Biochemical and Biophysical Research Communications 442 (2013) 133–138

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The transcription factor LEF-1 induces an epithelial–mesenchymal transition in MDCK cells independent of β-catenin

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ARTICLE INFO

Article history:
Received 5 November 2013
Available online 19 November 2013

Keywords:
EMT
LEF-1
β-Catenin
Slug
ZEB1

ABSTRACT

The epithelial–mesenchymal transition (EMT), a key process in the tumor metastatic cascade, is characterized by the loss of cell–cell junctions and cell polarity, as well as the acquisition of migratory and invasive properties. LEF-1 is a member of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family of DNA-binding transcription factors, which interact with nuclear β-catenin and act as central transcriptional mediators of Wnt signaling. To investigate the role of LEF-1 in EMT, we generated stable LEF-1 transfectants using MDCK cells. The transfectants had a spindle-shaped mesenchymal morphology, and enhanced migration and invasiveness relative to control cells. These EMT changes were accompanied by the downregulation of an epithelial marker protein, E-cadherin, and the upregulation of mesenchymal marker proteins, vimentin and N-cadherin. Consistent with these observations, the mRNA levels of Slug, ZEB1, and ZEB2—EMT-related transcription factors—increased significantly. Although the N-terminally deleted mutant LEF-1 cannot interact with β-catenin, it retained the ability to induce EMT. Consistent with these observations, neither the expression of a dominant negative β-catenin/engrailed chimera, nor the expression of a cytoplasmic domain of E-cadherin that sequesters β-catenin from binding to LEF/TCF, reversed LEF-1-induced EMT. Together, these data indicated that the nuclear function of β-catenin was not necessary for the induction of Slug, ZEB1, and ZEB2 expression leading to EMT.

1. Introduction

The loss of epithelial characteristics and the gain of a mesenchymal phenotype—a process referred to as an epithelial-to-mesenchymal transition (EMT)—is considered to be a hallmark of neoplasmic transformation. A key initial step in EMT is the downregulation of E-cadherin, which at the transcriptional level is repressed by several factors: namely, ZEB1, ZEB2, Snail, Slug, and Twist [1]. The loss of E-cadherin is accompanied by the upregulation of mesenchymal markers, such as N-cadherin, fibronectin, and vimentin. Concomitant with these molecular changes, cells acquire a spindle-shaped mesenchymal morphology, and display enhanced migration and invasive properties.

LEF-1 is a member of the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family of DNA-binding transcription factors, which interacts with nuclear β-catenin and acts as a central transcriptional mediator of Wnt signaling, regulating cell cycle- and growth-related genes, e.g., *Cyclin D1* and *c-myc* [2]. The N-terminal 53–76 amino acids of all LEF/TCF members show significant similarity to one another and are necessary and sufficient for β-catenin binding [3–5]. Deletion of the equivalent regions of LEF/TCF has been shown to act as an inactive or a dominant negative mutation [5].

Immunohistochemical analysis of small B-cell lymphomas [6] and lung adenocarcinomas [7] revealed that the universal nuclear expression of LEF-1 absent the nuclear localization of β-catenin correlates with a poor prognosis, indicating that LEF-1 acts independently of β-catenin in these settings. Interestingly, N-terminal LEF-1 mutations that impair β-catenin binding are found in human sebaceous skin tumors [8], and the expression of an N-terminal-deleted LEF-1 mutant that lacks the β-catenin binding domain leads to sebaceous skin tumors in the mouse [9].

In the present study, we show that cells expressing either the full-length LEF-1 or a mutant, ΔNLEF-1, lacking the β-catenin-binding site, increase the expression of Slug, ZEB1, and ZEB2 and induce EMT. To directly test whether the EMT induced by LEF-1 is mediated by β-catenin/LEF-1 signaling, we used two constructs that have been used to inhibit β-catenin-dependent LEF/TCF transcriptional activity. One encoded β-catenin as a chimera fused to the engrailed repressor domain (β-EngMT); its gene product inhibits β-catenin-dependent LEF/TCF mediated transcription [10]. The other construct encoded the cytoplasmic domain of cadherin that sequesters β-catenin from binding to LEF/TCF; thus, expression of its gene product also inhibits β-catenin-dependent LEF/TCF

Abbreviations: EMT, epithelial–mesenchymal transition; LEF-1, lymphoid enhancer-binding factor-1; TCF, T-cell factor.

