SNAIL1 employs β-Catenin-LEF1 complexes to control colorectal cancer cell invasion and proliferation

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The transcription factor SNAIL1 is a master regulator of epithelial-to-mesenchymal transition (EMT), a process entailing massive gene expression changes. To better understand SNAIL1-induced transcriptional reprogramming we performed time-resolved transcriptome analysis upon conditional SNAIL1 expression in colorectal cancer cells. Gene set variation analyses indicated that SNAIL1 strongly affected features related to cell cycle and Wnt/β-Catenin signalling. This correlated with upregulation of LEF1, a nuclear binding partner of β-Catenin. Likewise, transcriptomes of cell lines and colorectal cancers, including poor-prognosis mesenchymal tumours, exhibit positively correlated SNAIL1 and LEF1 expression, and elevated LEF1 levels parallel increased patient mortality. To delineate the functional contribution of LEF1 to SNAIL1-induced EMT, we used the CRISPR/Cas9 system to knock-out LEF1 in colorectal cancer cells, and to engineer cells that express LEF1 mutants unable to interact with β-Catenin. Both complete LEF1-deficiency and prevention of the β-Catenin-LEF1 interaction impaired the ability of SNAIL1 to elicit expression of an alternative set of Wnt/β-catenin targets, and to promote cancer cell invasion. Conversely, overexpression of wildtype, but not of mutant LEF1, stimulated alternative Wnt/β-Catenin target gene expression, and caused cell-cycle arrest. Moreover, like SNAIL1, LEF1 retarded tumour growth in xenotransplantations.

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Author Contributions: VF, KrR, JM, PF and KaR designed and performed experiments, acquired data, and analysed them with contributions from HB, MB, RZ and AH. VF, HB, MB and AH prepared figures and wrote the manuscript. AH conceived and supervised the project.

Additional Supporting Information may be found in the online version of this article.

Key words: cell cycle control, epithelial-to-mesenchymal transition, genome editing, metastasis, Wnt, β-Catenin signalling

Abbreviations: ChIP: chromatin immunoprecipitation; CMS: consensus molecular subtype; CRC: colorectal cancer; Dox: doxycycline; EMT: epithelial-to-mesenchymal transition; GSVA: gene set variation analysis; HA: hemagglutinin; HMG-box: high-mobility group box; KI: knock-in; KO: knock-out; LEF1: lymphoid enhancer-binding factor 1; PCR: polymerase chain reaction; qPCR: quantitative PCR; qRT-PCR: quantitative reverse transcription PCR; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCF: T-cell factor; TF: transcription factor; v/v: volume/volume; WT: wildtype

Conflict of Interest: The authors declare no conflict of interest.

Grant sponsor: Bundesministerium für Bildung und Forschung; Grant numbers: DeCaRe, FKZ 01ZX1409B; Grant sponsor: Deutsche Forschungsgemeinschaft; Grant numbers: CRC-850, subprojects B5, C5, Z1, EXC306, GSC-4, Spemann Graduate School (SGBM); Grant sponsor: European Research Council; Grant number: GVHDUCURE

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DOI: 10.1002/ijc.32644

History: Received 11 Sep 2018; Accepted 6 Aug 2019; Online 28 Aug 2019

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Int. J. Cancer: 146, 2229–2242 (2020) © 2019 The Authors. International Journal of Cancer published by John Wiley & Sons Ltd on behalf of UICC
Thus, LEF1 phenocopies SNAIL1 with respect to several critical aspects of EMT. Indeed, comparative transcriptomics suggested that 35% of SNAIL1-induced transcriptional changes are attributable to LEF1. However, LEF1 did not autonomously induce EMT. Rather, LEF1 appears to be a strictly β-Catenin-dependent downstream effector of SNAIL1. Apparently, SNAIL1 employs β-Catenin-LEF1 complexes to redirect Wnt/β-Catenin pathway activity towards pro-invasive and anti-proliferative gene expression.

What’s new?

Epithelial-to-mesenchymal transition (EMT), which plays a critical role in cancer progression, is underpinned by massive alterations in gene expression. While the transcription factor SNAIL1 induces many of these changes, the process of transcriptional reprogramming in EMT remains poorly understood. This study, using a colorectal cancer model, shows that SNAIL1 redirects Wnt/β-Catenin signaling to initiate EMT. SNAIL1, via LEF1 upregulation, diverts β-Catenin-dependent transcription toward target genes, including genes that promote invasion and cell-cycle arrest, despite oncogenic CTNNB1 and KRAS mutations. The findings delineate a role for β-Catenin-LEF1-driven subprograms in EMT induction and show that this process includes unexpected anti-proliferative activity.

Introduction

Epithelial-to-mesenchymal transition (EMT) facilitates several steps during metastasis by altering cell–cell and cell–matrix interactions, migration and the invasive potential of cancer cells. Additionally, EMT reduces proliferation which likely accounts for increased chemoresistance of mesenchymal cancer cells. A mainstay for the induction of EMT is the activation of a core network of transcription factors (TFs), comprising SNAIL, TWIST and ZEB family members. These core EMT TFs are upregulated in response to various environmental signals and pathological conditions. Upon induction, core EMT TFs trigger widespread alterations in epithelial and mesenchymal gene expression. Intriguingly, the number of genes that are deregulated in the presence of core EMT TFs vastly exceeds the number of their direct targets. Therefore, an important question becomes to decipher the molecular mechanisms whereby the full extent of EMT-associated transcriptional changes is brought about. A potential scenario for this could be that core EMT TFs reside at the apex of a regulatory hierarchy to prompt changes in the activity of an expanding set of downstream effectors, whereby the full EMT process gradually unfolds. Which signalling cascades and associated gene regulatory factors act in concert with or downstream of the core EMT TFs to effectuate transcriptional reprogramming and phenotypic conversion during EMT is largely unclear.

