Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism

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Wnt and Notch signaling have long been established as strongly oncogenic in the mouse mammary gland. Aberrant expression of several Wnts and other components of this pathway in human breast carcinomas has been reported, but evidence for a causative role in the human disease has been missing. Here we report that increased Wnt signaling, as achieved by ectopic expression of Wnt-1, triggers the DNA damage response (DDR) and an ensuing cascade of events resulting in tumorigenic conversion of primary human mammary epithelial cells. Wnt-1-transformed cells have high telomererase activity and compromised p53 and Rb function, grow as spheres in suspension, and in mice form tumors that closely resemble medullary carcinomas of the breast. Notch signaling is up-regulated through a mechanism involving increased expression of the Notch ligands Dll1, Dll3, and Dll4 and is required for expression of the tumorigenic phenotype. Increased Notch signaling in primary human mammary epithelial cells is sufficient to reproduce some aspects of Wnt-induced transformation. The relevance of these findings for human breast cancer is supported by the fact that expression of Wnt-1 and Wnt-4 and of established Wnt target genes, such as Axin-2 and Lef-1, as well as the Notch ligands, such as Dll3 and Dll4, is up-regulated in human breast carcinomas.

Breast cancer is a complex malignancy comprising 18 distinct histopathological entities (1) that are thought to arise through a series of mutations (2). Although a large number of genes have been found mutated or misexpressed in breast carcinomas, it is unclear to what extent these changes contribute to tumorigenesis. The problem is linked to the fact that the earliest identifiable lesion, the carcinoma in situ, already contains most genetic changes and is difficult to study.

Transgenic mouse models are used extensively to model human breast cancer (3). Species-specific properties do not allow one to directly extrapolate findings in mice to humans; in fact, growth control pathways are wired differently in the two species (4). Moreover, genes frequently activated in mouse mammary carcinomas because of insertion of the mouse mammary tumor virus, such as Wnt-1, Notch1, and Notch4 (5), are distinct from the most common targets of mutation in human breast cancers (6). However, recent evidence suggests that Wnt or Notch signaling may also be deregulated in human breast cancer. Thus, expression of different Wnts is increased (7); an extracellular inhibitor of Wnt signaling, secreted Frizzled-related protein 1, is down-regulated in 80% of breast carcinomas (8, 9); and the positive regulator Disheveled is up-regulated (10). Similarly, a tumor suppressor function was suggested for the Notch inhibitor Numb (11).

Wnt signaling is initiated by the interaction of a Wnt ligand with a seven-transmembrane-domain Frizzled receptor and leads to stabilization of β-catenin, one of the central components of the pathway, by inhibiting glycogen-synthase kinase-3β. In the absence of Wnt signaling, β-catenin is targeted for degradation by phosphorylation through a complex of glycogen-synthase kinase-3β, adenomatous polyposis colon protein, protein phosphatase 2A, and Axin-1 (12). Stabilized β-catenin translocates to the nucleus, where it forms a bipartite complex with transcription factors of the T cell factor family and activates target gene expression (13). Notch signaling is triggered by the binding of one of five different membrane-bound ligands, Delta (Dll1, Dll3, Dll4, Jagged1, and Jagged2), to one of four Notch proteins on a neighboring cell. The interaction leads to two proteolytic cleavages of the receptor, resulting in the release of the Notch intracellular domain, which translocates to the nucleus, binds to a highly conserved DNA-binding transcription factor of the CSL family (RBP-Jk/CBF1 in mammals), and recruits coactivators to form a transcriptional activation complex (14).

Here we find that increased Wnt signaling in human mammary epithelial cells (HMECs), as achieved by ectopic Wnt-1 expression, elicits a DNA damage response (DDR), which has recently been shown to be an early event in human carcinogenesis (15, 16), followed by a cascade of events including Notch activation, resulting in transformation to a tumorigenic state. These findings suggest that deregulation of Wnt signaling may be an early event in mammary epithelial transformation.

