

Paracrine Wnt signaling both promotes and inhibits human breast tumor growth

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Contributed by Roeland Nusse, March 7, 2013 (sent for review December 9, 2012)

Wnt signaling in mouse mammary development and tumorigenesis has been heavily studied and characterized, but its role in human breast cancer remains elusive. Although Wnt inhibitors are in early clinical development, it is unclear whether they will be of therapeutic benefit to breast cancer patients, and subsequently, to which ones. To address this, we generated a panel of Wnt reporting human breast cancer cell lines and identified a previously unrecognized enrichment for the ability to respond to Wnt in the basal B or claudin-low subtype, which has a poor prognosis and no available targeted therapies. By co-injecting Wnt3A expressing human mammary fibroblasts with human breast cancer cell lines into mouse mammary fat pads, we showed that elevated paracrine Wnt signaling was correlated with accelerated tumor growth. Using this heterotypic system and a dual lentiviral reporter system that enables simultaneous real-time measurement of both Wnt-responsive cells and bulk tumor cells, we analyzed the outcome of elevated Wnt signaling in patient-derived xenograft (PDX) models. Interestingly, the PDX models exhibited responses not observed in the cell lines analyzed. Exogenous WNT3A promoted tumor growth in one human epidermal growth factor receptor 2-overexpressing PDX line but inhibited growth in a second PDX line obtained from a patient with triple-negative breast cancer. Tumor suppression was associated with squamous differentiation in the latter. Thus, our work suggests that paracrine Wnt signaling can either fuel or repress the growth of human breast cancers depending on yet to be determined aspects of the molecular pathways they express.

tumor stroma | luciferase | Wnt responsive reporter

Wnt signaling plays myriad roles during development and is widely implicated in human diseases. The complexity of Wnt signaling derives from a plethora of ligands (19 Wnts) and receptors [10 Frizzleds, 2 low-density Lipoprotein related receptor protein (Lrp) coreceptors, and 3 receptor tyrosine kinases (RTK): Ror1, Ror2, and Ryk] and deepens with cross-talk between canonical (β -catenin dependent) and noncanonical (β -catenin independent) signaling mechanisms. Canonical Wnt signaling is initiated when a Wnt protein binds to a transmembrane Frizzled receptor and the coreceptor Lrp5/6. This association triggers a cascade of events leading to the stabilization of the intracellular transducer β -catenin, which accumulates, translocates to the nucleus, and serves as a transcriptional coactivator with the T cell factor (Tcf)/lymphoid enhancer-binding factor (Lef) family of transcription factors (1). Wnt was first identified for its ability to induce mammary tumors in mice (2), and Wnt is unique in its ability to transform human mammary epithelial cells when overexpressed alone (3).

Human breast cancers are routinely classified into subtypes in the clinic by immunohistochemical (IHC) analysis of their expression of the estrogen, progesterone, and human epidermal growth factor receptor 2 (Her2) receptors. Low or undetectable expression of these three targetable receptors defines the triple-negative breast cancer (TNBC) subtype, which has the worst prognosis and the fewest treatment options. This heterogeneous group of tumors has been associated with active Wnt signaling by IHC analysis of β -catenin in tumor samples and by expression profiling studies

(reviewed in ref. 4), raising the possibility that blocking Wnt signaling could provide a treatment for TNBC.

Part of the controversy surrounding the role of Wnt signaling in human breast cancer stems from the rarity of pathway-activating mutations in human breast tumors (5, 6), as opposed to colon cancer, for which such mutations are common (7). Although mutations are rare, negative regulators, particularly soluble inhibitors such as secreted frizzled related proteins, Dickkopf, and Wnt inhibitory factors, frequently exhibit reduced expression via methylation (reviewed in refs. 8 and 9). This suggests that Wnt activity in vivo may be regulated in a paracrine fashion, consistent with the secreted nature of Wnt ligands. The potential source of Wnt ligands in vivo is likely to be in close proximity to the cancer cells because Wnts diffuse poorly owing to their strong association with membranes and extracellular matrix, their instability at physiological temperatures, and their lack of solubility. Interestingly, Wnt signaling contributes to the pathogenesis of several human breast cancer cell lines (10–13). The effects of pathway activation in trans have not been investigated.

