Dissecting the roles of WNT signaling in breast cancer using *in vitro* and *in vivo* experimental models

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Summary

Canonical WNT pathway regulates expression of target genes by modulating intracellular amount of β-catenin. Without WNT pathway activation, a so-called “destruction complex” including APC and Axin facilitates the degradation of β-catenin. Upon binding of WNT ligand to its receptor Frizzled, the destruction complex is antagonized and β-catenin is stabilized. Stabilized β-catenin goes to the nucleus, binds to the TCF/LEF family of transcription factors and initiates the new gene expression program.

De-regulation of the WNT signaling pathway via mutations in APC and Axin, proteins that target β-catenin for destruction, or in β-catenin itself have been linked to various types of human cancer. These genetic alterations rarely, if ever, are observed in breast tumors. However, various lines of evidence suggest that WNT signaling may also be de-regulated in breast cancer. Most breast tumors show hypermethylation of the promoter region of secreted Frizzled-related protein 1 (sFRP1), a WNT antagonist, leading to downregulation of its expression. As a consequence WNT signaling is enhanced. We hypothesized that autocrine activation of WNT signaling plays an important role in breast cancer and loss of sFRP1 expression is one of the critical events leading to constitutively active WNT signaling in breast cancer formation.

We show that de-regulation of the WNT signaling pathway appears to occur by autocrine mechanisms in a panel of breast cancer cell lines and that interference with WNT signaling in breast cancer cell lines reduces their proliferative ability. Furthermore, ectopic expression of sFRP1 suppresses autocrine WNT signaling in MDA-MB-231 human breast cancer cells and leads to dramatically impaired outgrowth of these cells as xenografts in nude mice. A microarray analysis led to the identification of two genes encoding *CCND1* and *CDKN1A* whose

expression level is selectively altered in sFRP1 expressing tumors. The corresponding proteins, cyclin D1 and $p21^{Cip1}$ were down- and up-regulated, respectively in sFRP1 expressing tumors, suggesting that they are downstream mediators of WNT signaling. In addition to the effect on cell proliferation, we show that WNT stimulates the migratory ability of T47D human breast cancer cells. Conversely, ectopic expression of sFRP1 decreases the migratory potential of MDA-MB231 cells, suggesting that WNT activation not only promotes cell growth, but also stimulates cell motility.

In summary, these results suggest that the WNT pathway has an impact on various biological characteristics of human breast cancer cell lines. Throughout these studies, we revealed the possibility that interference with WNT signaling at the ligand-receptor level is a valid therapeutic approach in breast cancer.

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I. INTRODUCTION

1. Breast cancer

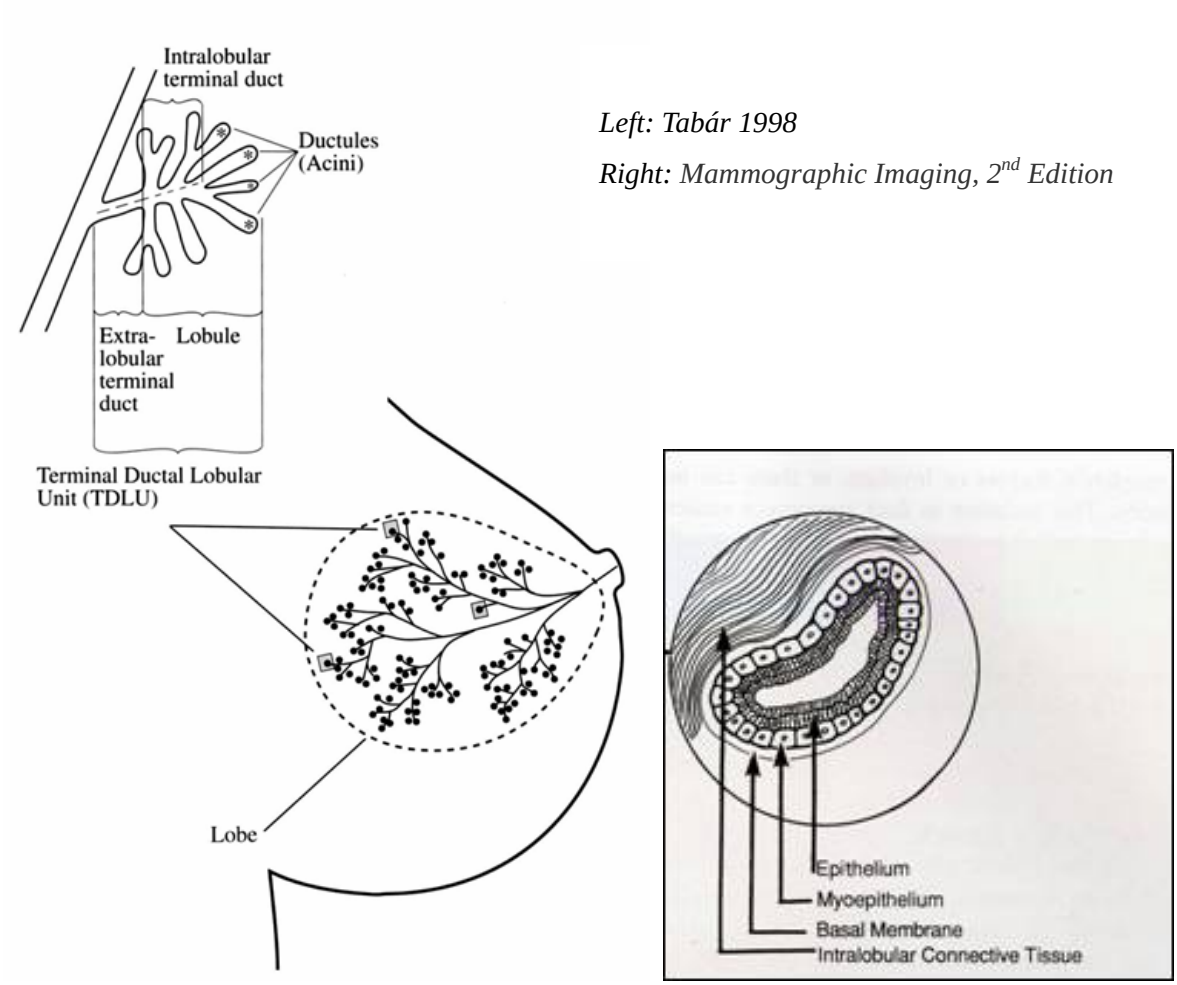
1.1. Normal female breast

1.1.1. Anatomy of normal female breast

Mammalian females in general have modified skin appendages that provide complete nourishment and immunologic protection for the newborns. In humans, paired mammary glands rest on the pectoralis muscle on the upper chest wall. The breast is composed of specialized epithelium and stroma. Six to ten major ductal systems originate at the nipple. The duct is lined by a double-layered epithelium. Although the breast ductal system is comprised of domains with distinct morphology and function, this layered architecture is found throughout the mammary gland from the nipple to the terminal alveoli. Successive branching of the large ducts eventually leads to the terminal duct lobular unit (TDLU). In the adult woman, the terminal duct branches into a grapelike cluster to form a lobule (Fig.1).

In the normal breast, ducts and lobules are lined by two cell types, myoepithelial cell and luminal cells. The mypepithelial cell is a contractile cell containing myofilaments and it is a component of a low, flattened discontinuous layer lying on the basement membrane. It assists milk ejection during lactation and has an important role in maintenance of the normal structure and function of the lobule and the basement membrane [1]. Luminal cells are components of the second (inner) layer of epithelial cells which line the lumens. Only the luminal cells of the terminal duct and the lobule produce milk, while those lining the large duct system do not produce milk. A committed stem cell in the terminal duct gives rise to both luminal and myoepithelial cells [2].

In humans, the majority of breast stroma consists of fibrous connective tissue mixed with adipose tissue (interlobular stroma). Lobules are enclosed by a breast-specific hormonally responsive stroma that contains a scattering of lymphocytes (intralobular stroma).



Left: Tabár 1998

Right: Mammographic Imaging, 2nd Edition

Figure 1. Anatomy of human female breast

Left: Sagittal section of human adult breast and illustration of the TDLU

Right: Structure of the breast lobule

Double-layered cuboidal epithelium, luminal cell and myoepithelial cell are surrounding the lumen. This epithelial sheet is sequestered from stroma by basement membrane.

1.1.2. Life cycle changes

The breast is not fully formed at birth, undergoes cyclic changes during the life span in which a female has menstruation, undergoes full differentiation when a female gives a birth, and starts to involute long before menopause.

During mid-embryogenesis, the specialized mesenchyme of the breast fat pad condenses around the epithelium of the breast bud. Via a complex interaction between stromal and epithelial cells, cords of cells "invade" the stroma to form the elementary ductal system. The continuing cross-talk between epithelium and stroma promotes normal tissue structure and function throughout life. The prepubertal breast has a minimal lobule formation. At the beginning of menarche in women, the terminal ducts give rise to lobules, and the interlobular stroma increases in volume. However, there is not yet much adipose tissue at that time.

The breast changes during the menstrual cycle [3]. In the first half of the cycle, which is called the follicular phase, the lobules are relatively quiescent. After ovulation, under the influence of estrogen and rising progesterone levels, cell proliferation and the number of acini per lobule increase, and vacuolization of epithelial cells occurs. Intralobular stroma markedly swells. When menstruation occurs, estrogen and progesterone levels fall, leading to epithelial cell apoptosis, disappearance of the stromal edema, and overall regression in the size of the lobules.

It is only with the onset of pregnancy that the breast completes morphologic maturation and functional activity. Lobules increase both in number and in size. As a consequence, by the end of the pregnancy, the breast is composed almost entirely of lobules separated by a relatively small amount of stroma. By the third trimester, secretory vacuoles of lipid material are found within the epithelial cells of the TDLU, but milk production is inhibited by the high levels of progesterone. Immediately after birth, the breast produces colostrum (high in protein), which changes to milk (higher in fat and calories) within the first 10 days as progesterone levels drop. Breast milk not only provides complete nourishment, but also provides maternal antibodies (mainly IgA), cells (neutrophils, lymphocytes, and macrophages), and other proteins (e.g., cytokines, fibronectin, and lysozyme) to protect the infant against infection and allergies [4] [5].

1.2. Carcinoma of the breast

Around 80% of cancer-related deaths in the Western world are due to carcinoma, which arises from epithelia. Breast cancer is not the exception since most breast tumors arise from epithelia. Among them, the majority of human breast cancers arise from the TDLU and not from the ductal system [6]. Here, as I write about breast cancer, I start with describing the etiology and pathogenesis, in order to make it easy to understand the mechanism underlying breast cancer development.

1.2.1. Etiology and pathogenesis

1.2.1.1. Risk factors of breast cancer

Carcinoma is the most common malignancy of the breast. A woman who lives to age 90 has a one in eight chance of developing breast cancer. Because of the frequency of this disease in women, extensive studies on risk factors for developing breast cancer have been made.

Four of the most common risk factors for the development of breast cancer are, age, age at menarche, first live birth and first-degree relatives. Age is a risk factor common with almost all types of cancers, because long life itself accumulates not only DNA damages, but also the other risk factors. On the other hand, age at menarche and first live birth are risk factors remarkable for breast cancer.

The younger a woman's age at menarche, the higher her risk to suffer from breast cancer [7]. Women who reach menarche at 12 years old or younger have 23% increased risk of developing breast tumor compared to women who reach menarche after 15 years old [8] (table 1). Late menopause also increases the risk. For every 5-year difference in age at menopause, the risk for breast cancer changes by about 17% [9]. There is also a report showing that women who reach natural menopause after age 55 have double of the risk of developing breast tumor compared to women who reach natural menopause before age 45 [10] (table 2). However, among postmenopausal women, the increased risk associated with late age at natural menopause is generally not seen until age 65, suggesting that the effect of age at menopause is not seen for 10-20 years after menopause [11]. The increased risks associated with early menarche and late menopause suggest that the longer the exposure to sex hormones through a woman's life, the higher the risk of breast cancer [12]

.

Another risk factor is age at first pregnancy. The younger a woman is when she has her first full-term pregnancy, the lower her risk of developing breast cancer [7]. Women with a first full-term pregnancy at younger than 20 years old have half the risk of women who do not give birth all through their life or women with a first full-term pregnancy at age of over 35 [13] (table 3). It is hypothesized that pregnancy results in terminal differentiation of epithelial cells, removing them from the potential pool of cancer precursors. However, the biologic basis of such differentiation has not been determined.

The risk of breast cancer also increases with the number of affected first degree relatives (mother, daughter or sister). However, not a majority of women suffering from breast cancer have such backgrounds. Only 13% of women with breast cancer have one affected first-degree relative, and only 1% have two or more affected first-degree relatives. This means that around 87% of women with a family history will not develop breast cancer [14].

TABLE 1. Odds ratios for breast cancer, by age at menarche*

Age (years) at menarche	Odds ratio
<12	1.00†
12	0.91
13	0.82
14	0.85
≥15	0.77

TABLE 2. Odds ratios for breast cancer, by age at natural menopause*

Age (years) at natural menopause	Odds ratio
<45	1.0†
45–49	1.3
50–54	1.5
≥55	2.0

TABLE 3. Adjusted odds ratios for the association between age at first full-term pregnancy and breast cancer among parous women aged 20–54 years, Cancer and Steroid Hormone Study*

Age (years) at first full-term pregnancy†	Adjusted odds ratio‡
<18	1.0§
18–19	1.0
20–21	1.0
22–23	1.1
24–25	1.1
26–27	1.0
28–29	1.2
30–31	1.4
32–34	1.5
≥35	1.6

Table 1-3
Relation between risk of developing breast tumor and age at menarche, natural menopause and first full term pregnancy

Reviewed by Jennifer L. Kelsey et al. 1993

1.2.1.2. Molecular alterations in breast cancer

Breast cancer is remarkable for its heterogeneity and currently it is not possible to provide a full list of all potential molecular alterations causing breast cancer. However, some are well known. Among them, here, I start with describing the tumor suppressor genes, *BRCA1* and *BRCA2,* molecules altered in breast cancer, because mutation in *BRCA1* and/or *BRCA2* is the most common event occurring in familial breast cancer patients. Then I will mention about estrogen receptor and ErbB2/HER2 receptor as most notable molecules altered in sporadic breast cancers. In fact,

estrogen receptor positive breast cancer and ErbB2/HER2 overexpressing breast cancer account for a large portion of all breast cancers, 70% and 20% respectively. Furthermore, currently breast cancer is roughly classified into three subgroups for clinical reason, estrogen/progesterone receptor positive, ErbB2/HER2 receptor overexpressing, and the others.

1.2.1.2.1. BRCA1 and BRCA2

About 25% of familial breast cancers, meaning around 2-3% of all breast cancers, can be attributed to two autosomal genes, *BRCA1* and *BRCA2* [14]. BRCA1 and BRCA2 act as tumor suppressors. The probability of breast cancer is associated with a mutation in these genes.

BRCA1 and BRCA2 do not show sequence homology, however, they function in similar pathways and interact with the same multi protein complexes. A wide variety of functions have been suggested for these proteins, including transcriptional regulation, cell-cycle control, ubiquitin-mediated protein degradation pathways, and chromatin remodeling.

A key function for both BRCA1 and BRCA2 appears to be their role in protecting the genome from damage by halting the cell cycle and promoting DNA damage repair in a complex process that is not yet fully understood. BRCA1 is phosphorylated in response to DNA damage and may transduce DNA damage signals from checkpoint kinases to effector proteins [15]. BRCA2 can bind directly to DNA and function in homologous recombination, which ensures the error-free repair of double strand DNA breaks [16].

Studies have shown that mutations of the *BRCA1* and *BRCA2* genes increase the risk of early onset breast carcinoma. BRCA1 mutation carriers have an 18% risk and BRCA2 mutation carriers have a 15% risk for developing breast cancer before the age of 39 years, and the risk increases to 59% for BRCA1 mutation carriers and 34% for BRCA2 mutation carriers at ages 40–49 years old[17] [18]. In hereditary carcinomas, one mutant *BRCA* allele is inherited, and the second allele is inactivated by somatic mutation. Although *BRCA1* and *BRCA2* mutations are rarely if ever found in sporadic tumors, about 50% of such tumors have decreased or absent expression of BRCA1. In most cases, this is accomplished by a combination of loss of heterozygosity (LOH) and methylation of the promoter to inactivate both alleles [19].

