Chapter II. EGF-mediated Tcf/Lef transcriptional activity is essential but not sufficient for cell cycle progression in non-transformed mammary epithelial cells

Abstract

Because β -catenin target genes such as *cyclin D1* are involved in cell cycle progression, we examined whether β -catenin has a more pervasive role in normal cell proliferation, even upon stimulation by non-Wnt ligands. Here, we demonstrate that epidermal growth factor (EGF) stimulates T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcriptional activity in nontransformed mammary epithelial cells (MCF-10A), and that its transcriptional activity is essential for EGF-mediated progression through G_1/S phase. Thus, expression of dominant-negative Tcf4 blocks EGF-mediated Tcf/Lef transcriptional activity and bromodeoxyuridine uptake. In fact, the importance of EGF-mediated Tcf/Lef transcriptional activity for cell cycle progression may lie further upstream at the G_1/S phase transition. We demonstrate that dominant-negative Tcf4 inhibits a reporter of cyclin D1 promoter activity in a dose-dependent manner. Importantly, dominant-negative Tcf4 suppresses EGF-mediated cell cycle activity specifically by thwarting EGF-mediated Tcf/Lef transcriptional activity, not by broader effects on EGF signaling. Thus, although expression of dominant-negative Tcf4 blocks EGF-mediated TOPFLASH activation, it has no effect on either EGF receptor or ERK phosphorylation, further underscoring the fact that Tcf/Lef-mediated transcription is essential for cell cycle progression, even when other pro-mitogenic signals are at normal levels. Yet, despite its essential role, Tcf/Lef transcriptional activity alone is not

sufficient for cell cycle progression. Serum also stimulates Tcf/Lef transcriptional activation in MCF-10A cells but is unable to promote DNA synthesis. Taken together, our data support a model wherein EGF promotes Tcf/Lef transcriptional activity, and this signal is essential but not sufficient for cell cycle activity.

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1. Introduction

β-catenin is a 90-kDa intracellular protein whose functions range from stabilization of cell-cell adhesion to control over gene expression. These functions are tightly regulated through its association with various proteins such as the transmembrane protein Ecadherin and Tcf/Lef transcription factors (1,2). E-cadherin is a major constituent of adherens junctions where it promotes epithelial cell-cell contact through homotypic interactions mediated by its extracellular domain (3). Meanwhile, its cytoplasmic domain binds to β-catenin, whose association with α-catenin and other structural proteins bridges E-cadherin-mediated contacts to the actin cytoskeleton (4). In addition to regulation by sequestration to the plasma membrane, β-catenin is tightly regulated by cytosolic degradation via a multiprotein complex consisting of Axin, APC, and glycogen synthase kinase 3β (GSK3β) (5). Signaling events that inhibit this cytosolic degradation machinery, such as those initiated by a subset of Wnt family ligands, help to stabilize βcatenin, which then translocates to the nucleus where it serves as a transactivator for the Tcf/Lef family of transcription factors.

Mutations that abnormally stabilize β -catenin occur in a diverse range of cancer types. In colorectal carcinomas and melanomas, these mutations include the loss and/or truncation of *APC* and mutations among critical N-terminal serine residues of β -catenin whose phosphorylation flags it for ubiquitin-mediated degradation (5-7). Evidence of β catenin stabilization has also been shown in hepatomas and prostate cancers wherein loss of *axin* and *PTEN*, respectively, leads to accumulation of nuclear β -catenin and increased Tcf/Lef-mediated transcription (8,9). In the mammary gland, transgenic expression of Wnt family ligands induces mammary adenocarcinomas in mice (10). Consistent with this finding, mammary-tissue-specific overexpression of a constitutively stable β -catenin mutant induces hyperplasia and adenocarcinoma in the mammary gland (11). Finally, studies using stabilized mutants of β -catenin or Tcf/Lef-VP16 fusion constructs have affirmed the capacity of this signaling pathway to transform established cell lines and primary cells (12-14).

In fact, antagonizing β -catenin signaling appears to be an effective method to curb the growth of cancer cell lines afflicted by elevated levels of nuclear β -catenin. Exogenous expression of APC, axin, or PTEN reinstates β -catenin turnover and suppresses growth of hepatocellular and prostate carcinoma cells (8,9). Inhibition of integrin-linked kinase, a serine/threonine kinase that inhibits GSK3 β and thereby stabilizes β -catenin, reduces growth in prostate cancer lines (15). Finally, overexpression of proteins, such as full-length E-cadherin or a truncated mutant possessing just the C-terminal β/γ -catenin-

binding domain, sequesters stabilized β -catenin, precludes its association with Tcf/Lef transcription factors, and effectively inhibits proliferation of colorectal cancer cell lines (16-18). Although the transformation potential of β -catenin has been closely examined, the role of β -catenin and Tcf/Lef transcription factors in cell cycle progression among *normal* mammalian cells is just now beginning to emerge. Immunohistochemical data have shown that self-propagating precursor cells in the intervillus regions of the small intestine epithelium – but not the well-differentiated cells at the villi tip – exhibit nuclear β -catenin and express several Tcf/Lef target genes, including c-myc and CD44 (19). In addition, Tcf4 knock-out mice lack proliferating stem cells and possess only differentiated villus cells, suggesting a causal role for Tcf/Lef in governing stem cell lineage commitment (20). In addition to intestinal epithelia, Tcf/Lef signaling is involved in lineage commitment of human epidermal stem cells (21-25), hematopoietic stem cells (26), and embryonic stem cells (27). However, the ligand(s) implicated in stimulating Tcf/Lef signaling and dictating stem cell fate are largely unknown, although Wnt is clearly involved in some instances (26,28).