Immortalized cell lines may acquire or be selected for stromal independence upon adaptation to in vitro culture. Therefore, we developed a system to investigate the role of paracrine Wnt signaling in both cell lines and patient-derived xenograft (PDX) models. PDX lines are generated by implanting human tumor tissue orthotopically into immunocompromised mice and maintained by serial transplantation in vivo to bypass in vitro adaptation. We developed a dual lentiviral vector system that enables simultaneous detection and discrimination of bulk tumor cells from those cells with active Wnt signaling to facilitate functional analyses using in vivo imaging. We used these vectors to generate a panel of double-transgenic human breast cancer cell lines and determined that Wnt responsiveness is associated with the subset of TNBCs called “basal B” or “claudin-low.” Transgenic human breast cancer cell lines and PDX models were coinjected with Wnt-secreting human reduction mammary fibroblasts (RMFs) into the mouse mammary fat pad to determine the outcome of activating Wnt signaling in trans. This system enabled real-time analyses of paracrine Wnt signaling in vivo and led to the unexpected finding that Wnt signaling can either promote or inhibit human breast tumorigenesis.

Results

Dual Lentiviral Wnt-Reporter System. To directly couple Wnt activity to bulk cell population analysis, we developed a dual lentiviral vector system that allows bulk tumor cells and cells with active Wnt signaling to be simultaneously and quantitatively monitored and distinguished.

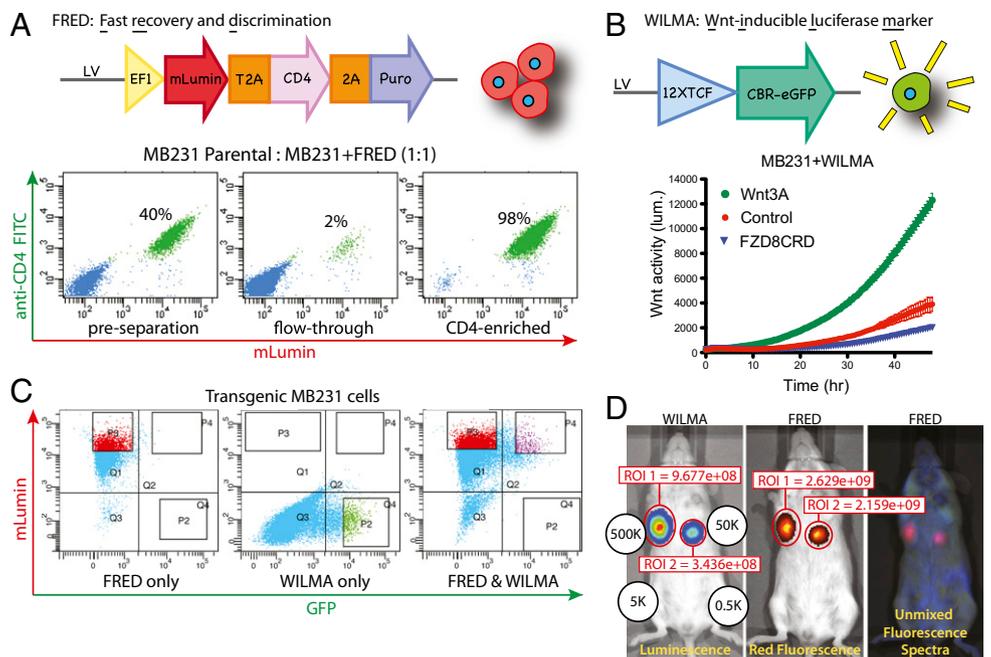
Author contributions: J.L.G., R.N., M.T.L., and G.M.W. designed research; J.L.G., J.L., K.W.Y., P.D., L.-W.R., X.Z., and M.L. performed research; J.L.G., M.T.L., and G.M.W. contributed new reagents/analytic tools; J.L.G., M.L., M.T.L., and G.M.W. analyzed data; and J.L.G., J.L., R.N., M.T.L., and G.M.W. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1303671110/-DCSupplemental.

