**Mammary Tumor Regression Elicited by Wnt Signaling Inhibitor Requires IGFBP5**

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**Abstract**

Wnt ligand–driven tumor growth is inhibited by the soluble Wnt inhibitor Fzd8CRD, but the mechanism through which this effect is mediated is unknown. In the MMTV-Wnt1 mouse model, regression of mammary tumors by Fzd8CRD treatment coincides with an acute and strong induction of insulin-like growth factor (IGF)–binding protein IGFBP5, an antagonist of IGF signaling that mediates involution of mammary gland in females after offspring are weaned. In this study, we show that repression of this IGF inhibitory pathway is crucial for Wnt-driven growth of mammary tumors. We found that IGFBP5 regulation was mediated by the β-catenin–dependent Wnt pathway. Wnt, in addition to IGF ligands, facilitated tumor growth by paracrine communication among tumor cells. In addition, Fzd8CRD caused precocious induction of IGFBP5 in normal mammary glands undergoing involution, implying an acceleration of the involution process by inhibition of Wnt signaling. The molecular and phenotypic parallel between tumor regression and mammary gland involution suggests that Wnt-driven mammary tumors use the same growth mechanism as proliferating normal mammary glands. *Cancer Res; 72(6): 1–11. ©2012 AACR.*

**Introduction**

Wnt signaling is an evolutionary conserved pathway that is crucial for developmental processes, stem cell maintenance, and cell fate determination. Misregulation of the Wnt pathway is implicated in various human diseases, particularly in cancers in which hyperactivation of the β-catenin–dependent canonical pathway is involved. Mammalian Wnt ligand was originally identified as a protooncogene in viral induced mouse mammary gland tumors; overexpression of Wnt1 ligand under the control of the mouse mammary tumor virus (MMTV) promoter leads to hyperplasia and, ultimately, adenocarcinoma in the mammary gland (1). MMTV-Wnt1 tumors have characteristic histologic features and are composed of a variety of cell types, including distinct tumor luminal epithelial and basal/myoepithelial cells and host-derived stroma cells (2). Importantly, the existence of basal/myoepithelial cells in Wnt tumors is reminiscent of human basal–like breast cancers (3), which are often associated with a poor prognosis and the lack of effective treatment. A recent report also showed that Wnt signaling activation is enriched in basal-like breast cancers (4). Therefore, the MMTV-Wnt1 tumor is a useful model for studying breast cancer tumorigenesis as well as for therapeutic evaluation (1). Disruption of Wnt signaling by administration of a soluble frizzled8 receptor-immunoglobulin G (IgG) Fc fusion protein (Fzd8CRD) that competes for Wnt1 binding results in regression of the tumors (5, 6).

While examining the events following inhibition of Wnt activity in established MMTV-Wnt1 tumors, we found that the rapid regression of Wnt1 mammary tumors caused by Fzd8CRD treatment coincided with acute and strong induction of insulin-like growth factor (IGF)–binding protein 5 (IGFBP5). IGFBP5 is a member of IGF-binding proteins (IGFBPs) and a key regulatory molecule during mammary gland involution (7). IGFBP5 is expressed throughout the mammary epithelium during puberty, but greatly reduced during lactation, and reexpressed during early mammary gland involution before the onset of epithelial apoptosis (8). Overexpression of IGFBP5 results in an increase in apoptotic cells in the mammary glands, whereas mice with mutant IGFBP5 exhibit a decrease in apoptotic cells and a delay in mammary gland involution (8, 9). The predominant role of IGFBP5 in these processes is to bind IGF ligand and inhibit its signaling, but the functions independent of IGF signaling have also been reported (8, 10). IGF is a known growth and survival factor in mammary epithelial cells, and hyperactive IGF signaling is found in various human tumors including breast cancer (11, 12). IGF signaling employs 2 ligands, IGF-I and IGF-II, that can bind to IGF1 receptor (IGF1R) to activate downstream intracellular signaling pathways, providing cells with mitogenic and antiapoptotic signals.

