Regulation of Cyclooxygenase-2 and Periostin by Wnt-3 in Mouse Mammary Epithelial Cells*

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The Wnt family members are critical in developmental processes and have been shown to promote carcinogenesis when ectopically expressed in the mouse mammary gland. The gene expression pattern mediated by Wnt is pivotal for these diverse responses. The Wnt pathway has been conserved among different species. Genetic studies have shown that Wnt effects are mediated, at least in part, by β-catenin, which regulates transcription of “downstream genes.” Wnt stimulation inactivates glycogen-synthase kinase-3β (GSK-3) with subsequent stabilization of β-catenin, which after heterodimerizing with lymphocyte enhancer factor-1/T-cell factor cofactors stimulates transcription. To establish whether Wnt-stimulated transcription is mediated solely by β-catenin, a comparison was made of gene expression profiles in response to Wnt-3, overexpression of β-catenin, and inhibition of GSK-3. Infection of cells with Wnt-3 and inhibition of GSK-3 regulate a set of genes that include cyclooxygenase-2 and periostin. Interestingly, overexpression of β-catenin or reducing β-catenin levels with antisense oligonucleotide transfection did not have any effect on cyclooxygenase-2 or periostin expression, thereby defining a Wnt pathway, which cannot be mimicked by β-catenin overexpression.

The Wnt proteins are a family of secreted cysteine-rich glycoproteins that play an essential role in directing developmental processes such as cell adhesion, cell fate, and cell proliferation (1, 2). The Wnt signaling pathway appears to be highly conserved across different species, and genetic and biochemical studies in Caenorhabditis elegans, Drosophila, Xenopus and mammals have contributed to increased understanding of the pathway (for review see Ref. 3). Some of the Wnt genes have been shown to promote mammalian carcinogenesis. Wnt-1 and Wnt-3 were initially identified as mouse mammary oncogenes that became tumorigenic by the insertion of a mouse mammary tumor virus (4, 5). Even though a role for Wnt proteins in breast tumorigenesis has been established in mice, this link has not yet been made in human breast cancer. However, components of the Wnt signaling pathway such as adenomatous polyposis coli (APC), a tumor suppressor protein, and β-catenin are clearly involved in other forms of human cancers including melanoma, colon, and hepatocellular cancer (6).

Stimulation of cells with Wnt-1 protein results in an increase of cytosolic levels of β-catenin as a consequence of the inhibition of glycogen-synthase kinase-3β. β-Catenin then heterodimerizes with a member of the Lef-1/T-cell family of transcription factors and induces gene transcription. APC can bind to β-catenin and facilitate its degradation when phosphorylated by GSK-3. Deletions in the tumor suppressor protein APC found in colon cancer preclude β-catenin degradation, and in so doing the increased level of β-catenin can stimulate transcription as described above. Further, stabilizing mutations or deletions in the regulatory N-terminal domain of β-catenin also result in increased transcription (3). How this increase in transcription relates to cancer is unclear, but some recently identified β-catenin target genes cyclin D1 and the protooncogene c-myc may contribute to neoplastic transformation (7, 8).

A number of potential Wnt-1 target genes have been identified in different organisms, including ultrabithorax and engrailed in Drosophila (9, 10), nodal-related 3 and siamois in Xenopus (11–13), Connexin 43 (14), Wisp (15), cyclin D1 (16), and Cox-2 (17) in various mammalian cell lines or tissues. Furthermore, it is not clear whether the entire transcriptional response to the stimulation by Wnt-1 or Wnt-3 is mediated by β-catenin-dependent processes. To explore whether there is any evidence for Wnt-stimulated pathways that are independent of β-catenin, we compared gene profiles regulated by Wnt-3, β-catenin, and inhibition of GSK-3. Cox-2 was up-regulated and periostin (formerly named osteoblast-specific factor-2) was down-regulated by both Wnt-3 and inhibition of GSK-3. However, in contrast, β-catenin did not have an effect on the regulation of these two genes, even though it did affect expression of other genes. Importantly, the data show that the genes regulated by Wnt-3 involve multiple pathways downstream of GSK-3, not all of which are dependent on β-catenin.