Fig. 1. Dual lentiviral Wnt reporter system. (A) Schematic of the FRED lentiviral vector (*Upper*). All FRED-transduced cells express red fluorescence, represented by the cartoon cluster of red cells to the right. (*Lower*) Magnetic separation of FRED-transduced MB231 cells from parental MB231 cells. Cells were mixed, incubated with magnetic particles coupled to anti-CD4 antibody, subjected to magnetic separation, and then stained with anti-CD4 (FITC). (B) Schematic of the WILMA lentiviral vector (*Upper*). Only WILMA-transduced cells with active Wnt signaling will be luminescent and green, represented by the cartoon of a luminescent green cell to the right. (*Lower*) Specificity of the WILMA reporter was determined by a real-time bioluminescence monitoring assay. WILMA-transduced MB231 cells were cocultured with iCHO cells secreting WNT3A (green circles) or Fzd8CRD (blue triangles), or parental iCHO cells (control, red circles), and luminescence was measured every 30 min for 48 h. (C) FACS profiles of transgenic cells. FRED-transduced cells were selected with Puromycin, thus all of the cells express mLumin (*Left*). To collect WILMA-transduced cells, GFP⁺ cells were collected 24 h after treatment with the GSK-3 inhibitor XV (*Center*). Double transgenic cells expressing mLumin and GFP were collected after XV treatment (*Right*). (D) MB231-FW cells were injected into the third and fourth mammary pads in the quantities indicated. Two weeks later, bioluminescence and fluorescence were measured. Signal intensity within the region of interest is shown in units of Efficiency (luminescence) or Radiance Efficiency (fluorescence).



The first lentiviral vector enables fast recovery and discrimination (FRED) of transduced cells (Fig. 1A). A constitutively expressed multicistronic ORF encodes the red fluorescent protein mLumin (14), a truncated CD4 surface protein (to enable magnetic separation), and a Puromycin resistance gene. We evaluated FRED functionality in MDA-MB-231 cells (MB231-F for FRED transduced). Stable MB231-F cells were mixed 1:1 with parental MB231 cells and gently separated using magnetic microbeads coupled to anti-CD4 antibodies (Fig. 1A). This strategy enables 98% efficient separation of FRED transduced from nontransduced cells.

The second vector encodes a Wnt-inducible luciferase marker (WILMA) (Fig. 1B). A Wnt-responsive promoter element comprising 12 copies of the TCF binding sequence (15) regulates expression of a click-beetle red luciferase/eGFP (CBR-eGFP) fusion protein (16). CBR luciferase emits red-shifted light compared with firefly luciferase and thus suffers less from absorption and scattering through tissue, making it more useful for *in vivo* imaging. Fusion of CBR to eGFP enables identification of individual Wnt-responsive cells by both flow cytometry and microscopy.

We tested the Wnt-responsiveness of WILMA by transducing MB231, known to be Wnt-responsive (11), with the WILMA lentivirus. MB231-W cells were then exposed to WNT3A or the soluble Wnt inhibitor FZD8CRD produced by inducible CHO (iCHO) cells in coculture (17) (Fig. 1B). As expected, WNT3A induced reporter expression as measured by luciferase activity. The transduced population showed a basal level of luciferase activity that was suppressed by exposure to FZD8CRD, consistent with previous reports that MB231 cells have some endogenous Wnt signaling (11). To recover cells transduced with WILMA, we transiently activated signaling downstream of the receptor by treating the cells with the GSK-3 inhibitor Factor XV. Twenty-four hours after XV treatment, cells with the most intense green and red fluorescence were collected by FACS and expanded *in vitro* (Fig. 1C).

The suitability of these vectors for *in vivo* imaging was assessed by injecting double transgenic (MB231-FW) cells into mouse mammary fat pads. Both luminescence and fluorescence were detected after injecting 500,000 or 50,000 cells, but not 5,000 or 500 cells (Fig. 1D). Autofluorescence in the mouse makes

fluorescence imaging less sensitive, so we grew six MB231-FW tumors and performed fluorescence imaging *ex vivo* (in a dish) to determine whether red fluorescence can be used as a surrogate for cell number (Fig. S1). Cell number was strongly correlated with *ex vivo* red fluorescence of intact tumors ($r^2 = 0.98$).

Basal B (Claudin-Low) Cell Lines Are Wnt-Responsive. Although many human breast tumors display evidence of Wnt pathway activity, the majority of human breast cancer cell lines do not display constitutively active Wnt signaling (10, 11). We hypothesized that this discrepancy could be due to the *in vitro* context in which cell lines are grown and that some cell lines may be able to activate Wnt signaling in response to exogenously applied Wnt ligands.