Wnt/β-Catenin signalling plays critical roles in development and adult tissue homeostasis. Deregulated Wnt/β-Catenin activity often occurs in carcinogenesis, most notably colorectal cancer (CRC) where a hyperactive Wnt/β-Catenin pathway drives tumour cell proliferation. A major aspect in Wnt/β-Catenin signalling is the transcriptional regulation of gene expression. This is mediated by heteromeric protein complexes formed by β-Catenin and members of the T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family which in humans consists of TCF7, TCF7L1, TCF7L2 and LEF1. Wnt/β-Catenin signalling controls complex formation with TCF/LEF proteins through regulated proteolysis and nuclear entry of β-Catenin. Exposure of cells to Wnt growth factors, and also mutational inactivation of inhibitory pathway components like the tumour suppressor APC, prevent β-Catenin degradation and promote its binding to TCF/LEF proteins. Although all TCF/LEF family members can interact with β-Catenin, individual TCF/LEF proteins possess highly different gene regulatory potential. Therefore, variations in the expression of TCF/LEF family members decisively shape the range of Wnt/β-Catenin-regulated genes and contribute to context-dependent transcriptional Wnt/β-Catenin responses. EMT and Wnt/β-Catenin signalling appear to be interconnected at several levels. β-Catenin-TCF/LEF complexes can promote expression of core EMT TFs, identifying the Wnt/β-Catenin pathway as an upstream inducer of EMT. Reciprocally, core EMT TFs modulate Wnt/β-Catenin target gene expression by physically interacting with β-Catenin, although the significance of this finding in the context of EMT was not determined. Additionally, Wnt/β-Catenin pathway components could function downstream of core EMT TFs. For instance, LEF1 was observed to be upregulated in several EMT models. LEF1 was even proposed to be an autonomous EMT-inducer. However, it is controversial whether LEF1 can elicit EMT in cells that are not pre-conditioned to become mesenchymal due to resident expression of EMT core TFs. Likewise, there are conflicting results concerning the requirement for β-Catenin as interactor of LEF1 in EMT processes. Consequently, it remains to be determined whether or not LEF1 acts as an effector of the Wnt/β-Catenin pathway in an EMT setting. Furthermore, the relevant transcriptional targets of LEF1, and the cellular processes which could potentially be influenced by LEF1 in the course of EMT, have yet to be elucidated.

Aiming to better understand the molecular basis of EMT, we have undertaken time-resolved transcriptome profiling using a model based on EMT-naïve colorectal cancer cells and doxycycline-(Dox-) inducible expression of murine SNAIL1 (mSNAIL1).
Here, we report that mSNAIL1 broadly alters the transcriptional output of the Wnt/β-Catenin pathway which coincides with the upregulation of LEF1. Through functional studies with wildtype (WT) and mutant LEF1, incapable of interacting with β-Catenin, we unequivocally show that LEF1 is a strictly β-Catenin-dependent downstream effector of mSNAIL1. LEF1, however, is not sufficient to induce EMT on its own. Rather, mSNAIL1 engages β-Catenin-LEF1 complexes to redirect Wnt/β-Catenin pathway activity toward an alternative set of target genes which support pro-invasive and, unexpectedly, anti-proliferative EMT subprograms.

Materials and Methods

Cell culture

LS174T (RRID: CVCL_1384) and HT29 (RRID: CVCL_0320) cells were obtained from the German Cancer Research Center Cell Line Service. The identity of the parental cells and derivatives thereof was confirmed by SNP-profiling at Multiplexion Inc. (Friedrichshafen, Germany). In contrast to LS174T cells used by others,20 our cells express E-Cadherin. Cells were cultivated in DMEM supplemented with 10% (v/v) FCS, 10 mM HEPES, 1% (v/v) penicillin/streptomycin and 1% (v/v) MEM non-essential amino acids at 37°C and 5% CO₂. LS174T cells expressing Dox-inducible mSNAIL1-HA, mLEF1-HA, mLEF1m5-HA (Lt-Lm5), and mLEF1-FLAG were described before19,21 or generated correspondingly. To obtain mLEF1m5-HA, previously described mutations22 were introduced into the mLEF1-HA plasmid21 by polymerase chain reaction (PCR) with mutagenic primers (5'-GAAGGGATCATCAGCCGGGTGGAGAGAGAG-3', 5'-CTCTGCGCCACCGCGAGGATGTCCGAGG-3') and cloned into the addgene plasmid #41824 as before.24

For Cas9-turboRFP expression, GFP was excised from plasmid #44719 (addgene) and replaced by turboRFP using Gibson assembly. The donor plasmid for the knock-in was constructed by PCR amplification of portions of the LEF1 locus from LS174T cells and transferring the sequences into pUC18. The m5 mutation was introduced in the process using mutagenic PCR primers. The PCR fragments were fused via recombinant PCR. All plasmids were sequence-verified. For genome editing 0.5 μg Cas9-turboRFP and 1 μg gRNA1 or 0.5 μg Cas9-turboRFP, 0.5 μg gRNA T1, 0.5 μg gRNA T2 combined with 2.5 μg donor plasmid were introduced into pN1-LS174T cells using the Cell line Nucleofector Kit L (#VCA-1005, Lonza). TurboRFP™ cells were single cell-sorted 72 hr post nucleofection.

