**Molecular and Cellular Pathobiology**

**Acquired Expression of NFATc1 Downregulates E-Cadherin and Promotes Cancer Cell Invasion**

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**Abstract**

NFATc1 is a transcription factor that regulates T-cell development, osteoclastogenesis, and macrophage function. Given that T cells, osteoclasts, and macrophages in the tumor microenvironment are thought to modulate tumor progression, tumor cells may acquire NFATc1 expression through fusion with these NFATc1-expressing normal cells. We here revealed that a small proportion of tumor cells in human carcinoma specimens expressed NFATc1. To investigate the consequences of NFATc1 acquisition by tumor cells, we established A549 and MCF7 cell lines expressing a constitutively active form of NFATc1 (NFATc1CA) in an inducible manner. The expression of NFATc1CA promoted cancer cell invasion in association with changes in cell morphology. Analysis of gene expression and RNA interference experiments revealed that NFATc1CA suppressed E-cadherin expression by upregulating the transcriptional repressors Snail and Zeb1 in a manner independent of TGF-β signaling. Induced expression of NFATc1CA also downregulated E-cadherin expression and increased invasive activity in tumor xenografts in vivo. Our results thus suggest that the acquisition of NFATc1 expression contributes to tumor progression. *Cancer Res; 73(16); 5100–9. ©2013 AACR.*

**Introduction**

Tumor progression involves intricate interactions of tumor cells with a variety of nonmalignant cells in the tumor microenvironment. These latter cells include bone marrow-derived cells, fibroblasts, and endothelial cells, and they facilitate tumor cell survival, growth, invasion, dissemination, and colonization of distant organs by secreting growth factors, chemokines, and proteases (1, 2). Normal cells are recruited to the tumor microenvironment as a consequence of a chronic inflammatory state induced by the tumor (3). Recent evidence suggests that inflammation promotes the fusion of cardiomyocytes, skeletal muscle cells, hepatocytes, or Purkinje neurons with bone marrow-derived cells or lymphocytes in vitro (4, 5). This fusion offers another potential mechanism (transfer of genetic material) by which normal cells might contribute to tumor progression. Indeed, macrophage–melanoma cell, osteoclast–melanoma cell, and macrophage–B-lymphocyte, or T-lymphocyte colon cancer cell hybrids have been detected in vivo (6–9).

Transcription factors of the nuclear factor of activated T cells (NFAT) family, which were originally identified as activators of T lymphocytes (10), play key roles in inflammatory and immune responses (11). Activation of NFATc1 has been shown to be induced in macrophages by prolonged exposure to TNF-α, a cytokine released during chronic inflammation (12). NFATc1 was also shown to be the master regulator in osteoclast differentiation (13). A potential target of NFATc1, Tks5, mediates cell–cell fusion (14). The fusion of tumor cells with normal cells in the inflammatory tumor microenvironment results in exposure of the tumor cell nucleus to various cytosolic factors of normal cells, including NFATc1. Tumor cells initially lacking NFATc1 expression might acquire NFATc1 by this mechanism. The consequences of this acquisition on tumor cell activities such as invasion and metastasis are unclear. Only recently, however, studies have begun to characterize the properties of tumor cell hybrids (7, 9, 15–17).

In this study, we analyzed protein expression in human carcinoma specimens and found that about half of the specimens included normal cells positive for NFATc1. Furthermore, a limited number of tumor cells in these specimens also expressed NFATc1, suggesting that tumor cells might gain this transcription factor by fusing with normal cells. To examine the effects of acquired NFATc1 expression on tumor cells, we established cancer cell lines expressing a constitutively active form of this protein (NFATc1CA) in an inducible manner. We here revealed that the introduction of NFATc1CA into cancer cells induces expression of the transcriptional repressors Snail and Zeb1, resulting in downregulation of E-cadherin expression and changes in cell morphology both in vitro and in vivo.
Materials and Methods

Human subjects
A human multiorgan carcinoma tissue array (BC000119) was purchased from US Biomax. Patients who underwent surgical resection for breast cancer at Keio University Hospital (Tokyo, Japan) were enrolled in this study. The specimens were subjected to immunostaining. This study was approved by the Keio University Hospital Ethical Review Board and the permission was obtained (ID number 20120316).

