Twist Is Up-Regulated in Response to Wnt1 and Inhibits Mouse Mammary Cell Differentiation

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ABSTRACT

Wnt1, initially identified as a mammary oncogene, can activate transcription via β-catenin/TCF complexes. Twist, a transcription factor of the basic helix-loop-helix class, has also been suggested to have oncogenic properties. The aim of this study was to determine whether Twist is regulated by Wnt1 and might thus be a novel mediator of Wnt signaling. We found that Twist was up-regulated in C57MG and HC11 murine mammary epithelial cells in response to Wnt1 expression. Additionally, we detected Twist expression in normal mammary gland and found elevated Twist expression in ~70% of mammary tumors from Wnt1 transgenic mice. A murine Twist promoter fragment was shown to be responsive to β-catenin, and its activity was enhanced by coexpression of c-jun and Ets factors of the PEA3 family. Both PEA3 factors and c-jun were highly expressed in tumors from Wnt1 transgenic mice and may therefore contribute to the increased Twist expression observed in these tumors. To evaluate functional consequences of Twist induction, we examined the effect of Twist on mammary cell differentiation. Strikingly, overexpression of either Wnt1 or Twist in HC11 mammary epithelial cells completely suppressed induction of the milk protein β-casein in response to lactogenic hormones. Additionally, Wnt1, but not Twist, partially abrogated induction of WDNM1, another marker of lactogenic differentiation. Taken together, our data indicate that Twist expression is regulated by Wnt/β-catenin signaling and that both Wnt1 and Twist can function as inhibitors of lactogenic differentiation, an effect that could contribute to mammary tumorigenesis.

INTRODUCTION

Wnt proteins are secreted signaling factors that play multiple roles during development, including essential functions in gastrulation, limb patterning, brain morphogenesis, kidney formation, and placental development. Activation of Wnt signaling is also associated with tumorigenesis. Wnt1, the founder member of the Wnt gene family, was initially identified as a mammary oncogene insertionally activated by mouse mammary tumor virus (1–3). Overexpression of Wnt1 in vivo results in mammary tumorigenesis (4) and in vitro causes morphological transformation of mammary epithelial cell lines such as C57MG and RAC311 (5, 6). Additionally, several WNT genes are misexpressed in human breast cancer (7–9), and mutation activation of the Wnt signaling pathway occurs in numerous human epithelial cancers (8, 10).

The canonical Wnt signaling pathway involves stabilization of a cytoplasmic β-catenin pool (11, 12), which can modulate transcription by interaction with transcription factors, including those of the TCF family (13, 14). Inappropriate β-catenin accumulation with consequent transcriptional activation can result from Wnt gene misexpression but also occurs as a consequence of mutation of the β-catenin gene (CTNNB1) itself or of other components of the Wnt/β-catenin signaling pathway such as AXIN or APC (10). Thus, there is considerable interest in identifying transcriptional targets of Wnt/β-catenin signaling, particularly those that contribute to tumorigenesis. Several candidate genes have been identified, including cyclin D1 (15, 16), c-myc (17), Matrilysin (18, 19), PPAR (20), WISP-1 (21), c-jun, and fra-1 (22). We are interested in identifying additional Wnt transcriptional targets and understanding their roles in Wnt-activated processes.

We focused on Twist as a candidate Wnt target gene based on evidence from Drosophila developmental genetics. Twist was first described in Drosophila as a gene essential for dorsoventral polarity (23, 24) and encodes a transcription factor of the bHLH family. Ventral Twist expression in early embryos is regulated by the Rel family Dorsal (25–27). Additionally, Twist expression is diminished in Drosophila embryos that are deficient in wingless, the Drosophila homologue of Wnt1 (28), suggesting that Twist might also be regulated by Wnt signaling. In mammals, Twist contributes to morphogenesis of the cranial neural tube: Twist-null mice die at E11.5 with unfused cranial neural folds, as well as defects of the head mesenchyme, somites, and limb buds (29). Embryonic Twist expression patterns are consistent with contributions to neural and limb development (30–32). Germ-line mutations at the Twist locus in humans cause Saethre-Chotzen syndrome, an autosomal-dominant craniosynostosis syndrome that results in premature closure of the coronal sutures of the skull (33, 34). Additionally, Twist shows some oncogenic properties, promoting colony formation of mouse embryonic fibroblasts in soft agar and antagonizing p53-induced growth arrest (35).