It is unclear whether non-Wnt ligands also utilize the Tcf/Lef pathway to regulate proliferation. Recently, a correlation between serum-mediated proliferation and Tcf/Lef transcriptional activity has been suggested in a study using an engineered mammary cell system (29). These cells express a c-Fos-estradiol receptor fusion protein that permits switching from epithelial to fibroblastoid phenotype upon estradiol-mediated activation of c-Fos (30). In both phenotypes, conditions that inhibited proliferation, such as serum starvation, also downregulated β -catenin transcriptional activity. However, a causal role for β -catenin in serum-induced cell cycle progression was not clearly established in the epithelial cell phenotype. Taken together, inducible activation of c-Fos, which is a component of the AP-1 transcriptional machinery and itself critically involved in cell cycle control (31), and the inability of β -catenin suppression to consistently inhibit proliferation preclude an assessment of whether β -catenin nuclear activity is mechanistically involved in proliferation.

Interestingly, several reports have indicated that specific growth factors such as insulin and insulin-like growth factor I induce β -catenin transcriptional activity (32). Although these studies were conducted with cancer cell lines lacking normal β -catenin degradation machinery, HGF and certain members of the Wnt family of ligands induce β -catenin transcriptional activity in normal cells (33,34). Although the importance of HGF-mediated β -catenin signaling for normal cell cycle progression has not been examined, certain members of the Wnt family of ligands regulate proliferation in a β -catenin-dependent manner (35). Nevertheless, because β -catenin target genes include *c-myc* and *cyclin D1*, whose protein products are ubiquitously crucial for cell cycle progression (36-38), the untested hypothesis remains that β -catenin has a more pervasive role in normal epithelial cell proliferation, even in response to growth-stimulating cues from non-Wnt ligands.

We examined this hypothesis pertaining to the role of β-catenin in cell cycle progression in the normal mammary epithelial cell line MCF-10A. We demonstrate that EGF stimulates Tcf/Lef transcriptional activity and that this transcriptional activity is necessary but not sufficient for cell cycle progression of normal epithelial cells. Thus, inhibition of Tcf/Lef transcriptional activity using dominant-negative Tcf4 prevents EGFmediated cell cycle progression. Since dominant-negative Tcf4 inhibits cyclin D1 promoter activity and BrdU uptake without affecting other EGF-mediated signals such as ERK that also regulate proliferation, we conclude that Tcf/Lef-mediated transcription is required for cell cycle progression.

2. Experimental Procedures

2.1. Antibodies

The following antibodies were used in this study: anti-actin (Santa Cruz), anti-BrdU (Roche Applied Science), anti-ERK2 (Santa Cruz), anti-GSK3β (BD Transduction Laboratories), anti-phospho-Ser9-GSK3β (BIOSOURCE), monoclonal and polyclonal anti-FLAG (Sigma), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology), anti-phosphotyrosine (Santa Cruz), and anti-Tcf4 (Upstate Biotechnology, Inc.).

2.2. Cell Culture

SW480 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine (Invitrogen), 10% (v/v) fetal bovine serum (Invitrogen), and 1% (v/v) penicillin/streptomycin (Invitrogen). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and Lglutamine (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 0.5 µg/ml hydrocortisone (Sigma), 0.1 µg/ml cholera toxin (Sigma), 10 μ g/ml insulin (Sigma), and 1% (v/v) penicillin/streptomycin. For serum starvation, the cells were washed twice in PBS and then cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 1% (v/v) penicillin/streptomycin and 0.1% bovine serum albumin (Sigma) for 24 h.

2.3. Plasmid Constructs

pcDNA-myc-ΔN-Tcf4 was generously provided by K. W. Kinzler (Johns Hopkins University) (7). pPGS and pPGS-ΔNTcf4 were kindly donated by E. Fearon (University of Michigan, Ann Arbor) (14). VSV-G and gag-pol vectors were gifts from D. Schaffer (University of California, Berkeley). Luciferase-based reporters pTOPFLASH and pFOPFLASH were purchased from Upstate Biotechnology, Inc., whereas 1745CD1 was a gift from R. Pestell (Georgetown University, Washington, D.C.) (39).

2.4. Retroviral Infection

Retrovirus was produced by either by single transfection of the packaging cell line 293GPG with 15 µg of retroviral plasmid (40) or by triple transfection of 293T cells with 5 µg each of VSV-G, gag-pol and a retroviral vector using LipofectAMINE (Invitrogen). For infection, MCF-10A cells were incubated with retrovirus-containing medium and 8 µg/ml polybrene for 24 h.

2.5. GSK3^β Serine 9 Phosphorylation Assay

MCF-10A cells were plated at a subconfluent density (10^5 cells/35-mm dish) and allowed to adhere for 48 h, followed by serum starvation for 24 h. The cells were stimulated with either full growth medium or serum-free medium supplemented with either 10 µg/ml insulin or 20 ng/ml EGF and then lysed in modified RIPA buffer at desired times.

2.6. ERK Signaling Assay

MCF-10A cells were plated at a subconfluent density (10^5 cells/35-mm dish), allowed to adhere for 24 h, and then infected with retrovirus encoding pPGS or pPGS-FLAG- Δ N-Tcf4 at multiplicity of infection equal to 1. Twenty-four hours after infection, the cells were starved in serum-free medium for 24 h, stimulated with 20 ng/ml EGF in serum-free medium, and then lysed in modified RIPA buffer at desired times.

2.7. Cell Lysis

The stimulated cells were washed twice in ice-cold PBS and scraped into cold lysis buffer. After incubating on ice for 15 min, the cell lysates were clarified by centrifugation, and the supernatant was collected as whole cell lysate. The protein concentrations were determined using BCA reagents (Sigma). The samples prepared to assay EGF-mediated activation of the ERK pathway were lysed in modified RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM β-glycerophosphate (pH 7.3), 10 mM NaPP, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Finally, cell lysis for all reporter measurements was performed in 1X passive lysis buffer provided by the manufacturer (Promega).

