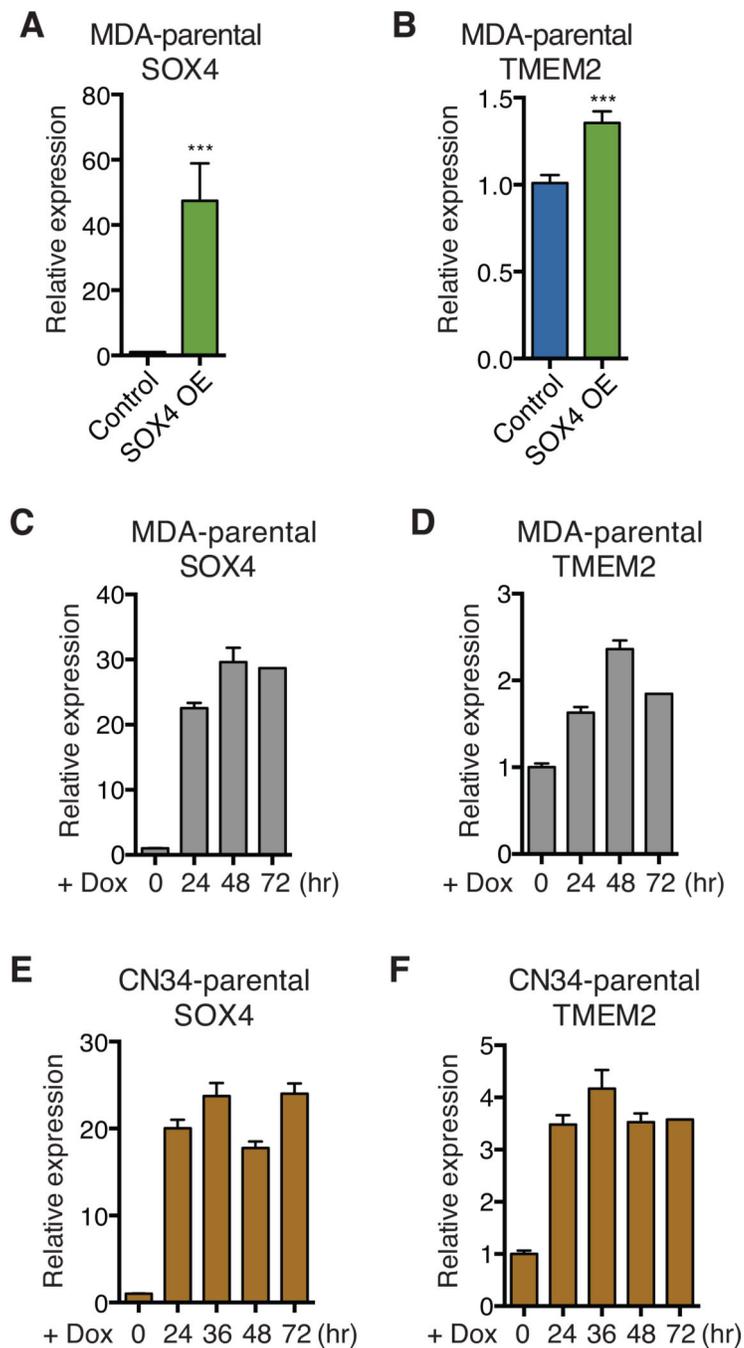
Figure 4. SOX4 is necessary and sufficient for regulation of TMEM2
Induction of TMEM2 transcript levels by SOX4 overexpression were measured by qRT-PCR. (A–B) Relative mRNA levels for SOX4 (A) and TMEM2 (B) in control or SOX4 overexpressing MDA-parental cells (n=3). (C–D) Time course of Doxycycline treatment in Doxycycline inducible SOX4 overexpressing MDA-parental cells depicting relative mRNA levels for SOX4 (C, n=2), and TMEM2 (D, n=2). (E–F) Same as (C–D) in CN34-parental cells (n=2). Error bars represent standard error of the mean (s.e.m.). ****, $P < 0.0001$; ***, $P < 0.0005$.