Results

Effects of Wnt-1 Expression on Primary HMECs. Wnt-1 was originally cloned as a frequent integration site for the mouse mammary tumor virus (17) and is a strong oncogene in the mouse mammary epithelium (18). It is functionally equivalent to other Wnts (19–21), some of which are overexpressed in human breast carcinomas (7). To assess potential effects of increased Wnt signaling in HMECs, we ectopically expressed Wnt-1 in primary HMECs. Specifically, we infected HMECs 10 days after they were derived from reduction mammoplasties, at passage 2 (P2) or P3, with high-titer retroviruses expressing full-length Wnt-1 cDNA or LacZ. Forty-eight hours later infection rates were determined by X-Gal staining to be ~70%. To eliminate uninfected cells, G418 selection was applied. Ten days after infection, the Wnt-1-expressing cell populations showed increased proliferation compared with LacZ controls (Fig. 1A). Wnt-1 protein expression was readily detectable, and biological activity was ascertained in a reporter assay (data not shown). Thirty days after infection, LacZ-expressing cells and uninfected controls

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Abbreviations: HMEC, human mammary epithelial cell; DDR, DNA damage response; Pn, passage n.

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flattened out, showed vacuoles, and began to senesce, as expected (Fig. 1B). In contrast, in the Wnt-1-infected cultures, a subset of cells continued to grow, and some of the cells started to detach from the dish (Fig. 1C). The detached cells began to form spheroid structures around day 40, which continued to grow into more complex structures (Fig. 1D). Cells that were dissociated from these structures were capable of reforming them within 1–3 days, growing indefinitely thereafter (currently P36 after infection). The same observations were made on cells derived from four independent reduction mammoplasties (A, D, E, and K) (Fig. 1A); the cell strains obtained by ectopic Wnt-1 expression will be referred to below as Wnt-1-HMECs.

Tumor Formation by Wnt-1-HMECs. The prolonged lifespan and ability to grow independent of substrate of Wnt-1-HMECs suggested they might be transformed to a tumorigenic state. To test whether these cell populations were indeed tumorigenic, we injected them into mammary glands of 3-month-old immunocompromised RAG2−/− female mice (22). Twenty days after the injection, palpable tumors were detected, which grew to 1 cm in diameter within 6 weeks (Fig. 1E). The efficiency of tumor formation was high for all of the three cell strains tested, with tumors arising in 68% of the injected glands within 2 months (Table 1, which is published as supporting information on the PNAS web site). The cell strains obtained by ectopic Wnt-1 expression will be referred to below as Wnt-1-HMECs.

Histological analysis revealed that Wnt-1-HMEC tumors have round borders and are surrounded by a pseudo capsule (Fig. 1F, arrow). Tumor cells grow as sheets and do not form glandular structures or tubes. Cell nuclei were highly pleiomorphic, and several mitotic figures per high-powered field were found (Fig. 1G, arrows). Necrosis was frequent. All of these features are diagnostic criteria for typical medullary carcinoma of the breast, a histological subtype that represents ~2% of breast carcinomas (26). To assess whether Wnt-1-HMEC tumors resemble medullary carcinomas at the molecular level, we examined expression of characteristic markers by immunohistochemistry. Like typical medullary carcinomas, the Wnt-1-HMEC tumors were positive for cytokeratin 18 and negative for cytokeratin 14, estrogen receptors, and progesterone receptors (Fig. 5, which is published as supporting information on the PNAS web site).
Strikingly, >70% of the tumor cell nuclei stained intensely for p53 (Fig. 3), as is characteristic of typical medullary carcinomas (27). A further hallmark of medullary carcinomas, a prominent lymphocytic infiltrate, was absent from Wnt-1-HMEC tumors, as was expected, because the \( RAG2^{-/-} \) mice lack mature B and T cells (22). Thus, the Wnt-1-HMEC form tumors in mice that closely resemble a subtype of human breast tumors, medullary carcinomas, both morphologically and molecularly.

**Mechanisms of Wnt-1-Induced Transformation.** These observations raised the question of how increased Wnt signaling triggers oncogenic transformation. Recent work indicates that a DDR similar to that caused by double-stranded breaks is activated at very early stages of tumorigenesis in the breast and other tissues (15, 16). Central mediator between insult and cellular response is the ATM (ataxia telangiectasia mutated) kinase (28). It autophosphorylates upon activation and proceeds to phosphorylate downstream effectors such as Chk2 (29) and histone H2AX (30). As a result, checkpoints are activated that ensure that a cell either repairs inflicted damage or undergoes apoptosis.

To assess whether Wnt-1 elicits a DDR, we compared levels of phospho Chk2 and phospho H2AX in Wnt-1-HMECs and parental control cells. Both proteins are phosphorylated in the Wnt-1-HMECs but not in the parental cells, whereas total H2AX levels are comparable, indicating that DDR is constitutively active in Wnt-1-transformed cells (Fig. 2A). We also assessed phospho H2AX levels in cells infected with Wnt-1 or LacZ control virus before the appearance of a transformed phenotype (P5). Whereas total H2AX protein levels were similar in the two cell populations, phospho H2AX was detectable in cells ectopically expressing Wnt-1 but not in control cells (Fig. 2B). Thus, the DDR triggered by increased Wnt signaling is an early event before overt signs of transformation.