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http://dx.doi.org/10.1016/j.bbrc.2013.11.031

transcriptional activity [11]. Neither of the constructs had any effect on EMT induced by LEF-1 expression. Therefore, β-catenin-dependent LEF-1 transcriptional activity did not appear to have a role in the LEF-1-induced EMT of MDCK cells.

2. Materials and methods

2.1. Plasmids

Mouse LEF-1 cDNA was provided by Rolf Kemler (Max-Planck-Institute for Immunobiology, Germany). PCR was used to construct HA-tagged LEF-1 derivatives. The following primers were used: LEF-1 (full-length) (CCGGATCCACCATGCCCCAACTTTCC and ATCGATGTAGGCAGCTGTCAT), ΔNLEF-1 (N-terminal deletion of residues 1–56) (CCGGATCCACCATGAACGAGTCCGAAATCATC and ATCGATGTAGGCAGCTGTCAT), ΔNLSLEF-1 (C-terminal deletion of residues 372–397) (CCGGATCCACCATGCCCCAACTTTCC and ATCGCCATAATTGTCTCGCGC). The PCR products were digested with BamH I, cloned into the BamH I and EcoRV sites of Bluescript II KS (+) vector, and then cloned into the Not I and EcoRV sites of the pCAGGS-neoHA vector [12]. The expression vector for human β-catenin has been described [13]. A stabilized form of β-catenin lacking N-terminal residues 15–91 (ΔNβ-catenin) was produced by digesting the full-length cDNA with Nco I and Sac I followed by reaction with T4 DNA polymerase and then ligation. The construct was cloned into the Not I and EcoR V sites of the pCAGGSneoFlag vector. The pTOPflash and pFOPflash reporter constructs [14] were provided by Marc van de Wetering (University Hospital, Utrecht). pC-DsRedECTN and pC-DsRedECTC, the N-terminally DsRed-tagged and C-terminally Flag-tagged E-cadherin cytoplasmic domain (ECT) constructs, were made by PCR using the following combinations of primers: DsRedECTN (CCTCGAGG-GAGAACGGTGGTCAAA GA and ATGAAATTGGAAACTTCATCGAT) and DsRedECTC (CCTCGAG GAAACTTCATCGATGAAAACCT and CGGTGGCGAGGACGACGAT). The products were digested with Xho I and cloned into the Xho I and EcoR V sites of the pC-DsRedFlag vector. The β-catenin–engrailed chimera (β-EngMT) [10] was provided by Pierre D. McCrea (University of Texas MD Anderson Cancer Center).

2.2. Transfection

MDCK cells were transfected with expression vectors using the calcium phosphate method [12]. After selection with G418, single colonies were isolated and analyzed by immunofluorescence staining and immunoblot analysis using anti-HA antibodies. To obtain cells transfected with additional constructs, cells were co-transfected with a vector containing the hygromycin resistant gene (pHMR272) and selected with hygromycin B. At least three independent clones were selected for each construct to ensure that any observed effects were not due to the phenotypic variability inherent to transfection. The experiments with these clones gave essentially the same results.

2.3. Antibodies

Mouse mAbs against E-cadherin, β-catenin, N-cadherin, fibronectin, and p120-catenin were purchased from Transduction Laboratories (Lexington, KY). A mouse mAb against vinculin was purchased from Sigma (St. Louis, MO). A mouse mAb against vimentin and a rat mAb against HA were purchased from Zymed Laboratories (South San Francisco, CA) and Roche Diagnostics GmbH (Mannheim, Germany), respectively. A mouse mAb against Flag (DYKDDDDK) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A rabbit mAb against Slug was purchased from Cell Signaling Technology Japan (Tokyo, Japan). A mouse mAb against myc (9E10) and goat pAb against ZEB1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.4. Immunofluorescence

Cells were grown on coverslips for 24 h, fixed with 3% paraformaldehyde in PBS for 20 min, and permeabilized with 0.1% Triton X-100 in PBS. Then, the fixed cells were incubated with primary and secondary antibodies as described previously [12], and analyzed using an Olympus fluorescence microscope (Tokyo, Japan) equipped with a CD72 camera (Olympus).

2.5. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblot analyses were carried out as previously described [12]. Briefly, cells were lysed in a buffer containing 0.5% sodium deoxycholate, and were immunoprecipitated [12]. Then, affinity-captured proteins were separated by polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk, and then incubated with a primary antibody for either 2 h at room temperature or overnight at 4 °C, followed by incubation with a peroxidase-conjugated secondary antibody. Bound antibody was visualized by enhanced chemiluminescence (ECL; Amersham International, Little Chalfont, UK).