2.8. Reporter Assays

SW480 or MCF-10A cells were plated at a subconfluent density (10^5 cells/35-mm dish) and co-transfected with 1 µg of the appropriate reporter and 0.1 µg of pRL-TK using FuGENE 6 (Roche Applied Science). SW480 cells were always maintained in growth medium and lysed 48 h after transfection. Meanwhile, MCF-10A cells were

serum-starved for 24 h, stimulated with appropriate medium, and lysed at desired times. In both cases, reporter activity was measured using the dual luciferase assay according to the manufacturer instructions (Promega). To normalize for potential variations in transfection or lysis efficiency, luciferase signals were normalized to control *Renilla* luciferase signal.

2.9. Integrated Reporter Response

The reporter signal response above its initial value was integrated numerically over time as follows,

$$\int_{0}^{12} R(t) - R(t_0) dt = \left(\left(\frac{1}{2} \sum_{k=1}^{n} R(t_k) + R(t_{k-1}) \right) - (n-1)R(t_0) \right) \Delta t$$
 (Eq. II-1)

where R(t) is the reporter signal, $R(t_0)$ is its basal, initial value, t_k is the time ranging from 0 to 12 h in discrete intervals of Δt (3 h), *n* is the number of time points (n = 5), and *k* is the index of summation.

2.10. Western Blotting

Whole cell lysates were resolved by SDS-PAGE on 7.5-10% gels and blotted onto polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked overnight and then incubated sequentially with primary and corresponding horseradish peroxidase-conjugated secondary antibody. The blots were treated with SuperSignal West Femto Substrate (Pierce) and imaged on VersaDoc 3000 (Bio-Rad) using Quantity One software (Bio-Rad).

2.11. DNA Synthesis

DNA synthesis was assayed by either [³H]thymidine or BrdU incorporation. In both cases, MCF-10A cells were seeded at the indicated cell densities. After 24 h, the cells were either infected with retrovirus or left in growth medium. On the following day, the cells were serum-starved. Notably, the 48 h of duration between cell seeding and serum starvation was chosen to match the time required for plating and transfecting cells in reporter assays, allowing direct comparison between DNA synthesis and reporter experiments. Following 24 h of serum starvation, the cells were stimulated with appropriate medium. Sixteen hours after stimulation, the medium was replaced with identical medium supplemented with either 10 μ Ci/ml [³H]thymidine (ICN Biomedicals) or 10 µmol/liter BrdU (Roche Applied Science) and further incubated for 6 h. In the case of [³H]thymidine incorporation, the cells were washed twice in ice-cold PBS, incubated in 5% trichloroacetic acid for 20 min at 4 °C, washed twice with cold 70% ethanol, and incubated with 0.1 M NaOH, 2% Na₂CO₃, and 1% SDS for 30 min at 37 °C. The solution was collected and mixed with CytoScint (ICN Biomedicals) for scintillation counting. For BrdU detection, the cells were fixed and co-stained with DAPI, anti-BrdU antibody, and polyclonal anti-FLAG antibody. The number of nuclei stained positive for BrdU and FLAG were quantified in 3-10 different fields on 2-5 independent trials using the Zeiss Axiovert 200M microscope.

2.12. Immunofluorescence

For Tcf/FLAG co-staining, the cells grown on glass coverslips were washed three times in ice-cold PBS, fixed in 4% formalin in PBS, and permeabilized in 0.2% Triton X-100. After blocking overnight in BB (10% goat serum, 0.1% bovine serum albumin in PBS), the coverslips were sequentially incubated with primary and corresponding Alexa dye-labeled secondary antibodies (Molecular Probes). Following antibody incubations, the coverslips were stained with DAPI (Sigma) and mounted using Prolong Anti-Fade (Molecular Probes). For BrdU/FLAG co-staining, the cells were fixed and permeabilized in 4% formalin and 0.2% Triton X-100, respectively, and then incubated with polyclonal FLAG antibody and Alexa 594-conjugated secondary antibody. The antibodies were then fixed in 4% formalin, followed by a second fixation in 15 mM glycine in 70% ethanol (pH 2). The coverslips were incubated with monoclonal BrdU antibody and then Alexa 488-conjugated secondary antibody, stained with DAPI, and mounted as described above.

3. Results

3.1. Re-entry into the Cell Cycle Correlates with Tcf/Lef Reporter Activity

Target genes for Tcf/Lef include *cyclin D1* and *c-myc*, suggesting a role for this family of transcription factors in cell cycle progression not only among cancer cells with stabilized nuclear β -catenin, but also among normal epithelial cells. Therefore, we determined whether a correlation exists between Tcf/Lef transcriptional activity and cell cycle progression in nontransformed mammary epithelial (MCF-10A) cells. Tcf/Lef transcriptional activity was monitored with TOPFLASH reporter (7), a plasmid containing consensus Tcf-binding sites upstream of the *luciferase* gene. In contrast, the negative control FOPFLASH reporter carries mutations at these Tcf/Lef-binding sites. Performance of TOPFLASH and FOPFLASH reporters was confirmed in SW480 colon carcinoma cells in which TOPFLASH, but not FOPFLASH, is constitutively active because of a truncation of the *APC* gene and consequent stabilization of β -catenin (7) (Fig. II-1*A*). Subconfluent MCF-10A cells transfected with TOPFLASH or FOPFLASH reporters were growth-arrested by serum starvation and then stimulated to re-enter the cell cycle by treatment with growth medium. As shown in Fig. II-1*B*, growth medium stimulation activated TOPFLASH reporter, which gradually increased to a nearmaximum level within the first 9 h. Meanwhile, FOPFLASH negative control reporter did not respond to growth medium stimulation. Taken together, this establishes a correlation between re-entry into the cell cycle and Tcf/Lef-mediated transcription.