Wnt signaling also plays an important role in normal mammary gland development. Although the exact role of Wnt
signaling in postnatal mammary development is not well understood, there is evidence to suggest that Wnt signaling provides growth/survival stimulus for the mammary epithelial cells during the late stages of pregnancy. Inhibition of Wnt signaling increases apoptosis in mammary glands of late pregnant and lactating mice (13, 14), whereas activation of the pathway delays mammary gland involution, presumably by hindering apoptotic events (15). Thus, the survival stimuli provided by Wnt signaling during normal mammary gland development and in established Wnt1 tumors may have some similarities. By studying the mechanism of Wnt inhibitory molecule, we uncovered the analogy of growth/survival mechanism in Wnt1 tumors, human basal-like breast cancer, and involuting mammary glands facilitated by IGFBP5.

Materials and Methods

Mice and allograft tumors
All mouse tumors are passaged as described previously (2). All experimental protocols using mice were approved by the Institutional Animal Care and Use Committee at Genentech Inc.

Immunohistochemistry
Immunofluorescence staining was conducted on formalin-fixed paraffin-embedded tumor sections (2). For image analysis, SlideBook software was used to measure pixel intensities and calculated as previously described (16). Five random areas per section from 3 separate sections for each treatment group were analyzed.

Quantitative real-time PCR
Real-time PCR was carried out by TaqMan reaction as previously described (2). All the primers used in this study were listed in the Supplementary Methods.

IGF1R-blocking antibody and recombinant IGFBP5
IGF1R-blocking antibody was derived from phage library screening against human IGF1R protein. The antibody exhibits partial activity against the mouse receptor, as shown by the inhibition of IGF1R phosphorylation in NMuMG cells (Supplementary Fig. S3B). Escherichia Coli derived recombinant IGFBP5 protein was purchased from BioVision. IGFBP5-human IgG Fc fusion protein (IGFBP5-Fc) was expressed and purified from Chinese hamster ovary cells; the inhibitory activity of IGFBP5 to IGF-1 and IGF-II in MCF7 cells was verified in vitro (Supplementary Fig. S3C).

Treatment of mice and doses
Dissociated tumor cells (0.5 × 10^6) were resuspended in HBSS:Matrigel (1:1) and injected into the #3 mammary fat pads of 8- to 12-week-old NCR-nude mice. Fzd8CRD protein (10 mg/kg) was given by intraperitoneal injections. Anti-IGF1R (20 mg/kg) was given by intraperitoneal injections twice per week. Recombinant IGFBP5 was injected intratumorally (10 mg/kg), and the injection was repeated 2 days following the first injection.

shRNA knockdown of IGFBP5 in Wnt1 tumor cells
Wnt1 tumor cells were plated on collagen 1-coated plates (BD Bioscience) in mouse mammary epithelial cell culture media (Stem Cell Technologies). Cells were infected with lentivirus expressing puromycin and shRNA to IGFBP5 or to GFP (Sigma-Aldrich; NM_010518.1-1285s1c1 and NM_010518.1-1530s1c1) at multiplicity of infection of 2.5 in media containing 8 μg/mL of polybrene. Infected cells were selected in medium containing 4 μg/mL of puromycin for 3 days. Puromycin-resistant cells were resuspended in HBSS: Matrigel (1:1) and injected into the mammary fat pads of nude mice (0.5 × 10^6 cells per mouse).

Fluorescence-activated cell sorting isolation of different types of cells in Wnt1 tumor
Cell dissociation method is previously described (2) and in the Supplementary Fig. S5A. P-cadherin and EpCAM antibodies were used for separating luminal and basal/myoepithelial cells.

In vitro experiments of human breast cancer cell lines
All cell lines were obtained from American Type Culture Collection. MDA-MB-468 and HCC38 cells were cultured in RPMI-1640/10% FBS containing Wnt3A (100 ng/mL; R&D systems) or Fzd8CRD (20 μg/mL). For β-catenin siRNA transfection, plated cells were transfected with 20 nmol/L of siRNA (Dharmacon) by using DharmaFECT transfection reagent. Wnt3A or Fzd8CRD treatment was done 48 hours after the transfection.

Fzd8CRD treatment of pregnant mice
Pregnant C57BL/6 mice were injected with Fzd8CRD (20 mg/kg) or PBS at days 9, 12, and 16 of pregnancy. Mammary glands were harvested at day 17 of pregnancy, immediate postpartum (lactating), and at 6, 12, and 24 hours after weaning (involution). The time from last Fzd8CRD injection to collection of last mammary gland sample was 3 days.

Statistical analysis
Two-tailed paired Student t test was calculated by Excel software. Prism software was used for one or 2-way ANOVA.