2.6. RT-PCR analysis

Total RNA was extracted from cells using the Isogen kit (Wako, Osaka, Japan), and then reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan). The resulting cDNA was used as a template for PCR reactions using GoTaq DNA polymerase (Promega, Madison, WI). PCR conditions were optimized for each primer pair as previously described [15]. The primer pairs used were ZEB1 (ACAAGTAGAACCTCTTGATCTTTC and ACTGGGCAGTGACTGTAGG-TAT); ZEB2 (GCTACGACCATACCCAGGAC and CTTGTCACATAAGT-CACATGCAT); Slug (GCGTTTTCCAGACCCTGGTT and GTGCTACA-CAGCAGCCAGATT); and GAPDH (ACCACCGTCCATGCCATCAC and TCCACCACCCGGTTGCTGTA).

2.7. Promoter assay

The assays were performed as previously described [12]. Briefly, HEK293T cells (1×10^5/well) were seeded in a 24-well plate for 24 h prior to transfection. The reporter constructs were transfected into cells along with the pCAGGSneo vector, containing either LEF-1 cDNA, ΔNLEF-1 cDNA, or no insert (total 500 ng), and with 40 ng of the pRL-CMV vector using Lipofectamine 2000 (Invitrogen). After 48 h, both firefly (FL) and *Renilla* luciferase (RL) activities were measured using a Dual Luciferase Reporter Assay kit (Promega). FL activity was normalized to RL activity. The experiments were performed in triplicate [12]. The following quantities of each construct were used for transfection: LEF-1, ΔNLEF-1, TOPflash, and FOPflash (200 ng); ΔNβ-catenin (100 ng); and neomycin (100 ng or 300 ng).

2.8. Wound healing assay

Cells were plated on 35 mm dishes and grown to confluency. Then, the cell monolayer was manually scratched with a pipette tip, washed with PBS, and incubated for 4 h. A phase contrast microscope was used to photograph the cells at 0 h and 4 h after performing the scratch.

2.9. Invasion assay

Invasion was measured using BioCoat MatriGel Invasion Chambers (BD Biosciences) according to the manufacturer's instructions. The lower chambers were filled with DME medium containing 5% FBS as a chemoattractant. The upper insert chambers were seeded with 2.5×10^4 cells in serum-free DME medium. After incubation for 22 h at 37 °C, the cells were fixed, stained, and counted.

3. Results

3.1. Expression of LEF-1 in MDCK cells induces EMT

MDCK (Madin–Darby canine kidney) cells are epithelial cells that are sensitive to the induction of EMT by either TGFβ [16] or by transcription factors such as Snail [12] or ZEB2 [17]. We introduced an expression vector encoding LEF-1, or an empty control vector (neo), into MDCK cells to generate stable transfectants after selection in G418. The ectopic expression of LEF-1 (Fig. 1A) induced morphological changes; notably, the cells became fibroblastic (Fig. 1B). LEF-1 was detected in the nucleus as revealed by immunodetection of the HA-tag appended to the LEF-1 C-terminus (Fig. 1B). Immunoblot analysis revealed the downregulation of E-cadherin, the upregulation of N-cadherin and vimentin, and the alteration of p120 splicing (Fig. 1C). These changes are characteristic of cells undergoing EMT [1]. A mutant LEF-1 lacking the C-terminal nuclear localization signal (ΔNLSLEF-1) failed to accumulate in the nucleus (Fig. 1B) and did not induce the changes characteristic of EMT (Fig. 1B and C). However, another mutant LEF-1 lacking the N-terminal β-catenin-binding site (ΔNLEF-1) did induce the morphological (Fig. 1B) and biochemical (Fig. 1C) changes associated with EMT.

Cells undergoing EMT lose cell–cell adhesion and increase their motility. Wound-healing assays demonstrated significant differences in the directional migration and motility of MDCK cells transfected with the control vector and MDCK cells transfected with either the LEF-1 or the ΔNLEF-1 vectors (Fig. 1D). MDCK cells expressing LEF-1 or ΔNLEF-1 closed the scratched area faster than did cells expressing the control neo gene (Fig. 1D). Furthermore, the MatriGel invasion assay revealed a greatly increased invasion ability of MDCK cells expressing either LEF-1 or ΔNLEF-1 (Fig. 1E). Together, these parallel results supported the hypothesis that LEF-1 drives EMT in MDCK cells.