MATERIALS AND METHODS

Cell Lines—The mouse mammary epithelial cell line C57MG was grown in Dulbecco’s modified Eagle’s medium (4.5 mg/ml l-glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 10 μg/ml insulin (Sigma). Wnt-3 was expressed in C57MG cells after infection with a murine leukemia virus-based retroviral vector (LNCX, Invitrogen). The vector expresses Wnt-3, which was cloned from a mouse brain library, under the control of the cytomegalovirus promoter and the bacterial neomycin phosphotransferase gene (neo). The same vector was used for expression of a β-catenin deletion mutant (ΔN89β-catenin) that lacks the potentially destabilizing phosphorylation sites for GSK-3. Infected cell populations were selected for 3 weeks in 400 μg/ml Genetecin (G418). At that time partial transformation to a more spindle-like cell shape was easily detectable in the Wnt-3 cells. About 400 G418 resistant clones designated Wnt-3, ΔN89β-catenin, or control (empty vector) were pooled and expanded for total RNA preparation and protein lysate preparation.

Differential Display—Total RNA was isolated from Wnt-3 or vector control cells according to manufacturer’s conditions (Qiagen) and then...
further treated with DNase using the MessageClean (GenHunter) protocol.

Based on a method developed by Liang and Pardee (18), 20 primers provided by the Hieroglyph mRNA profile kit for differential display analysis (Genomyx Corp.) were used to amplify 12 different pools of reverse-transcribed PCR reactions. A total of 240 primer pairs were used to profile gene expression. Products from these PCR reactions were run in duplicate with two sets of independently prepared total RNA on 4.5% sequencing gels using a genomyx LR sequencer (Genomyx Corp.). The dried gels were exposed to Kodak XAR-2 film (Eastman Kodak Co.).

A total of 50 differentially expressed cDNA fragments were excised, reamplified according to the manufacturer’s protocol (Genomyx Corp.), and sequenced. The length of the PCR products ranged from 0.3 to 1.0 kilobases. Only bands that were reproduced in duplicate lanes for two independent total RNA preparations were excised and reamplified.

cDNA Microarray—The cDNA microarray consisted of 26 cDNA fragments identified in the differential display analysis and control genes such as actin and tubulin.

Poly(A)+ RNA was purified with the Oligotex mRNA mini kit (Qiagen) from total RNA isolated from Wnt-3-expressing and control cells. 0.5 μg of mRNA was oligo(dT)- and nonamer-primed at 70 °C for 5 min, cooled on ice, and then reverse-transcribed with 600 units of SuperScriptII reverse transcriptase for 2 h at 42 °C in 1× first strand buffer, 10 mM dithiothreitol, 2 mM each dNTP, 1 mM dCTP (all reagents, Life Technologies Inc.), and either 1 mM Cy3 or Cy5 (Amersham Pharmacia Biotech) for fluorescent labeling of the cDNA in a 20-μl total volume. The mRNA was digested with 1 unit RNaseH (Life Technologies Inc.) and 0.5 unit RNase ONE (Promega) for 30 min at 37 °C. Unincorporated nucleotides were removed using Qiaquick PCR cleanup spin columns (Qiagen), and the volume was reduced to 9.5 μl. The aminosilane-coated slides were prehybridized for 3–4 h at 42 °C in 5× SSC, 0.1% SDS, and 50% formamide solution after UV-cross-linking and baking of the slides for 1 h at 80 °C. The probe was made in 5× SSC, 0.1% SDS, 50% formamide, 1 μg COT DNA (Roche Molecular Biochemicals), 1.25 μg of poly(A) oligo, and 4.25 μl of the fluorescently labeled cDNA in a volume of 25 μl and hybridized to the slides overnight at 42 °C. The next day the slides were washed once in 1× SSC, 0.2% SDS, twice in 2× 0.1 SSC, 0.2% SDS, and dried after a wash in deionized water.