As shown recently, the activation of a DDR at early stages of tumorigenesis provides selective pressure for inactivation of the G1/S-p16/Rb and the p53 checkpoints (15, 16). Consistent with cell-cycle checkpoint inactivation in Wnt-1-HMEC strains, hyperphosphorylated Rb protein was readily detected but below detection limit in the parental cells (Fig. 2C). To determine the mechanism that might account for the Rb hyperphosphorylation we analyzed the protein levels of different cyclins by immunoblotting. Cyclin D1 and D2 were present in control HMECs but below detection limits in Wnt-1-HMECs, whereas the expression levels of cyclin D3, cyclin A, and the mitotic cyclin B1 were increased in the Wnt-1-transformants (Fig. 2C). Wnt-1 transformation was accompanied by a substantial increase of p16 expression rather than down-regulation, as was previously reported for HMECs (31, 32). Thus, the Rb checkpoint is disrupted by a mechanism involving increased cyclin D3, cyclin A, and cyclin B1 expression and increased p16 levels.

Our finding that p53 protein levels are increased in the Wnt-1-HMEC tumors suggested that p53 is stabilized as a consequence of the DDR through mutations impairing its function (33). To test whether p53 protein levels are already changed during in vitro transformation or only later during in vivo growth, we determined p53 expression in cultured Wnt-1-HMECs and the parental control cells. p53 protein levels were found to be strongly up-regulated in all of the Wnt-1-HMEC strains compared with their parental cells (Fig. 2D). To assess whether p53 function is concomitantly disrupted, we measured mRNA expression levels of the p53 target gene p21 (34). In the presence of high p53 protein levels there was no up-regulation of p21 mRNA in the Wnt-1-HMECs compared with the parental controls (Fig. 2D); protein levels were actually decreased (data not shown). Together, these observations indicate that p53 protein levels are increased whereas its function is compromised during Wnt-1-induced transformation.

Inactivation of checkpoints results in genomic instability with abnormal karyotypes, a characteristic feature of most malignant tumors. Mitotic spreads from each of the four cell strains revealed two distinct cell populations: a larger one of near triploid cells (Fig. 2E) and a smaller one of diploid cells. At later stages of the transformation process telomerase activity is up-regulated to ensure continued adequate chromosome replication (2). Using a real-time quantitative telomeric repeat amplification protocol (35) to measure telomerase activity, we found telomerase activity close to detection limit in the parental control cells (P4 or P5). Wnt-1-HMECs showed a 1,000-fold increase in telomerase activity, comparable to that seen in HeLa cells (Fig. 2F).

**Notch Signaling Activity in Wnt-1-HMECs.** Activation of the DDR is a strong selective stimulus for genomic instability but is not sufficient to transform HMECs given that other oncogenes, such as SV40 large T, elicit a DDR (data not shown) yet fail to transform primary HMECs. To gain further insights into the molecular basis of Wnt-induced transformation we compared the gene expression profiles of Wnt-1-HMECs and parental cells. The Notch target
Notch2 expression did not change, and Notch1 was below detection published as supporting information on the PNAS web site).

Different Wnt-1-HMECs than in the controls (Fig. 3)

The specific activity of this promoter, as determined after normalization of mRNA expression of the endogenous Notch target genes HES-1 and HES-5 in the parental HMECs and Wnt-1-HMECs. All real-time RT-PCR values are expressed as relative arbitrary units after internal normalization for 18S rRNA. (B) Notch signaling activity in different Wnt-1-HMECs (P15–P18) and parental HMECs (P4 and P5) derived from different reduction mammoplasties (D, A, and E) assayed with reporter plasmids containing an artificial Notch/RBP-Jk-binding sites (pGAmut) provides a useful measure of Notch-RBP-Jk signaling components, Dll1, Dll4, Jagged1, and Jagged2, as well as Notch3 and Notch4 in parental HMECs (P4 and P5) and Wnt-1-HMECs (P15–P18) derived from different reduction mammoplasties (D, A, and E).

HES-1 was among the most highly up-regulated genes. Quantification of the mRNA levels of the Notch target genes HES-1 and HES-5 (36, 37) by quantitative RT-PCR showed 10- and 250-fold increases, respectively, suggesting that the Notch signaling pathway might be involved in the transformation (Fig. 3A).