Results
Wnt inhibitor rapidly triggers regression of MMTV-Wnt1 tumors
Fzd8CRD is a fusion protein of extracellular cysteine-rich domain (CRD) of Frizzled8 receptor and human IgG Fc domain (5). Fzd8CRD inhibits Wnt signaling and has been shown to inhibit Wnt tumor growth in vivo (5). Notably, a single injection of Fzd8CRD (10 mg/kg) resulted in a rapid decrease in the
volumes of MMTV-Wnt1 tumor (Fig. 1A). The same treatment had no effect on the MMTV-ΔN-β-catenin (15) or MMTV-Her2 tumors (Supplementary Fig. S1). Assessment of cell proliferation by anti-Ki67 staining of Wnt1 tumor sections (Fig. 1A) revealed an abundance of Ki67⁺ cells in PBS-treated controls, but the number of Ki67⁺ cells was dramatically reduced at 24 hours after Fzd8CRD treatment and declined further 48 hours posttreatment (Fig. 1B). The decrease in cell proliferation following Fzd8CRD treatment was accompanied by an increase in cells undergoing apoptosis, as determined by activated caspase 3 antibody staining (Fig. 1A). Western blot analysis of Wnt1 tumors also confirmed an increase in activated caspase 3 levels following 24- and 48-hour post-Fzd8CRD treatment (Fig. 1C).

Inhibition of Wnt signaling results in an immediate induction of IGFBP5 in the Wnt1 tumors

To understand the striking effect of Wnt signaling inhibition in vivo, we examined the gene expression changes in Wnt1 tumors that were treated with either Fzd8CRD or PBS. We found IGFBP5 mRNA to be consistently and strongly upregulated in tumor samples treated with Fzd8CRD. By quantitative reverse transcription PCR (qRT-PCR), we detected a 2-fold increase in IGFBP5 transcript level compared with controls as early as 5 hours post-Fzd8CRD treatment, with a further elevation noted at 12 and 24 hours post-Fzd8CRD treatment (Fig. 2A). The increase in IGFBP5 transcript coincided with a decrease in Wnt signaling, as determined by the levels of the Wnt-responsive gene transcripts, Axin2 and MMP7 (20, 21). These changes were not seen in Fzd8CRD-treated ΔN-β-catenin and Her2 tumors (Supplementary Fig. S2A). The levels of other IGFBPs were not changed following Fzd8CRD treatment, with an exception of a smaller increase in IGFBP6 transcripts (Supplementary Fig. S2B). Western blot revealed a significant elevation in IGFBP5 in Wnt tumors following 24 and 48 hours of Fzd8CRD treatment compared with PBS (Fig. 2B), but not in ΔN-β-catenin or Her2 tumors (Supplementary Fig. S2C).

To address whether the effect of Fzd8CRD is on canonical or noncanonical Wnt signaling, we tested an antagonistic antibody against LRP6, the Wnt coreceptor that is thought to
function only in canonical signaling. Monoclonal LRP6 antibody (clone YW210.09) was derived from human synthetic antibody phage libraries using recombinant human LRP6 protein. This antibody blocks the binding of Wnt1 to LRP6, thereby promoting the rapid regression of Wnt1 tumors (18).

Similar to Fzd8CRD, anti-LRP6 also increased the IGFBP5 transcript and protein levels in Wnt1 tumors compared with control antibody-treated tumors (Fig. 2C and Supplementary Fig. S2D), showing that disruption of canonical Wnt signaling is sufficient to increase IGFBP5 levels.

Because IGFBP5 is known as a negative regulator of IGF signaling, an increase in IGFBP5 expression following Fzd8CRD treatment may lead to a decline in IGF signaling in the Wnt1 tumors. We monitored phosphorylation of IGF1R in the Wnt1 tumors after Fzd8CRD treatment using anti-phospho-IGF1R (Y1161). The cells stained with phospho-IGF1R in Wnt tumor seemed to be in localized areas. In Fzd8CRD-treated tumors, we detected less phospho-IGF1R–stained regions compared with PBS-treated tumors (Fig. 2D). Because anti–phospho-IGF1R was shown to cross-react with phosphoinsulin receptor (Y1185), we immunoprecipitated total IGF1R from tumor lysates using IGF1R-specific antibody and analyzed the levels of phosphorylated IGF1R by anti–phosphotyrosine. We detected less phosphorylated IGF1R in Fzd8CRD-treated tumors compared with the control tumors (Fig. 2E). These results indicated that IGFBP5 induction upon Fzd8CRD may lead to the reduction of IGF signaling in the Wnt tumor.