Antibodies and reagents
Primary antibodies for immunofluorescence, immunohistochemical, or immunoblot analysis included the following: mouse monoclonal anti-NFATc1 and rabbit polyclonal anti-Tks5 (Santa Cruz Biotechnology); mouse monoclonal anti-E-cadherin and anti-N-cadherin (BD Biosciences); rabbit monoclonal anti-E-cadherin, anti-p53, anti-Zeb1, and mouse monoclonal anti-Snail (Cell Signaling Technology); rabbit polyclonal anti-vimentin (Abcam); mouse monoclonal anti-CD3 and anti-CD68 (Dako); and rabbit monoclonal anti-Ki67 (Thermo Scientific). SB431542 (Sigma) or A-83-01 (Wako) was used to inhibit TGF-β signaling. TGF-β was purchased from Peprotec, and TNF-α was purchased from R&D Systems.

Plasmid construction and retroviral gene transduction
The cDNA for a constitutively active form of human NFATc1 (NFATc1CA; ref. 18) was kindly provided by N. Clipstone (Department of Pharmacology, Loyola University, Chicago, IL) and cloned together with the DNA sequence for an NFATc1CA cells using TRIzol reagent (Invitrogen) and was further purified using an RNaseasy Mini Kit (Qiagen). The purified RNA was quantified by measuring absorbance using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies), and its quality was monitored with an Agilent 2100 bioanalyzer (Agilent Technologies). RNA preparations with a 260/280 nm absorbance ratio ≥2 and an integrity number ≥6.5 were analyzed. Cy3-labeled cRNA probes were prepared using a QuickAmp labeling kit (Agilent). Labeled cRNA (1650 ng) was hybridized with an Agilent whole human genome microarray, 4 × 44K. Raw intensity data for each experiment were analyzed with GeneSpring GX software (Tomy Digital Biology).

RT-PCR analysis
Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and portions (0.5–1 μg) of the RNA were subjected to reverse transcription with SuperScriptII polymerase (Invitrogen). TaqMan reverse transcription PCR (RT-PCR) primers for CDH1, NFATc1, SNAI1, ZEB1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ACTB were obtained from Applied Biosystems for quantitative PCR analysis. Quantitative PCR analysis was conducted using a 7500 Fast real-time PCR system (Applied Biosystems), and the abundance of target mRNAs was normalized to that of mRNAs derived from the housekeeping gene GAPDH or ACTB. The primers (forward and reverse, respectively) for semi quantitative PCR analysis included those for SNAI1 (5′-TCTCTGAGGCCAAGGATCTC-3′ and 5′-ATTCCATGGCAGTGACAAGG-3′), ZEB1 (5′-AGACATGTCGCACTTAAATTGATACTTATGG-3′ and 5′-GCAGTCTGGG-3′), and ACTB (5′-TCTTCCAGGCTCTTCTCTTCT-3′ and 5′-AAAGCATGTCGCAATCTCTCATC-3′).

Matrigel invasion assay
A549-NFATc1CA cells (4.0 × 10⁴) or MCF7-NFATc1CA cells (3.0 × 10⁴) cultured with or without doxycycline (1 or 0.5 μg/mL), respectively for 72 hours and then deprived of serum for 8 to 12 hours were isolated by exposure to trypsin. They were then transferred to a BioCoat Matrigel invasion chamber (BD Biosciences) and cultured for 24 hours under the same conditions, in RPMI medium. The cell lines were obtained from the Research Resources of Keio University School of Medicine.

Microarray analysis
Microarray analysis was conducted by the Collaborative Research Resources of Keio University School of Medicine. Total RNA was isolated from A549-NFATc1CA or MCF7-NFATc1CA cells using TRIzol reagent (Invitrogen) and was further purified using an RNeasy Mini Kit (Qiagen). The purified RNA was quantified by measurement of absorbance using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies), and its quality was monitored with an Agilent 2100 bioanalyzer (Agilent Technologies). RNA preparations with a 260/280 nm absorbance ratio ≥2 and an integrity number ≥6.5 were analyzed. Cy3-labeled cRNA probes were prepared using a QuickAmp labeling kit (Agilent). Labeled cRNA (1650 ng) was hybridized with an Agilent whole human genome microarray, 4 × 44K. Raw intensity data for each experiment were analyzed with GeneSpring GX software (Tomy Digital Biology).