The best characterized mechanism by which Notch activation controls transcription is by converting the DNA-binding protein RBP-Jk/CBF-1 from a transcriptional repressor into an activator (14). Activity of an artificial promoter with concatemerized RBP-Jk-binding sites (pGAwrt) or a control reporter (pGAmut) without RBP-Jk-binding sites, plotted after internal normalization. (C) Immunoblot of Notch signaling components, Dll1, Dll4, Jagged1, and Jagged2, as well as Notch3 and Notch4 in parental HMECs (P4 and P5) and Wnt-1-HMECs (P15–P18) derived from different reduction mammoplasties (D, A, and E).

Fig. 3. Notch signaling in Wnt-1-HMECs and in primary HMECs. (A) RT-PCR analysis of mRNA expression of the endogenous Notch target genes HES-1 and HES-5 in the parental HMECs and Wnt-1-HMECs. All real-time RT-PCR values are expressed as relative arbitrary units after internal normalization for 18S rRNA. (B) Notch signaling activity in different Wnt-1-HMECs (P15–P18) and parental HMECs (P4 and P5) derived from different reduction mammoplasties (D, A, and E) assayed with reporter plasmids containing an artificial Notch/RBP-Jk-responsive promoter (pGAmut) or a control reporter (pGAmut) without RBP-Jk-binding sites, plotted after internal normalization. (C) Immunoblot of Notch signaling components, Dll1, Dll4, Jagged1, and Jagged2, as well as Notch3 and Notch4 in parental HMECs (P4 and P5) and Wnt-1-HMECs (P15–P18) derived from different reduction mammoplasties (D, A, and E).

Wnt Signaling Activity in Human Breast Cancer. To test whether there is an increase in Wnt signaling in clinically occurring tumors that may be linked to up-regulation of Notch Dll ligand expression, we initially assessed levels of Wnt signaling activity in a panel of 34 human breast carcinomas by determining mRNA levels of the direct and specific Wnt target genes Axin-2 (41–43) and Lef-1 (44–46). Expression of these genes, as determined by real-time RT-PCR, was consistently higher in breast carcinomas than in normal breast tissue samples (on average 8- and 12-fold) (Fig. 4). Comitantly, expression of the Notch ligand Dll4 was remarkably increased in >90% of the tumors (on average 25-fold), with expression of Dll3 being also augmented in a third of them. Of the existing 16 human Wnt family members, Wnt1, 2, 4, 5a, 5b, 7a, 8b, 9a, 9b, 10b, and 11 are expressed in normal human breast samples (our unpublished observations). Of these, in parallel with the increased Wnt signaling, Wnt-1 and Wnt-4 were overexpressed in tumors, whereas the other family members were not (Fig. 4).

Discussion

Taken together, our results show that increased Wnt signaling is sufficient to cause transformation of primary HMECs, with early activation of the DDR followed by a cascade of events resulting in the tumorigenic phenotype. Expression of Notch ligands Dll1, Dll3, and Dll4 is increased, and Notch activation is required for Wnt-induced transformation both in vitro and in vivo. Finally, analysis of a substantial number of human breast carcinomas indicates that these findings are likely to be relevant to the clinical situation.

The transforming effects of increased Wnt signaling are unique, in that neither expression of SV40 small and large T antigens nor limit. Interestingly, expression of the ligands of the Jagged family was reduced, probably reflecting differences in amplified cell populations as discussed below.
Besides the DDR and ensuing biochemical events, Wnt-1-induced transformation of HMECs is associated with increased Notch signaling through a mechanism that is likely to involve up-regulation of Notch ligands of the Dll family. Our findings that dll3 and 4 are overexpressed in human breast cancers suggest that this observation is likely to be clinically relevant. In the case of Jagged ligands, a recent report shows that high Jagged1 expression in human breast carcinomas correlates with poor prognosis (49). The tumors that we obtained by Wnt-1 transformation have low levels of Jagged1 and resemble typical medullary carcinomas of the breast, which are characterized by a good prognosis despite their high-grade characteristics (nuclear morphology and high mitotic index). Thus, they fall into the categories of breast tumors that are expected to show relatively low Jagged expression. Additionally, in the normal human breast, at least Jagged1 expression is localized specifically to myoepithelial rather than luminal cells (49). In Wnt-1-HMEC cultures, immunostaining revealed expressed of the luminal marker cytokeratin 18, whereas in the control cultures 30 days after infection <5% of the cells express this marker, with the remaining population expressing cytokeratin 14, a marker of myoepithelial cells (S. Gass and C.B., unpublished observations). Thus, the loss of myoepithelial cells during Wnt-1-induced transformation may also account for the low Jagged1 levels, which contrast with the up-regulation of Dll expression and associated increase in Notch signaling activity.