**Attenuating IGF signaling inhibits Wnt1 tumor growth**

The reduction of IGF signaling in the Wnt1 tumors following Fzd8CRD treatment prompted us to assess the direct
contribution of IGF activity on the growth/survival of Wnt1 tumors. Accordingly, we injected Wnt1 tumor–bearing mice with an IGF1R inhibitor, PPP. Although the precise mechanism of action is not known, PPP was shown to selectively inhibit IGF1R tyrosine kinase activity without affecting insulin receptor (19). The other mechanisms such as downregulating IGF1R protein are also proposed (22). With a single injection of PPP at 30 mg/kg, the average tumor volume was reduced by 31% on the next day and continued to regress to a maximum of 43% reduction (Fig. 3A). The same treatment had no effect on Her2 tumors. We detected activated caspase 3 in the Wnt1 tumor lysates from mice treated with PPP, but not in the tumor lysates from vehicle-treated mice (Fig. 3B). IGFBP5 protein level was not affected by the PPP treatment (Fig. 3B).

We employed an additional approach to inhibit IGF1R using a functional blocking antibody. Monoclonal IGF1R antibody (Clone YW 95.6) was derived from human antibody phage library, screened against recombinant human IGF1R ectodomain. The moderate inhibitory effect was observed on IGF1R phosphorylation in normal mouse mammary epithelial cell line NMuMG (Supplementary Fig. S3). Injection of IGF1R antibody at 20 mg/kg, twice per week, resulted in a significant inhibition of Wnt1 tumor growth compared with that of the anti-gD–treated group (Fig 3C). By contrast, IGF1R antibody treatment had no significant effect on Her2 tumor growth.

Because inhibition of IGF signaling upon Fzd8CRD treatment is correlated with an increase in IGFBP5 expression, we tested whether IGFBP5 alone is sufficient to induce regression of Wnt1 tumors in vivo. The first injection of IGFBP5-Fc at 10 mg/kg into the Wnt1 tumor site resulted in a slight reduction in the tumor volume by day one postinjection, and the average tumor volume continued to decrease more significantly on day 2, approximately by 23% of the original volume (Fig. 3D). Similar levels of tumor regression were seen when we injected IGFBP5 purified from E. coli.

Induction of IGFBP5 expression is required for regression of Wnt1 tumors upon Fzd8CRD treatment

Our data suggested that Wnt1 tumors rely upon IGF signaling and that Fzd8CRD promotes tumor regression by interfering with this activity through induction of IGFBP5. This model predicted that a failure to induce IGFBP5 would abrogate tumor regression by Fzd8CRD. To directly address this, we employed lentivirus expressing short hairpin RNA (shRNA) to knockdown the expression of IGFBP5 (ShIGFBP5) in Wnt1 tumor cells. The basal levels of IGFBP5 were slightly decreased in the ShIGFBP5-infected tumors compared with the tumors derived from ShGFP-infected cells. When tumor-bearing mice were treated with Fzd8CRD, the tumors derived from ShIGFBP5-infected cells were greatly impaired in their ability to upregulate IGFBP5 expression compared with control tumors (Fig. 4A). More importantly, this impairment in IGFBP5 expression was accompanied by a failure of Wnt1 tumors to undergo regression upon Fzd8CRD treatment (Fig. 4B, Supplementary Fig. S4B). Lack of tumor regression was also consistent with the decreased levels of activated caspase 3 in Fzd8CRD-treated ShIGFBP5-infected tumors relative to Fzd8CRD-treated ShGFP-infected tumors (Fig. 4C).