BRCA1-associated breast cancers are more commonly poorly differentiated compared to sporadic breast carcinomas and do not express hormone receptors or overexpress ErbB2/HER2 receptor, while *BRCA2*-associated breast carcinomas do not have a distinct morphologic appearance [20] [21] [22]. Most *BRCA1*-associated breast carcinomas are characterized by the expression of basal (myoepithelial) markers, such as cytokeratin 5/6 and or P-cadherin. Furthermore, RNA profiling has revealed that *BRCA1*, *BRCA2*, and subtypes of sporadic cancers can be recognized by their gene expression patterns [23] [24]. The mRNA profile of *BRCA1*-associated breast carcinoma is similar to a type of sporadic carcinomas termed "basal-like" carcinomas. These results suggest that a subset of sporadic carcinomas have biologic similarities to hereditary carcinomas [22].

1.2.1.2.2. Estrogen and ER

Approximately and 70-80% of sporadic breast carcinoma express estrogen receptor (ER) [25-27]. In normal breast, estrogen plays a role in the growth of mammary gland in females and it lasts till the menopause, however, many evidences support the hypothesis that cumulative and/or excessive exposure to endogenous estrogen across a woman's life span contributes to and may be a causal factor in breast cancer [28].

Estrogen is a general term for a group of steroid sex hormone which is mainly secreted from ovary. The major members of estrogen are estrone (E1), estradiol (17β-estradiol, E2) and estriol (E3). Estrogens are naturally occurring cyclopentanophenanthrene compounds whose synthesis begins with cholesterol. While all E1, E2 and E3 can bind estrogen receptor (ER), the most potent and dominant estrogen is E2.

Estrogens can function via multiple mechanisms (Fig.2) [28]. The classic mechanism is the binding of the estrogens to nuclear ERs. Then the estrogen-ER complexes bind as dimers to estrogen-response elements (EREs) in the regulatory regions of estrogen-responsive genes and associate with basal transcription factors, co-activators and co-repressors to alter gene expression. For example, E2 and ER complex can mediate the activation of proto-oncogenes and oncogenes (e.g. c-fos, c-myc), nuclear proteins, as well as other target genes and eventually drive the proliferation of premalignant lesions as well as cancers [29, 30]. Non-classical mechanism involves ER-mediated gene expression without direct binding to DNA but through modulation of protein-protein interactions with other DNA-binding transcription factors [31]. Like peptide growth factors, estrogen can also exert non-genomic actions that are too rapid to be accounted for by gene

transcription and RNA and protein synthesis. For this, membrane-bound ERs play roles. Binding of estrogens to membrane-bound form of ERs leads to activation of various protein kinases [32] [33] (Fig.2). Reported nongenomic effects of 17β-estradiol include direct or indirect activation of adenylate cyclase and production of cyclic AMP, MAPK, Akt, Src, Shc, the regulatory and endothelial nitric oxide synthase (eNOS), amoung otheres [34]. There are also cross-talks between the membrane ER signaling process and other signaling pathways, such as epidermal growth factor receptor and insulin-like growth factor 1 receptor signaling pathways [28]. Recent study also showed the presence of ERs in mitochondria of various cells and tissues. The mitochondrial genome contains potentially estrogen-responsive sequences and estrogen has increased mitochondrial DNA-encoded gene transcript levels [28]. Finally, amplified growth factor receptor signaling can post-translationally modify the ER and its coactivators, resulting in estrogen-independent transcriptional activity of ER at EREs [35].
Besides these effects of estrogen on intracellular signaling and/or gene translation, estrogen itself can cause DNA damage phisically. Metabolites of estrogen can cause mutations or generate DNA-damaging free radicals [36] [28].

ERs have been initially identified as ligand-activated transcription factors that belong to the nuclear hormone receptor super family. Today, the two receptors are known as ERα and ERβ. They are products of two distinct genes. However, they are highly similar to each other when compared at the amino acid level. The amino acid sequence identity between ERα and ERβ is approximately 97% in the DNA-binding domain and approximately 56% in the ligand-binding domain, whereas the N terminus is poorly homologous at 24% [37]. In breast, ERα is found in the ductal and lobular epithelial cells, but not in stroma [38]. On the other hand, ERβ is found in both ductal and lobular epithelial and stromal cells of the rodent [38]. The functional difference between ERα and ERβ has not yet been clearly understood.

Although currently only the ERα form is clinically measured for clinical decision-making and treatment, various ER transcripts have been found in breast carcinomas [38]. Protein products corresponding to variant ERs have been described previously [39]. Normal and cancer tissues display a variety of distinct profiles regarding ERα, ERβ, and splice variants at both mRNA and

protein levels [39]. This heterogeneity in ER isoform profiles is suggested to result in variations in estrogen signaling and might affect breast cancer risk, hormone responsiveness, and survival.

Currently, Tamoxifen and similar antiestrogens inhibitors are the first-line therapy for treatment of hormone-dependent breast cancer [40]. However, further study to understand the heterogeneous estrogen-ER complex function is needed to develop more efficient treatment for ER positive breast cancer patients.

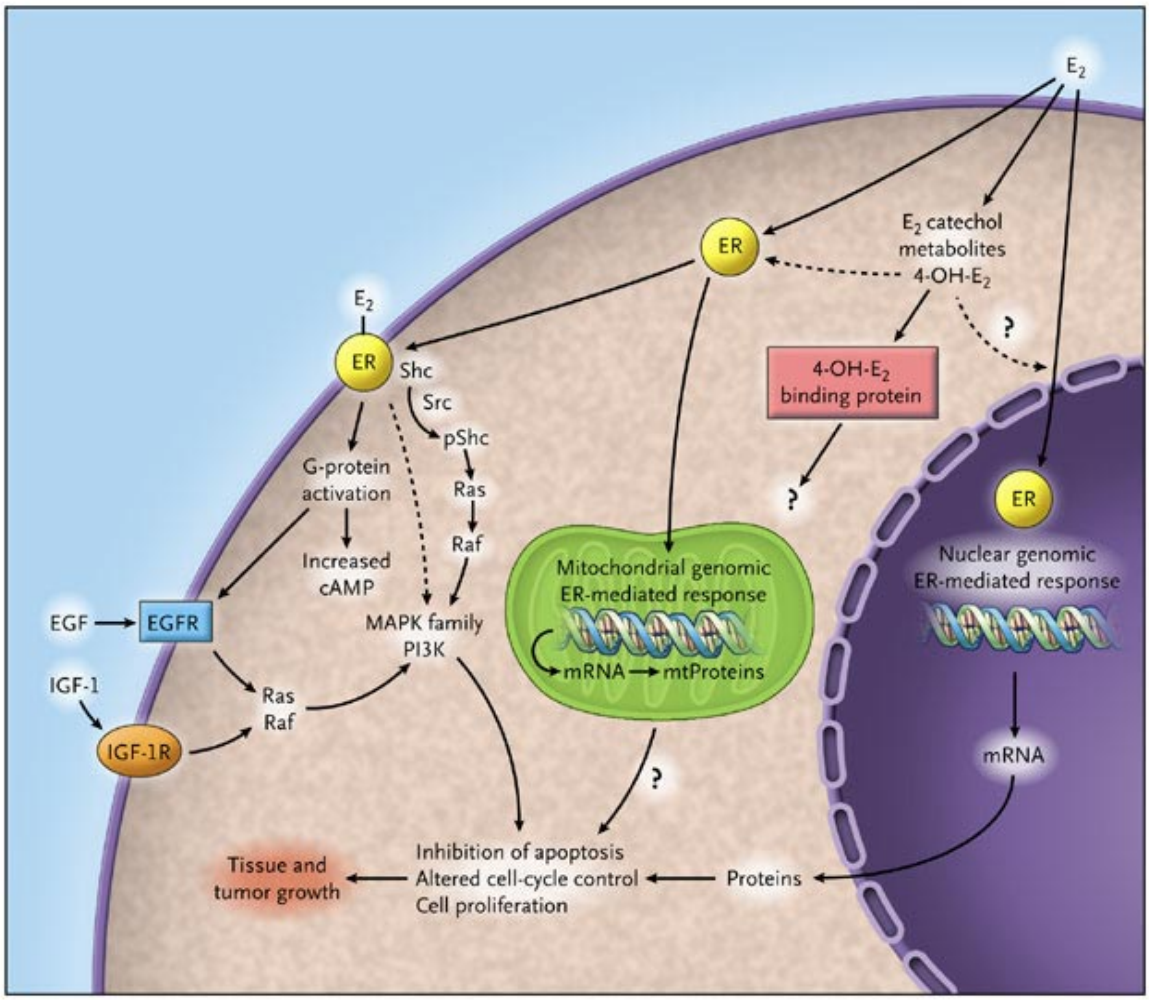


Figure 2. Estrogen-receptor-signaling pathways *Reviewed by James D.Yager et al. 2006*

1.2.1.2.3. ErbB2 receptor

In 1983, *v-erbB* was first cloned from avian eythroblastic leukemia virus [41] and in the next year, it was suggested that *v-erbB* was oncogenic form of the proto-oncogene coding EGFR [42]. Two years after the cloning of *v-erbB*, a gene coding tyrosine kinase receptor which also has a high homology to the gene coding human EGFR was cloned. The product of this gene was named HER2, an abbreviation of "human EGFR-related 2". At the same time, HER2 was found to be the same molecule coded by the oncogene, *neu* which had been found in rat neuroglioblastoma [43]. And around the same time, another group reported that a gene which has homology to *v-erbB* was amplified in human breast cancer cells [44]. This gene also had high homology to the gene coding human EGFR, but not the same gene. Then this new gene was named *c-erbB2*, while the gene coding human EGFR was named *c-erbB1*. Eventually, *neu* and *c-erbB2* were found to be the same gene [45]. This was the first report showing the relation between ErbB2/HER2 and breast carcinoma.

After these findings, a lot of researches on ErbB/EGFR have been made. Today, it is known that the ErbB family consists of four family members, EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. Homology between the molecules of this family is around 70-80%. All members have an extracellular ligand-binding region, a single membrane-spanning region and a cytoplasmic tyrosine-kinase-containing domain. All of these family members, except for erbB2 have their corresponding ligands. Binding of these ligands to each ErbB receptor leads to homo- or hetero-dimerization of the receptors and activation of their intracellular kinase domains. There are altogether 10 reported ligands. EGF, TGF-α, and amphiregulin (AR) bind specifically to ErbB1. Betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR) bind to ErbB1 and ErbB4. Neuregulin1 (NRG1) and neuregulin2 (NRG2) bind to ErbB3 and ErbB4. Neuregulin3 (NRG3) and neuregulin4 (NRG4) bind to ErbB4 (Fig.3).

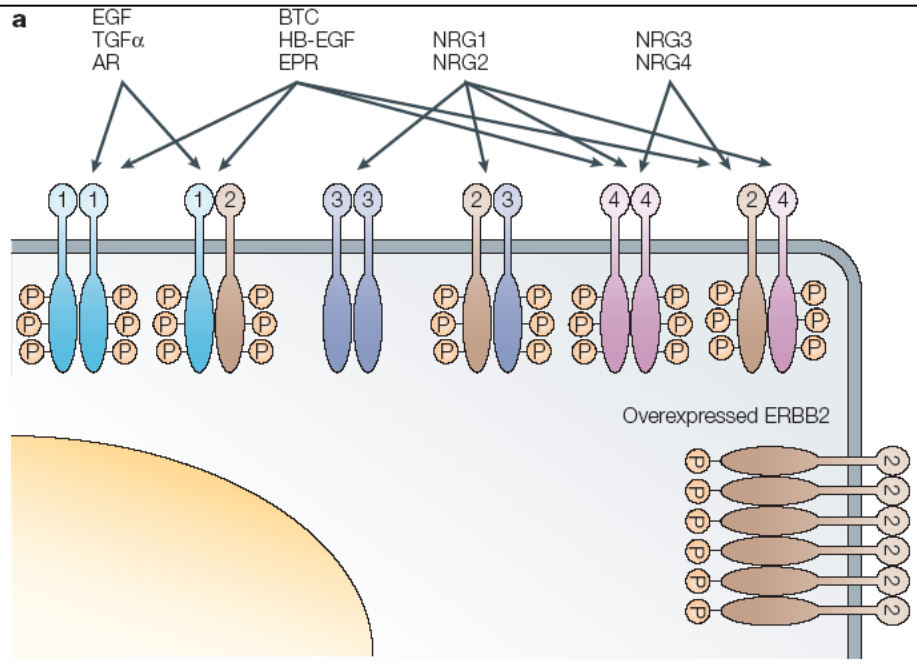


Figure 3. ErbB receptors and ligands *Reviewed by Hynes, NE. and Lane, H. 2005*

Structural studies suggest that biding of these ligands to ErbB receptor changes the conformation of the receptor so that the protruding arm from the extracellular domain of the erbB receptor becomes "opened-form". This protruded structure provides a direct receptor-receptor interaction. Because of this unique mechanism to form a receptor dimer, the two ligands are distant from each other even after dimerization [46] [47]. Although there are no reported soluble ligands which bind to ErbB2, ErbB2 can form heteromeric complexes with ErbB1, ErbB3, or ErbB4. This can be explained by the conformation of ErbB2. Even without ligand binding, ErbB2 shows "opened-form" of the extracellular protruded arm which provides a direct receptor-receptor interaction. The structure of ErbB2 is similar to that of the EGFR when it binds with its ligand [48]. Because of this structural reason, ErbB2 can form heterodimers with the other ErbB members without ligand binding.

Upon the dimerization of ErbB receptors, the intracellular kinase domains of the receptors get activated and signals are transferred to various intracellular signaling pathways via phosphorylation. The only exception, ErbB3 has impaired kinase activity and only acquires signaling potential when it is dimerized with another ErbB receptor and phosphorylated. Downstream process of ErbB

signaling is very complex. However, the two main pathways downstream of ErbB receptors are mitogen-activated protein kinase (MAPK) pathway and phosphatidylinosithol-3-kinase (PI-3K) pathway. Shc- and/or Grb2-activated MAPK pathway is a common target of all ErbB receptors [49]. Similarly, the PI-3K pathway is directly or indirectly activated by most ErbBs, but mainly due to ErbB3 [50].

Among all ErbB receptors, ErbB2 has very strong kinase activity [51] and notably, around 25% of breast tumor overexpress ErbB2 [52]. This overexpression of ErbB2 in breast tumor is mainly caused by gene amplification or by hyper-activated gene transcription. Furthermore, ErbB2 overexpression is associated with increased tumor aggressiveness, increased rates of recurrence, and increased mortality [53] [54]. Although mutations in kinase domain of ErbB2 were identified in a small subset of non-small cell lung cancer and subsequently in other tumor types including head and neck, ovarian, brain and gastric cancers [55] [56], no activating alterations of the *c-erbB2* gene have been found in human breast tumors [57]. ErbB2/ErbB3 heterodimer is reported to have the strongest signal transduction ability among all the homo- and heterodimers of ErbB receptor family members [51]. Besides the complex with ErbB3, the overexpression of ErbB2 also induces spontaneous receptor dimerization without the need of ligands. ErbB2/ErbB3 heterodimers and ErbB2/ErbB2 homodimers or higher order complexes are considered to play important roles in breast cancer progression.

Upon ErbB2 activation, signaling molecules are recruited to phosphorylated tyrosine residues triggering intracellular signaling cascades such as the Ras/Erk and PI3K/Akt pathways (Fig4-a). In addition to this classical route, although it is not so well accepted, ErbB2 was also shown to translocate to the nucleus and associate with specific sequences within the promoter region of proto-oncogenic genes to regulate their expression (Fig4-b) and furthermore, ErbB2 was demonstrated to control expression of specific proteins at the level of translation, via the Akt/mTOR pathway (Fig.4-c) [56]. Much more research should be made on the signaling pathways downstream of ErbB2 to understand the mechanism of tumorigenesis, however, it is clear that ErbB2 receptor signaling should be one of the most potent targets to treat breast cancer patients.