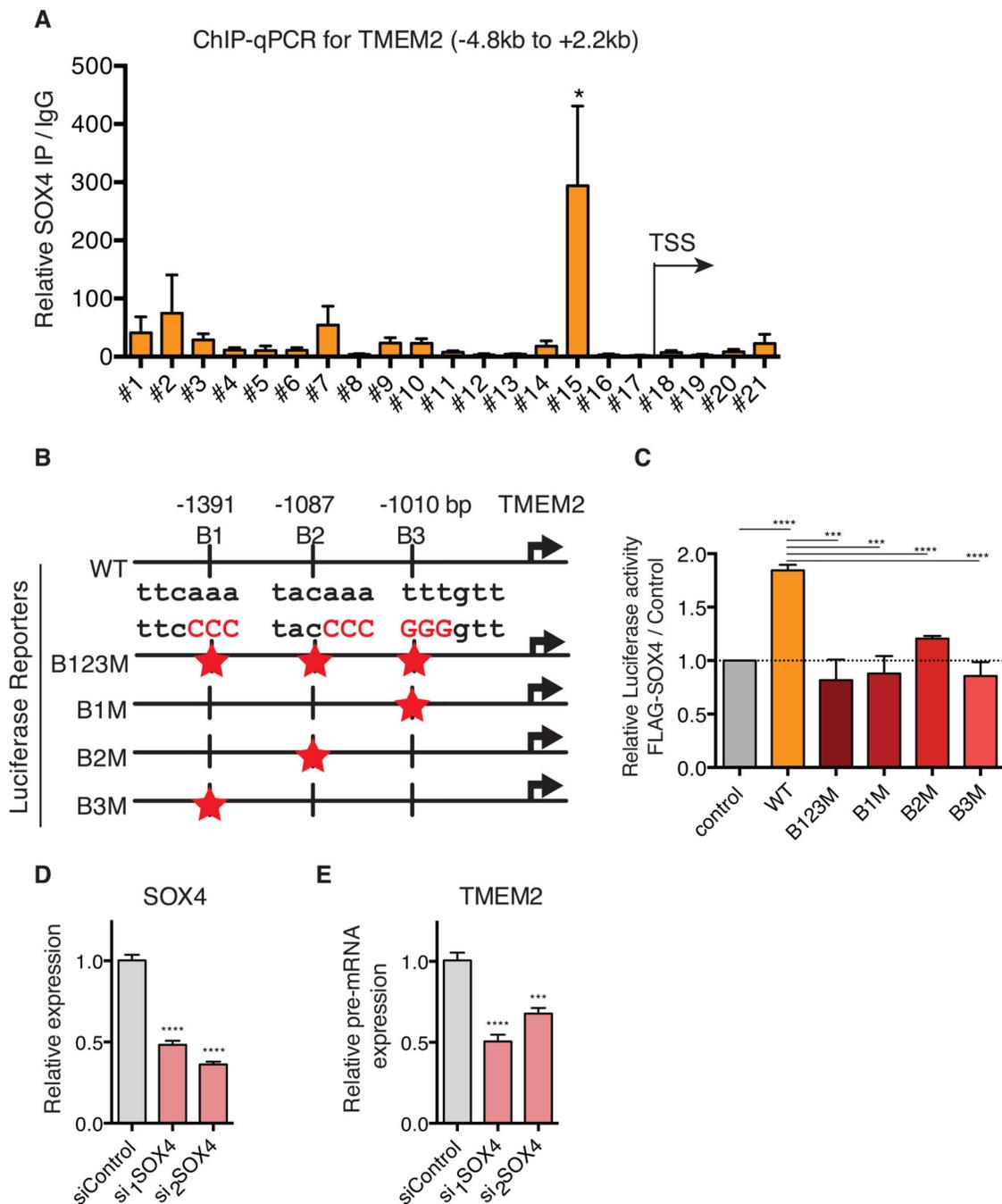
Figure 5. SOX4 transcriptionally regulates TMEM2

(A) ChIP-qPCR assessment of the *TMEM2* promoter region immunoprecipitated with an anti-SOX4 antibody or control IgG. Pairs of primers were design to cover a region from −4.8 kb to +2.2 kb from the Transcription start site (TSS). Sequences upstream of the TSS are marked as (−) and those downstream of the TSS are marked as (+). Experiments were performed in MDA-LM2 cells and data from biological triplicates are shown. Data represented as fold enrichment over SOX4 relative to a negative control IgG. (B) A schematic diagram depicting promoter region of *TMEM2* amplified by qPCR primer #15

used in (A). *TMEM2* promoter region from −1438 to −881 base pairs upstream of the *TMEM2* TSS have three putative SOX4 binding motifs marked by B1, B2, and B3. (C) Functional validation of the ChIP-qPCR by luciferase reporter assay. TMEM2 promoter region containing all three putative SOX4 motifs (WT) or sequences having mutations in all or each of the three motifs shown in (B) were cloned upstream of a luciferase reporter. The relative luciferase activity of the WT sequence compared