Immunofluorescence and immunohistochemical analyses
For immunofluorescence analysis, cells cultured on coverslips were fixed with 3.7% formaldehyde in PBS, permeabilized
with 0.1% Triton X-100 in PBS for 5 minutes, and incubated with primary antibodies for at least 60 minutes at room temperature. They were then washed with PBS and incubated with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) for 30 minutes. The cells were also stained with rhodamine–phalloidin (Invitrogen) to visualize F-actin and with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The cells were finally washed with PBS, mounted on glass slides, and examined with a fluorescence microscope (DMI 6000 with TCS SP5 and LAS AF software, Leica). For immunohistochemical analysis of the tumor xenografts, the tissue was fixed with 4% paraformaldehyde in PBS, embedded in paraffin, incubated with primary antibodies for overnight at 4°C, and detected using ImmPRESS reagents (Vector Laboratories) or Alexa Fluor 488- or 568-conjugated secondary antibodies (Molecular Probes). Paraffin-embedded human tumor specimens were incubated with primary antibodies for overnight at 4°C, including biotinylated NFATc1, and detected using ImmPRESS reagents, including Alexa Fluor 488- or 568-conjugated secondary antibodies or Alexa Fluor 591-conjugated avidin (Molecular Probes).

**Tumor xenografts**

Experiments were carried out with 6-week-old female nu/nu-BALB/c athymic nude mice (CLEA Japan). All experiments were approved by the Animal Care and Use Committee of Keio University and were carried out in accordance with the institute guidelines. MCF7-NFATc1CA cells (1.0 × 10^7) in 0.3 mL growth medium containing 50% volume for volume Matrigel (BD Biosciences) were subcutaneously injected into the flank of the mice. Although MCF7 cells require estrogen for efficient growth, the mice were not treated with estrogen or 17β-estradiol. Diet with doxycycline (625 mg/kg, LabDiet) was fed ad libitum.

**Statistical analysis**

Quantitative data are presented as mean ± SD. Comparisons between groups were conducted using Student t test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Identification of NFATc1-positive tumor cells in human carcinoma specimens**

Using a multiorgan carcinoma tissue array, we first examined whether human carcinoma specimens contain NFATc1-positive tumor cells. Immunohistochemical analysis revealed that a small proportion of breast (3 of 40), lung (2 of 40), prostate (1 of 40), and pancreatic (4 of 40) carcinoma specimens contained tumor cells positive for NFATc1 (Fig. 1). Intriguingly, those tumor cells with NFATc1 expression tended to express less E-cadherin, as observed by immunofluorescence analysis (Fig. 2A, arrowheads). On the other hand, 50% (20 of 40) breast, 67.5% (27 of 40) lung, 45% (18 of 40) colon, 30% (12 of 40) prostate, and 52.5% (21 of 40) pancreatic carcinoma specimens contained normal cells positive for NFATc1 expression in the tumor microenvironment (Fig. 1). Morphologic and immunohistochemical examinations suggested that NFATc1-expressing normal cells mostly consisted of CD68-positive macrophages (Fig. 2B). Some NFATc1-positive tumor cells were localized among groups of tumor cells negative for NFATc1 (Fig. 1, arrowheads and Fig. 2A). Fusion of tumor cells with hematopoietic or myeloid cells has been detected both in vitro and in vivo (19, 20). More recently, myofibroblasts have emerged as a new fusion partner for osteosarcoma or prostate cancer cells (16, 21). In addition, we detected CD68-positive tumor cells in the NFATc1-positive breast tumor specimens (Fig. 2C and Supplementary Fig. S1). Therefore, tumor cells may acquire NFATc1 expression through fusion...
with macrophages, although other possibilities, such as aberrant gene expressions in tumor cells, cannot be ruled out. We further investigated the correlation of NFATc1 expression in tumor cells with their malignancy using clinical breast tumor specimens and found that NFATc1 expression was observed predominantly in invasive tumors (5/19 specimens, 26%) compared with noninvasive tumors (1/15 specimens, 6.7%; Supplementary Fig. S1). In normal or benign lesion, NFATc1 expression was detected only in myoepithelial cells (Supplementary Fig. S1).