FIG. II-1. **TOPFLASH and FOPFLASH reporter activity in SW480 and MCF-10A cells** *A*, TOPFLASH, but not FOPFLASH, reporter is triggered in SW480 colon carcinoma cells. SW480 cells were co-transfected with 0.1 µg of pRL-TK and 1 µg of either TOPFLASH or FOPFLASH. Forty-eight hours after transfection, the cells were lysed, and the ratio of luciferase to *Renilla* luciferase signal was quantified. *B*, TOPFLASH, but not FOPFLASH, reporter is activated upon growth medium stimulation of normal mammary epithelial cells. MCF-10A cells were co-transfected with 0.1 µg of pRL-TK and 1 µg of TOPFLASH (\blacklozenge) or FOPFLASH (\bigcirc). After serum starvation, the cells were stimulated with growth medium and luciferase:*Renilla* luciferase signal ratio was quantified at desired time points. Reporter activity relative to the TOPFLASH response at 6 h is shown. The *error bars* represent ± S.E. from two to five independent experiments. The *asterisk* denotes p < 0.05 (Student's *t*-test) in comparing TOPFLASH signal to the zero time response.

To confirm further that the observed TOPFLASH signal was specifically monitoring Tcf/Lef transcription factor activity, a dominant-negative Tcf4 construct (myc- Δ N-Tcf4) was employed. This construct possesses the DNA-binding domain of Tcf4, but lacks the N-terminal 31 amino acids that mediate its association with its transactivating catenin partner (7). As expected, dominant-negative Tcf4 inhibited TOPFLASH activity in

SW480 cells (Fig. II-2*A*). Co-transfection of Δ N-Tcf4 into MCF-10A cells decreased growth medium-induced TOPFLASH response in a dose-dependent fashion (Fig. II-2*B*), indicating that the TOPFLASH signal was mediated specifically by Tcf/Lef transcription factors.



FIG. II-2. Dominant-negative Tcf4 effect on TOPFLASH reporter

A, dominant-negative Tcf4 inhibits TOPFLASH signal in SW480 colon carcinoma cells. SW480 cells were co-transfected with 1 µg of TOPFLASH, 0.1 µg of pRL-TK, and either 0.5 µg of empty vector (pcDNA) or dominant-negative Tcf4 (Δ N-Tcf4). Forty-eight hours after transfection, the luciferase:*Renilla* luciferase signal ratio was quantified. *B*, dominant-negative Tcf4 inhibits growth medium-mediated TOPFLASH signal in MCF-10A cells. MCF-10A cells were co-transfected with 1 µg of TOPFLASH, 0.1 µg of pRL-TK, and different amounts (0, 0.05, 0.1, and 0.5 µg) of dominant-negative Tcf4 (Δ N-Tcf4), always with a balancing amount (0.5, 0.45, 0.4, and 0 µg, respectively) of empty vector (pcDNA). Serum-starved cells were stimulated with growth medium for 9 h, after which the luciferase:*Renilla* luciferase signal ratio was quantified. Co-transfection with increasing amount of Δ N-Tcf4 correspondingly attenuated TOPFLASH induction by growth medium. The *error bars* indicate ± S.E. (*n* = 3). The *asterisk* indicates *p* < 0.01 (Student's *t*-test).

3.2. EGF Independently Induces Tcf/Lef Transcriptional Activity and DNA Synthesis

Because MCF-10A growth medium contains a complex mixture of stimuli, including serum factors, insulin, and EGF, it is unclear whether a single constituent is capable of inducing Tcf/Lef transcriptional activity and, moreover, whether the same constituent also functions as a mitogen. To address this issue, the cells were stimulated with each

constituent of growth medium separately, and Tcf/Lef transcriptional activity and DNA synthesis were assessed by measuring TOPFLASH reporter signal and [³H]thymidine uptake, respectively. EGF independently induced TOPFLASH signal to a level distinctly above the corresponding FOPFLASH control (Fig. II-3*A*). At early times, EGF-mediated TOPFLASH signal mirrors growth-medium-induced TOPFLASH activity. However, whereas full growth medium sustains TOPFLASH signal to 24 h (Fig. II-1*A*), EGF promotes a transient signal that reaches its peak intensity of nearly 3-fold above basal level at 3 h. Meanwhile, in contrast to EGF, insulin-mediated TOPFLASH activation more closely matches the FOPFLASH negative control, except at 3 h, where a transient signal that is 50% of the EGF-mediated TOPFLASH signal is observed. Taken together, growth medium constituents quantitatively vary in their ability to promote Tcf/Lef transcriptional activity, with EGF, more so than insulin, resembling the response to full growth medium.

The current paradigm for Wnt-mediated Tcf/Lef transcriptional activity involves inhibition of GSK3 β -mediated phosphorylation of β -catenin, which in turn stabilizes β catenin and ultimately enables its translocation into the nucleus (41-43). Interestingly, both EGF and insulin have been reported to inhibit GSK3 β kinase activity toward primed substrates by inducing phosphorylation of GSK3 β at serine 9 (44-48). Because there is conflicting evidence as to whether β -catenin qualifies as a primed or nonprimed substrate of GSK3 β (49-52), we investigated whether EGF, insulin, and growth medium affected GSK3 β phosphorylation at serine 9 in a manner that is quantitatively consistent with their differential effects on Tcf/Lef transcriptional activity. Within 10 min of stimulation, GSK3 β was phosphorylated at serine 9 among serum-starved MCF-10A cells in response to either insulin or EGF (Fig. II-3*B*). However, in insulin-stimulated cells, dephosphorylation of GSK3 β was evident by 60 min, whereas in EGF-stimulated cells, serine 9 phosphorylation of GSK3 β was more sustained. Stimulation with growth medium, which contains insulin, EGF, and serum factors, induced more sustained phosphorylation of GSK3 β than either EGF or insulin alone. Taken together, the stimuli (growth medium and EGF) that yield a qualitatively durable phosphoserine 9 GSK3 β signal also induce stronger Tcf/Lef transcriptional activity. This correlation suggests, but does not unequivocally demonstrate, a role for serine 9 phosphorylation of GSK3 β in EGF-mediated Tcf/Lef transcriptional activity, further raising the issue of whether β catenin is a primed or nonprimed substrate of GSK3 β .