Genome-edited cell clones were identified by Surveyor assays (#706020, Integrated DNA Technologies). For this, genomic DNA was isolated using thepeqGOLD Tissue DNA Mini Kit (#12–3,396, Plegab VWR), and used to PCR-amplify sequences surrounding the Cas9 cleavage sites (primers listed in Supporting Information Table S1). Single cell clones carrying a knock-in of the m5 mutation were identified by use of a PvuI cleavage site newly created upon donor sequence integration. Clones carrying deletions of the β-Catenin-binding domain and HT29 LEF1 knock-out clones were identified due to size differences of PCR products. LEF1 PCR products from all single cell clones of interest were subcloned and sequence-verified.

Work with RNA and whole transcriptome analysis

Procedures and kits used for experiments with RNA were the same as before,24 except that the qScript™ Flex cDNA Kit and the PerfeCTa® SYBR® Green SuperMix (95054, 95049; QuantaBio) were used for cDNA synthesis and qRT-PCR of RNA isolated from HT29 cell derivatives. For qRT-PCR, a cDNA amount equivalent to 10 ng RNA was used (primers listed in Supporting Information Table S1). Gene expression data were normalized using GAPDH/Gapdhl transcript measurements. Biotinylated RNA for microarray experiments was prepared with the Ambion MessageAmp kit for Illumina arrays according to the manufacturer’s protocol. Quality of RNA was controlled using RNA Nano Chip Assay on an Agilent 2100. cRNA was hybridized to Illumina HumanHT12-v4 BeadChips (Illumina) following the manufacturer’s protocol. Raw microarray data were processed chip-wise using the Bioconductor R package beadarray25 and subsequently quantile normalized together. Illumina Probes were mapped to Entrez IDs using the Illumina Human v4 annotation data (Version 1.26) from Bioconductor. If several probes mapped to the same Entrez ID, the one having the largest interquartile range was retained. To detect differentially regulated genes over time we generated a cubic spline curve over the measured time points with 3 and 5 degrees of freedom for the mLEF1 and mSNAIL1 overexpression data, respectively, and performed a moderated F-test on the interaction terms between the spline and the treatment/control groups. The analysis was done using the
R/Bioconductor package limma.\textsuperscript{26} p-values were corrected for multiple testing using Benjamini & Hochberg. Gene set variation analysis (GSVA) was used to detect differential pathway regulation over time.\textsuperscript{27} Gene set enrichment scores were calculated for each sample and their temporal significance assessed using a moderated F-test as above for the genes. For functional annotation, we used the HALMARK pathway collection from the Molecular Signatures Database (MSigDB, v. 6.1)\textsuperscript{28} as well as the cell cycle- and WNT signalling-related gene sets from the Gene Ontology and consensus PathDB, respectively.\textsuperscript{29}

**Pairwise correlation analyses**

Pairwise correlation analyses of publicly available data sets were conducted as previously described.\textsuperscript{21} GSE14333 and GSE59857 data were downloaded from the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). COADREAD TCGA data were obtained from the Xena Functional Genomics Explorer (https://xenabrowser.net/).

**TCGA data analysis**

The Xena Functional Genomics Explorer (https://xenabrowser.net/) was used to analyse the TCGA COADREAD data set. Samples were filtered for primary tumour data. \emph{SNAI1} and \emph{LEF1} gene expression levels were assessed based on RNA-seq results and are annotated as log2(norm_count+1). Kaplan–Meier-Plots for \emph{LEF1} and \emph{SNAI1} high or low expression were calculated by the tool implemented in the Xena browser. Expression data for the different genes in primary tumours were downloaded from the Xena website and correlation analysis was then conducted as described for the other data sets.

**Western blotting and immunoprecipitation**

For western blotting and immunoprecipitation whole cell lysates were prepared as described,\textsuperscript{20} or by using RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 1x Complete\textsuperscript{®} protease inhibitor) for cell lysis. For immunoprecipitations, aliquots with 500 µg of protein were incubated with 0.5 µg antibody and 30 µl protein G Dynabeads at 4°C overnight. After washing three times for 10 min with lysis buffer the beads were re-suspended in loading buffer and boiled for 5 min. Protein precipitates were analysed by SDS-PAGE and western blotting along with 75 µg of the starting material. Supporting Information Table S2 lists the antibodies used.

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed as described\textsuperscript{24} with modifications. Briefly, LS174T cells expressing FLAG epitope-tagged mLEF1 (Ls-L\textsuperscript{FLAG} cells) were crosslinked with 1% formaldehyde for 10 min at RT and then disrupted using a Bioruptor Plus device (Diagenode). Chromatin was isolated and sheared by sonication. Two micrograms of the monoclonal anti-FLAG M2 antibody (F1804, Sigma Aldrich) were used for the immunoprecipitations. qPCR was performed as described earlier using 1 µl of precipitated DNA and 2% of the input material.

**Organoid culture**

Organoid cultures were established from Apc\textsuperscript{SRS5/SRS5}, tgVillin-creERT2 mice with a mixed 129S6C7BL/6 background.\textsuperscript{31,32} Mice were handled in accordance with legal regulations at the Center for Experimental Models and Transgenic Service of the University of Freiburg Medical Center (project registration number: X-17/07S). To initiate and propagate the cultures, a previously published protocol\textsuperscript{33} was slightly modified. Briefly, the small intestine was opened longitudinally, washed thoroughly, and incubated with 2 mM EDTA in PBS. After washing once with PBS, the tissue was transferred to a 50 ml reaction tube containing 10 ml PBS. By vigorous shaking, intestinal crypts were detached from the tissue which was then transferred to a second 50 ml reaction tube containing 10 ml PBS. This procedure was repeated 10 times. All the fractions were collected and checked for their content of crypts. Fractions with the highest crypt numbers were combined, pelleted, and put on a 70 µm cell strainer (Corning Life Sciences). After another centrifugation step, the crypt pellet was re-suspended in Matrigel (3#356231, Corning Life Sciences) and seeded in 24-well plates. Crypts were cultured in advanced DMEM/F12 containing 1:100 (v/v) Glutamax, 10 mM HEPES, 1:100 (v/v) penicillin/streptomycin, 1:100 (v/v) N2 supplement, 1:50 (v/v) B27 supplement, 1 mM n-acetyl-cysteine, 50 ng/ml murine EGF, 100 ng/ml murine Noggin and 500 ng/ml human R-Spondin1. Apc was deleted \emph{in vitro} by adding 0.5 µM tamoxifen to the media, thereby activating the CreERT2 recombinase.