When LEF-1 was transiently expressed in HEK293T cells, binding to co-transfected β-catenin was detected by immunoprecipitation (Fig. 2A). By contrast, ΔNLEF-1 was unable to co-immunoprecipitate β-catenin (Fig. 2A). A β-catenin-dependent LEF/TCF reporter gene assay (TOPflash luciferase reporter) [14] revealed that the reporter was strongly transcribed in cells co-transfected with β-catenin- and LEF-1-expression plasmids, but not in cells transfected with the plasmids individually, or in cells co-transfected with β-catenin- and ΔNLEF-1-expression plasmids (Fig. 2B). These results indicated that the interaction of the NH_2-terminus of LEF-1 with β-catenin leads to transcriptional activation.

To determine the β-catenin-dependent LEF/TCF activity in MDCK cells expressing either LEF-1 or ΔNLEF-1, cells were transfected with the TOPflash luciferase reporter plasmid alone or together with β-catenin. The results showed that the activity of MDCK cells expressing LEF-1, but not ΔNLEF-1, was significantly enhanced by co-transfection with β-catenin (Fig. 2C).

3.2. Expression of either LEF-1 or ΔNLEF-1 in MDCK cells upregulates the production of EMT-related transcription factors

Previous studies reported that transcription factors, e.g., Slug and ZEB1, are targets of β-catenin-dependent LEF/TCF signaling [11,18]. Using an Agilent Whole Canine Genome microarray, we compared the gene expression profiles in MDCK cells expressing LEF-1, ΔNLEF-1, or ΔNLSLEF-1. RT-PCR was used to confirm the microarray data. The mRNA expression levels of Slug, ZEB1, and ZEB2 were significantly increased in MDCK cells expressing LEF-1 and ΔNLEF-1 (Table 1 and Fig. 3A). Furthermore, immunoblot analysis (Fig. 3B) revealed that cells expressing LEF-1 or ΔNLEF-1 also increased Slug and ZEB1 production at the protein level; and immunofluorescence staining revealed that these transcription factors were localized to the nucleus (Fig. 3C). Thus, it is very likely that the expression of LEF-1 or ΔNLEF-1 in MDCK cells induced the upregulation of Slug, ZEB1, and ZEB2, and that these transcription factors cooperated to downregulate E-cadherin expression and induced EMT.

3.3. Inhibition of β-catenin-dependent activity does not reverse EMT

ΔNLEF-1 lacks the β-catenin-dependent activity of LEF-1, and acts as a dominant-negative mutant on endogenous LEF/TCF transcription factors [5]. Furthermore, β-catenin levels in MDCK cells expressing LEF-1 and ΔNLEF-1 decreased significantly (less than 1/10 of that observed in control MDCK cells or in cells expressing ΔNLSLEF-1). Therefore, we surmised that the EMT induced by LEF-1 and ΔNLEF-1 may not be mediated by β-catenin. To exclude the possible involvement of β-catenin in LEF-1-induced EMT, we tested the effects of independently using two different inhibitors of β-catenin—a β-catenin–engrailed repressor chimera and the E-cadherin cytoplasmic domain—on MDCK cells that had undergone EMT. Since EMT is reversible [12,16], these inhibitors should have induced the conversion of mesenchymal to epithelial cells if β-catenin was necessary for that transition.

The β-catenin–engrailed chimera (β-EngMT) [10] was created by replacing the activation domain of β-catenin with the repression domain of engrailed, which is known to interact with transcriptional repressors to actively block transcription; the Myc-tag was installed at the N-terminus. This chimera was shown to specifically repress the transcriptional activity of β-catenin [10]. Expressing β-EngMT in MDCK cells that had undergone EMT mediated by LEF-1 or ΔNLEF-1 did not change the cell morphology (Fig. 4A) or the expression of EMT markers, e.g., E-cadherin, vimentin, and fibronectin (Fig. 4B).