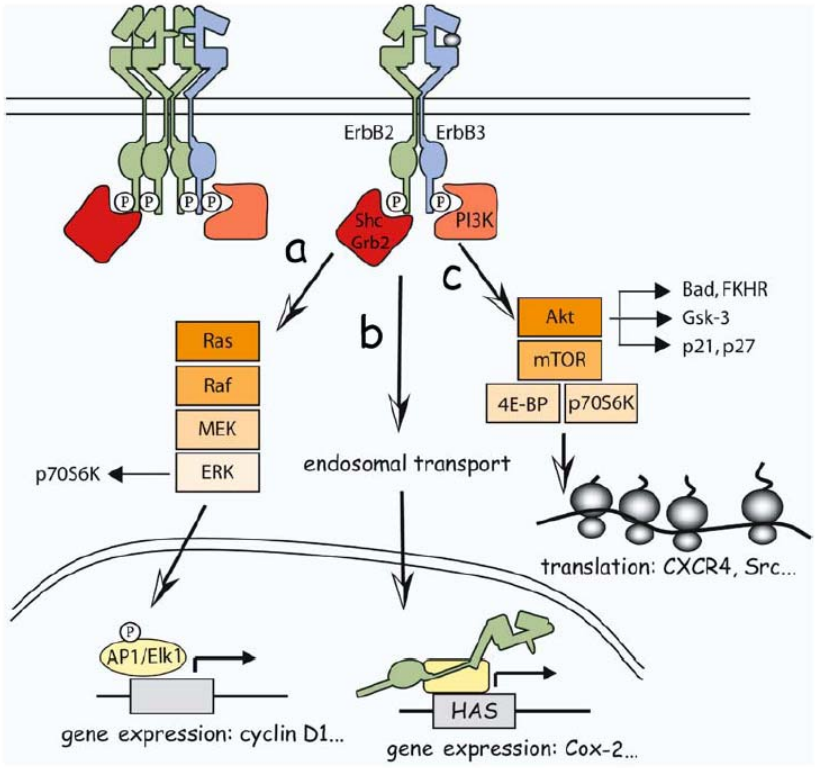


Figure 4. ErbB2-induced intracellular signaling pathways *Reviewed by Badache, A. et al. 2006*

In 1985, it was shown that the transformed phenotype of neu-transformed NIH3T3 cells is reverted to an untransformed phenotype by anti-neu antibody treatment [58]. This report prompted researchers to develop an antibody against human erbB2 for the use of breast cancer therapy. As a result of researchers' efforts, trastuzumab (Herceptin) has been developed and currently it is used to target ErbB2 in ErbB2 overexpressing breast cancer patients. Trastuzumab is a recombinant humanized monoclonal antibody directed against the extracellular domain of ErbB2. Although trastuzumab treatment is efficient in clinical studies, the mechanisms by which trastuzumab exert the effect on ErbB-overexpressing tumors are not fully understood. Initially, it was thought that upon the binding with trastuzumab, ErbB2 receptor is internalized and degraded. However, while ErbB2 down-modulation has long been considered as the primary event of trastuzumab inhibitory effect, some studies examining ErbB2 localization and trafficking suggest that trastuzumab does not actually down-modulate ErbB2 [59]. Thus this "ErbB2 internalization hypothesis" is not

satisfactory. On the other hand, another hypothesis is that after the binding with ErbB2, the Fc domain of trastuzumab triggers antibody dependent cellular cytotoxicity (ADCC). As another hypothesis, although this hypothesis is currently not well accepted, in 2004, it was reported that trastuzumab activates tumor suppressor protein, PTEN, resulting in the suppression of PI3-K [60]. PTEN binds to the plasma membrane via the C2 domain that is negatively regulated by Src-dependent tyrosine phosphorylation. Trastuzumab is hypothesized to interfere with the association of ErbB2 and Src, resulting in the inhibition of Src activation. If Src is not activated, PTEN is not phosphorylated. Without tyrosine phosphorylation, PTEN can go to the plasma membrane and there dephosphorylate PIP3, leading to the inhibition of the activation of AKT (PKB) [61]. These hypotheses are still under discussion and further research is necessary for the efficient use of trastuzumab treatment.

1.2.2. Classification of breast carcinoma

Breast cancer is remarkable for its heterogeneity and further investigation will need to answer the question, “how heterogeneous it is”. As a simple example, in normal human mammary grand, there are two distinct types of epithelial cell, luminal epithelial cells and basal (and/or myoepithelial) cells. These two cell types can be distinguished immunohistochemically by detecting the expression of specific keratins. Luminal epithelial cells can be stained with antibodies against keratin 8/18 [62], on the other hand, basal epithelial calls can be stained with antibodies against keratin 5/17 [63]. Character of a breast cancer, indeed, seems to differ depending on which lineage a breast cancer arise from. Furthermore, in addition to this histology-based classification, at the clinical level, breast carcinoma has been roughly categorized according to the status of estrogen receptor (ER), progesterone receptor (PgR), and ErbB2/HER2 receptor for clinical decision-making and treatment.

ER status is measured as a prognostic factor and a means to predict response to endocrine (anti-estrogen) therapy. PgR is an ER-regulated protein and the presence of PgR should indicate a functional ER pathway. In fact, the presence of PgR was associated with better response to endocrine therapy in some studies [64] [65] [66]. On the other hand, overexpressed ErbB2/HER2 in tumor tissue suggests a good response to therapies targeting the pathway activated by ErbB2/HER2, for example, treatment with trastuzumab (Herceptin).

However, not all the ER+/PgR+ breast carcinomas react to hormonal treatments and also not all HER2 overexpressing breast tumor is cured by trastuzumab treatment. Furthermore, 10-15% of breast cancers are neither ER/PgR positive nor overexpressing HER2. These remaining breast cancers are in a so-called "triple-negative" (also called "receptor-negative") category [67]. Breast cancer in this category is not treated with therapy targeted to ER or ErbB2 signaling. The lack of targets prompted researchers to find more precise classification of breast cancer in order to target candidate pathways to develop efficient treatment for individual breast cancer patients.

In 2000, Perou and co-workers showed the possibility to classify breast cancers based on gene expression profiles using DNA microarray technology [68]. Within a couple of years after this report, the same group reconfirmed the robustness of this DNA microarray-based classification and showed that there are at least 5 subtypes of breast cancer based on gene expression profiles, Luminal A, Luminal B, Basal-like, ERBB2+, and Normal Breast-like subtypes [69, 70].

Luminal subtypes are ER positive and characterized by the relatively high expression of many genes expressed by breast luminal cells including keratin 8/18. Within the luminal subtypes, luminal A shows the highest expression of the ERα gene, on the other hand, luminal B shows low to moderate expression of the luminal specific genes including the ER cluster. The other groups are all characterized by low to absent gene expression of the *ER* and several additional transcriptional factors expressed in the luminal/ER+ cluster. The basal-like subtype is characterized by high expression of keratins 5 and 17, laminin and fatty acid binding protein 7. Because of the expression of the basal keratins 5 and 17, this group is called "basal-like". ErbB2+ subtype is characterized by high expression of several genes in the *ERBB2* amplicon at 17q22.24 including *ERBB2* and *GRB7*, suggesting that a potent mechanism of overexpressing ErbB2 in breast cancer is gene amplification. Normal breast-like group shows the highest expression of many genes known to be expressed by adipose tissue and other nonepithelial cell types and also shows strong expression of basal epithelial genes and low expression of luminal epithelial genes[68] [69].

This method to classify breast cancer subtype according to DNA expression profile brought us new aspects of breast cancer classification. The proportion of each subtype in all breast cancer cases are calculated and shown on table 4 [70]. Three independent studies show almost same distribution of the five novel breast cancer subtypes. The prognoses for each subtype are also distinctive. The

prognosis is the best for luminal A breast cancer, on the other hand, basal-like breast cancer has the worst prognosis (Fig.5).

Data set	Basal	ERBB2	Luminal A	Luminal B	Normal breast-like	No subtype
Norway/Stanford	17	10	24	10	3	36
van't Veer *et al.*	21	6	25	18	5	25
West *et al.*	37	4	39	14	0	6

Sorlie, T. et al. 2003

Table 4. Distribution of tumors (%) from three different studies across five tumor subtypes

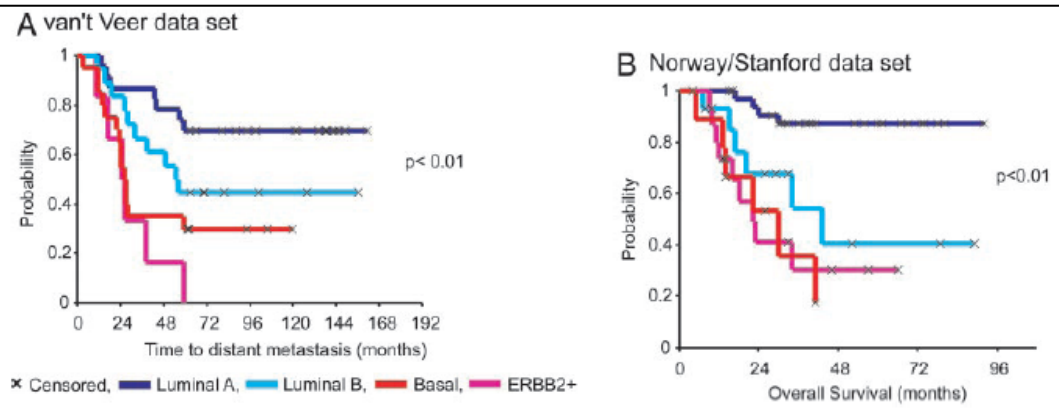


Figure 5. Prognoses of different subtypes of breast cancer *Sorlie, T. et al. 2003*
(Data was obtained without Herceptin treatment)

Interestingly, Carcinomas arising in women with *BRCA1* mutations also cluster with basal-like group. *BRCA1* carcinomas are similar to basal-like carcinomas in being poorly differentiated, lacking ER and HER2/neu expression, and expressing basal-like keratins. However, most women with basal-like carcinomas do not have germ-line *BRCA1* mutations [70] [67].

Currently, there are no treatment targeting basal-like breast cancer and it has the worst prognosis as described above. There are still many things to be uncovered in order to find a good strategy to treat basal-like breast cancer, however, the character of basal-like breast cancer is still largely unknown. For example, we do not have a clear answer even to the simple question, "where does basal-like breast cancer come from?" First of all, in the breast, the term "basal" has acquired two meanings. In one context it has become the word having the same meaning of "breast myoepithelium" and in the

other context, it defines a specific subpopulation of “basal” cytokeratins, CK5, CK14 and CK17, expressing cells [6]. However, in the latter meaning, the origin of “basal” cell is still controversial. Because most basal-like breast tumors still express luminal cytokeratin 8/18, suggesting that basal-like cancers also possess characteristics of luminal lineage. Furthermore, several classic myoepithelial markers were rarely expressed in basal-like tumors. These findings provide evidence against the hypothesis that basal-like breast cancers are derived directly from myoepithelial cells. Instead, these finding could suggest the possibility that basal-like breast cancer cells is differentiated directly from a stem cell [67]. This hypothesis is currently still under the discussion, however, further study on this hypothesis might bring a breakthrough to target basal-like breast cancer.

1.2.3. Breast cancer research Model

1.2.3.1. Breast cancer cell lines

Breast cancer cell lines have been the most widely used models to investigate how proliferation, apoptosis and migration become deregulated during the progression of breast cancer. Established cell lines are easily propagated, relatively easy to manipulate genetically, and generally yield reproducible results under well-defined experimental conditions. Compared to rodent cells, human cells are more frequently used. The main reason for this is, first of all, human cells have more relevance to human disease. Furthermore, it has been suggested that same genetic alterations might not transform both mouse and human epithelial cells [71]. Various breast cancer cell lines are used for breast cancer research. One of the most comprehensive studies on these breast cancer cell lines is the report by Neve R. M. and his colleagues [72]. There, cells are characterized according to their ER, PgR, HER2, and TP53 (p53) status.

1.2.3.2. Mouse models

The growth of breast cancer cell lines as xenografts makes it possible to investigate the breast cancer growth and progression in the *in vivo* environment, which includes the complex tumor-stromal cell interactions that facilitate tumor formation and progression. As immunocompromised mouse experimental model, nude mice and SCID mice are widely used.

The nude mouse has been a major breakthrough for cancer research because it allows human tumors to be studied in another animal. The nude mice were discovered in 1962 as a hairless mutant. They

have a spontaneous deletion in the *nu* (*FOXN1*) gene on the chromosome 11. As a result, the nude mouse lacks or has a deteriorated thymus and also does not have hair. Because they lack thymus, nude mice cannot generate mature T lymphocytes. Therefore, they are unable to mount most types of acquired immune responses including antibody formation that requires CD4+ helper T cells, cell-mediated immune responses which require CD4+ and/or CD8+ T cells, delayed-type hypersensitivity responses which require CD4+ T cells, killing of virus-infected or malignant cells which requires CD8+ cytotoxic T cells, and graft rejection which requires CD4+ ad CD 8+ T cells.

SCID mice were discovered in 1983. These mice were suffering severe combined immune deficiency (SCID). They have a mutation in the gene, *scid* (*Prkdc*), which is located on the chromosome 16. Because of this mutation, SCID mice can not complete the rearrangement of genes coding IgG and T cell receptor during lymphoid maturation. This leads to the lack of both B cells and T cells in SCID mice. Thus, SCID mice are even more immunodeficient than nude mice, which lack only T cells.

Experiments using these mice have brought us a lot of useful knowledge about breast cancer, however, there are some technical aspects that affect the utility of xenograft models. The most important one is that xenografts must be established in immunocompromised mice as described above. The absence of an intact immune system in xenograft experiments may profoundly affect tumor development and progression.

2. WNT signaling

2.1. Discovery of Wnt1

The research on WNT signaling had started more than 30 years ago. In early-mid ‘70s, a *Drosophila* mutant which lacks wing was found. This mutant fly embryo showed severely impaired segmentation of the epidermis as evidenced by abnormalities in the overlying ventral cuticle. In contrast to the wild-type cuticle, which exhibits alternating denticle and naked belts, the mutant cuticle was completely covered with denticles. These phenotypes were caused by a mutation which inactivates a segment polarity gene, which is in general responsible for specifying anterior posterior polarity within individual embryonic segments. The gene responsible for the phenotype of the mutant fly was named “*wingless* (*Wg*)” [73] [74].

On the other hand, an oncogene which causes mouse mammary tumors was identified through the research on mouse mammary tumor virus (MMTV). It is known that the majority of mammary tumors in mice are caused by MMTV. MMTV is a retrovirus which can be transmitted endogenously or vertically via milk. It does not code an oncogene itself, however, when the virus RNA is reversely transcribed into DNA and inserted inside or near an oncogene of host genome, the expression of the oncogene can be changed and cause a cancer [75]. During the study on MMTV, in 1982, a mouse gene that is induced by (MMTV) was identified and referred as "*int-1*' [76].

These two independent studies unexpectedly met each other in 1987. Rijsewijk and colleagues isolated the *Drosophila* homolog of *Int-1*, *Dint-1*. And found that *Dint-1* and the segment polarity gene *wingless* are identical and map to the same location [77]. Then this protein started to be called as "Wnt-1" (Wingless + int-1). Now it is known that Wnt1 is a modified glycoprotein which is secreted from expressing cells.

2.2. Pathway background

Biochemical properties of Wnt proteins, ligands in Wnt signaling, and receptors in Wnt signaling are well summarized in the review by Kikuchi and his colleagues [78] and the phenotypes of Wnt-related gene knockout mice are well summarized in the review by Amerongen and Berns [79].