Although ongoing work is focused on parsing the role of serine 9 phosphorylation of GSK3 β and other mechanisms by which EGF stimulates TOPFLASH activity, we focused in this work on understanding the downstream significance of EGF-mediated Tcf/Lef transcriptional activity. Since the kinetics of TOPFLASH reporter response varied among stimuli, we calculated the time integral of each signal as a single quantitative metric capable of capturing effects on both signal magnitude and dynamics (53). Using this metric, we determined whether the ability to induce Tcf/Lef transcriptional activity quantitatively relates to the mitogenic potency of the stimulus as measured by DNA synthesis using a [³H]thymidine uptake assay. EGF independently induces DNA synthesis and accounts for 70% of the mitogenic activity of complete growth medium (Fig. II-3*C*); insulin, however, fails to promote DNA synthesis. Thus,



FIG. II-3. Growth medium constituents vary in the ability to induce Tcf/Lef transcriptional activity, GSK3β phosphorylation, and DNA synthesis.

A, EGF transiently stimulates Tcf/Lef transcriptional activity. MCF-10A cells were co-transfected with 0.1 ug of pRL-TK and 1 ug of TOPFLASH (filled symbols, solid line) or FOPFLASH (open symbols, dotted *line*). After serum starvation, the cells were stimulated with full growth medium (\blacklozenge and \diamondsuit) or serum-free medium supplemented with 20 ng/ml EGF (\blacksquare and \Box), 5% horse serum (\blacktriangle and \triangle), or 10 µg/ml insulin (\bullet and \circ). The concentration of each supplement was chosen to match the concentration of the supplement in growth medium. Luciferase: Renilla luciferase signal ratio was quantified at the desired time points and is reported relative to its initial, basal level. The *error bars* represent \pm S.E. from two to five independent experiments. B, insulin, EGF, and growth medium induce serine 9 phosphorylation of GSK3β with qualitatively distinct time courses. Serum-starved MCF-10A cells were stimulated with either full growth medium (GM) or serum-free medium supplemented with 10 µg/ml insulin or 20 ng/ml EGF and then lysed at indicated times. Western blotting of cell lysates with a phospho-specific antibody shows that serine 9 of GSK 3β is phosphorylated within 10 min of stimulation with insulin, EGF, or growth medium. After 10 min, the levels of phosphorylated GSK3β decrease in insulin-stimulated cells, whereas cells stimulated with EGF show relatively more sustained phosphorylation (compare the 30-min bands). Finally, the cells stimulated with growth medium possess the most sustained phosphorylation response (compare the 60-min bands). Taken together, EGF and growth medium promote a longer lifetime of serine 9 phosphorylation of GSK3ß than insulin. Blotting with a total GSK3ß antibody confirmed equal protein loading. The data shown are representative of two independent trials. C, stimuli that promote DNA synthesis also activate TOPFLASH reporter, but not all stimuli that induce TOPFLASH promote DNA synthesis. TOPFLASH data (white bars) are expressed as the integrated response over a 12-h stimulation period. Both TOPFLASH reporter response and [³H]thymidine incorporation (*black bars*) are expressed relative to the EGF response. Although EGF, growth medium, and horse serum induced significant TOPFLASH responses, only EGF and growth medium stimulate [³H]thymidine incorporation.

the ability of EGF, insulin, and full growth medium to induce Tcf/Lef transcriptional activity strictly correlates with their ability to induce DNA synthesis, because stimuli that induce high levels of Tcf/Lef transcription also promote DNA synthesis (e.g., EGF and growth medium), whereas those stimuli that do not induce Tcf/Lef-mediated transcription (e.g., insulin) do not promote DNA synthesis. The exception to this apparent correlation between TOPFLASH response and DNA synthesis involves serum stimulation, which induced strong integrated TOPFLASH signal but failed to promote DNA synthesis. Taken together, stimuli that induce DNA synthesis also promote Tcf/Lef transcriptional activity (e.g., EGF), but the converse is not necessarily true (e.g., serum). Therefore, Tcf/Lef transcriptional activity is by itself insufficient to promote proliferation.

3.3. Tcf/Lef Transcriptional Activity Is Required for EGF-mediated DNA Synthesis

Although Tcf/Lef transcriptional activity is not sufficient for proliferation, we examined whether its activity is required for cell cycle progression using dominantnegative Tcf4. MCF-10A cells were infected with retrovirus encoding FLAG-tagged, dominant-negative Tcf4 (pPGS-FLAG- Δ N-Tcf4), and expression was confirmed by Western blot (data not shown) and immunofluorescence (Fig. II-4*A*). Exogenous dominant-negative Tcf4 was clearly identifiable among Δ N-Tcf4-infected cells because of distinct anti-FLAG staining, which was only observed at background levels in control (pPGS)-infected cells. Staining with anti-Tcf4 antibody revealed that both endogenous Tcf4 and FLAG- Δ N-Tcf4 were localized to the nucleus, and expression of the dominantnegative construct greatly enhanced the intensity of anti-Tcf4 stain, consistent with the expected overexpression of this exogenous protein. To determine the role of Tcf/Lef in



FIG. II-4. Dominant-negative Tcf4 blocks DNA synthesis.