Imaging and Data Analysis—Fluorescence intensities of the immobilized probes were determined from images taken with an Arrayscanner GenerationII (Molecular Dynamics) confocal microscope with laser excitation sources and interference filters appropriate for Cy3 and Cy5 fluores. Separate scans were taken for each fluor at the resolution of 80 μm2/pixel. The image intensities were quantified using ImageQuant software (Molecular Dynamics). A minimal threshold value was set, and spots with values below this threshold were considered negative. The average for the 16 repeats of immobilized probes was taken to calculate the average intensities. The ratio of the average intensities determined the differences of gene expression in the different probes. Control spots, e.g. tubulin and actin cDNA, gave ratios close to 1.

Western Blotting—Control cells were treated with increasing concentrations of LiCl (0.1–20 mM) for 7 h. Lysates were prepared by treating cells with lysis buffer, consisting of 120 mM Tris/HCl, pH 6.8, 5% SDS (w/v), 20% glycerin (v/v), and 1 mM dithiothreitol. The lysates were run through QiaShredder membranes (Qiagen) and stored at −20 °C until used. For cytosolic fractions, cells were lysed in hypotonic buffer (10 mM Hepes, pH 7.2, 1.2 mM EGTA, 1.5 mM MgCl2, 10 mM KCl, and protease inhibitor mixture (Roche Molecular Biochemicals)) by passing through 27G1/2 gauge needles. After centrifugation at 6000 rpm for 10 min, supernatants were spun in a Ultracentrifuge (Optima TLX, Beckman) at 100,000 rpm for 35 min at 4 °C. The supernatant, which contained the cytosolic fraction, was collected, and equal amounts were prepared for SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 10–12% polyacrylamide gels or using NuPAGE polyacrylamide gels with Mes-based running buffer. Resolved proteins were transferred from the gel onto nitrocellulose (Amersham Pharmacia Biotech).

After blocking, membranes were incubated with β-catenin mouse monoclonal antibody (Transduction Laboratories), Cox-2 goat polyclonal antibody (Santa Cruz Biotechnology), and 14-3-3 rabbit polyclonal antibody (Santa Cruz Biotechnology). The membranes were then incubated with a peroxidase-conjugated secondary antibody followed by development with an enhanced chemiluminescence (ECL plus) mixture (Amersham Pharmacia Biotech).

Northern Blot Analysis—Total RNA was isolated from transduced C57MG cells using the RNeasy kit (Qiagen). 10–15 μg of each RNA sample was fractionated by agarose/formaldehyde gel electrophoresis, transferred to nylon membranes, and cross-linked by exposure to UV light.

Cox-2 and periostin-specific probes corresponding to nucleotides 2760–3102 and 651–1026, respectively, were amplified by PCR and labeled with biotinylated dNTPs according to the manufacturer’s protocol (North2South Biotin Random Prime DNA Labeling kit, Pierce). The hybridization and detection were done by following the protocol for the North2South chemiluminescence hybridization and detection kit.
(Pierce), which uses a streptavidin horseradish peroxidase-coupled antibody for detection. Detection of GAPDH mRNA helped to determine differences in RNA loading for each lane.

*Lef-1 Reporter Gene Assay*—8 × 10⁴ cells were grown overnight in 12-well plates. The cells were transfected with LeF-1, LeF-1 reporter, and Renilla plasmid using LipofectAMINE Plus (Life Technologies, Inc.) 24 h later and after cell lysis, luciferase reporter activity (Promega) was measured.

**GSK-3 Kinase Assay**—To measure GSK-3 kinase activity in vitro, recombinant GSK-β was incubated in 50 mM Tris, pH 7.5, 1 mM dithiothreitol, and 10 mM MgCl₂ with 0.4 μg of pre phosphorylated P-cAMP-response element-binding protein peptide (SGSGKREILSR-RSPgYR) with varying concentrations of LiCl (1–20 mM). The kinase reaction was started by adding an ATP mixture including [γ-³²P]ATP. After a 20-min incubation at room temperature, the reaction mixture was pipetted onto phosphocellulose filters. The filters were washed four times in 75 mM H₃PO₄ and air-dried, and radioactivity was measured in a scintillation counter. Each kinase reaction was done in triplicate.