2.2.1. WNT ligands

WNT family are secreted, glycosylated and palmitoylated peptides that interact with seven-transmembrane receptors of the Frizzled (FZD) family [80]. The primary amino-acid sequence of Wnts suggests that they should be quite soluble. However secreted Wnt proteins are hydrophobic and are mostly found associated with cell membranes and the extracellular matrix (ECM) [81]. After the purification of active Wnt proteins, mass spectroscopy analyses revealed that Wnt proteins are lipid modified by the attachment of a palmitate moiety on the first absolutely conserved cysteine residue within the protein family [82]. It was shown that Wnt proteins are palmitoylated by the acyltransferase named porcupine and anchored to the membrane of ER [83]. This step is suggested to be critical, because Wnt secretion is completely abrogated in the absence of porcupine [84]. Then ER-anchored Wnt proteins are glycosylated and transported to the cell membrane to be secreted [85].

The hydrophobic character of Wnts strengthens their interaction with the cell membrane and extracellular matrix, favoring short-range autocrine and juxtacrine signaling [84]. A number of diverse signaling pathways are activated upon WNT/FZD binding. Genome sequencing has revealed that mammalian species have roughly 20 (human has 19) secreted Wnt proteins, which can be divided into 12 conserved Wnt subfamilies (Fig.6) [86]. Each human *Wnt* gene exists on an independent gene locus, however, some of the members are located close to each other. Namely, *Wnt3*and *Wnt9b* are on 17q21, *Wnt3a* and *Wnt9a* are on 1q42, *Wnt2* and *Wnt16* are on 7q31, *Wnt1* and *Wnt10b* are on 12q13, *Wnt6* and *Wnt10a* are on 2q35. Among them, notably, *Wnt1* and *Wnt10b,* and *Wnt6* and *Wnt10a* are very close to each other and the expression of these closely-located genes might be regulated in harmony.

2.2.2. WNT receptors

Frizzled (FZD) receptors

Wnt ligands interact with the cell surface receptor, Frizzled (FZD). FZD receptors are seven-pass transmembrane receptors which have cycteine-rich domains (CRD) in their N-terminus. Through the CRD, FZD receptor binds Wnt ligands [87]. In general, it is thought that a monomeric FZD receptor transmit signals downstream upon binding with Wnt ligand, however, the crystallographic resolution of the structure of the mouse FZD8 and sFRP3 CRD domains suggested that CRDs might be able to homodimerise or heterodimerise [87]. Furthermore, there are reports showing that dimerisation of FZD receptor activates the Wnt/β-catenin pathway [88] and that FZD form specific homo- and hetro-oligomers [89]. These reports suggest the wide possibility of the signal transmission mechanism downstream of FZD receptor.

Upon the binding of Wnt to FZD receptor, the intracellular amino sequences, K-T-X-X-X-W directly binds to Dishevelled proteins [90]. There are 10 reported human frizzled receptors. Phylogenetically, the Frizzled receptors fall into four groups. Frizzled-1, 2 and 7, and Frizzled-3 and 6 make up two related groups, while Frizzled-5 and 8 comprise a third group, and Frizzled-4, 9 and 10 generate a distant fourth group [91].

LRP5, LRP6

There also exist co-receptors of FZD receptor. A genetic study using flies showed that a single-pass trans-membrane receptor, Arrow, is required to establish a segment polarity triggered by Wg signaling [92]. Arrow is homologous to two members of the mammalian low-density lipoprotein

receptor (LDLR)-related protein (LRP) family, LRP5 and LRP6. LRP5/6 function as co-receptors of FZD receptor and binding of Wnt ligand to both FRZ receptor and LRP5/6 co-receptor activates Wnt/β-catenin pathdway [93].

Kremen

A single-pass trans-membrane receptor, Kremen, was initially identified as a binding partner of a negative regulator of Wnt/β-catenin signaling, Dkk1. Upon binding to Dkk1, Kremen is internalized by endocytosis with LRP5/6, leading to a suppression of Wnt/β-catenin pathway [94].

Ror2

The Ror family of receptor tyrosine kinases (RTK) consists of two structurally related proteins, Ror1 and Ror2. They have an extracellular CRD, a membrane proximal kringle (KR) domain, and intracellular cytoplasmic tyrosine kinase domain and a proline-rich domain near the c-terminus [95]. Ror2 has been shown to act as an alternative receptor or co-receptor for Wnt5a [96] [97]. In addition to its ability to bind Wnt5a, Oishi and colleagues reported the ability of Ror2 to bind some FZD receptors as well [97], suggesting that Ror2 might play a role as a co-receptor. The extracellular domain of Ror2 associates with Wnt5a but not with Wnt3a. Furthermore, Ror2 mediates Wnt5a signaling by activating the Wnt/JNK pathway and/or inhibiting the β-catenin/Tcf pathday. It has also been shown that Ror2 interacts with filamin A and that it mediates Wnt5a-dependent cell migration [98].

Strabismus / Van Gogh-like Protein

Strabismus is a four-pass trans-membrane protein. There are two Strabismus genes in mouse and human. In human, they are known as human Strabismus-1 (also known as Van Gogh-like Protein-2) and human Strabismus-2 (also known as Van Gogh-like Protein-1). Although Strabismus does not bind with Wnt ligands, it can bind the PDZ domain of Dvl adopter proteins. This leads to the activation of JNK in PCP pathway and suppression of Wnt/β-catenin pathway [99].

Ryk

Ryk is a single-pass transmembrane RTK and Ryk can interact at least with Wnt1 and Wnt3a [100]. Ryk family members have been shown to be required for Wnt signaling in several contexts. For

example, knockdown of Ryk reduces the Wnt1-dependent TCF activation in HEK-293 cells [100]. However, whether Ryk mediates Wnt signaling in concert with Fz-LRP5/6 or independently is not clear and also how Ryk activates the intracellular signaling cascade after binding to Wnt ligands has not yet been uncovered.

2.2.3. Conservation of the WNT pathway

Members of the WNT family of lipid-modified glycoproteins are found in most metazoans, ranging from the sea anemone *Nematostella* (starlet sea anemone) to humans [86] [101]. After the first finding of *wingless* in *Drosophila*, further studies revealed that the gene *wingless* is genetically related to other segmentation polarity genes, *dishevelled* (*dsh*), *shaggy*, *armadillo*, and *pangolin*. These genes were found to possess orthologs in mammalian. Namely, *dishevelled* is a homolog of mammalian gene *Dvl*, *shaggy* is *GSK-3β*, *armadillo* is *β-catenin,* and *pangolin* is *TCF*. These facts suggested that WNT signaling is well conserved during the evolution.

On the other hand, from the embryologic point of view, in 1989, it was demonstrated that body axis of Xenopus early embryo (4-cell stage) is duplicated by the injection of mouse Wnt1 mRNA [102]. Together with the results from the genetic experiments in *Drosophila*, this observation supported the notion that WNT signaling is shared between vertebrates and invertebrates.

A recent genome-wide study has revealed that mammalian species have roughly 20 secreted Wnt proteins, which can be divided into 12 conserved Wnt subfamilies (Fig.6). Of these, 6 subfamilies have counterparts in ecdysozoan animals such as *Drosophila* and *Caenorhabditis.* In contrast, at least 11 of the Wnt subfamilies occur in the genome of a cnidarian, the simplest metazoans with a nervous system, such as *Nematostella* (starlet sea anemone). While this suggests that some Wnt subfamilies were lost during the evolution of the ecdysozoan lineage, the most important thing suggested by these facts is that a complex Wnt factors had existed in multicellular animals well before the Cambrian explosion (550 million years ago) [86] [101].

Being in line with these genetic, embryologic and genome-phylogenic findings, it has been revealed that WNT signaling plays important roles in many biological events in most metazoans. WNT signaling plays roles not only in embryonic development, but also in cell proliferation and differentiation in adult tissues.

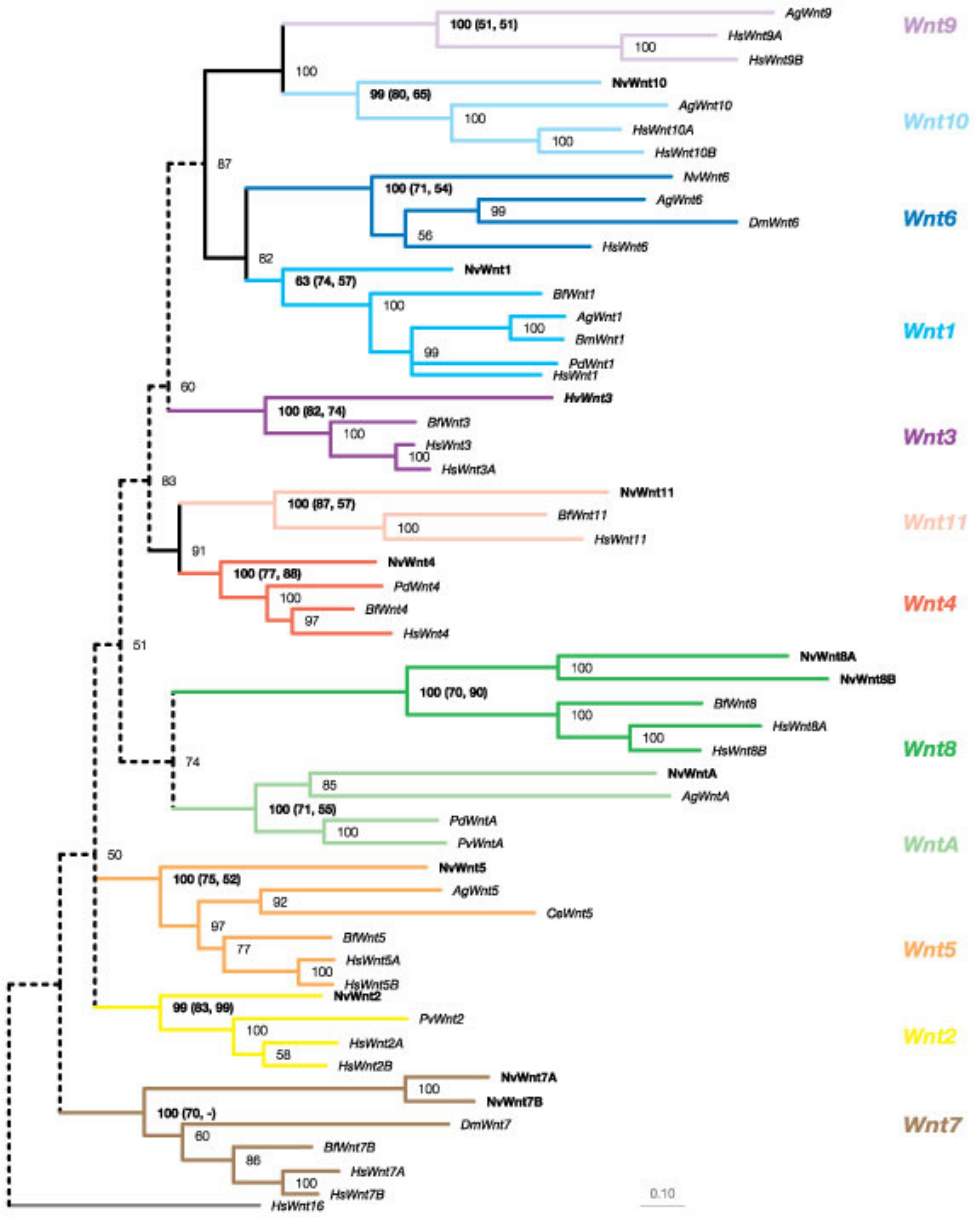


Figure 6. Bayesian inference consensus tree of the Wnt gene family

Bf=Branchiostoma floridae (amphioxus), Bm=Bombyx moori (insect), Ce=Caenohabditis elecans, Dm=Drosophila melanogaster, Hs, Homo sapiens, Hv=Hydra vulgaris, Nv=Nematostella vectensis (sea anemone), Pd=Plathynereis dumerlii (Polychaete), Pv=Patella vulgata (mollusc), Ag=Anopheles gambiae, Bilaterian genes are italicized, N. vectensis genes are in bold, the H. vulgaris gene is italic and bold.

Kusserow et al. 2005

2.2.4. WNT pathways

Upon binding of Wnt to its receptor, either Frizzled or a complex comprising Frizzled and LRP5/6, a signal is transduced to the cytoplasmic phosphoprotein Dishevelled (Dsh). There are three Dsh proteins in mammals (Dsh-1, Dsh-2 and Dsh-3). The ligand/receptor interaction has been shown to induce the phosphorylation of the Dsh family by casein kinase 1ε and -2 and PKCα. [103-105]. This event was reported to be a component of all Wnt-induced signaling pathways [106, 107]. At the level of Dsh, the Wnt signal branches into roughly three separate pathways, the canonical pathway (Wnt/β-catenin pathway), planer cell polarity (PCP) pathway and calcium pathway. Most importantly, the way in which Dsh couples and distributes Wnt signaling into the three signaling branches remains at best poorly understood. Furthermore, compared to the canonical pathway, PCP pathway and calcium pathway are largely unknown. Here, I will describe these three pathways.

2.2.4.1. Canonical pathway (Wnt/β-catenin pathway)

Historically called the "Canonical pathway" signaling is initiated when Wnt ligands engage their cognate receptor complex consisting of FZD family and a member of the LDL receptor family, LRP5/6. The central player is a cytoplasmic protein termed β-catenin, the stability of which is regulated by the destruction complex. When Wnt receptors are not engaged, two scaffolding proteins in the destruction complex, adenomatous polyposis coli (APC) and axin, bind newly synthesized β-catenin. CKI and GSK3, two kinases residing in the destruction complex, then sequentially phosphorylate a set of conserved Ser and Thr residues in the N-terminus of β-catenin. The resulting phosphorylated footprint recruits a β-TrCP-containing E3 ubiquitin ligase, which targets β-catenin for proteasomal degradation. In the presence of Wnt ligands, receptor occupancy inhibits the kinase activity of the destruction complex by an incompletely understood mechanism involving the direct interaction of axin with LRP5/6, and/or the actions of an axin-binding molecule, Dsh (Fig. 7) (Fig.8a). As a consequence, β-catenin accumulates, travels into the nucleus where it engages the N-terminus of DNA-binding proteins of the Tcf/Lef family [108] and initiates gene expression of target genes such as Cyclin D1 and c-Myc [109-111]. The vertebrate genome encodes four highly similar Tcf/Lef proteins. It is also reported that in the absence of a Wnt signal, certain Tcfs repress target genes through a direct association with co-repressors such as Groucho. The interaction with β-catenin transiently converts Tcf/Lef factors into transcriptional activataors. As a

consequence, the canonical pathway translates a Wnt signal into the transient transcription of a Tcf/Lef target gene program [112] [113],

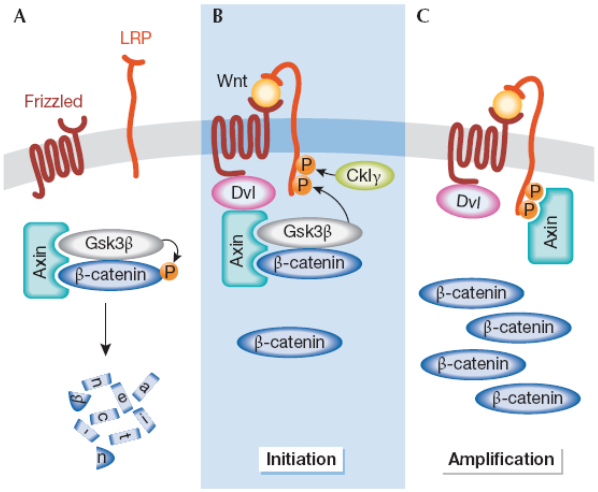


Figure 7. Model for the activation of the Wnt/β-catenin pathway

(B) On binding of Wnt to the receptors, FZD and LRP, Dvl binds to FZD and recruits the destruction complex through interaction with axin. Subsequently, LRP is phosphorylated and acts as docking site for axin. (C) Binding of axin to LRP leads to inhibition of the destruction complex and stabilization of β-catenin.