Here we demonstrate that Twist is up-regulated in response to Wnt1 expression in mouse mammary epithelial cell lines and tumors. Analysis of Twist promoter regulation revealed responsiveness to β-catenin, c-jun, and Ets factors of the PEA3 family. All of these are elevated in Wnt1-expressing mammary tumors and may therefore contribute to the observed Twist up-regulation in these tumors. We also found that overexpression of either Wnt1 or Twist abrogated prolactin-stimulated β-casein induction in a cell culture model. Furthermore, up-regulation of an additional lactogenic marker, WDNM1, was also diminished in Wnt1-overexpressing cells. Since suppression of terminal differentiation is associated with tumorigenesis, Wnt-mediated suppression of lactogenic differentiation could contribute to Wnt-induced mammary tumorigenesis.

MATERIALS AND METHODS

Plasmids. A single genomic clone containing the Twist gene was isolated from a lambda Fix II C3H mouse genomic library (Stratagene, La Jolla, CA). The library was probed with a 268-bp fragment generated by PCR with primers designed to amplify a region of the 3′ untranslated region straddling an intron. Primers were 5′-CGGAGACCTAGATGTCATTGTTCCC-3′ and 5′-GGG-

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6 The abbreviations used are: bHLH, basic helix-loop-helix; MG, mammary gland; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DIP, dexamethasone/insulin/prolactin; FBS, fetal bovine serum; WAP, whey acidic protein.
GACACAAACGAGTGTTCAG-3′. A clone of ~9 kb was isolated and subcloned into pBluescript SK+ (Stratagene). A 2-kb NcoI fragment containing ~70 bases from exon 1 (30) plus 1.9 kb of upstream sequence was subcloned from this construct into Smal-cut pGL2basic (Promega, Madison, WI) to generate the promoter reporter construct Twist-LUC. An additional promoter reporter construct Rev-Twist-LUC was also generated in which the 2-kb promoter fragment was inverted in the reverse orientation, pmV-Twist was constructed by subcloning a PCR product encompassing the entire coding region of Twist into the retroviral vector pMV7 (PCR primers; 5′-CCGGA-ATTCATGATCCAGGCT-3′ and 5′-CCCGAATCTAGTGGAGCGCAGC-3′). pSK-Twist was generated by subcloning a blunt 859-bp SacII fragment of genomic Twist comprising the entire Twist open reading frame into NotI-digested pBluescript SK+ using NotI linkers. pRC-Twist was constructed by subcloning a BangHI-AviII fragment from pSK-Twist containing the entire coding sequence of Twist into pRC-CMV (Invitrogen, Carlsbad, CA). All constructs were verified by DNA sequencing by the DNA/Protein Technology Center (Rockefeller University, New York, NY). The plasmids used for transfection of 293 cells (pMT23, pMT2β-catenin, pCANAN9β-catenin, pCANmycPEA3, pCDNA-ER81, pCANmycERM, Ets-1, pSG5-Ets-2, pCMX-c-jun, pRL-TK, and the stromelysin-1 promoter construct p75TR-Luc) were as described previously (18, 36).

Mouse Tissue and Tumor Harvesting. A breeding colony of Wnt1 transgenic mice (4) was maintained by crossing Wnt1 transgenic B6/SJL males (Jackson Laboratory) with strain-matched females. Mice were genotyped by PCR analysis of tail-tip DNA, as described previously (36). (Jackson Laboratory) with strain-matched females. Mice were genotyped by PCR analysis of tail-tip DNA, as described previously (36).

RESULTS

Twist Expression is Up-regulated in Response to Wnt1. Our interest in Twist as a candidate Wnt-regulated gene was stimulated by the observation that Twist expression is diminished in wingless mutant Drosophila embryos (28). One potential explanation for this observation is that Twist is transcriptionally regulated by wingless, the Drosophila homologue of Wnt1. Therefore, we asked whether mammalian Twist is a target of Wnt/β-catenin signaling. As an initial approach, we evaluated Twist expression in Wnt1-expressing C57MG cells because C57MG cells have previously proved useful as a model cell line for identifying Wnt target genes relevant to carcinogenesis (21, 37, 41). Northern blot analysis demonstrated significant up-regulation of Twist expression in C57/Wnt-1 cells relative to expression levels in C57/MV7 cells (Fig. 1A), and a corresponding increase in Twist protein was observed (Fig. 1B). We had previously made the novel observation that Twist is expressed in adult murine MG during a PCR-based screen designed to identify BHLH proteins involved in the process of mammary differentiation (data not shown). Therefore, we also examined Twist expression in wild-type MG and in mammary tumors from Wnt1 transgenic mice. Interestingly, we observed increased expression of Twist RNA in five of seven tumors tested relative to that observed in normal MG (Fig. 1, C and D). Taken together, these data suggest that Twist is regulated by Wnt signaling.