**Antisense Oligonucleotide Transfection**—Antisense oligonucleotides against mouse β-catenin (antisense β-cat: 5′-GGAGGTTCACACAA-CAGCCAGTCCC-3′) or reverse control oligonucleotides (reverse control β-cat: 5′-CCTGACGACACACCAATTTGAGG-3′) were transfected into C57MG cells at a final concentration of 100 nM by using a cationic peptoid reagent (19). Cells were collected for quantitative RT-PCR in RNA lysis buffer, protein lysis buffer for Western blot analysis, or passive lysis buffer for the LeF-1 reporter gene assay.

**Quantitative RT-PCR**—Using a RT reaction kit (Perkin-Elmer), 1 μg of total RNA from different antisense oligonucleotide transfection experiments was reverse transcribed. After 10% of the RT reaction was amplified in a quantitative PCR using the LightCycler System (Roche), the product was quantified using a standard curve that correlated each cycle number at which the amplification of the product was in the linear phase with a value. This value was then normalized to the value of the internal standard GAPDH or actin for each probe.

**Prostaglandin Assay**—4 × 10⁴ cells were grown for 48 h in 24-well plates. Conditioned medium was centrifuged for 5 min at 6000 rpm. 25–50 μl of the supernatant was assayed for prostaglandin E₂ (PGE₂) in an enzyme immunoassay according to the manufacturer’s protocol (Amersham Pharmacia Biotech).

**RESULTS**

To identify Wnt-3 target genes, a reverse transcriptase-PCR-based differential display analysis was used to compare mRNA levels from two C57MG cell lines that were transduced with either Wnt-3 or control vector. The mammary epithelial cell line C57MG displays a morphological response to expression of Wnt-3 and several other Wnt proteins (20) and has been used extensively for studies on the Wnt pathway. The cells acquire an elongated spindle-like cell shape (Fig. 1A) and show increased levels of cytosolic β-catenin (Fig. 1B) in response to Wnt-3 expression.

Preparations of total RNA from Wnt-3 transduced cells and from control cells were reverse-transcribed and amplified in PCR reactions with 240 primer pairs to profile gene expression. Out of the 26 sequences that were differentially expressed, 19 were up-regulated and 7 were down-regulated in Wnt-3 cells versus control cells. From those 26 genes, 14 sequences were known, 11 were ESTs and one sequence was unknown. Sequenced fragments were considered as known if they showed a sequence identity greater than 90% with any gene in the NCBI nucleotide data base.

The differential expression pattern was validated by microarray and Northern blot analyses. For the microarray analysis, the 26 cDNA fragments were cross-linked to a glass slide. Using labeled Cy3/Cy5 cDNA probes from Wnt-3 and control cells, respectively, Cox-2 gave a 3-fold up-regulated and periostin a 10-fold down-regulated difference in signal (Fig. 2). These data were confirmed by Northern blot analysis of mRNA from Wnt-3 and vector-transduced C57MG cells (Fig. 3, A and B). Unexpectedly however, periostin mRNA levels did not change in response to ΔN89β-catenin overexpression (Fig. 3C).

Whether the observed levels of mRNA were reflected in protein levels was assessed for Cox-2 in lysates prepared from Wnt-3-expressing cells when compared with lysates from vector control cells (Fig. 4A). Levels of periostin protein could not be measured, because no periostin-specific antibodies were available. In another unexpected result, C57MG cells ectopically expressing ΔN89β-catenin did not have increased Cox-2 protein levels (Fig. 4A). The presence of ΔN89β-catenin in the cytosol was confirmed by Western blot analysis (Fig. 4D), and its transcriptional activity was verified as a 54-fold increase in the β-catenin-LeF-1-dependent reporter gene expression (Fig. 4C).