Fuerer, C and Nusse, R 2008

2.2.4.2. Planar cell polarity (PCP) pathway and Ca^{2+} pathway

In the PCP pathway Wnt signaling through FZD receptors mediates asymmetric cytoskeletal organisation and the polarization of cells by inducing modifications to the actin cytoskeleton. Two independent pathways, which are initiated by Dsh trigger the activation of the small GTPases Rho and Rac. Activation of Rho requires Daam-1 and leads in turn to the activation of the Rho-associated kinase ROCK. Rac activation is independent of Daam-1 and stimulates Jun Kinase (JNK) activity (Fig.8b) [114] [115]. In vertebrates, Strabismus is a four-TM molecule that recruits Dsh to a PDZ domain on its cytoplasmic tail [99]. Dsh interaction with Strabismus interferes with β-Catenin signaling and activates the PCP pathway. Evidence suggests that it is Wnt itself that determines the pathway involved. Canonical Wnts such as Wnt-1 and Wnt-3a block Strabismus binding of Dsh, and encourage its association with Frizzled. Wnt-5a and Wnt-11, however, promote Dsh interaction with Strabismus and block its association with Frizzled [116].

Wnt signaling via FZD receptors can also lead to the release of intracellular calcium. FZD co-receptors involved in this pathway include Knypek and Ror2. Other intracellular second messengers associated with this pathway include heterotrimeric G-proteins, phospholipase C (PLC) and protein kinase C (PKC). The exact genes activated by the Wnt/Ca^{2+} pathway are unknown, but NFAT, which is a transcription factor regulated by the calcium/calmodulin-dependent protein

phosphatase, calcineurin, appears to be involved. The Wnt/Ca^{2+} pathway is important for cell adhesion and cell movements during gastrulation (Fig.8c) [115, 117].

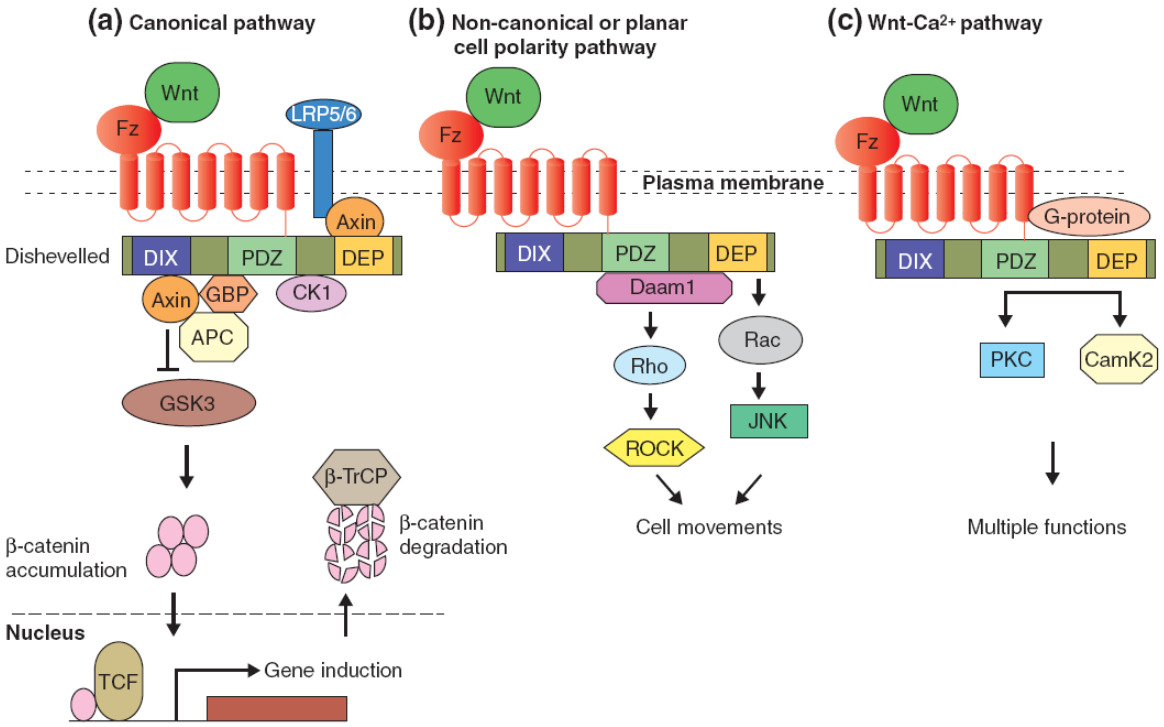


Figure 8. Three Wnt pathways branch at the level of Dsh

(A) Canonical pathway requires DIX domain and PDZ domain of Dsh and leads to β-catenin stabilization. (B) In PCP pathway, activation of Rho requires Daam1 that binds to the PDZ domain of Dsh. Rac activation is independent of Daam1, but requires DEP domain of Dsh. (C) In calcium pathway, the signal via FZD mediates activation of heteromeric G proteins, which engage Dsh, PLC, CamK2 and PKC. This pathway also uses the PDZ and DEP domains of Dsh. *Habas et al. 2005*

2.2.5. WNT negative regulators

2.2.5.1. sFRPs (Secreted Frizzled-related Proteins)

First, I will describe sFRPs in general. Afterwards, I will also describe specifically sFRP1 in detail, since I examined the effect of sFRP1 on breast cancer during my PhD study.

The sFRPs are a group of Wnt-binding glycoproteins that resemble the transmembrane receptor FZD. Their actions are mainly considered to be inhibitory to Wnt activity, however, there are also some reports showing their actions stimulatory to Wnt activity at low concentrations [118].

There are presently eight known members of the family, sFRP1 to sFRP5, Sizzled, Sizzled2 and Crescent. On the basis of sequence homology, sFRP1, sFRP2 and sFRP5 form a subgroup, as do sFRP3 and sFRP4, which are quite distantly related to the other sFRPs. Sizzled, Sizzled2 and Crescent form a third group, but they have not been identified in mammals and Drosophila [119] [120].

All sFRPs are secreted and derived from unique genes and none are alternate splice forms of the FZD family [120]. They share sequence similarity with the Frizzled receptor CRD (cysteine rich domain), but lack the transmembrane and intracellular domains [121] [122] [123]. Through its CRD, sFRP exhibits the ability to bind Wnt. Furthermore, the CRD of sFRP1 also appears to interact with itself to make dimmers or multimers and with FZD [124]. Thus, sFRPs may block Wnt signaling either by interacting with Wnt proteins to prevent them from binding to FZD receptors or by forming nonfunctional complexes with FZD receptors [125] [126] [124].

sFRP1

Human sFRP1 is also known as SARP2 (secreted apoptosis-related protein 2) and FrzA (Frizzled in aorta). In some human colorectal tumors, sFRP1 is found to be mutated. First, mutations in its gene create a stop codon at precursor position (3 out of 10 samples). Second, there can be a 1 aa insert at position 13, leading to the addition of an extra alanine residue (31% from colorectal cancer-free individuals and 35% from colorectal cancer patients). Additionally, there is an alternate splice form that removes the extreme seven C-terminal aa and replaces them with a new stretch of 30 aa. This new addition is suggested to serve as a membrane "anchor", creating a membrane-anchored receptor [127]. In addition to gene-directed changes, proteolytic processing creates multiple sFRP1 isoforms. In particular, the N-terminus is reported to begin at Ser4, Asp14, and Phe23 of the mature segment. The two shortest forms represent up to 17% of all sFRP1. Further, the C-terminal lysine is known to be selectively cleaved. The significance of this is unknown [128].

sFRP1 has been reported to bind to Wnt1 [129] [124], Wnt2 [124, 130], Wnt8 [131], Wnt4 and Wnt3a [132] [133]. However, it does not bind to Wnt5a [129] [130]. In any event, in binding to Wnts, sFRP1 would seem to act primarily as an inhibitor of Wnt signaling [129] [118]. sFRP1 binding to Wnt1 is reported to be antagonistic to Wnt activity [124]. sFRP1 also has been reported to protect cells from apoptosis, but this may be context dependent [134] [132] [135] [136]. Other functions associated with sFRP1 include endothelial cell migration and capillary tube formation [136], myofibroblast recruitment and collagen deposition, and a sFRP-induced decrease in MMP-9 activity [134].

2.2.5.2. WIF1 (Wnt-inhibitory fancor 1)

WIF1 is a Wnt binding protein secreted by variety of tumors and embryonic tissues. WIF1 has an N-terminal signal sequence, a unique WIF domain (WD) that is highly conserved across species, and five EGF-like repeats. Although WIF1 does not share any sequence similarity with the CRD domain of FZD or sFRPs, it can bind to Wnt ligands [119]. It apparently does so by forming a non-covalent complex with Wnt8 and Wnt1 [137].

2.2.5.3. Dkks (Dickkopfs)

The Dkk family comprises four structurally-related members (Dkk1 to Dkk4) and a unique Dkk3-related protein named Soggy (Sgy), which possesses homology to Dkk3. Dkks contain two characteristic cysteine-rich domains separated by a linker region of variable length [138] [139]. Dkk1 is a negative regulator of Wnt-mediated LRP signaling. Dkk1 interacts with LRP5/6 and a single-pass transmembrane proteins Kremen1 (Krm1) and Kremen2 (Krm2), which are endocytosable molecules [94]. Using these interactions, Dkk1 can form a “bridge” between LRP and Kremen leading to the endocytosis of Kremen accompanied by internalization of Dkk/LRP. This internalization blocks LRP deactivation/destabilization of axin and results in the phosphorylation/degradation of β-catenin [119] [140] [141] (Fig.9). Thus, Dkk1 acts as a negative regulator of Wnt/β-catenin signaling, but not PCP signaling or calcium signaling.

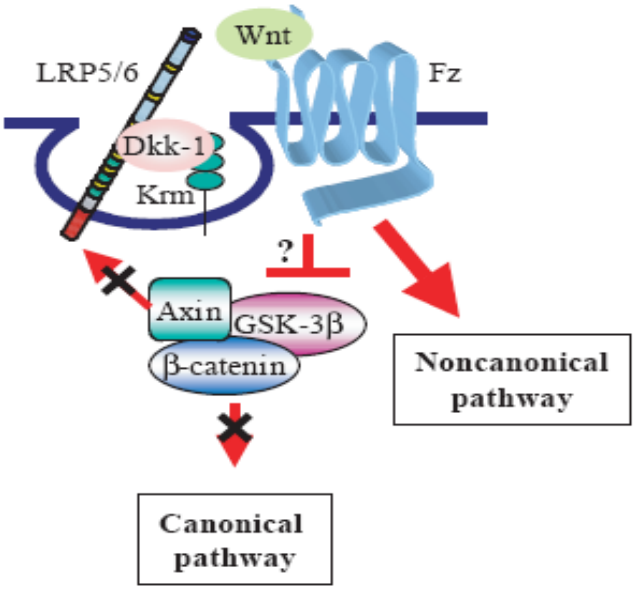


Figure 9. Dkk1 inhibitory effect on Wnt/β-catenin signling

Dkk1 interacts with LRP5/6 and the co-receptor Kremen1/2, and this triggers LRP5/6 endocytosis, thereby preventing formation of the LRP5/6-Wnt-FZD complex. As a result, b-catenin is degraded.

Kawano et al. 2003

3. Role of WNT in cancer

3.1. WNT signal abnormalities in human cancers

A wide range of human cancers carry mutations in at least one component of the canonical Wnt/β-catenin pathway leading to a ligand-independent stabilization of β-catenin. One of the well known examples is colorectal cancer (CRC), in which approximately 85% of cases harbor loss-of-function mutations in the tumor suppressor gene, APC [142, 143]. Loss of APC function is seen at an early stage in colorectal carcinogenesis and is believed to be the initiating event for formation of adenomatous polyps [144]. Although APC is widely expressed in other tissues, the incidence of the mutations in APC is relatively rare [142, 143]. However, mutation in β-catenin is a more common event in other types of human cancers including colorectal, gastric, hepatocellular and ovarian cancers. Their incidence ranges from a few persent to as much as 80% [142, 143]. These mutations affect the N-terminal phosphorylation sites and thus render β-catenin resistant to phosphorylation and ubiquitination. Mutations in Axin is also found in 5-10% of hepatocellular carcinomas and in a small number of colorectal cancers lacking mutations in APC or β-catenin [142, 143].

A principle that emerges from these studies is that potentially any mutations that results in elevated β-catenin levels without decreasing cell viability may contribute to a premalignant condition or tumorigenesis. Aberrant activation of the other PCP and calcium pathways in malignant tissue is less well characterized and their relevance to human cancer is largely unknown.

3.2. WNT activation in breast cancer

As described in former section, the discovery of *Wnt1* as a mammary oncogene was brought from the study on mouse mammary tumor virus (MMTV) [145] [76]. MMTV acts as an insertional mutagen in mouse mammary tissue and in many MMTV-induced tumors, integration of proviral DNA results in transcriptional activation of the gene we know as *Wnt1* [146].

The immediate consequence of Wnt1 expression in the mouse mammary gland is hyperplasia rather than carcinoma. This is also evident from the phenotype of MMTV-Wnt1 transgenic mice, which show extensive lobulo-alveolar hyperplasia and subsequently develop focal mammary carcinomas after a long latency period [146, 147]. Despite this long latency, *Wnt1* does have an oncogenic potential.

In humans, there is no equivalent viral etiology demonstrated for breast cancer. Furthermore, although, N-terminal mutations of β-catenin have been detected in 45% (15 of 33) of breast fibromatosis cases, which are benign, stromal lesions [148](table 5), mutations in APC, Axin or β-catenin are rarely, if ever, detected in human breast carcinomas [149] [150] [151] [152]. However, there is documented overexpression of Wnts 1, 2, 3a, 4, 5A, 7B, 10B, 13 and 14 in human breast cancer tissues or cell lines (some of them are summarized on table 5) [153] [154, 155]. Furthermore, multiple FZD receptors are reported to be expressed in human breast cancer cell lines and primary tumors [156] [155]. Moreover, DVL1, a central regulator of WNT signaling, has been observed to be up-regulated in breast cancer (11 of 24 patients; 46% in mRNA level, 6 of 10 patients; 60% in protein level) [157]. Finally, around 60% of primary breast tumors show cytoplasmic or nuclear β-catenin localization rather than membrane localization [158] [159] [160] [161] and this was correlated with poor patient outcomes (Fig. 10) [160]. Taken together, these observations suggest that WNT signaling may frequently be de-regulated in breast cancer.

However, the mechanisms by which breast cancer tissue shows aberrantly activated WNT signaling pathway have not yet been clearly understood. One possibility is an inactivation of genes coding

negative regulator of WNT signaling pathway. Since aberrant hypermethylation of gene promoters is a major mechanism associated with inactivation of tumor-suppressor genes in cancer [162], promoter hypermethylation of *SFRP1* gene suggests one mechanism that might contribute to WNT pathway activity. In agreement with this hypothesis, promoter methylation of the gene coding sFRP1 is one of the most consistent observations in many cancers including breast cancer. There have been reports of the suppression of sFRP1 expression via promoter methylation in colon cancer [127, 163], ovarian cancer [164], bladder cancer [165] [166], mesothelioma [167], prostate cancer [168], lung cancer [169] and breast cancer [170].

In primary breast tumors, expression of sFRP1 is reported to be lost in a high percentage, both at the RNA level (>75%) [171] [172] and at the protein level (>40%) [173]. Moreover, methylation of the promoter region of the *SFRP1* gene was reported both in primary breast tumors (>60%) and breast cancer cell lines [170] [174] [175]. Interestingly, loss of sFRP1 protein expression and promoter hypermethylation is associated with disease progression and poor prognosis [173] [170]. Since aberrant hypermethylation of gene promoters is a major mechanism associated with inactivation of tumor-suppressor genes in cancer [162], promoter hypermethylation of *SFRP1* gene suggests one mechanism that might contribute to WNT pathway activity.