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Coordinate Regulation of the Twist Promoter by β-Catenin, c-jun, and PEA3 Factors. To investigate transcriptional regulation of the Twist gene, we generated a Twist promoter reporter construct (Twist-LUC) by insertion of a 2.0-kb murine Twist promoter fragment into the luciferase reporter construct pGL2basic. Alignment of this promoter fragment with the previously cloned murine Twist gene (30), and the human Twist gene (42) is shown in Fig. 2A. At the 5′ end, our sequence extends the previously characterized murine sequence (30) by ~1280 bases. At the 3′ end, the fragment extends into exon 1 but contains no coding sequence.

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Transient transfection experiments were performed using Twist-LUC to test whether the Twist promoter was responsive to overexpressed β-catenin. The Twist promoter construct Twist-LUC exhibited...
Twist expression is increased in response to Wnt1. A, total RNA was prepared from C57/MV7 and C57/Wnt-1 cells, and 20 μg of RNA were analyzed by Northern blotting. The blot was probed sequentially with a murine Twist probe and a GAPDH probe. Transcript sizes are 1.7 and 1.4 kb for Twist and GAPDH, respectively. B, cell lysates were prepared from C57/MV7 and C57/Wnt-1 cells, and 50 μg were subjected to SDS-PAGE and Western blotting with anti-Twist antibody. The positions of molecular mass markers (in kDa) are indicated. A Twist protein band was detected in C57/Wnt-1 but not in C57/MV7 cells.

Fig. 1. Twist expression is increased in response to Wnt1. A, total RNA was prepared from C57/MV7 and C57/Wnt-1 cells, and 20 μg of RNA were analyzed by Northern blotting. The blot was probed sequentially with a murine Twist probe and a GAPDH probe. Transcript sizes are 1.7 and 1.4 kb for Twist and GAPDH, respectively. B, cell lysates were prepared from C57/MV7 and C57/Wnt-1 cells, and 50 μg were subjected to SDS-PAGE and Western blotting with anti-Twist antibody. The positions of molecular mass markers (in kDa) are indicated. A Twist protein band was detected in C57/Wnt-1 but not in C57/MV7 cells.

Fig. 6. A, Western blot analysis of Twist expression in mammary tumors. Although c-jun transcription can be activated by β-catenin in human colorectal carcinoma cell lines (22), we did not observe increased c-jun expression in C57/Wnt-1 relative to control C57/MV7 cells (data not shown). However, c-jun was highly expressed in all six tumors tested (Fig. 5). These data indicate that both c-jun and PEA3 factors may contribute to the Twist up-regulation observed in a subset of Wnt1 transgenic mammary tumors. However, because both c-jun and PEA3 are uniformly expressed at high levels in all tumors tested (Fig. 5; Ref. 36), whereas Twist expression was increased in only five of seven tumors examined (Fig. 1, C and D), the murine Twist promoter may also be subject to regulation by other factors yet to be identified.

Twist Expression Antagonizes β-Casein Induction by Lactogenic Hormones. Twist has previously been demonstrated to antagonize differentiation during mammalian myogenesis and osteogenesis (44, 45). Given our novel observation of Twist expression in mammary cell lines and tissues, we speculated that Twist might similarly repress mammary cell differentiation. To test this hypothesis, we used HC11, a murine mammary epithelial cell line. HC11 cells exhibit up-regulation of milk protein genes in response to lactogenic hormones, with no requirements for extracellular matrix components or cocultivation with other cell types (39). This makes them a convenient in vitro model for studying lactogenic differentiation. HC11 cells overexpressing Twist were generated by retroviral infection with MVTwist, and Twist overexpression was confirmed by Western blotting (Fig. 6A). No Twist protein was detectable in the control infected HC11/MV7 cells or in the parental HC11 cells (Fig. 6A).