**Cell migration, invasion and population dynamics**

HT29 derivatives were cultured with or without Dox for 96 hr, before being seeded into ibidi\textsuperscript{®} 2-well cell-culture inserts (5 × 10⁴ cells per well). Cells were further cultivated for 24 hr with or without Dox. Then the ibidi\textsuperscript{®} insert was removed, and the cells were incubated with 0.5 µM mitomycin C (M4287, Sigma-Aldrich) for 40 min. Thereafter, pictures were taken every hour in a JuLI\textsuperscript{™} Stage Real-Time Cell History Recorder (NanoEnTek) for 24 hr. The degree of wound closure was determined using the JuLI\textsuperscript{™} STAT software taking the 0 hr Dox value as 100%. Spheroid invasion assays were performed as described\textsuperscript{19} except that pictures were taken 72 hr after embedding in collagen I. Population dynamics were monitored by crystal violet staining as described\textsuperscript{19} with minor modifications. Cells were seeded in 24-well plates. Four hours thereafter, Dox-treatment was initiated. At the desired time points, cells were stained with 0.5% crystal violet in 20% methanol. After incubation for 10 min and washing four times with H₂O, crystal violet was extracted from the cells using 100% methanol for 30 min. Absorbance at 595 nm was measured in duplicates of 1:100 dilutions of the extracted dye.

**Cell cycle analyses**

For cell cycle analyses, 5 × 10⁵ cells were seeded in 6-well plates. After a 24 hr Dox-treatment, cells were trypsinised, transferred to FACS tubes and washed with PBS. For permeabilisation and fixation, cells were re-suspended in 70%
EtOH and stored at 4°C for at least 1 hr. After washing twice with PBS, the cells were re-suspended in PBS containing 10 μg/ml RNase A and 20 μg/ml propidium iodide and incubated for 30 min at 37°C. After washing again with PBS the cells were re-suspended in PBS for flow cytometry.

**Luciferase reporter assays**

For reporter gene assays, 0.5 × 10^5 Ls-V and Ls-S cells were seeded in 24-well plates. Cells were transfected with the pSuper8xTOPflash and pSuper8xFOPflash constructs as described, and treated with Dox for 48 hr starting 24 hr after transfection. Reporter gene expression was determined as described.

**Xenotransplantations**

Rag2^−/− γc^−/− mice, obtained from the Center for Experimental Models and Transgenic Service of the University of Freiburg Medical Center, were injected subcutaneously with 1 × 10^6 cells following animal protocols approved by the local animal welfare committee (G13-116 and G17-049). Seven days after transplantation, tumours were palpable and Dox-treatment was started. Twelve days later, tumours (n = 39) were excised, formalin-fixed and paraffin-embedded. Xenografts were cut in serial sections, transferred to glass slides, deparaffinised, subjected to antigen retrieval and analysed by immunofluorescence staining. After a blocking step with 5% horse serum in PBS-Tween, the samples were incubated with primary antibodies at 4°C overnight. After washing three times with PBS, slides were incubated with appropriate secondary antibodies for 1 hr at room temperature. After washing three times, the samples were mounted using VECTASHIELD Antifade Mounting Medium with DAPI (+H-1200, VECTOR Laboratories). The samples stained for mSNAIL1 were incubated with HRP-conjugated anti-rabbit antibody. After three washing cycles, they were treated with Alexa Fluor 488 Tyramide Reagent (#B40953, Invitrogen) for 5 min, washed three times with PBS and mounted as above. Pictures were taken using an Axio Observer Z1 fluorescence microscope. Brightness was adjusted using the Canvas™ X 2017 software. All images were edited identically.

**Data availability**

The microarray data that support the findings of our study are openly available at the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under the accession ID GSE115716.