Table 5. Selected studies of WNT signaling pathway components in breast cancer and benign breast lesions

Adapted from the review of Howe and Brown, 2004

Signaling component	Reported abnormality	% cases	Tumor type or samples
sFRP1	loss/underexpression	80%	carcinomas
WIF1	reduced immunostaining	60%	carcinomas
Wnt2	overexpression, RNA	45%	carcinomas
Wnt2	overexpression, RNA	22%	1 degree breast cancers
Wnt2, Wnt4	overexpression, RNA	80%	fibroadenomas
Wnt5a	overexpression, RNA	36%	carcinomas
Wnt5a	loss of immunostaining	36%	carcinomas
Wnt7b	overexpression, RNA	10%	carcinomas
Wnt10b	overexpression, RNA	6%	carcinomas
Wnt13/2b	overexpression, RNA	14%	carcinomas
Wnt14	overexpression, RNA	11%	1 degree breast cancers

APC	truncation mutation	4%	Breast ca. cell lines
APC	truncation mutation	0.5%	carcinomas
APC	truncation mutations	6%	carcinomas
APC	reduced immunostaining	41%	carcinomas
APC	truncation mutations	18%	fibroadenomas
β-catenin	nuclear and/or cytoplasmic staining	60%	1 degree breast cancers
β-catenin	nuclear and/or cytoplasmic staining	63%	1 degree breast cancers
β-catenin	increased protein, but no mutation	13%	carcinomas
β-catenin	nuclear staining, but no mutation	72%	Phyllodes tumors
β-catenin	activating mutations	45%	fibromatoses

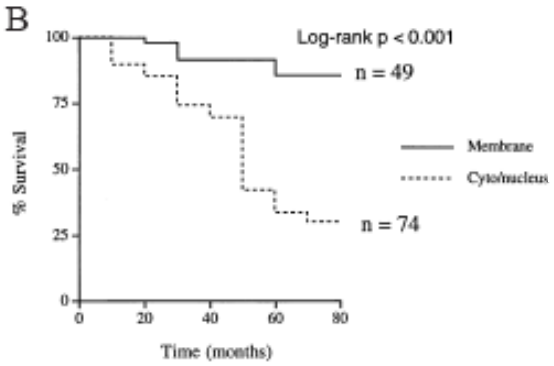


Figure 10.
Aberrantly-localized β-catenin association with poor patient survival rate *Lin et al 2000*

II. AIMS OF THE WORK

Currently, targeted therapeutics for breast cancer patients are endocrine treatment for ER+ breast tumors and trastuzumab treatment for ERBB2+ breast tumors. However, around 50% of breast cancer cases are neither ER+ nor ERBB2+. Furthermore, basal-like breast cancer, which shows the worst prognosis, accounts for 17-37% of breast cancer cases [70]. Thus, more targets are needed for breast cancer therapy.

On the other hand, around 60% of primary breast tumors show cytoplasmic or nuclear β-catenin localization rather than membrane localization [158] [159] [160] [161] and this was correlated with poor patient outcome [160], suggesting that WNT signaling pathway is aberrantly activated in many

breast cancers. Since multiple WNT ligands are known to be expressed in breast cancer [153], one potential mechanism contributing to pathway activity might be loss of negative modulators of WNT signaling [176]; decreased expression of sFRP1 is well documented in human breast cancer [171] [173] [170] [174] [175].

These reported evidences prompted us to hypothesize that activated WNT signaling pathway plays important roles in breast cancer formation and interference with WNT pathway by sFRP1 treatment might have effect on breast cancer cell proliferation and tumor growth. Based on these hypotheses, the main aims of my work have been, 1; to evaluate the importance of WNT pathway activation in breast cancer and 2; to evaluate whether sFRP1 treatment can block the breast cancer cell proliferation, tumor formation and tumor growth.

III. RESULTS

In the paper published by us in 2007 (Schlange et al. [177]), we showed a panel of breast cancer cell lines that have autocrine WNT signaling activity. Furthermore, interference with the activated autocrine loop using sFRP1 conditioned medium (CM) resulted in a decrease of active β-catenin and suppressed proliferation. In another project, we showed interference of autocrine WNT pathway by sFRP1 treatment affect cell migration and ectopic expression of sFRP1 suppresses *in vivo* tumor formation and tumor growth in xenograft model.

Research article

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Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation

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Abstract

Background De-regulation of the wingless and integration site growth factor (WNT) signaling pathway via mutations in APC and Axin, proteins that target β-catenin for destruction, have been linked to various types of human cancer. These genetic alterations rarely, if ever, are observed in breast tumors. However, various lines of evidence suggest that WNT signaling may also be de-regulated in breast cancer. Most breast tumors show hypermethylation of the promoter region of secreted Frizzled-related protein 1 (sFRP1), a negative WNT pathway regulator, leading to downregulation of its expression. As a consequence, WNT signaling is enhanced and may contribute to proliferation of human breast tumor cells. We previously demonstrated that, in addition to the canonical WNT/β-catenin pathway, WNT signaling activates the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in mouse mammary epithelial cells via epidermal growth factor receptor (EGFR) transactivation.

Methods Using the WNT modulator sFRP1 and short interfering RNA-mediated Dishevelled (DVL) knockdown, we interfered with autocrine WNT signaling at the ligand-receptor level. The impact on proliferation was measured by cell counting, YOPRO, and the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay; β-catenin, EGFR, ERK1/2 activation, and PARP (poly [ADP-ribose]polymerase) cleavages were assessed by Western blotting after treatment of human breast cancer cell lines with conditioned media, purified proteins, small-molecule inhibitors, or blocking antibodies.

Results Phospho-DVL and stabilized β-catenin are present in many breast tumor cell lines, indicating autocrine WNT signaling activity. Interfering with this loop decreases active β-catenin levels, lowers ERK1/2 activity, blocks proliferation, and induces apoptosis in MDA-MB-231, BT474, SkBr3, JIMT-1, and MCF-7 cells. The effects of WNT signaling are mediated partly by EGFR transactivation in human breast cancer cells in a metalloprotease- and Src-dependent manner. Furthermore, Wnt1 rescues estrogen receptor-positive (ER^+) breast cancer cells from the anti-proliferative effects of 4-hydroxytamoxifen (4-HT) and this activity can be blocked by an EGFR tyrosine kinase inhibitor.

Conclusion Our data show that interference with autocrine WNT signaling in human breast cancer reduces proliferation and survival of human breast cancer cells and rescues ER^+ tumor cells from 4-HT by activation of the canonical WNT pathway and EGFR transactivation. These findings suggest that interference with WNT signaling at the ligand-receptor level in combination with other targeted therapies may improve the efficiency of breast cancer treatments.

4-HT = 4-hydroxytamoxifen; ADAM = A Disintegrin And Metalloprotease; CM = conditioned medium; CRC = colorectal cancer; DMEM = Dulbecco's modified Eagle's medium; DVL = Dishevelled; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; ER = estrogen receptor; ERK1/2 = extracellular signal-regulated kinase 1/2; FCS = fetal calf serum; FZD = Frizzled; Gα = heterotrimeric G protein subunit α; GFP = green fluorescent protein; GPCR = G protein-coupled receptor; HRP = horseradish peroxidase; IP = immunoprecipitation; MEF = mouse embryonic fibroblast; MMP = matrix metalloprotease; PARP = poly(ADP-ribose)polymerase; PgR = progesterone receptor; PTX = pertussis toxin; sFRP1 = secreted Frizzled-related protein 1; shRNA = short hairpin RNA; siRNA = short interfering RNA; TCF = T-cell factor; TKI = tyrosine kinase inhibitor; Tyr = tyrosine; WNT = wingless and integration site growth factor.

Introduction

Growth factors of the wingless and integration site growth factor (WNT) family are secreted, glycosylated, and palmitoylated peptides that interact with seven-transmembrane receptors of the Frizzled (FZD) family. Diverse signaling pathways are activated upon WNT/FZD binding. The ligand/receptor interaction has been shown to induce the phosphorylation of scaffolding proteins of the Dishevelled (DVL) family by casein kinase Iε and -2 and PKCα [1-3]. This event was reported to be a component of all WNT-induced signaling pathways [4,5]. The so-called canonical WNT signaling pathway leads to stabilization of β-catenin through inactivation of a protein complex consisting of, amongst others, the tumor suppressors APC and Axin. This destruction complex normally triggers rapid β-catenin phosphorylation, inducing its ubiquitination and degradation. In the presence of canonical WNT ligands, β-catenin is stabilized, binds transcription factors of the LEF-1/T-cell factor (TCF) family, and stimulates target gene transcription [6].

Aberrant activation of the WNT signaling pathway plays an important role in the development of many human cancer types. In colorectal cancer (CRC), mutations in APC, axin, or β-catenin itself promote β-catenin stabilization and transcription of target genes encoding cancer-associated proteins [7]. In contrast to CRC, WNT pathway mutations rarely, if ever, are detected in breast tumors [8]. However, various lines of evidence suggest that, in breast cancer, the WNT pathway may be de-regulated by loss of expression of negative pathway regulators. For example, expression of the extracellular inhibitor of WNT signaling, secreted Frizzled-related protein 1 (sFRP1), which competes with FZD receptors for ligand binding, is downregulated in many breast tumors and is associated with poor prognosis [9-11]. Furthermore, many studies have reported that WNT ligands and FZD receptors are expressed in human breast cancer cell lines and primary tumors [7,12-14]. Finally, β-catenin is frequently found stabilized and nuclear in human breast tumors and this finding has been associated with poor prognosis [15]. Taken together, these observations suggest that WNT signaling may frequently be de-regulated in breast cancer.

We have previously described a novel crosstalk between WNT signaling and epidermal growth factor receptor (EGFR) [16]. The mechanism, which we have shown to involve activation of zinc-dependent membrane-associated metalloproteases [16] that control the cleavage and availability of ERBB ligands [17], appears to be analogous to that described for transactivation of EGFR triggered by stimulation of G protein-coupled receptors (GPCRs) [18]. GPCR-mediated EGFR transactivation involves various heterotrimeric G protein α subunits, activation of PKC and/or Src kinase, as well as ADAMs (A Disintegrin And Metalloprotease) (reviewed recently in)[19]) or matrix metalloprotases (MMPs) [20].

In this study, we provide evidence for constitutive autocrine WNT signaling in human breast cancer cells. We show that sFRP1 blocks proliferation of many breast tumor cell lines through interference with pathway activation that is presumably driven by endogenous WNT ligands. Thus, our study clearly demonstrates that sFRP1 fulfills its proposed tumor suppressor function [21]. Downstream of the WNT ligand/FZD receptor interaction, knockdown of DVL expression using short interfering RNA (siRNA) also results in a proliferative reduction and the induction of apoptosis in many human breast cancer cell lines. Our results, showing that Wnt1 transactivates EGFR in tumor cell lines, imply that, in breast cancer, constitutive WNT signaling might impact not only on the canonical pathway, but also on EGFR activity by stimulating ligand availability. Considering that constitutive ERBB receptor activation is an important mechanism promoting cancer cell proliferation, migration [22,23], and sensitivity to anti-cancer therapies [24], approaches to target WNT pathway activity might be appropriate as an anti-cancer strategy.

Materials and methods

Reagents

The following antibodies were used in this study: extracellular signal-regulated kinase 1/2 (ERK1/2), p-ERK1/2, total β-catenin, poly(ADP-ribose)polymerase (PARP), EGFR, p-EGFR (tyrosine [Tyr] 845), and p-Tyr-100 (Cell Signaling Technology, Inc., Danvers, MA, USA); c-MYC (9E10), DVL2 and DVL3, EGFR 528, 1005, and R1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Wnt1 and DVL1 (R&D Systems Europe, Abingdon, UK); active β-catenin (anti-ABC; Upstate, now part of Millipore Corporation, Billerica, MA, USA); and α-Tubulin (Lab Vision Corporation, Fremont, CA, USA). As secondary antibodies, α-rabbit and α-mouse (GE Healthcare, Little Chalfont, Buckinghamshire, UK, and LI-COR Biosciences, Lincoln, NE, USA) or α-goat (DAKO A/S, Glostrup, Denmark) coupled to horseradish peroxidase (HRP) were used. Mouse Wnt1 in the retroviral vector pLNCX was obtained from Andrew McMahon (Harvard University, Cambridge, MA, USA); the cDNA encoding human sFRP1 in pcDNA was provided by Jeffrey Rubin (National Cancer Institute, Bethesda, MD, USA). The retroviral vector for the expression of short hairpin RNA (shRNA) constructs pSUPERretro Neo green fluorescent protein (GFP) was provided by Francois Lehembre (DKBW, Basel, Switzerland). PKI166 and AEE788 were provided by Peter Traxler (Novartis Pharma AG, Basel, Switzerland); CGP77675 was provided by Jonathan Green and Mira Susa Spring (Novartis Pharma AG), and CGS27023A was provided by Ulf Neumann (Novartis Pharma AG). 4-Hydroxytamoxifen (4-HT) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture, transfections, and retroviral infections

The human breast cancer cell lines T47D, MCF-7, ZR-75.1, SkBr3, BT474, and MDA-MB-231 (American Type Culture Collection, Manassas, VA, USA) and JIMT-1 (DSZM, Braun-

schweig, Germany) were cultivated in Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal calf serum (FCS) (Amimed, Allschwil, Switzerland) supplemented with penicillin and streptomycin. HC11 and HC11/Wnt1 cells were maintained in RPMI 1640, 10% FCS, penicillin/streptomycin, epidermal growth factor (EGF) (Collaborative Research Co., Bedford, MA, USA) and insulin (Sigma-Aldrich). HC11/Wnt1 cells were kept under selection in 1 mg/mL G-418 (Life Technologies, Inc., now part of Invitrogen Corporation, Carlsbad, CA, USA). HEK 293 cells were transfected with a vector encoding myc/HIS-tagged human sFRP1 using Lipofectamine according to the manufacturer's guidelines. Cells were kept for 3 weeks in medium containing 1.5 mg/mL G-418, and clones were selected. T47D and SkBr3 cells were stably transfected with Wnt1 or empty pLNCX as control by Lipofectamine Reagent (Invitrogen Corporation) according to the manufacturer's instructions. Clones of Wnt1-expressing cells were selected with 0.5 mg/mL G-418. The expression of Wnt1 ligand was verified by Western blotting, and biological activity was assayed in a co-culture assay with HEK 293/8× SUPERTopFlash cells, using 300,000 cells each in a six-well overnight culture before the assay was performed. Knockdown of β-catenin was achieved by retroviral infection [25] with pSUPERretro Neo GFP containing a short-hairpin targeting β-catenin [26]. A construct targeting bacterial LacZ (sense strand gCggCTgCCggAATTTACCdTdT) was used as control. Clones and a pool of cells with low levels of β-catenin were analyzed for their response to Wnt1 condition medium (CM). $Src^{-/-}$ mouse embryonic fibroblasts (MEFs), provided by Kurt Ballmer (Paul Scherrer Institut, Villigen, Switzerland), were transfected with empty vector or a c-Src-expressing vector, and clones were selected. Src re-expressing MEFs were generated by Monilola Olayioye (University of Stuttgart, Germany).

siRNA transfections

Five hundred thousand cells per well were seeded in a six-well plate the day before transfection and were transfected with either 50 nM control RNA duplex targeting bacterial LacZ (sense strand gCggCUgCCggAAUUUACCdTdT) or a mixture of two siRNA duplexes (25 nM each; Qiagen GmbH, Hilden, Germany) targeting bases 1420 to 1440 (gCUCAACAAgAUCACCUUCUdTdT) in human DVL1 (NM_004421) and bases 1754 to 1774 and 1579 to 1599 (gUCAACAAgAUCACCUUCUdTdT) in human DVL2 (NM_004422) and DVL3 (NM_004423), respectively, using HiPerfect (Qiagen GmbH) according to the manufacturer's instructions. The DVL target sequences were chosen based on the high conservation in all three human DVL homologues. The cells were cultured for 72 hours, and knockdown efficiency was monitored by Western blotting.