DIP was used as a lactogenic stimulus to induce HC11 differentiation (39). Expression of several milk proteins commonly used as markers of lactogenic differentiation was examined, including WDNM1, β-casein, and WAP (46). We were unable to detect WAP expression in either control or DIP-treated HC11 cells (data not shown). However, both WDNM1 and β-casein were up-regulated in HC11/MV7 cells in response to lactogenic stimulation with DIP (Fig. 6, B and C). DIP-mediated up-regulation of the WDNM1 transcript
Fig. 2. Murine Twist promoter. A, alignment of 2-kb murine Twist promoter fragment with published sequences. Features depicted include: open reading frame (●), exons (■), and potential TATA boxes (black rectangles), of which only the 3′ TATA box is functional in human Twist (42). The entire sequence can also be roughly aligned with residues 15391–13419 of the human Twist gene (65). B, nucleotide sequence. Three potential TCF binding sites are shown in bold. Of these, the site at bases 76–83 corresponds well to a consensus TCF site (5′-CCTTTG,A/T,A/T-3′), but the sites at bases 896–902 and bases 962–970 do not precisely match the consensus TCF binding site. None of these are conserved in the human promoter. Thirty-four Ets binding core sequences (5′-GGA,A/T-3′) are boxed in gray.
was unaffected by Twist overexpression (Fig. 6B). Strikingly, however, induction of β-casein RNA in response to DIP was completely absent in HC11/Wnt cells (Fig. 6C). Thus, overexpression of Twist was sufficient to block hormone-stimulated β-casein expression in HC11 cells.

**Ectopic Wnt1 Expression Antagonizes β-Casein Induction.**

Taken together, our observations that Twist can regulate β-casein induction and that Twist itself is regulated by Wnt signaling suggested that Wnt1 might also modulate cellular responsiveness to lacticogenic hormones. To test this possibility, we generated Wnt1-expressing HC11 cells (HC11/Wnt-1) and control cells (HC11/MV7) using retroviral expression vectors. Interestingly, Wnt1 expression in HC11 cells led to morphological transformation (data not shown), as has previously been described for both C57MG and RAC311 cells (5, 6). Expression of Wnt1 in HC11 cells caused an increase in Twist protein and RNA (Fig. 7, A and B), consistent with our observations in C57MG-derived cell lines (Fig. 1). The effect of Wnt1 expression on both β-casein and WDNM1 induction was compared in HC11/MV7 and HC11/Wnt-1 cells, again using DIP as a lacticogenic stimulus. Control HC11/MV7 cells showed robust WDNM1 expression in response to DIP, but Wnt1 expression significantly abrogated WDNM1 induction in response to hormonal stimulation (Fig. 7C). Additionally, we examined the effect of Wnt1 overexpression on β-casein induction (Fig. 7D). Although β-casein was up-regulated by DIP in control HC11/MV7 cells, there was no induction of β-casein in HC11/Wnt-1 cells. Thus, Wnt1 expression, similarly to Twist overexpression, completely abolished the ability of HC11 cells to activate β-casein transcription in response to DIP. Because Wnt1 causes induction of Twist in these cells, their failure to respond to lacticogenic stimuli by activating β-casein transcription may thus be attributable to Twist.

**DISCUSSION**

Twist Regulation by Wnt1. Here, we report that Twist is up-regulated in Wnt1-expressing mouse mammary epithelial cell lines (Figs. 1 and 7). Additionally, we have shown that Twist is expressed in adult murine MG and that Twist expression is significantly increased in mammary tumors from Wnt1 transgenic mice (Fig. 1). To our knowledge, this is the first demonstration that Twist is up-regulated in response to Wnt signaling and that Twist is expressed in mammary tissue. Several Wnt genes exhibit dynamic expression patterns during mammary development (47–50). In particular, the mammary expression profile of Wnt2 resembles that which we have observed for Twist (48).

8 Wnt2 activates the canonical Wnt/β-catenin signaling pathway (12, 51), and we have observed elevated Twist expression in C57/Wnt2 cells (data not shown). Taken together, these data suggest that Wnt2 could contribute to the regulation of endogenous Twist expression in the MG.