Expression profiling analysis of human breast tumors has enabled categorization into at least five “intrinsic” subtypes: luminal A, luminal B, Her2-enriched, basal-like, and claudin-low (18–20); however, similar analysis of large panels of immortalized breast cancer cell lines identified only three subsets: luminal, basal A, and basal B, with the basal B subset corresponding to claudin-low tumors (21–23). We generated a panel of double transgenic (FW) human cell lines in which each subset was represented, plus the nontransformed human mammary epithelial cell line MCF10A. To confirm successful transduction and intact signaling machinery downstream of β -catenin, potential reporter cell lines were pre-screened by treatment with Factor XV (Fig. S24). Cell lines that failed to induce WILMA under these conditions were excluded from the panel. The cell line panel was then assayed for response to WNT3A by real-time bioluminescence coculture assays with WNT-producing iCHO cells (Fig. 2A and Fig. S2B and C). Response to WNT3A was normalized to reporter induction by Factor XV, to control for variable copy number of the WILMA transgene. WNT3A induced WILMA expression in 7 of 16 cell lines: MB231, HCC1395, Hs578T, BT549, CAL51, HCC1187, and MB468, which are all TNBC. Interestingly, we found that the ability to respond to exogenously applied Wnt ligands segregated between the subsets, with a response observed in five of five basal B, two of five basal A, and none of five luminal cell lines (Fig. 2B).

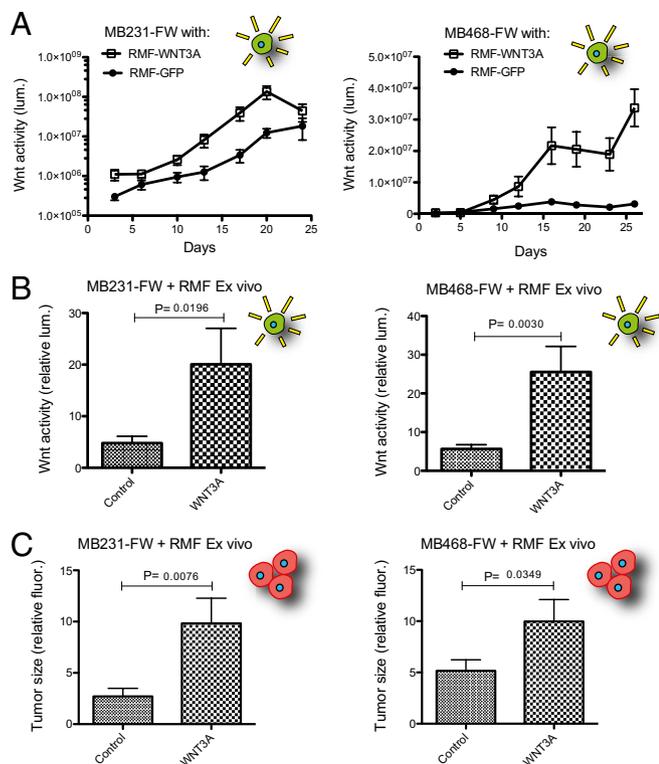


Fig. 4. WNT3A promotes tumorigenesis of breast cancer cell lines. Orthotopic heterotypic recombination experiments as in Fig. 3C using MB231-FW (Left) or MB468-FW (Right) cell lines, $n = 8$ (MB231-FW) or $n = 10$ (MB468-FW). (A) Bioluminescence was measured twice per week for 3–4 wk. (B) Tumors were harvested at day 27 (MB231-FW) or day 33 (MB468-FW), and luminescence was measured ex vivo. Data were normalized to the lowest value. (C) Ex vivo red fluorescence (*mLumin*) intensity of the harvested tumors, as in B.

that the tumor cells were never exposed to selective pressures in vitro (Fig. S4A). Luminescence was measured biweekly, and the transgenic FW tumor lines were stable for at least five passages at the time of writing (Fig. S4B). Although the relatively intense signal from the primary tumors prevented detection of micro-metastases in vivo, luminescence was seen in resected lungs and lymph nodes ex vivo after collection of the primary tumors (Fig. S4C). These xenograft models were used specifically for in vivo experimentation, because primary breast tumor cells typically undergo limited proliferation in culture.