**Expression of a constitutively active form of NFATc1 (NFATc1CA) promotes cancer cell invasion**

To investigate the effects of acquired NFATc1 expression on cancer cells that did not originally express this protein, we established stable lines of A549 pulmonary carcinoma and MCF7 breast carcinoma cells that express a constitutively active form of NFATc1 tagged with the FLAG epitope (FLAG-NFATc1CA) under the control of a doxycycline-responsive promoter (A549-NFATc1CA and MCF7-NFATc1CA cells, respectively). Induced expression of NFATc1CA in either A549 or MCF7 cells promoted migration (Supplementary Fig. S2) and invasion into the Matrigel (Fig. 3A and B), suggestive of functional similarity between NFATc1 and NFATc2 (22, 23). Morphologic examination revealed that induction of NFATc1CA expression resulted in loosening of cell–cell contacts and marked rearrangement of the actin cytoskeleton (Fig. 3C and D), suggesting that NFATc1CA might promote cancer cell invasion by triggering the epithelial–mesenchymal transition (EMT). During EMT, upstream signals, such as growth factors, are thought to promote the expression of mesenchymal markers and the acquisition of invasive properties. Our findings suggest that NFATc1CA may contribute to the invasion of cancer cells by promoting the EMT process.

![Figure 3](https://example.com/figures/figure3.png)
factors produced by the tumor stroma, induce the expression of transcriptional repressors, including Snail, Slug, Twist, and Zeb1, which directly and indirectly inhibit transcription of the E-cadherin gene (encoded by \( CDH1 \); refs. 24–28). Indeed, E-cadherin expression was downregulated in both A549-NFATc1CA and MCF7-NFATc1CA cells after induction of NFATc1CA expression (Fig. 3E), although typical mesenchymal markers such as N-cadherin and vimentin were not induced.

Figure 3. Induction of a constitutively active form of NFATc1 (NFATc1CA) promotes cancer cell invasion. A and B, A549 (A) or MCF7 (B) cells that express NFATc1CA under the control of a doxycycline (Dox)-responsive promoter (A549-NFATc1CA and MCF7-NFATc1CA cells, respectively) were cultured in the absence (−) or presence (+) of Dox (1 or 0.5 \( \mu \)g/mL, respectively) for 72 hours and then deprived of serum for 8 to 12 hours. The cells were then transferred to a Matrigel chamber and assayed for invasive activity. The cells that invaded through the Matrigel were stained with crystal violet, and the area occupied by the stained cells in 3 distinct regions of a single chamber was quantified. Data are mean ± SD from 3 independent experiments. **, \( P < 0.01 \). C and D, A549-NFATc1CA (C) or MCF7-NFATc1CA (D) cells cultured in the absence or presence of doxycycline (1 or 0.5 \( \mu \)g/mL, respectively) for 72 hours were stained with rhodamine-phalloidin (red) to detect F-actin, with antibodies to NFATc1 (green) and with DAPI (blue) to visualize nuclei. Bars, 50 \( \mu \)m. E and F, A549-NFATc1CA or MCF7-NFATc1CA cells cultured in the absence or presence of doxycycline (1 or 0.5 \( \mu \)g/mL, respectively) for 72 hours were subjected to immunoblot analysis with the indicated antibodies. The E-cadherin and \( \gamma \)-tubulin bands were quantified by using ImageJ software (E-cad and \( \gamma \)-tub, respectively), and normalized E-cad/\( \gamma \)-tub ratio is indicated. Data are mean ± SD from 3 independent experiments. *, \( P < 0.05 \).
NFATc1CA suppresses CDH1 expression independent of TGF-β signaling