**Results**

**mSNAIL1 alters the output of the Wnt/β-Catenin pathway**

We used LS174T colorectal adenocarcinoma cells with Dox-inducible expression of mSNAIL1 with a hemagglutinin (HA) epitope tag (Ls-S cells) to assess global effects on gene expression at different time points after mSNAIL1 induction. Over time, more than 2000 genes were up- and downregulated (Supporting Information Fig. S1a, Table S3). GSVA revealed alterations in a variety of cellular pathways involved in metabolism and signal transduction (Supporting Information Fig. S1b, Table S4). We also detected initial upregulation of cell cycle-related gene sets, which was superseded by an increase in Wnt/β-Catenin pathway features (Fig. 1a, Supporting Information Table S4). Indicative of the latter was the upregulation of APCDD1, NOTUM, NDK1 and DKK4 (Supporting Information Fig. S1a). The question of how mSNAIL1 can alter the transcriptional output of the Wnt/β-Catenin pathway. As potential explanation we focused on LEF1, which was strongly upregulated in the presence of mSNAIL1 (Supporting Information Fig. S1a, Table S3). By use of the Xena Functional Genomics Explorer for the analysis of the TCGA COADREAD data we determined that SNAIL1 and LEF1 were both expressed at higher levels in primary tumours compared to normal tissue (Fig. 1b). Also, high expression of SNAIL1 and LEF1 correlated with worse overall survival (Fig. 1c). Correlation analyses based on publicly available transcriptome data from human colorectal cancers and CRC cell lines revealed strong tendencies of co-expression for SNAIL1 and the mesenchymal markers VIM, FNI and CDH11 (mesenchymal gene group), and for APCDD1, AXIN2, NOTUM, NDK1 and DKK4 (Wnt/β-Catenin target group). Interestingly, LEF1 was positioned at the transition zone between the two gene clusters, showing positive correlations with both (Fig. 1d). Comparatively higher and seemingly coordinate expression of the Wnt/β-Catenin target group and the mesenchymal gene group was also evident when comparing transcriptomes from consensus molecular subtype 2 (CMS2) colorectal cancers, featuring marked Wnt/β-Catenin pathway activation, and from poor prognosis, mesenchymal CMS4 tumours (Supporting Information Fig. S1d). Association with the mesenchymal gene group is consistent with the upregulation of LEF1 in several EMT models and with FNI being a Wnt/β-Catenin target gene that exhibits preferential activation by LEF1. Association with the Wnt/β-Catenin target group additionally ties LEF1 to a set of genes which are negative feedback regulators of the Wnt/β-Catenin pathway. The segregation of the two gene clusters indicates that LEF1 may affect the Wnt target group also outside of an EMT context. Altogether, these observations support the idea that LEF1 significantly contributes to gene expression dynamics that accompany mSNAIL1-induced EMT.

**LEF1 is required for an altered Wnt/β-Catenin pathway output and full-blown EMT upon mSNAIL1 expression**

To explore the role of LEF1 in mSNAIL1-induced EMT, we knocked-out LEF1 in LS174T cells (Fig. 2a, Supporting Information Figure S2). Additionally, to confirm that LEF1 acts as a β-Catenin-dependent Wnt pathway effector, we abrogated its interaction with β-Catenin by introducing a 111 bp internal deletion and by knocking-in the m5 mutation (Fig. 2a, Supporting Information Figure S3). We then used retroviral transduction to restore...
conditional mSNAIL1 expression in the genome-edited clones. As controls, two WT clones were used which had undergone Cas9 and gRNA transfection but had not suffered LEF1 sequence changes. Altogether, this yielded Ls-SLΔ (LEF1 with internal deletion), Ls-SLm5KΔ (LEF1 m5 knock-in), Ls-SLKO1 and Ls-SLKO8 (LEF1 knock-out), as well as Ls-SLWT1 and Ls-SLWT8 (LEF1 WT) cells. Dox-inducible mSNAIL1 expression and concomitant upregulation of WT and mutant LEF1 was confirmed (Fig. 2b, Supporting Information Figure S4a). No LEF1 was found in Ls-SLKO1 and Ls-SLKO8 cells. Disruption of β-Catenin-LEF1 complex formation in Ls-SLΔ and Ls-SLMΔ cells was verified (Supporting Information Fig. S5a). We then examined how the LEF1 status affected mSNAIL1-induced transcriptional changes. Although we observed some fluctuations in gene expression levels that are more likely
attributable to clonal variation, it appears that downstream of mSNAIL1 the induction of FN1 and the Wnt/β-Catenin target group critically depends on LEF1 and β-Catenin-LEF1 complex function (Fig. 2b, c; Supporting Information Figure S4a). In contrast, deregulation of the epithelial marker CLDN3 and the mesenchymal markers VIM and CDH11 does not require LEF1 (Supporting Information Fig. S4b). LEF1 was reported to participate in CDH1 repression. However, this was not reproduced in our model.
Figure 3. mLEF1 expression alters the output of the Wnt/β-Catenin pathway. (a) Western blot analysis of LEF1/mLEF1 expression after 72 hr of Dox-treatment in Ls-S and Ls-L cells. The detection of RNA polymerase II (RNA-Pol II) served as a loading control. Mw, molecular mass. (b) Venn diagrams showing the overlap between the genes regulated in Ls-S and Ls-L cells after 24 hr and 72 hr of Dox-treatment. (c) GSVA of Ls-L cells. (d) Normalised qRT-PCR expression analysis of the genes indicated in Ls-S and Ls-L cells at different time points after Dox-treatment (0 hr, 24 hr, 48 hr, 72 hr and 96 hr). For comparison, LS174T cells stably transduced with an empty expression vector (Ls-V) were included in the analyses. Values represent mean ± SEM. n ≥ 3. (e) Normalised qRT-PCR expression analysis of the genes indicated in Apcfl/fl and ApcΔ/Δ small intestinal organoids. Values represent mean ± SEM. n = 3.
Nonetheless, β-Catenin-LEF1 complexes are essential mediators of a subset of mSNAIL1-induced transcriptional changes. Next, we investigated a requirement for LEF1 in mSNAIL1-induced cellular invasion by growing LS174T cell derivatives as spheroids in three-dimensional collagen I matrices. Compared to LEF1 WT cells, Ls-SL$^{KO1}$, Ls-SL$^{KO8}$, Ls-SL$^{LD}$ and Ls-SL$^{mSK1}$ spheroids formed fewer and shorter invasive sprouts (Fig. 2f). In particular, the number of single cells detaching from the bulk of cells
and invading the surrounding matrix was reduced in LEF1 knock-out and mutant cells (Fig. 2c). Thus, β-Catenin-LEF1 complexes are necessary for the complete execution of mSNAIL1-induced EMT.