Variable Effects of WNT3A on Tumorigenesis. To determine whether the outcome of elevated Wnt signaling in PDX models is the same as that observed in immortalized cell lines, we subjected our transgenic FW tumor lines to the orthotopic heterotypic recombination assay described above. Like MB231-FW and MB468-FW, WNT3A-RMFs reproducibly increased luminescence and fluorescence of BCM-3963-FW, one of our double-transgenic PDX lines, relative to

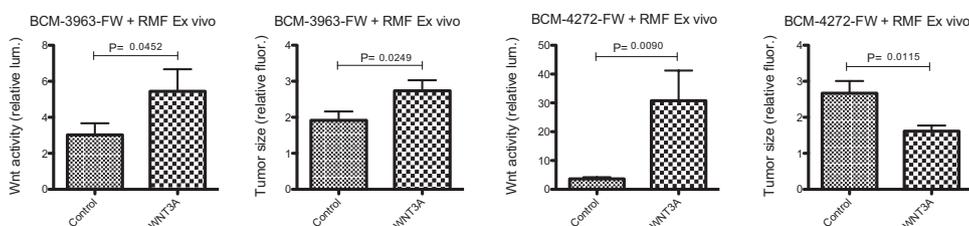


Fig. 5. Wnt signaling in PDX lines. Orthotopic heterotypic recombination experiments as in Fig. 4 using the transgenic PDX lines BCM-3963-FW and BCM-4272-FW, $n = 10$. Tumors were harvested at day 19 (BCM-3963-FW) or day 21 (BCM-4272-FW), and luminescence and fluorescence were measured ex vivo. The entire experiment was repeated with both PDX lines (Fig. S3).

control conditions (Fig. 5 and Fig. S3). Thus, the oncogenic effects of WNT3A in human breast cancer are not limited to immortalized cell lines. Interestingly, BCM-3963-FW was derived from a patient with HER2⁺ breast cancer, and elevated HER2 expression is stable in the transgenic xenograft line (Fig. 6A). Stimulation of BCM-3963-FW by WNT3A contrasts with our cell line panel where none of five HER⁺ cell lines were Wnt responsive. Also unexpectedly, WNT3A-RMFs had the opposite effect on a second PDX line, BCM-4272-FW. Here, luminescence was increased, indicating Wnt pathway activation, but red fluorescence was decreased, indicating that tumor growth was inhibited (Fig. 5). This effect was reproducible in two independent experiments with 10 mice in each group (Fig. S3). The BCM-4272-FW tumors grown in the presence of RMF-WNT3A displayed metaplasia (Fig. 6B), with prominent areas of large round cells with abundant pale cytoplasm that resembled squamous cells. These cells stained positive for cytokeratin 10 (Fig. 6C), a marker for squamous differentiation (26). Furthermore, the mean Ki67 proliferating index was significantly lower ($P = 0.002$) in RMF-WNT3A tumors ($26.8\% \pm 3.2\%$ Ki67 positive cells, $n = 3$) vs. RMF-GFP tumors ($40.6\% \pm 1.4\%$, $n = 3$), consistent with the smaller tumor size. It is not clear whether these effects are direct or indirect; for example, WNT3A could be stimulating secretion of another factor responsible for the observed tumor inhibition. Nevertheless, these results suggest that activated Wnt signaling can either promote or inhibit human breast cancer growth.

Discussion

The dual lentiviral vector system presented here combines a sensitive signaling pathway reporter with cutting edge molecular imaging and magnetic separation modalities. We used this system to generate and characterize multiple state-of-the-art cell line and PDX models of Wnt signaling in breast cancer. Using Wnt signaling as a paradigm, we further showed that it is possible to modulate paracrine signaling pathways in vivo using a relevant cellular source of ligand and to simultaneously monitor the effectiveness of pathway modulation in real time. If used preclinically, these models would enable one to monitor signaling activity in the cells of interest in vivo while simultaneously assessing whether potential therapeutics affect signaling and determining the biological consequences of signaling activation or inhibition.

In our panel of 16 transgenic human breast cancer cell lines, all of the Wnt-responders were triple negative, consistent with literature reports that Wnt-responsiveness correlates with the triple-negative phenotype in human tumors. We improved the resolution of categorizing Wnt-responsiveness by identifying basal B (claudin-low) as the predominant triple-negative subset that is activated by Wnt. We characterized five of five basal B-cell lines as Wnt responsive. The commonality of Wnt responsiveness suggests that this characteristic may be intrinsic to the basal B classification and, by extension, claudin-low tumors. The claudin-low subtype is associated with a core epithelial-to-mesenchymal gene expression signature (27), and Wnt signaling is reported to promote epithelial to mesenchymal transition (EMT) in breast cancer cell lines (28, 29). One explanation for the association we found between the claudin-low subset and Wnt-responsiveness is that Wnt-induced EMT contributes to the EMT signature, and perhaps to the pathology, of claudin-low tumors. Alternatively, claudin-low tumors may originate in mesenchymal-like cells that are naturally Wnt-responsive. Furthermore, because

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