Consistent with the notion that NFATc1CA expression induces EMT, we found that CDH1 expression was significantly downregulated by doxycycline treatment in both A549-NFATc1CA and MCF7-NFATc1CA cells (Fig. 4A). To determine whether TGF-β, a potent inducer of EMT in epithelial cells (29), is required for the downregulation of CDH1 expression by NFATc1CA, we examined the effects of 2 different TGF-β receptor blockers, SB431542 and A-83-01. Neither of them affected the inhibition of CDH1 expression by NFATc1CA in MCF7-NFATc1CA cells (Fig. 4B). Parental MCF7 cells require TNF-α in addition to TGF-β for downregulation of CDH1 expression. However, the combination of these factors did not increase NFATc1 gene expression in these cells (Fig. 4C). These results thus suggested that NFATc1CA suppresses CDH1 expression independent of TGF-β signaling.

Zeb1 and Snail mediate NFATc1CA-dependent suppression of CDH1 expression

Expression of CDH1 is partly controlled by transcriptional repressors such as Snail, Slug, Twist, Zeb1, Zeb2, E12, and E47. To characterize the molecular signature of NFATc1CA-induced EMT, we compared the gene expression profiles of nontreated and doxycycline-treated A549-NFATc1CA or MCF7-NFATc1CA cells by microarray analysis (Fig. 5A and Supplementary Table S1). Doxycycline treatment increased the expression of 3 genes encoding EMT-related transcriptional repressors of CDH1 more than 2-fold in MCF7-NFATc1CA cells: ZEB1 (20.9-fold), SNAI1 (encoding Snail; 8.7-fold), and SNAI2 (encoding Slug; 6.8-fold). In contrast, only ZEB1 expression was upregulated more than 2-fold (2.9-fold) in A549-NFATc1CA cells. We confirmed the doxycycline-induced upregulation of ZEB1 and SNAI1 expression in MCF7-NFATc1CA cells at the mRNA level by semiquantitative (Fig. 5B) and quantitative (Fig. 5C) RT-PCR analysis and at the protein level by immunoblot analysis (Fig. 5D). However, we could not detect an NFATc1CA-dependent increase in the expression of SNAI2 or Slug in these cells (data not shown), which could represent inherent inaccuracies of the microarray techniques. Although upregulation of ZEB1 was also observed in A549-NFATc1CA cells, SNAI1 expression was decreased in these cells (Fig. 5C). We then investigated the importance of Zeb1 and Snail in MCF7-NFATc1CA cells by targeted RNAi. Two siRNAs specific for each of ZEB1 and SNAI1 effectively depleted the cells of the corresponding protein and reversed the downregulation of CDH1 expression and E-cadherin expression induced by NFATc1CA (Fig. 5E). These results suggest that both Zeb1 and Snail suppress CDH1 transcription downstream of NFATc1CA.
Expression of NFATc1CA alters tumor cell morphology in vivo

Finally, we tested the relevance of acquired NFATc1 expression in tumor cells in vivo. Nude mice were subcutaneously injected with MCF7-NFATc1CA cells and then fed a diet with or without doxycycline for 4 weeks. Visible subcutaneous tumors developed in all mice within 4 weeks after cell injection. Immunohistochemical analysis showed that the tumor cells expressed NFATc1 in their nuclei in a manner dependent on the presence of dietary doxycycline (Fig. 6A). Doxycycline also...
induced downregulation of E-cadherin immunoreactivity (Fig. 6A), consistent with our results obtained in vitro (Figs. 3E and 4A). Staining for Ki67 revealed that tumor cell proliferation was independent of doxycycline (Dox) for 4 weeks. The resulting tumors were fixed, embedded in paraffin, and subjected to immunohistochemical (A) or immunohistochemistry (B) staining with antibodies to NFATc1, E-cadherin, or Ki67. Nuclei were stained with hematoxylin (A) or DAPI (B). The small boxed regions in A are shown at higher magnification in the insets. Data are representative of 10 mice per group. Scale bars in A, 100 μm, and in B, 50 μm. Arrowheads in B indicate the cells without NFATc1CA expression in Dox(+) tumor.