Conditioned media

Confluent HC11/Wnt1 or parental HC11 cells were cultured for 3 days in RPMI 1640, 10% FCS, penicillin/streptomycin without EGF, insulin, and G-418. The supernatant was filtered through a 0.25-μm syringe filter (Sarstedt AG & Co., Nümbrecht, Germany). Biological activity of Wnt1 CM and control CM was assayed by their ability to induce β-catenin/TCF-dependent luciferase reporter activity in HEK 293/8× SUPERTopFlash cells (provided by Feng Cong, Novartis Institutes for BioMedical Research, Cambridge, MA, USA).

sFRP1 CM was obtained from HEK 293 cells transfected with myc/HIS-tagged human sFRP1 cDNA. CM was collected and sFRP1 activity was assayed by testing its ability to block the activation of β-catenin/TCF-driven transcription in a co-culture of T47D/Wnt1 cells and HEK 293/8× SUPERTopFlash cells and the reduction of DVL3 phosphorylation in T47D/Wnt1 cells. For treatment of breast cancer cell lines, confluent sFRP1-expressing HEK 293 cells were treated overnight with 10 mM sodium butyrate in 0.1% FCS to increase sFRP1 expression. The CM was concentrated, and sodium butyrate was removed by filtration with a Centricon Plus-70 filtration unit (Millipore Corporation). The resulting concentrate was diluted to the starting volume or used as a 2× concentrate and adjusted to 10% FCS accordingly. Cell proliferation was measured either by counting cell numbers manually or with a Vi-Cell XR cell viability analyzer (Beckman Coulter, Nyon, Switzerland), Cell Proliferation Kit I (MTT; Roche Diagnostics GmbH, Mannheim, Germany), or YOPRO cell viability assay (Invitrogen Corporation) according to manufacturer instructions.

Hybridoma cells secreting the EGFR monoclonal antibody C225 were cultured in DMEM, 10% FCS. Collected medium was cleared by centrifugation, filtered, and used undiluted on target cells for 2 hours prior to collection of cell lysates.

Purification of sFRP1

sFRP1 was purified by fast performance liquid chromatography from sFRP1 CM. After 1:10 dilution in 50 mM sodium phosphate loading buffer pH 7.0, the solution was loaded on a 1 mL HiTrap-HIS column (GE Healthcare) that was previously loaded with 1 mL 0.5 M $NiSO_4$ and washed with 10 column volumes of loading buffer. Elution was performed using 50 mM sodium phosphate, 100 mM NaCl pH 7.0 elution buffer with a 3-minute step-gradient of 10 to 500 mM imidazole. Fractions were collected, and 1-μL aliquots were analyzed by Western blotting using a c-MYC antibody for detection of the MYC-tag. Biological activity was assayed as previously described for sFRP1 CM, and the identity of the purified protein was determined by mass spectrometry.

Protein extraction, immunoprecipitation, and Western blotting

Cells were lysed in lysis buffer (1% Nonident P-40, 50 mM Tris pH 7.5, 120 mM NaCl, 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM EGTA [ethylene glycol tetraacetic acid], 2 mM sodium vanadate, 20 mM β-glycerophosphate, 10 μg/mL

aprotinin, 10 μg/mL leupeptin, 0.5 mM PMSF [phenylmethylsulphonyl fluoride], 50 mM NaF, and 1 mM dithiothreitol) for 5 minutes on ice, and lysates were collected. For a Western analysis, loading buffer was added to 30 to 50 μg of protein and the samples were denatured for 10 minutes at 95°C prior to separation on 10% polyacrylamide gels and blotting by semi-dry transfer for 90 minutes on polyvinylidene fluoride membrane (Millipore Corporation). Blots were pre-blocked using 10% horse serum in TBS-T buffer for 1 hour (0.2 M NaCl, 25 mM Tris pH 7.5, 0.5 mL/L Tween-20) and incubated with primary antibodies at room temperature for 1 hour or at 4°C overnight, followed by 30 minutes of incubation with secondary antibodies: α-rabbit-HRP, α-mouse-HRP, or α-goat-HRP (1:5,000). Detection of luminescence was carried out using ECL (enhanced chemiluminescence) (GE Healthcare) or SuperSignal West Dura (Pierce, Rockford, IL, USA) according to manufacturer instructions. Immunoprecipitations (IPs) and Western analyses were performed using standard procedures [24,27]. EGFR IP was performed with α-EGFR 528 and R1. Quantifications of Western blots were carried out using the ImageQuant TL version 2005 software package from Amersham Biosciences (now part of GE Healthcare).

Results

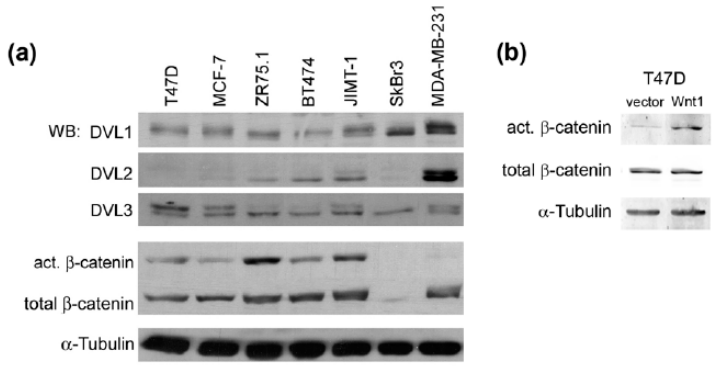
WNT pathway activity in human breast tumor cell lines

WNTs activate multiple intracellular signaling cascades, including the canonical pathway that promotes β-catenin stabilization and TCF-mediated transcription [6] and other non-canonical pathways, one being Wnt-mediated EGFR transactivation [16]. To explore the possibility that Wnt signaling is deregulated in breast cancer by autocrine pathway activation, we examined breast cancer cell lines for signs of canonical pathway activity and for crosstalk between WNT, EGFR, and ERK1/2 signalling. The panel includes the luminal, estrogen receptor-positive (ER^+) T47D, MCF-7, and ZR75.1 cells, the ERBB2-overexpressing SkBr3, JIMT-1, and BT474 cells, and the basal B, ER-negative MDA-MB-231 cells [28].

As a consequence of WNT binding to FZD, cytoplasmic scaffolding proteins of the Dishevelled family (DVL1, DVL2, and DVL3) become phosphorylated on serine and threonine residues. DVL phosphorylation is the most proximal signaling event downstream of the WNT-mediated activation of FZD and can be monitored by a decrease in the electrophoretic mobility of p-DVL [4]. To date, DVL phosphorylation has been shown to be mediated only by WNT signaling and DVL is upstream of all known WNT-induced signaling pathways. DVL1 and DVL3 were consistently expressed at relatively uniform levels in all the breast cancer cell lines, whereas DVL2 was expressed in a more differential manner (Figure 1a). Bands corresponding to p-DVL1 and/or p-DVL3 were detected in all of the cell lines. p-DVL2 was also high in MDA-MB-231 cells (Figure 1a). These results suggest that WNT signaling might be activated in an autocrine fashion in each of the examined breast cancer cell lines.

As a read-out for activation of the canonical WNT pathway, active, unphosphorylated β-catenin (act. β-catenin) was analyzed in these breast cancer cell lines and in a control T47D cell line engineered to ectopically express Wnt1 (Figure 1b). Control and T47D/Wnt1 cells have the same level of total β-catenin. Importantly, the Wnt1-expressing T47D cells have an approximately three-fold increase in active β-catenin levels compared with control cells (Figure 1b), attesting to the ability of the antiserum to measure canonical pathway activity. In the majority of the breast tumor cell lines, active β-catenin was

Figure 1



Autocrine WNT signaling in breast cancer cell lines. **(a)** Lysates from the indicated human breast cancer cell lines were analyzed by SDS-PAGE followed by immunoblotting for Dishevelled 1 (DVL1), DVL2, and DVL3 (the upper band indicates the phosphorylated form of each), active β-catenin, total β-catenin, and α-Tubulin as a loading control. **(b)** Lysates from T47D/Wnt1 cells and vector control were analyzed by SDS-PAGE/immunoblotting for active β-catenin, total β-catenin, and α-Tubulin. WB, Western blotting; WNT, wingless and integration site growth factor.

present at various levels (Figure 1a). Only in SkBr3 cells, which have very low total β-catenin levels, no active β-catenin protein was detected. These results imply that the canonical WNT signaling pathway is constitutively active in most breast tumor cell lines.

In vitro effects of sFRP1 on proliferation of human breast cancer cell lines, canonical β-catenin signaling, and ERK activity

Since sFRP1 expression is lost in primary breast tumors and tumor cell lines by promoter hypermethylation [10,29], this might be one mechanism contributing to WNT pathway activity. We therefore assessed the effect of blocking WNT pathway activity on *in vitro* proliferation of breast tumor cell lines. Treatment of T47D cells with either purified sFRP1 or sFRP1 CM blocked their proliferation by 30% (Figure 2a,b). Proliferation of JIMT-1, SkBr3, and MDA-MB-231 cells was also significantly inhibited (20% to 30%) by sFRP1 CM, whereas BT474 and MCF-7 cells were not significantly affected by the treatment (Figure 2a).

To analyze the signaling pathways involved in the anti-proliferative activity of sFRP1, we examined its effects on canonical WNT signaling, which, as shown above (Figure 1a), is constitutively active in most of the examined breast tumor cell lines. Treatment of T47D, BT474, and JIMT-1 cells with sFRP1 CM caused a 10% to 20% reduction in active β-catenin levels, whereas there was no observable decrease in MCF-7 cells (Figure 2c, quantified below). These results suggest that, in these three cell lines, β-catenin stabilization is at least partly due to autocrine activation of the pathway by WNT ligands that can be blocked from binding their cognate FZD receptor by sFRP1.

As we have previously shown that Wnt growth factors activate the ERK1/2 pathway in mouse mammary epithelial cells [16], we next examined the effect of sFRP1 on ERK1/2 activity. sFRP1 treatment lowered the basal level of p-ERK1/2 in all cell lines analyzed with the exception of MCF-7 (Figure 2c), which also showed no decrease in active β-catenin in response to sFRP1. These results are in good agreement with those showing that sFRP1 treatment reduced proliferation of T47D, JIMT-1, and SkBr3 cells, but not of MCF-7 cells. In summary, these results show that, in some breast cancer cell lines, both canonical and non-canonical Wnt signaling can be blocked by sFRP1 treatment. Furthermore, they suggest that sFRP1 has the potential to act as an anti-proliferative agent.

siRNA-mediated knockdown of DVL reduces c-MYC expression and induces apoptosis

Human breast cancer cells express multiple WNT ligands and FZD receptors [7,12-14], and it is likely that different sFRP family members interfere with only a subset of ligands [30]. Therefore, we hypothesized that knockdown of DVL homologues would lead to a stronger blockade of autocrine WNT signaling. Introduction of two siRNAs that target the three human DVL homologues (pan-DVL siRNA) achieved a strong knockdown of DVL2 and DVL3 in the cells; DVL1 was decreased approximately 50% with this siRNA (Figure 4d). siRNA-mediated DVL knockdown blocked proliferation of human breast cancer cells by 20% to 60% 7 days after transfection as determined by cell counting after viability staining (Figure 3a), with the most prominent effect in JIMT-1, SkBr3, and MDA-MB-231 cells, whereas BT474 and MCF-7 cells are less affected. As expected, DVL knockdown affects canonical WNT signaling activity since the level of active β-catenin decreases concomitantly with a reduction in c-MYC, a canonical WNT target (Figure 3a). SkBr3 cells show no reduction in c-MYC levels upon DVL knockdown, very likely because either *c-MYC* is amplified or canonical signaling is impaired since there is no active β-catenin in these cells. Finally, we observe an increase in PARP cleavage after DVL knockdown in all cell lines analyzed (Figure 3a), indicating that apoptosis is induced in all but BT474 cells. These data show that autocrine WNT signaling is required for proliferation and survival of human breast cancer cells.

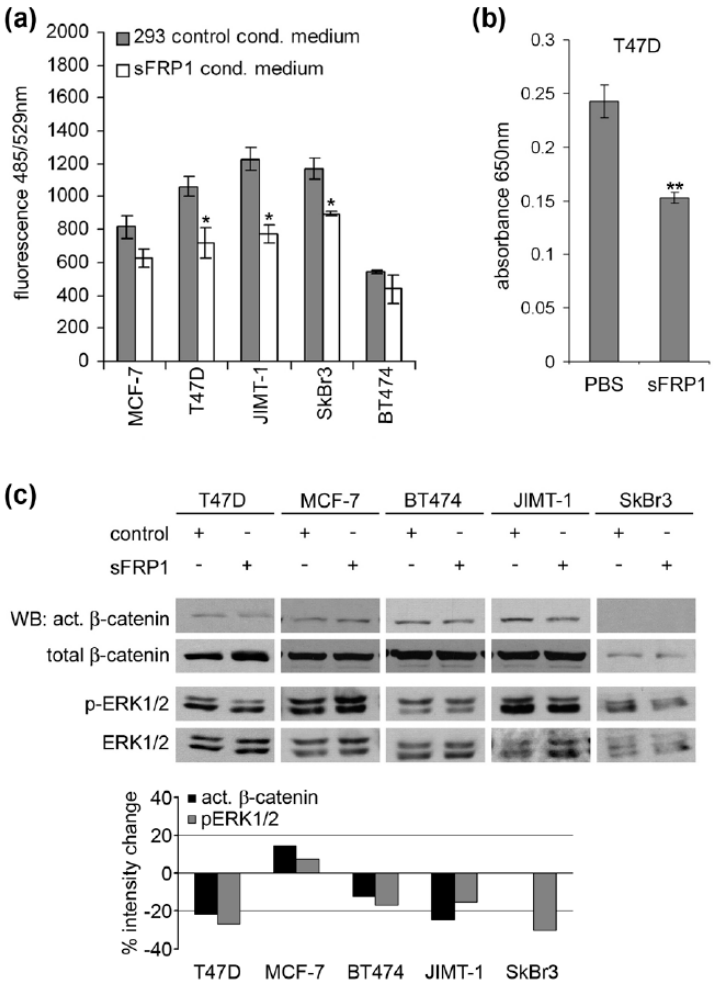
Downregulation of DVL in breast cancer cells lowers EGFR and ERK activity

Multiple mechanisms contribute to the autocrine ligand-induced EGFR activity [31] that is detected in many human tumors [32,33]. Given our previous results on WNT-induced EGFR transactivation [16], we considered it possible that WNT signaling might also play a role in some breast tumors. Thus, we asked whether WNT signaling also contributes to EGFR activity, concentrating on three cell lines, BT474, JIMT-1, and SkBr3, that in addition to ERBB2 overexpression have high levels of active EGFR and p-ERK1/2 (Figure 4a) [24,34,35]. pan-DVL knockdown lowered EGFR activity, as shown by a decrease in pY845 levels, and strongly reduced ERK1/2 activity in each of these cancer cell lines (Figure 3b). In summary, the results suggest that, in the examined breast cancer cell lines, WNT activity contributes to autocrine EGFR activation and ERK1/2 activity.

Wnt1 induces ERK1/2 activity independently of canonical WNT signaling

In light of these results, we asked whether WNT ligands induce EGFR/ERK1/2 activation in human breast cancer cells in a fashion similar to that in non-transformed mouse mammary epithelial cells [16]. Wnt1 is not commercially available in a bioactive form and our own efforts to purify the protein using published protocols have failed. Our approaches to prove the specificity of Wnt1 action on ERK1/2 activity relied on the use of CM in combination with the natural WNT inhibitor sFRP1 and on ectopic expression of Wnt1 in breast cancer cell lines. Furthermore, we knocked down expression of DVL, the central WNT signaling mediators downstream of WNT-ligand-triggered FZD activation.