Conditioned medium from cells transduced with either Wnt-3, ΔN89β-catenin, or the empty vector, were tested for PGE₂, which is one of the main Cox-2 products. Correlating with the Cox-2 protein expression levels (Fig. 4A), Wnt-3-expressing cells secreted 7–10-fold more PGE₂ compared with the control cells and ΔN89β-catenin-expressing cells (Fig. 4B). These data clearly show that Wnt-3 regulates Cox-2 and periostin expression levels in C57MG cells, most likely via a pathway that does not involve β-catenin.

Another component of the Wnt-3 signaling pathway is GSK-3. We tested whether Li⁺, an inhibitor of GSK-3 (21), alters Cox-2 and periostin expression in response to GSK-3 inhibition. Treatment of C57MG cells with increasing concentrations of LiCl resulted in a 3-fold increase of Cox-2 protein levels.
but not by LiCl, indicating that the GSK-3 enzyme activity is relieved. In the case of Wnt-3, the inhibition of GSK-3 pathway that is independent of β-catenin/TCF-mediated transcription has been shown.

The present study presents evidence that Cox-2 and perios-
tin are two genes whose expression is regulated by Wnt-3 in a pathway that is independent of β-catenin/TCF-mediated transcription. We have shown that Cox-2 and perios-
tin are regulated by the inhibition of GSK-3 by LiCl but not by β-catenin. This suggests that a pool of Wnt target genes exists that is independent of β-catenin signaling.

Studies performed to date have shown that β-catenin stabi-
lation seems to be required for Wnt-induced transformation. These data are consistent with studies showing that mutations in β-catenin, present in human tumors and cancer cell lines, result in a stabilized cytoplasmic pool (22, 23). Cytosolic β-catenin then complexes with the Lef-1/TCF transcription factor(s) and activates gene transcription. Interestingly, there is no
change in the morphology of C57MG cells when they are transduced with ΔN89β-catenin or incubated with LiCl in contrast to transduction by Wnt-3, which promotes a spindle-shaped morphology (Fig. 1A and data not shown). These differences in morphology suggest that even though, as previously reported, there is overlap between genes regulated by Wnt and by β-catenin as well as by Li⁺ inhibition of GSK-3, there must also be significant differences.

In the present study, a RT-PCR based differential display approach was used to examine the gene profiles regulated by Wnt-3. Cox-2 expression increased and periostin expression decreased in response to activation of the Wnt pathway(s). These genes were also regulated by inhibition of GSK-3 but overexpression of ΔN89β-catenin failed to mediate the same changes. In addition to overexpression of ΔN89β-catenin, the reduction of β-catenin expression levels by antisense oligonucleotides (Fig. 8A). Both Wnt-3 and inhibition of GSK-3 by LiCl suppress the mRNA levels of periostin. However, neither cells overexpressing

![Fig. 6. Reduction of β-catenin RNA levels by antisense oligonucleotide transfection has no effect on the induction of Cox-2 by inhibition of GSK-3. A, C57MG cells were transected with β-catenin antisense or reverse control oligonucleotides. After 24 h the cells were incubated with 30 mM LiCl for 9 h, and Cox-2 protein levels were analyzed by Western blotting. The lower panel shows 14-3-3 protein as a loading control. B, β-catenin mRNA levels were measured by quantitative PCR and normalized to GAPDH in each sample treated with antisense or reverse control oligonucleotides and LiCl as in A. RC, reverse control; AS, antisense.](image)

![Fig. 7. The decrease in periostin mRNA levels in response to LiCl treatment is independent of reduced levels of β-catenin. Total RNA from cells that were transected with β-catenin antisense or reverse control oligonucleotides and consequently treated with 30 mM LiCl for 7 h was reverse-transcribed, and mRNA levels of β-catenin and periostin were measured in a quantitative PCR reaction. Periostin and β-catenin mRNA levels were normalized to actin mRNA in each probe. RC, reverse control; AS, antisense.](image)