The other β-catenin inhibitor that we used was the cytoplasmic domain of E-cadherin, which sequesters β-catenin and prevents it from binding to LEF/TCF, thus inhibiting β-catenin-dependent LEF/TCF transcriptional activity [11]. The N-terminal half of the cytoplasmic domain carries the p120-binding site and the C-terminal half of the domain encodes the β-catenin-binding site. These domains were independently fused to the fluorescent protein DsRed, generating two chimeras: the N-terminal (DECTN) and C-terminal (DNCTC) fusion proteins (Fig. 4C). Although expression of the N-terminal chimera in LEF1-MDCK did not change the amount of β-catenin in the cells, expression of the C-terminal half of the domain (encoding the β-catenin-binding site) increased the β-catenin levels by more than 10-fold (Fig. 4D). However, these constructs had no effect on LEF-1-induced EMT (Fig. 4E). Together, these data strongly suggested that β-catenin-dependent LEF-1 transcriptional activity is not required for the LEF-1–induced EMT of MDCK cells.

4. Discussion

In the present study, we showed that the ectopic expression of LEF-1 in MDCK cells induced changes that are characteristic of EMT. The observed changes included the acquisition of a fibroblast-like morphology, the decreased expression of E-cadherin, and the increased expression of N-cadherin and vimentin. Since