to the control empty vector sequence was measured in the context of transient FLAG-SOX4 overexpression in HEK293T cells. Experiments were performed in triplicates in two different biological replicates. (D–E) pre-mRNA levels of TMEM2 in the MDA-LM2 cells expressing either siSOX4 or siControl were assessed by qRT-PCR with primers detecting intronic regions close to 3'UTR of TMEM2. Data from quadruplicates in two different biological replicates are shown. Error bars represent s.e.m. ****, $P < 0.0001$; ***, $P < 0.0005$; *, $P < 0.05$.
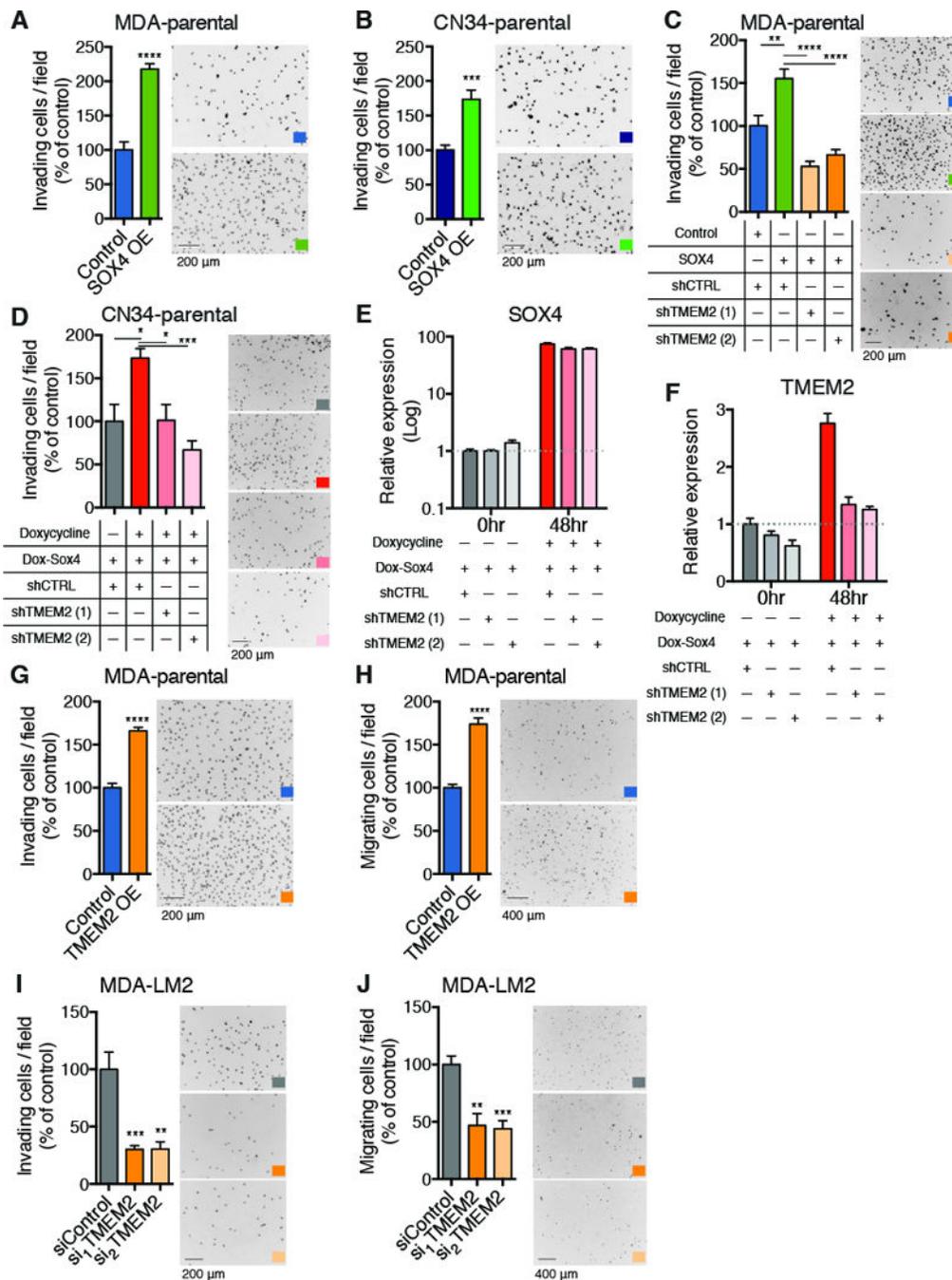
Figure 6. TMEM2 mediates the effects of SOX4 on cell invasion and migration

(A–B) Invasion assays by 5×10^4 MDA-parental cells expressing either a control vector or FLAG-SOX4 (A, n=6) and by 1×10^5 CN34-parental cells expressing either control or FLAG-SOX4 (B, n=5). Images of DAPI stained cells that invaded the matrigel were obtained at 10× magnifications and quantified by Image J. (C) Matrigel invasion by MDA-parental cells after knockdown of TMEM2 in the context of constitutive expression of FLAG-SOX4 (n=4–5). (D) Matrigel invasion by knockdown of TMEM2 in the context of doxycycline inducible FLAG-SOX4 overexpression in CN34-parental cells (n=3–4). (E–F)