To examine whether LEF1 contributed to EMT in another cell model, we Dox-inducibly expressed mSNAIL1 in HT29 CRC cells (HT-S) (Supporting Information Fig. S6). Again, LEF1 was strongly upregulated upon induction of mSNAIL1 (Supporting Information Fig. S6b,c). We then knocked-out LEF1 in HT-S cells by using the CRISPR/Cas9 system to introduce a deletion in the LEF1 gene extending from exon 2 to exon 3. Thereby, we obtained the HT-SLKO and HT-SLKO16 cell clones (Supporting Information Fig. S6a,b). To serve as a control in subsequent analyses, we also retained from the genome-editing experiment a LEF1 WT clone (HT-SWT29). Similar to our findings in LS174T cells, absence of LEF1 attenuated mSnail1-induced expression of FN1 (Supporting Information Fig. S6b,c). Expression of the Wnt/β-Catenin target group could not be analysed in the HT29 cell background due to lack of even basal expression. Nonetheless, LEF1-deficiency abated mSNAIL1-induced morphological changes, whereby HT-SLKO cells exhibited a more compact growth pattern and formed fewer protrusions compared to LEF1 WT cells (Supporting Information Fig. S6d). Furthermore, relative to HT-S and HT-SWT29 cells, the HT-SLKO cell clones had impaired migratory capacities (Supporting Information Fig. S6e). Altogether, we conclude that LEF1 plays a role in mSNAIL1-induced EMT also in HT29 cells.

GSVA of Ls-S cell transcriptome changes had shown down-regulation of cell cycle-related features around the time when Wnt/β-Catenin pathway activity increased. We therefore tested whether LEF1-deficiency influenced mSNAIL1-mediated changes in proliferation. However, like their LEF1 WT counterparts, Ls-SLKO and Ls-SLKO16 cell lines exhibited reduced population dynamics upon mSNAIL1 induction (Supporting Information Fig. S7a,b). Also, LEF1-deficiency did not overcome tumour growth inhibition by mSNAIL1 in vivo (Supporting Information Fig. S7a,c,d). This was not due to fortuitous restoration of LEF1 expression, because immunofluorescence stainings of xenografts confirmed the absence of LEF1 in Ls-SLKO and Ls-SLKO16 tumours (Supporting Information Fig. S7e). Consistent with their impaired growth, LEF1 WT and LEF1-deficient tumours exhibited diminished Ki67 staining in tumour areas where mSNAIL1 was expressed (Supporting Information Fig. S7e). Although these results do not strictly rule out that LEF1 participates in mSNAIL1-induced growth arrest, it appears that mSNAIL1 does not solely rely on LEF1 to control cell proliferation.

LEF1 controls a large fraction of SNAIL1-regulated genes including Wnt/β-Catenin targets

To follow the idea that LEF1 is responsible for the altered transcriptional output of the Wnt/β-Catenin pathway in Ls-S cells, we used cells which Dox-inducibly express murine HA-tagged mLEF1 (Ls-L cells) at levels comparable to endogenous LEF1 in Ls-S cells (Fig. 3a). Global gene expression profiling of Ls-L cells showed that mLEF1 triggered extensive gene expression changes (Fig. 3b, Supporting Information Fig. S8a, Table S3). Comparing LEF1 and SNAIL1-dependent transcriptomes revealed an overlap which vastly increased from 24 hr to 72 hr post induction demonstrating that LEF1 mediates up to 35% of the gene expression changes in Ls-S cells (Fig. 3b). Similar to what was seen in Ls-S cells, mLEF1 expression affected gene sets related to metabolism, cell cycle control and Wnt/β-Catenin signalling (Fig. 3c, Supporting Information Fig. S8b, Table S4). Genes that were regulated by mLEF1 in a manner largely independent of SNAIL1 include the Wnt/β-Catenin target group (Supporting Information Fig. S8, Table S3). No dysregulation of CDH1 and mesenchymal genes occurred in Ls-L cells. We confirmed by qRT-PCR that mLEF1 overexpression had no impact on CDH1 and FN1 expression in the absence of mSNAIL1, whereas expression of the Wnt/β-Catenin target group was autonomously induced by mLEF1 (Fig. 3d). To determine whether members of the Wnt target group were direct targets of mLEF1, we performed ChIP analyses. For this, we identified genomic regions potentially occupied by mLEF1 using as guide-line ChIP-seq data obtained for TCF7L2, another TCF/LEF family member (Supporting Information Fig. S9a-c). Indeed, mLEF1 was found to bind to previously described TCF7L2 ChIP-seq peak regions at the APCDD1, NDK1 and NOTUM loci (Supporting Information Fig. S9a-h), arguing that mLEF1 directly acts upon these genes.

Interestingly, upregulation of Lef1 in intestinal organoids triggered by deleting Apc was also accompanied by increased expression of the Wnt/β-Catenin target group (Fig. 3e). Expression of Snai1, Cdh1 and Tcf7l2, the key Wnt/β-Catenin pathway effector in the normal intestinal epithelium, did not change. However, Fni1 was upregulated in Apc-deficient organoids (Fig. 3e), arguing that Fni1 regulation is context-dependent and requires additional input which is present in ApcΔ/Δ organoids and Ls-S cells but absent from Ls-L cells. In contrast, for induction of the Wnt/β-Catenin target group it may suffice that LEF1 expression exceeds a certain threshold, although this requires mechanistic confirmation.