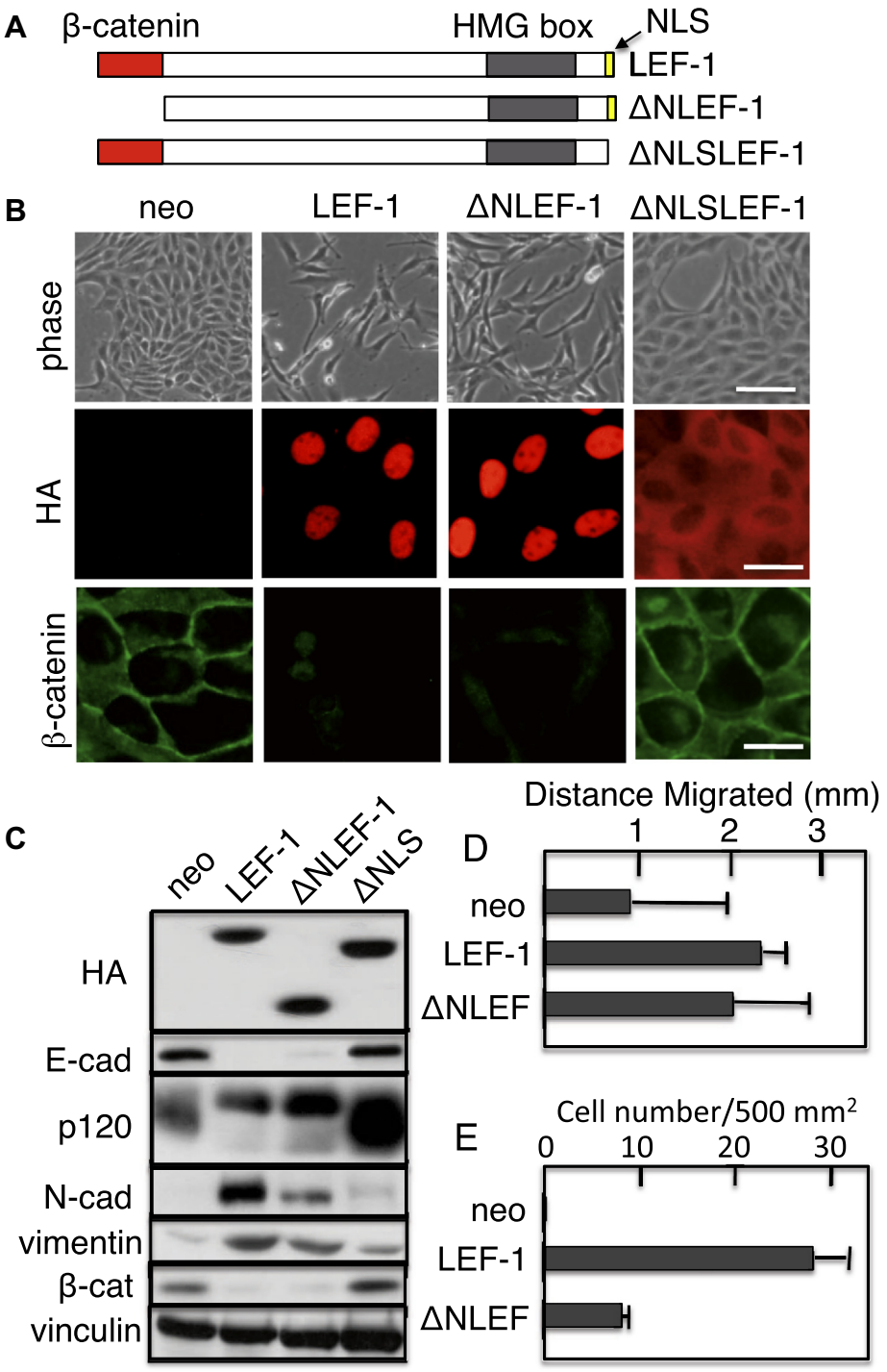

Fig. 1. Expression of LEF-1 or ΔNLEF-1 induces EMT in MDCK cells. (A) Schematic representation of the constructs used. LEF-1 has a β-catenin binding domain (red) at its N-terminus and a nuclear localization signal (NLS: yellow) at its C-terminus. The gray box indicates an HMG box, a DNA-binding domain. Since ΔNLSLEF-1 cannot localize to the nucleus and is thus nonfunctional, it can be used as a negative control. (B) Phase contrast microscopy shows that the expression of LEF-1 or ΔNLEF-1, but not ΔNLSLEF-1, induced morphological (epithelial to fibroblastic) changes. Bar, 100 μm. Staining with anti-HA antibody revealed the nuclear localization of LEF-1 and ΔNLEF-1, but not ΔNLSLEF-1. The membrane localization of β-catenin that was observed in control MDCK cells (neo) or cells expressing ΔNLSLEF-1 was abrogated in cells expressing LEF-1 or ΔNLEF-1. Bar, 50 μm. (C) Immunoblot analysis revealed that the expression of LEF-1 or ΔNLEF-1 resulted in the downregulation of E-cadherin (E-cad) and β-catenin (β-cat), a change in the isoform pattern of p120-catenin reflective of an epithelial to mesenchymal shift, and the up-regulation of N-cadherin (N-cad) and vimentin. Vinculin was used as a loading control. (D) A wound healing assay showed that cells expressing LEF-1 or ΔNLEF-1 increased their migrational activity approximately 2-fold as compared to cells expressing neomycin. (E) Cells expressing either LEF-1 or ΔNLEF-1 showed increased invasion as compared to cells expressing neomycin. In addition, cells expressing either LEF-1 or ΔNLEF-1 showed significant and reproducible differences in invasion activity as compared to each other. We do not know the reason for this at present; however, it was not due to clonal variation because three independent clones of each LEF-1 construct yielded the same results.

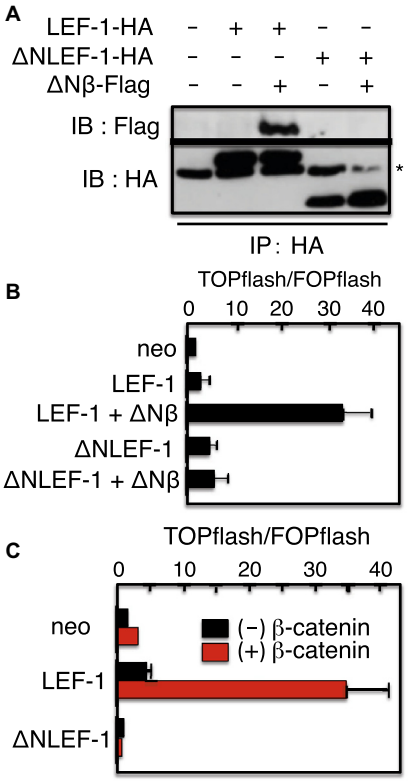

Fig. 2. LEF-1, but not ΔNLEF-1, binds β-catenin and activates the TOPflash reporter construct in the presence of β-catenin. (A) HEK293T cells were transfected with the indicated combinations of HA-tagged LEF-1, ΔNLEF-1, and the Flag-tagged stabilized form of β-catenin (ΔNβ-Flag). After cell lysis, proteins were immunoprecipitated with an anti-HA antibody and subjected to immunoblot analysis with either anti-HA or anti-Flag antibodies. An asterisk indicates the position of the immunoglobin heavy chain. (B) TOPflash/FOPflash reporter activity was measured in HEK293T cells transfected with the indicated combinations of constructs. (C) TOPflash/FOPflash reporter activity was measured in MDCK cells expressing LEF-1 or ΔNLEF-1. TOPflash or FOPflash constructs were transfected with or without ΔNβ-catenin.