Figure 3. Effect of IGF inhibitors on Wnt tumor growth. A, Wnt1 or Her2 tumor–bearing mice treated with PPP (30 mg/kg; ■) or vehicle (DMSO/vegetable oil; ○). B, immunoblot of IGFBP5 and activated caspase 3 in Wnt1 tumors treated in vivo with Fzd8CRD, PPP, or vehicle. C, Wnt1 or Her2 tumor–bearing mice treated with IGF1R antibody (20 mg/kg; ■) or anti-gD (○) twice per week. D, Wnt1 tumor–bearing mice treated twice with anti-gD (○), Fzd8CRD (■), IGFBP5-Fc (●, top), or IGFBP5 (●, bottom) at day 0 and 2 (10 mg/kg). Error bars represent ± SEM (N = 5 mice per group), and * P < 0.05.
The expression of Wnt1 ligand was not affected by viral infection of these tumor cells, and Wnt signaling in these infected tumors were faithfully inhibited by Fzd8CRD treatment, as indicated by a reduction in Wnt-responsive transcripts, Axin2 and MMP7 (Fig. 4D). This showed that Wnt1 expression and inhibition of Wnt activity by Fzd8CRD is unaffected by knocking down IGFBP5 expression, yet the tumors fail to regress.

Paracrine effect of IGF and Wnt signaling in Wnt1 tumors

MMTV-Wnt1 tumors contain different types of stroma and tumor epithelial cells which can be separated by fluorescence-activated cell sorting (FACS) using cell surface markers (ref. 2; Supplementary Fig. S5A). Mainly, we isolated CD45+ hematopoietic and CD45− stroma cells and 2 distinct tumor epithelial cells referred to as basal/myoepithelial and luminal cells. Sorted epithelial cells have been characterized by the expression of cytokeratin isotypes (CK5 for basal/myoepithelial cells and CK18 for luminal epithelial cells) and basal/myoepithelial cell marker, P-cadherin and smooth muscle actin (2).

By examining the transcripts from these isolated cell populations, we found that IGF-I was predominantly expressed by the tumor hematopoietic cells (CD45+), and IGF-II was expressed by the CD45− fibroblasts enriched stromal cells (Fig. 5A). In contrast, IGF1R transcripts and proteins were highly expressed by the Wnt1 basal/myoepithelial cells compared with the luminal cells (Fig 5). To determine whether IGF specifically enhances the growth/survival of basal/myoepithelial cells, we injected isolated tumor basal/myoepithelial or luminal cells into nude mice (30,000 cells per mouse), then treated with either IGF-I twice per week at 10 mg/kg. Injected basal/myoepithelial cells developed tumors significantly faster with IGF-I treatment compared with bovine serum albumin treatment, whereas IGF-1 treatment had no effect on the growth of luminal cells (Fig. 5C). When both basal/myoepithelial and luminal tumor cells were injected together, IGF-1 treatment also enhanced the tumor growth (Supplementary Fig. S5B). In addition, the growth of isolated tumor basal/myoepithelial cells in culture were slightly enhanced with IGF-1, but not the luminal cells (Supplementary Fig. S5B and C). These data suggested that stromal IGF selectively enhances the growth/survival of Wnt1 tumor basal/myoepithelial cells in which IGF1R is expressed.

When we examined the Wnt signaling activity in sorted cell populations, we found that expression of Wnt1 was restricted to the tumor luminal cells, whereas the expression of Wnt-responsive genes, axin2 and MMP7, was higher in the tumor myoepithelial cells (Fig. 5A). When Wnt1 activity was inhibited by Fzd8CRD treatment, axin2 and MMP7 transcript levels were significantly reduced in the myoepithelial cells, and concurrently, IGFBP5 expression was dramatically increased in these cells (Fig. 5D). Expression of IGF-I and IGF-II in the stroma was not affected by the Fzd8CRD treatment. Consistent with these in vivo data, isolated Wnt1 tumor myoepithelial cells cultured in media containing Wnt3A suppressed IGFBP5 expression.
Regulation of IGFBP5 by Wnt signaling in human basal-like breast cancer cells

Wnt3A or Fzd8CRD treatment can only affect IGFBP5 expression in basal/myoepithelial cells, and this Wnt regulation of IGFBP5 expression is not seen in luminal cells (Fig. 5D and E). When we examined Wnt signaling–responsive gene, MMP7, and IGFBP5 expression in human breast cancer tissues and cell lines in NCBI GEO database (23), accession GSE7904, (24, 25); we found MMP7 expression was significantly higher (P = 0.007) in the basal-like breast cancers than in nonbasal-like cancers (Fig. 6A and Supplementary Fig. S6A). Coincidentally, IGFBP5 expression was significantly lower in the basal-like than in nonbasal-like cancers.