Figure 4. mLEF1 overexpression induces growth arrest but no EMT. (a) Morphology of LS174T cells stably transduced with an empty vector control (Ls-V) compared to Ls-S and Ls-L cells. Pictures were taken after 72 hr of Dox-treatment. Scale bars: 100 μm. (b) Spheroid growth of LS174T cell derivatives in a three-dimensional collagen I matrix. Pictures were taken after 96 hr of Dox-treatment. Scale bars: 100 μm. (c,d) Population dynamics of LS174T cell derivatives with or without Dox-treatment for the indicated time spans. Representative images of crystal violet stainings (c) and corresponding quantifications (d). Values represent mean ± SEM. n = 4. **p < 0.001. (e) Flow cytometric cell cycle analyses after 24 hr of Dox-treatment. Values represent mean ± SEM. n = 3. **p < 0.01. (f,g) Expression of p21 (encoded by CDKN1A) and MYC on protein (f) and RNA (g) level in LS174T cell derivatives. Cells were treated for 0 hr, 24 hr, 48 hr, 72 hr, and 96 hr. mLEF1-HA and mSNAIL1-HA were detected using an anti-HA antibody, detection of α-Tubulin served as a loading control (f). Mw, molecular mass. Values represent mean ± SEM. n = 3.
β-Catenin-LEF1 complexes induce growth arrest but no EMT
To further explore how LEF1 contributes to EMT, we investigated consequences of elevated LEF1 expression at a cellular level. Unlike mSNAIL1, mLEF1 did not impart a mesenchymal morphology on LS174T cells grown on plastic (Fig. 4a). Also, in three-dimensional invasion assays, Ls-L cells formed
aggregates with smooth borders like control cells, and did not become invasive (Fig. 4b). However, two-dimensional and three-dimensional Ls-L colonies appeared significantly smaller than those formed by Ls-S cells. Quantitative analysis confirmed that Ls-L population dynamics were even more compromised than those of Ls-S cells which show reduced proliferation known to be associated with EMT (Fig. 4c,d).3,19 Cell cycle analyses by flow cytometry revealed opposite changes in G1 and S-phase cell numbers in Ls-L cells already after 24 hr of Dox-treatment, when the Ls-S cell cycle was not yet altered by mSNAIL1 expression (Fig. 4e). In agreement with the apparent G1/S arrest, we found up- and down-regulation of the cell cycle inhibitor CDKN1A and the proto-oncogene MYC, respectively, in Ls-L cells which closely resemble Ls-S cells in this regard (Fig. 4f,g).

In LS174T cells, MYC expression is positively regulated by β-Catenin-TCF7L2 complexes which interact with multiple regulatory elements at the MYC locus.38,42 As a potential explanation for the observed downregulation of MYC upon induction of mLEF1 expression we considered the possibility that LEF1 competes with TCF7L2 for binding to regions at the MYC locus similar to what we previously reported for the EPHB2 gene.21 Indeed, by ChIP we were able to determine that mLEF1 occupies the same chromosomal regions as TCF7L2, including Wnt responsive elements close to the MYC promoter and at −335 kb (Supporting Information Fig. S9i,j). These findings identify the cell cycle regulator MYC as a direct LEF1 target gene whose downregulation may involve displacement of TCF7L2 by LEF1.

Importantly, mLEF1-mediated upregulation of the Wnt/β-Catenin target group, cell cycle arrest and expression changes of CDKN1A and MYC all turned out to be strictly β-Catenin-dependent (Supporting Information Figs. S4a, S10) arguing that a β-Catenin-LEF1 complex is operating in Ls-L cells. However, unlike mSNAIL1, this complex does not induce EMT and only phenocopies mSNAIL1-induced cell cycle arrest.

**mLEF1 reduces tumour growth in vivo**

To study a potential growth inhibitory effect of mLEF1 in vivo we conducted xenograft experiments with Ls-L, Ls-S and Ls-V cells, which harbour an empty control vector (Fig. 5a). Expression of mLEF1, mSNAIL1 and mSNAIL1-induced endogenous LEF1 was confirmed in tumour lysates (Fig. 5b). However, these factors turned out not to be uniformly distributed in tumour sections. We observed areas in which the transgenes seemingly were not expressed, possibly because of insufficient Dox supply or detection limits. There were also small clusters of LEF1-positive cells in tumours formed by Ls-V cells (Fig. 5c). Nonetheless, mLEF1/LEF1-positive cell numbers strongly increased in Ls-L and Ls-S derived tumours. Importantly, the number of cells expressing the proliferation marker Ki67 was clearly diminished in Ls-L- and Ls-S-derived tumours, and the remaining Ki67-positive cells were locally restricted to tumour areas devoid of mSNAIL1 and mLEF1/LEF1. In agreement with a lower content of proliferating cells, Ls-L and Ls-S xenograft growth was retarded when compared to Ls-V tumours (Fig. 5d,e). The more pronounced and longer lasting suppression of xenograft growth by mSNAIL1 may be due to differences in expression levels or numbers of mLEF1/LEF1-positive cells in Ls-L versus Ls-S tumours. Alternatively, it could reflect more stringent cell cycle control by mSNAIL1 through multiple redundant mechanisms as suggested by the persistent mSNAIL1-induced growth arrest in Ls-SLKO1 and Ls-SLEFO cells (see earlier). Nonetheless, the transplantation experiments demonstrate that similar to mSNAIL1, mLEF1 can attenuate proliferation of CRC cells in vivo.

**Discussion**

At its core, EMT relies on widespread gene expression changes which cannot be explained solely by the activity of core EMT TFs. For instance, SNAIL1 mainly acts as transcriptional repressor, yet our time-resolved transcriptome profiling showed that during EMT similar numbers of genes are up- and downregulated with nearly identical kinetics. Our study shows that a sizeable fraction of SNAIL1-induced gene expression changes can be traced to the activity of β-Catenin-LEF1 complexes. Apparently, these complexes complement the gene-regulatory potential of SNAIL1 to shape EMT-associated gene expression patterns. Thus, we pinpoint β-Catenin-LEF1 complexes as significant, albeit not exclusive mediators of SNAIL1-induced EMT, and assign them to the control of cell proliferation and single cell invasion.