Expression of SOX4 (E) and TMEM2 (F) in CN34-parental cells expressing doxycycline inducible FLAG-SOX4 48 hr after doxycycline induction (n=2). (G) MDA-parental cells overexpressing TMEM2 showed enhanced matrigel invasion compared to control cells (n=4). (H) Increased trans-well migration by MDA-parental cells overexpressing TMEM2 compared to the control cells (n=4). (I–J) Reduced matrigel invasion (I) and trans-well migration (J) by knockdown of TMEM2 in MDA-LM2 cells (n=4). Error bars represent s.e.m. ****, $P < 0.0001$; ***, $P < 0.0005$; **, $P < 0.001$; *, $P < 0.05$.
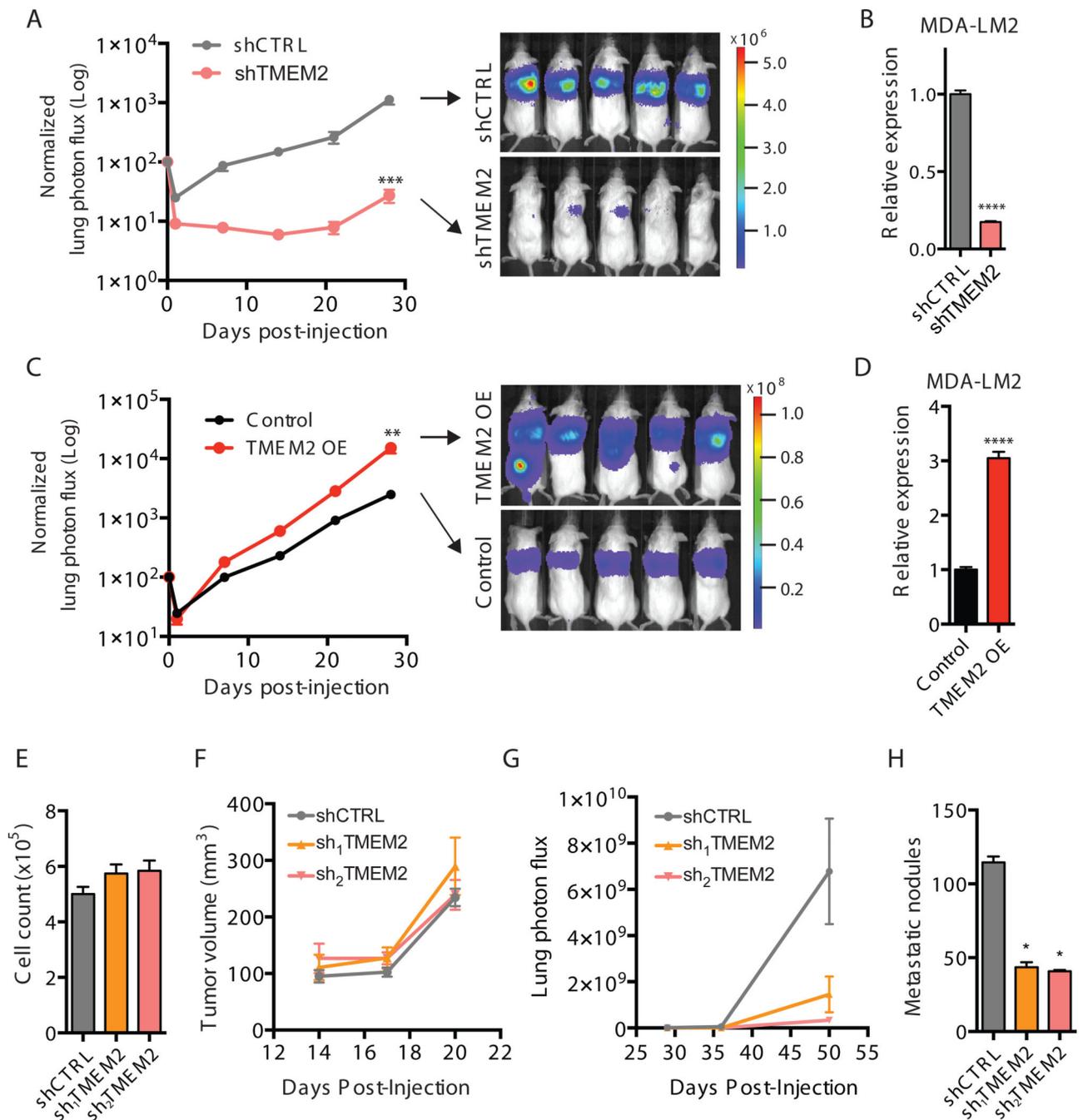
Figure 7. TMEM2 promotes *in vivo* metastatic lung colonization
(A) Bioluminescence imaging plot of lung metastasis by 4×10^4 MDA-LM2 cells expressing control shRNA or shRNA targeting TMEM2 (n=5). NOD-SCID gamma female mice were imaged every seven days and bioluminescence signals were normalized to the signal on the day of tail-vein injection (day 0). (B) Knockdown efficiency of TMEM2 measured by qRT-PCR in MDA-LM2 cells injected into the tail-vein of mice. (C–D) Bioluminescence imaging plot of lung metastasis by 4×10^4 MDA-LM2 cells expressing control or TMEM2 (C, n=5) and mRNA levels of TMEM2 measured by qRT-PCR in MDA-

LM2 cells injected into the tail-vein of NOD-SCID gamma female mice (D). Error bars represent s.e.m. ****, $P < 0.0001$; ***, $P < 0.0005$; **, $P < 0.001$. (E) *In vitro* cell proliferation by 5×10^4 MDA-LM2 cells expressing either shControl or shTMEM2. Cells were seeded in triplicate at day 0. Dead cells were excluded by Trypan blue and live cells were counted at day 5. Data from biological triplicates are shown (n=3). (F) *In vivo* primary tumor growth by 5×10^5 MDA-LM2 cells expressing either shControl or shTMEM2. Cells were bilaterally injected into the mammary fat pads of NOD-SCID gamma female mice at day 0. Primary tumor growth was monitored from day 14 to day 21 (control, n=5; TMEM2 KD #1, n=4; TMEM2 KD #2, n=4). (G–H) At day 21, tumors were resected and orthotopic metastasis was monitored. Lungs were harvested on day 50 (G), Hematoxylin-eosin stained, and the number of nodules per lungs was counted (H). *, $P < 0.05$ obtained using two-tailed Mann-Whitney test. Error bars represent s.e.m.