We further tested various basal-like breast cancer cell lines for regulation of IGFBP5 by Wnt3A or Fzd8CRD. We found that inducing Wnt signaling by Wnt3A in MDA-MB-468, HCC38, and MDA-MB-157 cells resulted in a decrease in IGFBP5 transcripts (Fig. 6B and Supplementary Fig. S6B). Conversely, inhibiting Wnt signaling by Fzd8CRD increased IGFBP5 transcripts. We also detected IGFBP5 protein in the culture media of these cells treated with Fzd8CRD, but not in the media with other treatments (Supplementary Fig. S6C). These findings suggested that both human breast cancer cells and the mouse mammary tumor share a similar mechanism of IGFBP5 regulation by Wnt activity.

To further investigate whether the regulation of IGFBP5 expression by Wnt is depended on B-catenin, we suppressed B-catenin expression using siRNA. MDA-MB-468 cells transfected with B-catenin siRNA can no longer suppress IGFBP5 expression upon Wnt3A treatment and further elevate IGFBP5 expression with Fzd8CRD treatments (Fig. 6C). Similar results were obtained from HCC38 cells. These data suggested that regulation of IGFBP5 expression by Wnt ligands is via a B-catenin–dependent mechanism.

Figure 5. Expression of signaling components in various types of cells in Wnt tumor. A, relative expression of various genes in the isolated basal/myoepithelial (myo), luminal, stromal, and hematopoietic cells from Wnt1 tumors. P value was calculated by 1-way ANOVA. B, expression of IGF receptors, CK5 (basal/myoepithelial marker) and CK18 (luminal marker) in sorted cells from Wnt tumor. C, the tumor growth of isolated epithelial cells with or without IGF-1. Error bars represent ± SEM (N = 10 per group). **, P < 0.01. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Inhibition of Wnt signaling accelerates induction of IGFBP5 expression in normal mammary gland during involution

Our finding that IGFBP5 is the key molecule during tumor regression by Wnt inhibitor is reminiscent of the effects of IGFBP5 on remodeling of the normal mammary gland during involution (8, 9). It prompted us to determine whether Wnt signaling influences the expression of IGFBP5 in the involuting glands. Accordingly, we treated pregnant mice with Fzd8CRD at days 9, 12, and 16 postcopulation. Upon inhibition of Wnt signaling, as indicated by the decrease in Wnt target genes, axin2, MMP7, and TROY (26) (Supplementary Fig. S7A), expression of IGFBP5 protein and mRNA was increased slightly in late pregnant glands (day 17) but was undetectable in lactating glands from both Fzd8CRD-treated and PBS control–treated mice (Fig 7A and B). During early involution, induction of IGFBP5 was easily detected as early as 6 hours postweaning in Fzd8CRD-treated glands, but remained undetectable in PBS-treated glands. At 24 hours postweaning, IGFBP5 was detected in the PBS-treated glands, although at considerably lower levels than that observed in Fzd8CRD-treated mice. The acceleration of IGFBP5 expression in the involuting glands suggested that inhibition of Wnt activity precedes reexpression of IGFBP5 during this stage of mammary gland remodeling.

To assess the consequences of Wnt inhibition on cell survival, we stained mammary glands with antiactivated caspase 3. A few activated caspase 3+ cells were seen in glands from Fzd8CRD-treated mice at day 17 pregnancy and lactation, whereas glands from PBS-treated mice had no detectable caspase 3+ cells (Fig. 7C and Supplementary Fig. S7B). During early involution, at 12 and 24 hours of postweaning, significantly more caspase 3+ cells were detected in glands of Fzd8CRD-treated mice than in PBS-treated glands. These data indicated the resemblance of Wnt tumor and normal mammary gland undergoing remodeling process.

Discussion

In studying potential mechanisms of action for the Wnt inhibitor Fzd8CRD, we found the induction of IGFBP5 in Wnt mammary tumors to be an immediate endogenous outcome of inhibiting Wnt activity. We detected an acute increase in IGFBP5 transcript and protein (Fig. 2A and B) coincided with a decrease in IGF signaling following Fzd8CRD treatment of Wnt tumors (Fig. 2D), suggesting a possible role of IGFBP5 to inhibit IGF signaling in the tumors. Using various inhibitory reagents, we showed that IGF signaling supports Wnt1 tumor growth. Our IGFBP5 knockdown experiments show clearly that the induction of IGFBP5 is required for Wnt1 tumor regression in response to disruption of Wnt activity (Fig. 4).