A, both endogenous Tcf4 and exogenous Δ N-Tcf4 localize to cell nuclei. MCF-10A cells were retrovirally infected with either the empty vector (pPGS) or FLAG-tagged dominant-negative Tcf4 (pPGS-\DeltaN-Tcf4) and costained for Tcf4 and FLAG. Among pPGS-infected cells, endogenous Tcf4 (green) was visible in the nucleus (DAPI, blue), whereas an anti-FLAG antibody (red) revealed only background staining within the cell body. Among cells infected with pPGS- Δ N-Tcf4, anti-FLAG staining produced intense signal from cell nuclei, demonstrating that dominant-negative Tcf4, like its endogenous counterpart, localizes to the nucleus. Consistently, Tcf4 staining in Δ N-Tcf4-expressing cells was significantly more intense than endogenous Tcf4 levels in pPGS-infected cells. B, ΔN -Tcf4 completely blocks BrdU incorporation at a single-cell level. MCF-10A cells were infected with either pPGS or pPGS-ΔN-Tcf4 at a multiplicity of infection less than 1. After serum starvation, the cells were stimulated to re-enter the cell cycle with either full growth medium, serum-free medium, or serum-free medium supplemented with 20 ng/ml EGF. After a 6-h pulse with BrdU, the cells were co-stained with anti-FLAG antibody (*red*), anti-BrdU (*green*) antibody, and DAPI (blue). Images depict immunofluorescence results of EGF-stimulated cells. The BrdU and FLAG images have been superimposed to demonstrate that cells incorporating BrdU never express FLAGtagged ANTcf4. The arrowheads denote FLAG-positive cells, and the asterisks denote BrdU-positive cells.



FIG. II-4. Dominant-negative Tcf4 blocks DNA synthesis.

C, quantification of immunostaining reveals that Δ N-Tcf4 completely inhibits BrdU incorporation in MCF-10A cells. The fraction of nuclei positive for BrdU was quantified from immunostained samples of cells treated with serum-free medium (*open bars*), growth medium (*black bars*), and EGF (*hatched bars*) medium as described above for *B*. Among pPGS-infected cells, both growth medium and EGF substantially increase the fraction of cells incorporating BrdU. Within the population targeted for infection by pPGS- Δ N-Tcf4, those cells lacking expression of FLAG-tagged, Δ N-Tcf4 responded to growth medium and EGF similar to pPGS-infected cells. Notably, FLAG-positive cells failed to incorporate BrdU upon stimulation by either EGF or growth medium. The *error bars* are ± S.E. (*n* = 6). The *asterisk* denotes *p* < 0.05 (Student's *t*-test) in comparing delineated data pairs.

cell cycle progression, EGF- and growth-medium-induced DNA synthesis were measured by assessing BrdU incorporation in control and dominant-negative Tcf4-infected MCF10A cells. Because the multiplicity of infection was less than 1, BrdU uptake was observed among both cell populations upon stimulation (Fig. II-4*B*). Importantly, costaining for FLAG revealed that cells clearly expressing FLAG-tagged, dominantnegative Tcf4 never incorporated BrdU, showing a strict Tcf/Lef requirement for DNA synthesis.

More quantitatively, both growth medium and EGF stimulation induced BrdU uptake in pPGS-infected cells (Fig. II-4*C*). Consistent with thymidine incorporation in uninfected cells (Fig. II-3*C*), growth medium was more mitogenic than EGF alone. In the case of pPGS- Δ N-Tcf4-infected cells, the fraction of nuclei positive for BrdU was determined among FLAG-positive and FLAG-negative subpopulations. Although the FLAG-negative cells incorporated BrdU at levels similar to control pPGS-infected cells, growth medium- and EGF-mediated BrdU incorporation in FLAG-positive cells was inhibited completely. This result quantitatively demonstrates that inhibition of Tcf/Lef-mediated transcription thwarts EGF-mediated S phase progression.

In addition to Tcf/Lef transcriptional activity, EGF induces other signaling pathways that regulate cell proliferation, including the ERK pathway. To verify that dominantnegative Tcf4 inhibited EGF-mediated DNA synthesis by specifically blocking Tcf/Lef signaling rather than by more globally affecting EGF receptor signaling, we examined EGF-mediated tyrosine phosphorylation and ERK signaling among control pPGSinfected cells and pPGS-ΔN-Tcf4-infected cells. Anti-phosphotyrosine Western blotting revealed that EGF receptor (180 kDa) becomes heavily tyrosine-phosphorylated within 15 min of EGF stimulation in pPGS-infected cells and that infection with pPGS-ΔN-Tcf4 alters neither the magnitude nor the dynamics of EGF receptor phosphorylation (Fig. II-5). Similarly, ERK1/2 undergoes rapid and sustained dual phosphorylation after EGF stimulation in both pPGS- and pPGS- ΔN -Tcf4-infected cells. Expression of FLAGtagged, dominant-negative Tcf4 was confirmed by Western blot (Fig. II-5) and immunofluorescence (data not shown). Taken together, this confirms that the inhibitory effect of dominant-negative Tcf4 on proliferation is specifically due to inhibition of EGFmediated Tcf/Lef transcriptional activity.



FIG. II-5. Dominant-negative Tcf4 does not affect EGF-mediated EGF receptor and ERK phosphorylation.