Irrespective of the specific ligands involved, Notch activation is likely to play an important role in breast carcinogenesis. In fact, we have shown that increased Notch activity is required for Wnt-induced transformation and is by itself sufficient to reproduce significant aspects of this process (formation of 3D structures). Both Wnt and Notch signaling pathways are important in maintaining and amplifying progenitor cells in different tissues (50–52), including the breast (53–55). Thus, in concomitance with the biochemical events described above, a further factor to be considered is the existence of subpopulations of HMECs with different susceptibility to malignant transformation that may be selectively amplified by activation of Wnt and/or Notch signaling pathways. The crosstalk between these pathways may impinge especially on early steps of breast carcinogenesis, with potential impact on novel treatments and/or prevention of breast cancer.

Methods

Cell Culture and Retroviral Infection. Normal human breast tissue was obtained from women undergoing reduction mammoplasties, with no previous history of breast cancer, who gave their informed consent. All samples were confirmed by histopathological examination to be free of malignancy. Primary HMECs were derived from these specimens as described (56). At P2 or P3, cells were spin-infected with high-titer amphotropic retroviruses as described (57) and selected with G418 (200 μg/ml). Notch intracellular domain cDNA was subcloned into MSCV2.2 (57) with a neomycin resistance gene. NIH 3T3 cells stably expressing Dll or Jagged (39) were grown in DME/10% calf serum and treated for 2 h with 5 μg/ml mitomycin before mixing with Wnt-1-HMECs.

Quantitative Real-Time PCR. Total RNA (1 μg) was reverse-transcribed by using reverse transcriptase (Invitrogen) and random hexamers (Roche). The resulting cDNAs were used for quantitative PCR analysis by using the iCycler apparatus (Bio-Rad) and the SYBR Green PCR Core Reagents system (Qiagen). Results were evaluated with iCycler IQ real-time detection system software (Bio-Rad). Samples were run in triplicate, and all quantifications were normalized to endogenous control 18S rRNA. For primer sequences, see Supporting Methods, which is published as supporting information on the PNAS web site.

Transient Luciferase Assays. Transient transfections were performed by using FuGENE (Roche) according to the manufac-
turer’s instructions. A total of 10^6 cells were cotransfected with 25 ng of luciferase reporter for RBP-Jκ activity, either pGAwt or pGAmut, and 25 ng of Renilla activity by using the dual-luciferase reporter assay system (Promega, E1910).

Western Blot Analysis. For details, see Supporting Methods.

Mice. RAG2^−/− (129SV/C57BL/6) mice were purchased from Taconic Farms and bred under conventional conditions with a 12-h cycle of light and dark in filter-top cages. They were supplied ad libitum with irradiated feed and water. Tumor size was measured every 3–4 days.

Tumor Assay. A total of 10^5 Wnt-1-HIMECs were resuspended on their own or mixed with 3 × 10^6 mitomycin-treated NIH 3T3 cells in 100 μl of PBS and injected into inguinal mammary glands of 3-month-old RAG2^−/− females.

RNA Isolation. Frozen tumor samples and reduced mammaryplasty tissue, enriched for epithelial components, were used for total RNA extraction with TRIzol (GIBCO). After RNAse (Qiagen) treatment, RNA was dissolved in RNAse-free water.

Histopathology. Samples were fixed in 10% buffered formalin and embedded in paraffin. Six-micrometer sections were stained with hematoxylin and eosin according to standard protocols. Antibodies against cytokeratin 18 and α1-proteinase inhibitors, progesterone receptor (Neomarkers, MS-142, RB-9020, RB-9101, and RB-9232), and α5 (Novocorsa NCL-p53D01) were diluted as indicated by the supplier and applied overnight at 4°C. Biotinylated secondary antibodies were detected with the VestaStain Elite Kit (Vector Laboratories).

Real-Time Quantitative Telomeric Repeat Amplification Protocol. Real-time quantitative telomeric repeat amplification protocol was performed as described in ref. 35 by using 0.25 μg of protein extract per 25-μl reaction with the SYBR Green PCR Core reagents system (Qiagen).

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