![Fig. 8. β-Catenin antisense oligonucleotide transfection blocks Lef-1 reporter gene activation in response to Wnt-3a and LiCl. A, C57MG cells were transected with antisense or reverse control oligonucleotides, and 48 h later treated with Wnt-3a-conditioned medium or 30 mM LiCl for 7 h. The fold induction compared with control of the Lef-1-dependent luciferase activity is shown. The corresponding β-catenin mRNA levels are measured by quantitative PCR and normalized to GAPDH mRNA levels in each probe (B). RC, reverse control; AS, antisense.](image)

![Fig. 9. Wnt-3 regulates periostin and Cox-2 mRNA levels independent of β-catenin mRNA levels. Wnt-3 expressing cells and control cells were transected with antisense (AS) or reverse control (RC) oligonucleotides. Total RNA from the cells was reversed-transcribed 48 h after transfection, and β-catenin, Cox-2, and periostin mRNA levels were determined by quantitative PCR and normalized to actin or GAPDH.](image)
ΔN89β-catenin or the reduction of β-catenin expression levels by antisense oligonucleotide transfection resulted in a change of periostin mRNA levels (Figs. 3C and 7).

Our findings suggest that the transcriptionally active β-catenin-Lef-1 complex is not sufficient to repress periostin levels or to induce Cox-2 and that other or additional factors are employed to mediate transcriptional responses to GSK-3 inhibition and Wnt-3 signaling. This is supported by data obtained from the antisense oligonucleotide transfection experiments. The fact that GSK-3 inhibition is a common regulatory mechanism in a number of ligand-stimulated pathways, for example by insulin and epidermal growth factor (25, 26), which do not increase cytosolic levels of β-catenin, indicates that there may also exist additional Wnt pathway(s) that are β-catenin-independent. A possible way by which the transcriptional effects of these growth factors could be mediated is via the transcription factor c-jun that can be inhibited by GSK-3 phosphorylation (24).

The increase in Cox-2 protein correlates with an increase in PGE2 synthesis and was seen after Wnt-1 expression, as observed previously by Hedgepeth et al. (24). Cox-2 mRNA levels and consequently prostaglandin synthesis are markedly increased in over 80% of human colorectal cancers (28, 29). The precise role for Cox-2 in intestinal tumorigenesis is not fully understood, but the variety of effects mediated by prostaglandins indicate the negative regulation of apoptosis in cells that are otherwise supposed to undergo cell death and induced release of angiogenic factors in endothelial cells (30, 31).

An indirect link has been made between Cox-2 and the Wnt pathway in a study where Cox-2 inhibitors displayed antineoplastic activities in a mouse model for familial adenomatous polyposis. In this model, loss of APC causes an increase of polyps in the colon of mice, and a Cox-2 inhibitor was able to attenuate this effect (32). Interestingly, despite the elevation of Cox-2 expression by LiCl, no link has been established in patients treated with Li+ for mental disorders and the development of cancer (33).

That periostin is a gene regulated by Wnt-3 signaling is described here for the first time. However, its function as a downstream target of the Wnt pathway still needs to be clarified. Initially, periostin was identified as a secreted factor in a screen of a mouse osteoblastic library. Its potential function in bone adhesion is based on its sequence similarity to the adhesion protein fascinulin 1 (34, 35) and because it is expressed in specialized connective tissues that form and support mineralized tissues (36). Interestingly, periostin was also identified in a subtractive hybridization experiment comparing embryonic rhabdomyosarcoma cells and primary lung fibroblasts (37). Periostin was down-regulated in the tumor cells compared with normal lung fibroblasts. The loss of periostin expression correlated with the transformed phenotype. This is directionally consistent with our findings, if one assumes that Wnt can promote transformation.

The data in the present study suggest that even though there is overlap between genes regulated by Wnt-3 and β-catenin, there are also significant differences. Distinguishing between pools of Wnt-regulated genes that can be induced via different routes will allow for a better understanding of the significance with respect to oncogenic β-catenin mutations that would specifically induce only a subset of Wnt-regulated genes as well as an independent pool of genes not regulated by Wnt. Because mutations in both β-catenin and APC are found in a high percentage of tumors, this knowledge could have important consequences for the identification of therapeutic targets.

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