Having demonstrated Wnt1-mediated Twist up-regulation, we next...
investigated the mechanism by which Wnt1 transcriptionally activates Twist. A Twist promoter reporter construct was generated containing 2.0 kb of 5′ untranslated sequence from the murine Twist gene (Fig. 2) linked to a luciferase reporter gene. This promoter fragment contains three potential TF binding sites (Fig. 2B). In addition, we identified 34 potential Ets binding sites based on the presence of the core binding site 5′-GGA,A/T-3′, of which approximately one-third were conserved between mouse and human. Analysis of the sequence using MatInspector revealed several API sites and numerous other potential transcription factor binding sites. Luciferase assays demonstrated that this Twist promoter construct was responsive to β-catenin (Fig. 3). Additionally, Ets factors of the PEA3 subfamily activated the Twist promoter, and c-jun enhanced the responses to both β-catenin and PEA3 (Figs. 3 and 4). The synergy between β-catenin, PEA3, and c-jun is particularly striking because similar findings have recently been reported with respect to the human Twist promoter. An additional target of Wnt1 is the Ets factor, PEA3, and c-jun is particularly striking because similar findings have recently been reported with respect to the Drosophila Even-skipped promoter, which contains a functional HNF4α site. This finding suggests that PEA3 factors may contribute to regulation of multiple target genes of the Wnt/β-catenin pathway. Consistent with this hypothesis, the promoters of several genes known to be β-catenin-responsive also contain consensus Ets binding sites. For example, the Drosophila gene Even-skipped is coordinately regulated via TCF and Ets binding sites, and the cdc2 promoter can be activated cooperatively by PEA3, β-catenin, and c-jun. Interestingly, the promoter of the Drosophila Twist gene also contains both Ets and TCF binding sites, suggesting that there may be a role for β-catenin and Ets factors in regulating its expression. This would represent a novel mechanism of Drosophila Twist activation, in addition to the previously established Dorsal-mediated regulation (25–27).

Both Wnt1 and Twist Inhibit Mammary Cell Differentiation. Twist and Wnt1 have previously been shown to antagonize the differentiation of certain cell lineages (44, 45, 53, 54). Therefore, we were interested in determining whether either Twist or Wnt1 might repress lactogenic differentiation in mammary cells. To address this possibility, responsiveness to lactogenic hormones was examined in HC11 cells, using the milk protein transcripts β-casein and WDNM1 as markers of lactogenic differentiation. Overexpression of Wnt1, but not of Twist, diminished WDNM1 up-regulation in response to lactogenic stimuli (Figs. 6 and 7). In contrast, β-catenin induction was effectively abolished by either Wnt1 or Twist (Figs. 6 and 7). Interestingly, the expression profile of Twist during postnatal murine mammary development is consistent with a role for Twist in suppressing β-casein expression in vivo. Mammary Twist expression is constant during early gestation but diminishes during mid pregnancy, exhibiting a reciprocal expression pattern with the milk protein β-casein. Taken together, these observations raise the possibility that Twist may function in vivo to negatively regulate β-casein expression.

The simplest interpretation of the ability of Twist to antagonize β-casein induction is that Twist is acting as a transcriptional repressor. Several mechanisms have been proposed to account for transcriptional repression mediated by mammalian Twist, mostly through analysis of its role in myogenic differentiation (55–57). Additionally, the ability of Twist to bind to and inhibit histone acetyl transferases may be important for Twist-mediated transcriptional repression (58).

Suppression of Differentiation and Tumorigenesis. The ability of Wnt1 to inhibit differentiation, as evidenced by diminished induction of lactogenic markers, may be a contributory factor to Wnt-mediated tumorigenesis. Suppression of terminal differentiation is thought to favor tumorigenesis, a paradigm particularly well illustrated by the HLH-containing Id proteins. Originally identified as inhibitors of myogenic differentiation, Id proteins are now known to function as dominant negative regulators of cell lineage commitment and differentiation in multiple cell types (59, 60). However, Id proteins are also associated with tumorigenesis (61–63), and indeed, expression of an Id1 transgene can induce formation of intestinal adenomas (64). By analogy, the ability of Wnt1 to suppress lactogenic differentiation may be important for its function as a mammary oncogene. This effect of Wnt1 may be mediated in part via Twist up-regulation because overexpressed Twist completely abolishes β-casein induction. However, the failure of Twist to repress WDNM1
induction clearly suggests a requirement for other factors to mediate this effect. In addition to altered differentiation, enhanced cellular proliferation and diminished apoptosis are frequently prerequisites for carcinogenesis. Of the Wnt/β-catenin transcriptional targets thus far identified, several are likely to contribute to these processes (15–17, 20–22). Thus, suppression of terminal differentiation by Wnt1 is only one of a panoply of responses that may contribute to Wnt-mediated tumorigenesis.

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