MCF-10A cells were plated at a subconfluent density $(1 \times 10^5 \text{ cells/35-mm dish})$, allowed to adhere for 24 h, and then retrovirally infected at a multiplicity of infection of 1 with empty vector (pPGS) or dominant-negative Tcf4 (pPGS- Δ N-Tcf4). Forty-eight hours after plating, the cells were serum-starved for 24 h, stimulated with 20 ng/ml EGF, and then lysed at indicated times afterward. The lysates were analyzed by Western blot using anti-phosphotyrosine and anti-phospho-ERK 1/2 antibodies. Expression of dominant-negative Tcf4, as confirmed by an anti-FLAG blot, did not affect either the magnitude or the dynamics of EGF receptor tyrosine phosphorylation (180-kDa band shown) and ERK phosphorylation. Furthermore, probing for total ERK2 revealed that expression level of this signaling protein was unaffected by dominant-negative Tcf4 expression. An anti-actin blot demonstrated equal loading of cell lysate.

3.4. EGF-mediated Activation of Tcf/Lef Transcriptional Activity Is Upstream of Cyclin

D1 Promoter Activity

Upstream of S phase entry, cyclin D1 regulates passage through mid-G₁ phase of the cell cycle and is also a Tcf/Lef target gene (36,37). Therefore, we examined whether Tcf/Lef regulates cyclin D1 induction using the 1745CD1 reporter that monitors cyclin D1 promoter activity (39). In serum-starved MCF-10A cells, stimulation with growth medium initiated cyclin D1 reporter activity at 12 h (Fig. II-6*A*). The observation that the time course of TOPFLASH activation (Fig. II-1*B*) precedes timing of cyclin D1 promoter activity is consistent with, but does not prove the fact, that Tcf/Lef lies upstream of cyclin D1 upregulation.



FIG. II-6. Tcf/Lef involvement in cyclin D1 promoter activity

A, growth medium treatment of MCF-10A cells stimulates cyclin D1 promoter activity. MCF-10A cells were co-transfected with 1 µg of 1745CD1 reporter and 0.1 µg of pRL-TK. After serum starvation, the cells were stimulated with growth medium, and luciferase:*Renilla* luciferase signal was monitored at different times. Maximum promoter activity occurred at 12 h after stimulation. *B*, dominant-negative Tcf4 blocks induction of cyclin D1 promoter activity. MCF-10A cells were transfected with 1 µg of 1745CD1 reporter, 0.1 µg of pRL-TK, and different amounts (0, 0.05, 0.1, and 0.5 µg) of dominant-negative Tcf4 (Δ N-Tcf4) in balance with empty pcDNA vector (0.5, 0.45, 0.4, and 0 µg, respectively). Following serum starvation, the cells were stimulated with growth medium for 12 h, and the luciferase:*Renilla* luciferase signal was measured. Increasing the dose of Δ N-Tcf4 reduced the induction of cyclin D1 promoter activity. The *error bars* are ± S.E. (*n* = 2), and the *asterisk* denotes *p* < 0.05 (Student's *t*-test).

To determine whether Tcf/Lef activity is in fact required for cyclin D1 promoter activity, different amounts of dominant-negative Tcf4 (pcDNA-myc- Δ N-Tcf4) balanced with empty vector (pcDNA) were co-transfected, and cyclin D1 promoter activity was measured 12 h after growth medium stimulation. The reporter response was abolished in MCF-10A cells co-transfected with myc- Δ N-Tcf4 in a dose-dependent fashion (Fig. II-6*B*), establishing a causal link between Tcf/Lef activity and cyclin D1 promoter activity.

4. Discussion

In this work, we demonstrate that EGF stimulates Tcf/Lef transcriptional activity in normal mammary epithelial cells and that its transcriptional activity is essential for EGF-

mediated cyclin D1 induction and DNA synthesis. Thus, expression of dominantnegative Tcf4 inhibits EGF-mediated Tcf/Lef transcriptional activity and also blocks EGF-mediated BrdU uptake. To our knowledge, this report offers the first demonstration that a specific growth factor, other than Wnt ligands, stimulates cell cycle progression in a Tcf/Lef-dependent manner in an untransformed epithelial cell line.

Although complex medium containing fetal calf serum has been shown to stimulate TOPFLASH reporter (29), the only specific, non-Wnt growth factors implicated in Tcf/Lef signaling among normal epithelial cells are HGF and Gas6 (34,54). Although Gas6 was shown to induce both Tcf/Lef-mediated transcription and proliferation, these results were correlative and did not establish a mechanistic role for β -catenin signaling in cell proliferation. Nevertheless, a role for Tcf/Lef in HGF-mediated proliferation may be inferred cautiously from the finding that overexpression of oncogenic MET and RON (receptors for HGF and macrophage-stimulating protein/HGF-like protein, respectively) induces transformation in a Tcf/Lef-dependent manner (55). Because these studies involved overexpression of oncogenic receptors, it is difficult to conclude whether HGFmediated Tcf/Lef signaling will have similar functional significance in normal cells. Indeed, our findings suggest that such extrapolation may prove quantitatively inaccurate, especially in the case of insulin-induced Tcf/Lef signaling. Insulin and insulin-like growth factor I stabilize β -catenin and induce TOPFLASH reporter activity in cancer cell lines with constitutive defects in β -catenin degradation machinery (32). However, our observations reveal that, in the context of normal β -catenin regulation, insulin stimulates Tcf/Lef transcriptional activity only to a relatively minor extent and to a level that is

insufficient to promote cell cycle activity.