Table 1
Relative expression levels of the EMT-related transcription factors in MDCK cells expressing LEF-1, ΔNLEF-1, or ΔNLSLEF-1 as determined by microarray analysis.

	LEF-1	ΔNLEF-1	ΔNLSLEF-1
Slug	38.32	5.89	1
ZEB1	1245.37	1312.73	1
ZEB2	41.54	17.91	1

A comparison of the gene expression profiles from the indicated cells was performed using Agilent Whole Canine Genome microarrays.

these changes are hallmarks of EMT [1], MDCK cells expressing LEF-1 could be classified as cells that had undergone EMT. As a result of EMT, cells acquire the ability to migrate into and invade surrounding tissues. Since the overexpression of LEF-1 in tumors is correlated with poor survival and the increased risk of metastasis [19], our observation that LEF-1 induces EMT sheds light on our understanding of tumor metastasis.

The possible involvement of a β-catenin-independent role for LEF-1 in tumor progression has been reported in small B-cell lymphomas [6], lung adenocarcinomas [7], and sebaceous skin tumors [8]. The formation of the latter tumors was specific to the expression of N-terminally deleted LEF-1 [9]. Our observation that both full-length and N-terminally deleted LEF-1 induced EMT in a β-catenin-independent manner may be relevant to the metastasis of lung adenocarcinomas [7], where LEF-1 seems to act independently of β-catenin, because EMT is a prerequisite for tumor metastasis.

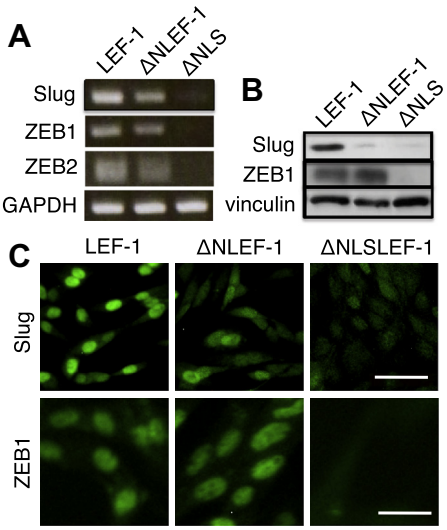

Fig. 3. Upregulation of EMT-related transcription factors in cells expressing LEF-1 or ΔNLEF-1. (A) RT-PCR analysis of Slug, ZEB1, and ZEB2 mRNA in MDCK cells expressing LEF-1, ΔNLEF-1, or ΔNLSLEF-1 (ΔNLS). GAPDH served as an internal loading control. (B) Immunoblot analysis using anti-Slug or anti-ZEB1 antibodies. Vinculin served as a loading control. (C) Immunofluorescent staining using anti-Slug or anti-ZEB1 antibodies. Bar, 50 μm.

Previous data showing the importance of nuclear β-catenin in the LEF/TCF-mediated transcription of Slug [11] or ZEB1 [18], and in the induction of EMT were obtained using SW480 cells, a colorectal cancer cell line with a mutated adenomatous polyposis coli (APC) gene. Since APC is involved in the GSK3β-mediated phosphorylation and subsequent ubiquitin-dependent degradation of β-catenin, SW480 cells display increased levels of β-catenin. Another study demonstrated that the expression of LEF-1 promotes EMT in DLD-1, a colon cancer cell line bearing an APC mutation; in contrast to our results, however, they did not observe LEF-1-mediated EMT in normal epithelial cell lines, e.g., human corneal epithelial cells or MDCK cells, containing functional APC and normal β-catenin levels [20]. One possible explanation for the disparity in our studies is a difference in the MDCK cells that were used. The other authors used type II MDCK cells, whereas we used type I MDCK cells in the present study. Thus far, our attempts to induce EMT in type II MDCK cells have been unsuccessful (M. Ozawa unpublished observation). Therefore, LEF-1 appears to induce EMT in a cell context-dependent manner.