We analyzed the signaling components and showed how Wnt and IGF signaling pathways are orchestrated in the various cell types in the tumors. Consistent with a previous report (27), expression of Wnt1 transgene is predominantly found in the luminal cells, whereas inhibition of Wnt activity by Fzd8CRD specifically affects the basal/myoepithelial cells (Fig. 5C, Supplementary Fig. S5). Mammary myoepithelial cells have been shown to respond to Wnt signaling, and this is likely due to the expression of LRP5/6 coreceptors in these cells (28–30). We also found that the basal/myoepithelial cells express IGFR1 and that stromal cells express IGF ligands (Fig. 5A and B), indicating paracrine IGF signaling in vitro.

Various studies suggest the connection between Wnt signaling and human basal–like breast cancers (3, 4). We also
noticed that human basal–like breast cancers show on average higher levels of one of the Wnt signaling–responsive gene, MMP7, and lower levels of IGFBP5 compared with nonbasal–like cancers (Fig. 6A and Supplementary Fig. S6A). We found that some human basal breast cancer cell lines have a similar regulation of IGFBP5 by Wnt activity to what we found in the mouse Wnt1 tumors (Figs. 5D, 6B, and Supplementary Fig. S5B). Although the precise mechanism has yet to be determined, we showed that regulation of IGFBP5 expression in human cells is dependent on the canonical Wnt signaling effector β-catenin, consistent with the results from anti-LRP6 treatment of Wnt1 tumors (Fig. 2C, 6D, and Supplementary Fig. S2D).

We found that IGFBP5 expression is dramatically induced upon inhibition of Wnt signaling before the onset of tumor regression, analogous to the phenomenon previously described for IGFBP5 in contributing to mammary involution (7). Therefore, we hypothesized that endogenous Wnt activity suppresses IGFBP5 expression during normal mammary development and were able to show that inhibition of Wnt signaling in normal mammary glands undergoing early involution induces precocious IGFBP5 expression and apoptosis (Fig. 7). This similarity in induction of IGFBP5 upon inhibition of Wnt activity in the Wnt1 mammary tumors and in the normal involuting glands suggests that regression of Wnt1 tumors uses a similar mechanism to that used by normal glands undergoing involution. Wnt1 tumors may still possess characteristics of normal mammary glands, but with continuous Wnt activity, the tumors remain in an aberrant developmental state that resembles pregnant glands without undergoing involution.

In summary, we showed that IGFBP5 induction is a critical response to inhibition of Wnt signaling in Wnt mammary tumor, and this regulation of IGFBP5 by Wnt signaling is also observed in human basal–like breast cancer cells. We also showed striking similarities between Wnt1 tumors undergoing regression upon Fzd8CRD treatment and involuting normal mammary glands. Our data suggests that oncogenic transformation by Wnt is mediated by paracrine Wnt signaling in vivo, with incorporating normal developmental signaling such as IGF/IGFBP5. Wnt signaling is considered to be an attractive

Figure 7. Effect of Fzd8CRD on normal pregnant and involuting mammary glands. IGFBP5 protein expression (A) or mRNA expression (B) in mammary glands at the different developmental stages with or without Fzd8CRD treatment. *, P < 0.05; **, P < 0.01. C, immunostaining of activated caspase 3 in mammary glands with or without Fzd8CRD treatment. Percentage of activated caspase 3+ cells in DAPI-stained cells are shown as a bar graph. DAPI, 4′,6-diamidino-2-phenylindole. ***, P < 0.01.
target for cancer therapeutics (5, 18, 31, 32), and our findings may also provide insights into a mechanism of Wnt pathway–targeted therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: B.Y. Liu, C. Sakanaka.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.Y. Liu, I. Soloviev, P. Chang, C. Sakanaka.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis): B.Y. Liu, I. Soloviev, P. Chang, X. Huang, J.A. Ernst, P. Polakis, C. Sakanaka.

Acknowledgments
The authors thank members of the FACS Core, Hai Ngu and Laszlo Komuves for their technical support, Yan Gong and Mike Costa, Somasekar Seshagiri, Jing Zha, Yan Wu, Scot Marsters for the reagents, and Pamela Cowin for MMTV-ANβ-catenin tumor.

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Received November 7, 2011; revised December 27, 2011; accepted January 16, 2012; published OnlineFirst February 3, 2012.

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Cancer Res  Published OnlineFirst February 3, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-3668

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