Discussion

Expression and activation of NFATc1 induces transcription of the c-Myc gene and thereby promotes cell proliferation and anchorage-independent growth in pancreatic cancer cells (30), resulting in invasion of the tumor cells into the surrounding stroma (Fig. 6A and B). However, we could not detect any distant metastasis at this point of time (data not shown). These results indicate that acquired expression of NFATc1CA in tumor cells alters their invasive properties in vivo.
suggested that NFATc1 may play a causative role in carcinogenesis. However, given that most carcinoma cells develop without NFATc1 expression (Fig. 1), the relevance and mechanism of its acquisition have remained unknown.

We have now revealed that introduction of an active form of NFATc1 into A549 or MCF7 cells confers an invasive phenotype. This acquired invasive potential is associated with E-cadherin downregulation, which occurs in a manner independent of TGF-β signaling. Snail and Zeb1, 2 well-characterized repressors of CDH1 transcription, are induced by NFATc1CA expression and mediate the downregulation of CDH1 expression. NFATc1 directly activates transcription of SNAI1, given that the human gene contains a consensus recognition sequence for NFATc1 located 524 bp upstream of the initiation codon and that this sequence was present in a chromatin immunoprecipitate of anti-NFATc1 antibody (Supplementary Fig. S3). Although Snail and Zeb1 each inhibit E-cadherin expression in mammary carcinoma cells (26, 31), NFATc1-dependent acquisition of invasive potential does not occur as a result of typical EMT, given that mesenchymal markers such as N-cadherin and vimentin were not induced by NFATc1CA expression in vitro or in vivo (Fig. 3F and data not shown). This might be attributed to the relatively weak induction of other CDH1 repressors, such as TWIST genes or ZEB2 (Supplementary Table S1), the products of which can directly induce CDH2 (encoding N-cadherin; ref. 32) or VIM (encoding vimentin; ref. 33), respectively. Our results thus reveal a new paradigm for the transition of epithelial cells to an invasive cell type triggered by NFATc1.

Other downstream target genes of NFATc1 that would endow the cells with invasive potential include ANGPTL2 (encoding angiopoietin-like protein 2; ANGPTL2) and EGF. ANGPTL2 was recently shown to be induced by NFAT transcription factors and to promote cell migration and metastasis (34). Indeed, ANGPTL2 was increased by a factor of about 1.2 or 30 in A549-NFATc1CA or MCF7-NFATc1CA cells, respectively, on doxycycline treatment (Supplementary Table S1). EGF triggers invasion by activating the EGF receptor–GEP100–Arf6 pathway (35). Treatment with doxycycline induced EGF by a factor of about 8.2 or 14.3 in A549-NFATc1CA or MCF7-NFATc1CA cells, respectively (Supplementary Table S1). Therefore, increased expression of these factors, in concert with reduced E-cadherin expression, might contribute to the NFATc1-induced invasive properties of the cells.

Macrophages and osteoclasts are candidate cell types for conferring NFATc1 expression on cancer cells by cell–cell fusion. Indeed, melanoma cell–macrophage and myeloma cell–osteoclast hybrids have been observed in vivo (6–8). Furthermore, breast cancer cells have been shown to fuse with endothelial cells both in vitro and in vivo (36). Therefore, it is plausible that the NFATc1-positive tumor cells found sporadically in tumor specimens are generated through cell–cell fusion with NFATc1-expressing normal cells such as macrophages and osteoclasts. As a proportion of malignant tumor cells are thought to generate invadopodia to degrade surrounding tissues (37) and invadopodia can also function as fusion machineries when fusing with osteoclasts or macrophages bearing podosomes (14), NFATc1-positive tumor cells with macropage features might be generated through invadopodia/podosome-mediated cell–cell fusion.

Such hybrid cells would be expected to manifest complex pathologic features. Some cells might experience growth arrest and cell death (38–40), whereas others might exhibit increased malignancy (9, 16), depending on their parental cell types and the tumor microenvironment. Identification and analysis of bona fide fused cells positive for NFATc1 expression in vivo will be necessary to validate a role for NFATc1 in tumor progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Oikawa
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