The mechanism of LEF-1-induced EMT is still undetermined. Cells that have been treated with targeted shRNA to reduce E-cadherin expression have been reported to upregulate the expression of the mesenchymal proteins, N-cadherin and vimentin, and the EMT-inducer proteins, Twist and ZEB1 [21]. LEF-1 has been reported to bind the E-cadherin promoter and repress its transcription [4]. However, in preliminary experiments with the human E-cadherin promoter [12], we observed no repression of the promoter activity by LEF-1 (W. Kobayashi, unpublished observation). Another group reported that LEF1, when functionally activated by phosphorylated Smad2 and Smad4 (rather than β-catenin), binds with the E-cadherin promoter and represses its transcription in response to TGFβ3 signaling [22]. Despite this observation, we have not yet detected the formation of a complex between LEF-1 and Smad2 or Smad4 (W. Kobayashi, unpublished observation). Therefore, we believe that another molecule(s) is involved in LEF-1-induced EMT.

In conclusion, we showed that LEF-1 induces EMT in a β-catenin-independent manner. The treated cells acquire highly motile

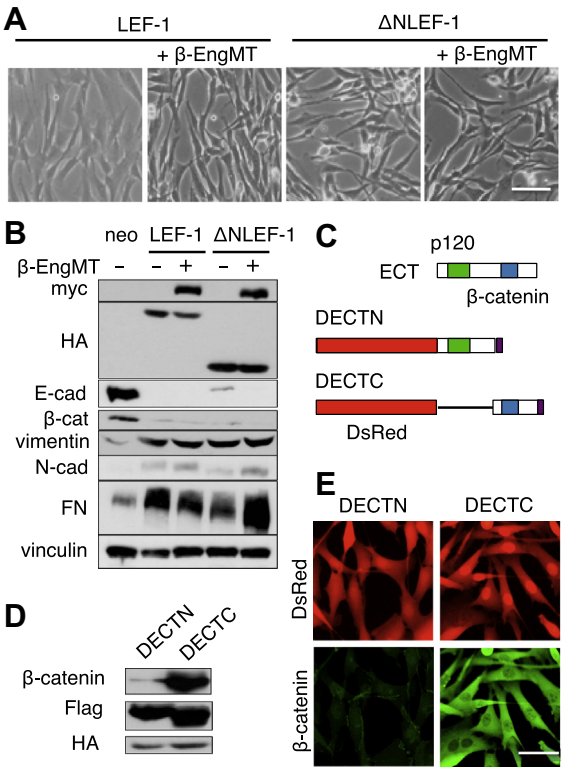

Fig. 4. Inhibition of β-catenin activity with either a β-catenin–engrailed repressor chimera, or the E-cadherin cytoplasmic domain, does not reverse EMT. (A) Morphology of MDCK cells expressing either LEF-1 or ΔNLEF-1 co-transfected with a Myc-tagged β-catenin–engrailed repressor chimera (β-EngMT) and a hygromycin-resistance gene vector. Bar, 100 μm. (B) Immunoblot analysis revealed no change in the expression of the epithelial markers, E-cadherin (E-cad) and β-catenin (β-cat), and the mesenchymal markers, vimentin, N-cadherin (N-cad), and fibronectin (FN). Vinculin served as a loading control. Although β-EngMT was shown to specifically repress the transcriptional function of β-catenin [10], the phenotype of LEF-1- and ΔNLEF-1-expressing cells did not change upon co-expression of β-EngMT. (C) Schematic representation of the E-cadherin cytoplasmic domain and its derivatives. ECT: E-cadherin cytoplasmic domain. The green and blue boxes indicate the p120-catenin and β-catenin binding sites, respectively. DECTN: a chimeric construct composed of DsRed and the N-terminal region of ECT containing the p120-catenin–binding site. DECTC: a chimera of DsRed and the C-terminal half of ECT containing the β-catenin-binding site. Purple boxes indicate the Flag-tag. (D) Immunoblot analysis of LEF-1-expressing cells that were co-transfected with either the DECTN or the DECTC construct. Although the expression of DECTN or DECTC did not affect LEF-1 protein levels, the expression of DECTC, but not DECTN, significantly increased the amount of β-catenin. Thus, the binding of β-catenin to DECTC increased its stability. (E) The increased levels of β-catenin were exclusively detected in the cytoplasm. Bar, 50 μm. Although constructs similar to DECTC were shown to specifically repress the transcriptional activity of β-catenin [11], the phenotype of LEF-1-expressing cells did not change upon co-expression of DECTC.

and invasive properties and these changes may be related to tumor cell metastasis.

Acknowledgments

This work was supported by the following Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M. O, and a Grant from Kodama Memorial Found for Medical Research. We thank Kumiko Sato for secretarial assistance, and the Joint Research Laboratory at the Kagoshima University Graduate School of Medical and Dental Sciences for the use of their facilities.

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