Interestingly, our results suggest a correlation between the level of EGF- and insulinmediated Tcf/Lef transcriptional activity and their differential ability to phosphorylate GSK3 β at serine 9. Serine 9 phosphorylation inhibits GSK3 β kinase activity toward primed substrates (49). However, published reports support both the possibility that β catenin is a primed substrate (51,52) and the possibility that it may be a nonprimed substrate (49,50) for GSK3β. In vitro kinase assays have demonstrated that mutations in GSK3 β that abolish kinase activity toward primed substrates do not affect its activity toward β -catenin (49). Consistent with this finding, Wnt-mediated stabilization of β catenin signaling does not coincide with phosphorylation of GSK3 β at serine 9, whereas insulin-mediated phosphorylation of GSK3ß at serine 9 correlates with its ability to activate a primed substrate, glycogen synthase (50). Taken together, these reports support a model where β -catenin is a nonprimed substrate whose phosphorylation is regulated by GSK3 β , but not in a serine 9 phosphorylation-dependent manner. In contrast, other reports have identified members of the casein kinase I family as priming kinases that are required for GSK3 β -mediated phosphorylation of N-terminal β -catenin serine residues (51,52). Thus, assuming β -catenin stabilization via inhibition of GSK3 β activity plays a crucial role in EGF-mediated Tcf/Lef transcriptional activation as it does in Wnt signaling, the mechanistic significance of our observed correlation between the level of phosphorylation of GSK3ß at serine 9 and the intensity of Tcf/Lef transcriptional activity will depend on whether EGF employs a primed or nonprimed mechanism to affect β -catenin stabilization. Further quantitative experiments to test this and other

hypotheses regarding the mechanisms by which EGF stimulates Tcf/Lef transcriptional activity are underway.

Although the mechanisms connecting EGF stimulation to Tcf/Lef transcriptional activity remain to be deciphered, this work focused on the essential role that EGFmediated Tcf/Lef activation plays in stimulating DNA synthesis. Further upstream within the cell cycle, we show that EGF-mediated induction of cyclin D1 promoter activity was blocked by expression of dominant-negative Tcf4, consistent with the fact that *cyclin D1* is a putative target gene for Tcf/Lef transcription factors (36,37).

Importantly, although Tcf/Lef transcriptional activity is essential for G₁/S phase progression, it alone is insufficient for DNA synthesis. Thus, serum induces TOPFLASH reporter signal but does not elicit DNA synthesis, clearly underscoring that other signaling pathways, such as PI3K and ERK, are likely to be important for the ultimate mitogenic response. Some clues to how this combination of signals impinges on cell cycle regulation are beginning to emerge. An important point of convergence may involve the upregulation of cyclin D1 during late G₁ phase of the cell cycle. The cyclin D1 promoter contains both Tcf/Lef-binding sites that are essential for β-catenin responsiveness and Ets and CREB sites that are essential for Ras-mediated activation (37). Studies using cyclin D1 promoter reporters carrying mutations in Tcf/Lef, Ets, and/or CREB sites revealed that exogenous expression of a stabilized β-catenin mutant stimulates cyclin D1 promoter activity independent of Ets/CREB sites, whereas RasV12mediated reporter stimulation is independent of the Tcf/Lef sites. Yet, maximal response was observed only when all sites were intact. Hence, these findings argue for an additive effect of Ras and β -catenin signaling on cyclin D1 promoter activity. More recently, PI3K has been implicated in regulating Tcf/Lef-dependent cyclin D1 induction, as inhibition of PI3K or its downstream target IKK α thwarts serum-mediated induction of cyclin D1 (56). Furthermore, the sensitivity of cyclin D1 induction to PI3K/IKK α signaling was traced to a single Tcf-binding site on the cyclin D1 promoter.



FIG. II-7. Proposed model for the strict requirement of Tcf/Lef signaling for EGFmediated cell cycle progression EGF-induced progression from G₁ into S phase is controlled by cyclin D1 upregulation and is known to require signals such as ERK. Here, we report that EGF also induces Tcf/Lef-mediated transcription and that this transcriptional activity is essential for cyclin D1 upregulation and DNA synthesis. Other studies have suggested that Tcf/Lef transcription may require PI3K/IKKa and/or PKC (50,56). Although ERK signaling is not affected by inhibition of Tcf/Lef transcription, ERK may either lie upstream of β/γ -catenin:Tcf/Lef transcription or act as a parallel signal. Taken together, both Tcf/Lef signaling and parallel signals such as ERK may cooperate to upregulate cyclin D1, which leads to cell cycle progression and, ultimately, cell proliferation.

Whether working additively with Ras-mediated signals, possibly including ERK, or synergistically via PI3K, Tcf/Lef transcriptional activity seems to be essential for EGF-mediated DNA synthesis, since blocking Tcf/Lef transcriptional activity using a dominant-negative Tcf4 inhibits EGF-mediated DNA synthesis (Fig. II-7). In addition to

the β/γ -catenin:Tcf/Lef signal, other canonical growth factor signals, such as ERK, are crucial mediators of cell cycle activity (57). Here, we demonstrate that inhibition of Tcf/Lef transcription by dominant-negative Tcf4 does not affect EGF-mediated activation of the ERK pathway (Fig. II-5). Thus, ERK signaling is not downstream of Tcf/Lefmediated transcription; however, the ERK pathway may work synergistically with Tcf/Lef signaling to provide multiple, essential signals that initiate cell cycle progression. Alternatively, ERK signaling may lie upstream of Tcf/Lef signaling. Although current work is focused on understanding the relationship among ERK, Tcf/Lef signaling, and cell cycle progression, our data demonstrate that Tcf/Lef-mediated transcription is one of several intracellular signals that are essential for cell cycle progression.

Taken together, our findings in MCF-10A normal mammary epithelial cells, along with recent reports in a range of stem cells (19,21,25-27), underscore the important role β/γ -catenin:Tcf/Lef signaling plays in normal cell proliferation. On the other extreme, mutations that constitutively aggrandize β -catenin nuclear activity lead to transformation (5). Thus, finding strategies that attenuate hyperactive β -catenin signaling in cancer cells (8,9,15-18), while minimizing deleterious effects in normal cells, will clearly be important to the success of this family of therapeutic strategies.

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6. References

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