SNAIL1-induced upregulation of LEF1 leads to its co-expression with TCF7L2, the main effector of the Wnt/β-Catenin pathway in the intestine. Based on our findings we assume that LEF1 might largely replace TCF7L2 to steer β-Catenin-mediated gene expression. This replacement apparently involves competitive displacement of TCF7L2 from regulatory DNA elements at genes such as MYC and EPHB2.21 Due to differences in transactivation potential,7,8 the LEF1/TCF7L2 effector switch results in repression of some Wnt/β-Catenin targets while expression of others is intensified, as exemplified by APCDD1 and NKD1. Further investigations are needed, however, to determine whether its distinctive DNA-binding properties enable LEF1 to additionally tap into a transcriptional programme which supports SNAIL1-induced EMT but which is entirely independent of TCF7L2.7–9

Previous work suggested that SNAIL1 activates TGFβ signalling to induce LEF1.15 This is unlikely to occur in our model, because LS174T cells have two defective TGFBR2 alleles.45 Alternatively, SNAIL1 may downregulate a repressor of LEF1, since LEF1 expression increases upon inactivation of the SNAIL1 target gene FOXA1.24 As LEF1 is a β-Catenin-responsive gene,46 its upregulation could also result from a boost in β-Catenin transcriptional activity.11 This could also explain the induction of Lef1 in Apc-deleted organoids. Even though these organoids do not express Snail1, Apc-deficiency
massively increases nuclear β-Catenin and thereby potentiates its transcriptional activity. Accordingly, we favour the idea that LEF1 expression soars once the transcriptional activity of β-Catenin exceeds a certain threshold.

LEF1 loss-of-function does not entirely abrogate SNAIL1-induced EMT and LEF1 activity accounts for only a fraction of the SNAIL1-mediated transcriptome changes. Thus, additional transcription factors and signalling pathways must exist whose activation and repression contributes to the aggregated gene expression changes in the course of EMT. FOXA and SMAD proteins, AP4, and PBX3 are transcription factors with roles in EMT suppression and execution. Therefore, the conversion of epithelial into mesenchymal gene expression likely follows a gradual and hierarchical pattern whereby core EMT TFs trigger sequential changes in the activity of an expanding number of signalling pathways and TFs. An intriguing question is whether the same EMT executioner mechanisms operate regardless of cellular background.

Similar to mSNAIL1, mLEF1 overexpression produced G1/S cell cycle arrest and impaired tumour growth – remarkable effects considering that LS174T cells harbour oncogenic versions of β-Catenin and KRAS. Of note, mLEF1 depended on β-Catenin to suppress proliferation, arguing that mLEF1 does not simply function as a dominant negative factor. Rather, β-Catenin-mLEF1 complexes seem to trigger specific anti-proliferative transcriptional responses. This is surprising since Wnt/β-Catenin signalling stimulates cell division in the intestine and during colorectal carcinogenesis. In these instances, however, TCF7L2 is the cognate binding partner of β-Catenin. We presume that mLEF1 causes a shift in target gene expression that blunts mitogenic Wnt/β-Catenin pathway activity. MYC and EPHB2 represent two genes known to promote proliferation when bound by TCF7L2 which exhibit reduced expression when occupied by LEF1. Aside from repressing drivers of cell cycle progression, mLEF1 may additionally affect proliferation through upregulating an array of Wnt/β-Catenin pathway feedback inhibitors which function at virtually every level of the pathway. Their inhibitory function may still be operative in tumour cells and contribute to the anti-proliferative effects of mLEF1.

Although LEF1 had been implicated in EMT before, it remained controversial whether LEF1 could induce an EMT on its own. The phenotypic changes after mLEF1 overexpression and LEF1 knock-out demonstrate that the β-Catenin-LEF1 complex fulfils crucial functions downstream of mSNAIL1. However, expression of mLEF1 in LS174T cells did not confer a mesenchymal phenotype and LEF1 loss-of-function did not fully suppress mSNAIL1-induced EMT. These findings argue against LEF1 being a self-sufficient EMT inducer which contrasts with previous reports. Cell-type-specific differences in EMT susceptibility may partially explain this discrepancy. Besides, we conducted our study in EMT-naïve cells which are not pre-sensitised by expression of endogenous core EMT TFs. This may have enabled a more accurate assessment of the EMT-inducing capacity of LEF1. Furthermore, we demonstrate the strict dependency of LEF1 on β-Catenin as effector in EMT. This had not unambiguously been achieved before because β-Catenin depletion does not allow to separate EMT-specific functions of β-Catenin as cooperation partner of LEF1 from its roles in cell adhesion and CRC cell viability. Taken together, our data suggest that β-Catenin-LEF1 complexes are important downstream effectors of mSNAIL1 whose activities account for a subset of EMT aspects in CRC cells. While LEF1 is not sufficient to autonomously induce a mesenchymal phenotype, LEF1 is required to facilitate mSNAIL1-induced transcriptional reprogramming by redirecting Wnt/β-Catenin pathway activity, thereby bringing about strongly increased invasive properties and allowing for full-blown EMT.

Acknowledgements

The authors are grateful to members of the Hecht lab for critical reading of the manuscript, to S. Jägle and O. Schnappau for the gift of reagents, to K. Geiger and D. Herchenbach for excellent cell sorting services, and to M. Bewerunge-Hudler and her team from the Genomics and Proteomics Core Facility at the German Cancer Research Center (DKFZ), Heidelberg, Germany for their microarray service.

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