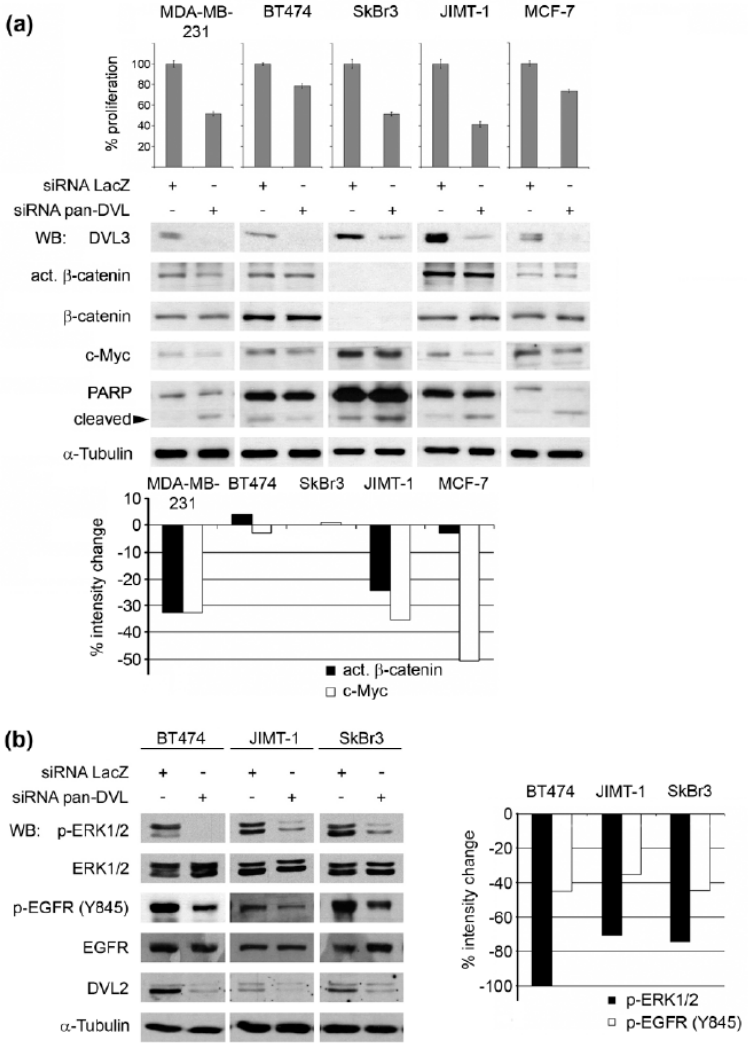
Figure 2



Treatment of human breast cancer cells with secreted Frizzled-related protein 1 (sFRP1) reduces proliferation and impairs canonical wingless and integration site growth factor signaling and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. **(a)** One thousand to 5,000 cells of the indicated cell lines were seeded in a 96-well plate, and proliferation was measured in a YOPRO assay after 3 days of treatment with sFRP1 conditioned medium (CM) or control CM. **(b)** T47D cells were treated for 3 days with 30 μg/mL purified sFRP1 or phosphate-buffered saline, and cell numbers were measured in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. The results represent the mean of three experiments (± standard error). *$p < 0.05$, **$p < 0.005$, unpaired Student *t* test, comparison to corresponding control-treated cell line. **(c)** The indicated human breast cancer cell lines were treated for 2 hours with concentrated sFRP1 CM, and cell lysates were analyzed by SDS-PAGE/immunoblotting for active β-catenin, total β-catenin, p-ERK1/2, and ERK1/2 (upper panel). The results were quantified using ImageQuant (lower panel).

Cells were treated for 20 minutes with Wnt1 CM or control CM, and p-ERK1/2 levels were examined (Figure 4a). The ER+ tumor cells T47D, MCF-7, and ZR75.1 have low basal p-ERK1/2 levels that strongly increased in response to Wnt1 treatment. The ERBB2-overexpressing tumor cells BT474 and SkBr3 have high basal p-ERK1/2, and both showed a further increase in ERK1/2 activity in response to Wnt1. p-ERK1/2 levels were not stimulated by Wnt1 treatment of MDA-MB-231 tumor cells, which have a *K-RAS* mutation and high basal ERK1/2 activity (Figure 4a) [36]. Wnt1 CM effects on ERK1/2 activity were blocked in T47D cells simultaneously treated with sFRP1 (Figure 4b). Similarly, when T47D/Wnt1 or

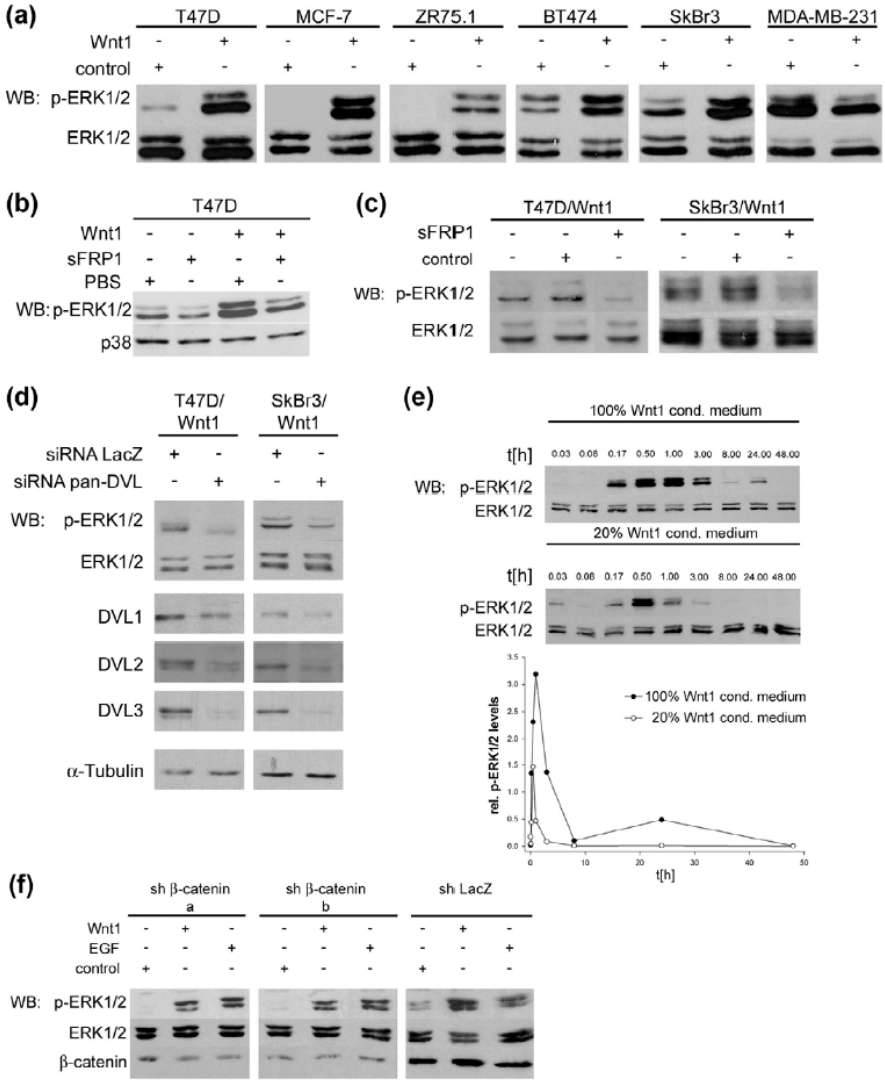
Figure 3



Short interfering RNA (siRNA)-mediated knockdown of Dishevelled (DVL) homologues results in decreased canonical wingless and integration site growth factor (WNT) signaling, a reduction in basal epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase 1/2 (ERK1/2) activation, and the induction of apoptosis in human breast cancer cells. **(a)** The indicated human breast cancer cell lines were transfected with pan-DVL siRNA. Two thousand to 5,000 cells were seeded in triplicate in 12-well plates the day after the transfection, and the cell number was counted after 7 days using a Vi-Cell XR cell viability analyzer. DVL knockdown was verified by SDS-PAGE/immunoblotting (only DVL3 is shown). The levels of act. β-catenin, total β-catenin, the WNT target c-MYC, and poly(ADP-ribose)polymerase (PARP) were analyzed by SDS-PAGE/immunoblotting. The lower band (80 kDa) in the blot probed for PARP represents the cleavage product upon induction of apoptosis. α-Tubulin was used as a loading control. For quantification, act. β-catenin levels were normalized to total β-catenin and c-MYC was normalized to α-Tubulin expression. **(b)** The indicated human breast cancer cell lines were transfected with pan-DVL siRNA and analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and EGFR Y845 phosphorylation. DVL2 levels are shown to monitor efficient knockdown of DVL, and α-Tubulin was used as loading control. For quantification, p-ERK1/2 was normalized to total ERK1/2 and p-EGFR Y845 was normalized to total EGFR expression.

SkBr3/Wnt1 cells were treated with sFRP1 for 2 hours prior to lysis of the cells, the level of ERK1/2 phosphorylation was strongly decreased (Figure 4c). This strongly suggests that the response in ERK1/2 phosphorylation toward Wnt1 treatment or stable Wnt1 expression is Wnt ligand-specific. This finding is supported by interference with WNT signaling down-

Figure 4



Wnt1 induces rapid phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in human breast cancer cells. **(a)** Cultures of the indicated cell lines were treated for 20 minutes with Wnt1 conditioned medium (CM) or control CM, and lysates were analyzed by SDS-PAGE followed by immunoblotting for p-ERK1/2 and ERK1/2. **(b)** T47D cells were treated with control CM or Wnt1 CM, which was previously incubated for 2 hours with 30 μg/mL purified secreted Frizzled-related protein 1 (sFRP1) or phosphate-buffered saline as control. Cell lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and p38 as loading control. **(c)** Stably Wnt1-transfected T47D and SkBr3 cells were treated for 2 hours with sFRP1 CM, control CM, or normal growth medium. Total lysates were analyzed by SDS-PAGE followed by immunoblotting for p-ERK1/2 and ERK1/2. **(d)** T47D/Wnt1 and SkBr3/Wnt1 cells were seeded at 300,000 cells per well in a six-well plate the day before short interfering RNA (siRNA) transfection with either a LacZ control siRNA or pan-DVL siRNA. The cells were cultured for an additional 48 hours under normal growth conditions and 24 hours in 0.1% fetal calf serum before harvesting. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2, ERK1/2, DVL1, DVL2, and DVL3, or α-Tubulin as a loading control. **(e)** T47D cultures were treated for the indicated times with 100% and 20% vol/vol Wnt1 CM. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2 (upper panel). ERK activation was quantified after normalization of signal intensity of p-ERK1/2 to total ERK1/2 using the ImageQuant software. ERK activity peaks at between 30 minutes and 1 hour (lower panel). **(f)** Wnt1-mediated effects are independent of β-catenin. T47D cells were infected with a retrovirus carrying an expression cassette for a short-hairpin RNA targeting β-catenin. Two independent shβ-catenin clones and a control pool infected with a short hairpin against bacterial LacZ were treated with control CM or Wnt1 CM for 20 minutes or 10 ng/mL EGF for 5 minutes. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2, ERK1/2, and β-catenin. DVL, Dishevelled.

stream of the FZD receptor level through DVL knockdown that abolishes the increase in ERK1/2 phosphorylation in T47D/Wnt1 and SkBr3/Wnt1 cells (Figure 4d).

To assess the involvement of canonical β-catenin-dependent WNT signaling in the activation of ERK1/2 pathway, we next examined the kinetics of Wnt1-induced ERK1/2 activation after treating T47D cells with concentrated and with five-fold diluted Wnt1 CM. In both cases, ERK1/2 activation was rapid, peaking at between 30 and 60 minutes and falling back to basal by 8 hours (Figure 4e). Whereas the p-ERK1/2 levels were lower in cells treated with diluted Wnt1 CM, the kinetics were identical (Figure 4e). The rapid nature of ERK1/2 phosphorylation in response to Wnt1 makes it unlikely that transcriptional activity driven by canonical WNT/β-catenin signaling contributes to transactivation. However, to directly exclude this, β-catenin was knocked down in T47D cells by infection with an shRNA vector. Two independent knockdown clones showing an approximately 70% decrease in β-catenin levels and a control LacZ shRNA were analyzed (Figure 4f). Treatment of both β-catenin knockdown clones and the control clone with Wnt1 CM led to a rapid increase in p-ERK1/2 levels to the same extent as seen in EGF-treated cells (Figure 4f). Taken together, these data demonstrate that, in human breast cancer cells, Wnt1 activates the ERK1/2 pathway in a WNT ligand- and DVL-dependent manner and this is independent of canonical signaling via β-catenin stabilization.

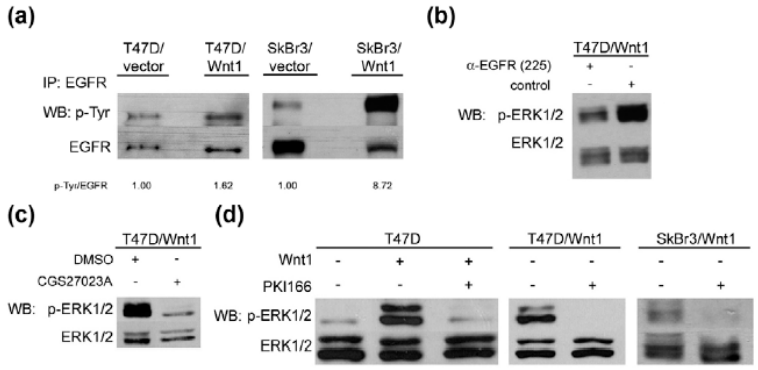
Wnt1-induced ERK1/2 phosphorylation is EGFR-dependent

We next explored whether activation of EGFR is induced by Wnt1 and acts upstream of the observed ERK1/2 phosphorylation. Overall EGFR phospho-tyrosine levels are 1.6 fold and 8.7 fold elevated in T47D/Wnt1 and SkBr3/Wnt1 cells over the level in corresponding control transfected cells (Figure 5a). Treatment of T47D/Wnt1 cells with an EGFR-blocking antibody (225) that prevents ligands from binding the receptor causes a decrease in p-ERK1/2 to basal levels in the cells (Figure 5b). Shedding of EGF-like ligands from their membrane-bound form requires the activity of metalloproteases of the MMP and/or ADAM families. MMP or ADAM activity is required for the activation of the ERK1/2 pathway downstream of Wnt1 as the inhibitor of metalloprotease activity CGS27023A lowered Wnt1-induced ERK1/2 activity to basal levels (Figure 5c). Finally, the Wnt1-mediated increase in ERK1/2 activity was blocked by either pre-treatment of T47D cells or treatment of T47D/Wnt1 and SkBr3/Wnt1 cells with PKI166, an EGFR tyrosine kinase inhibitor (TKI) (Figure 5d) [37]. Taken together, these data suggest that Wnt transactivates EGFR via metalloprotease-dependent ligand release)[19].

Wnt1-induced ERK phosphorylation requires Src kinase activity

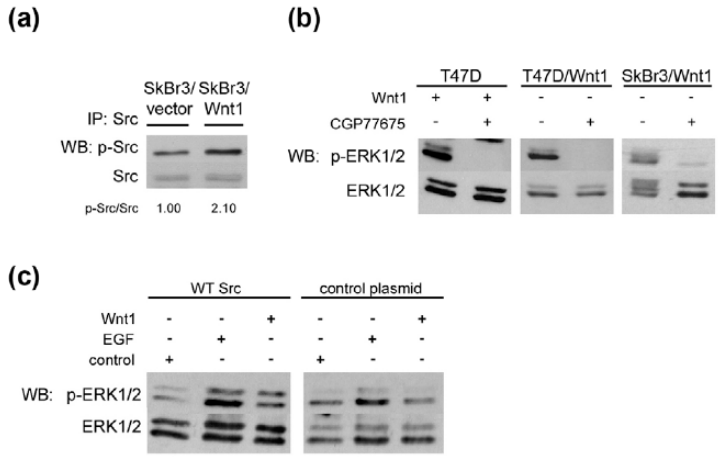
As FZD receptors are structurally related to GPCRs and members of the Src kinase family were reported to act in GPCR ligand-induced EGFR transactivation)[19], we explored the possibility that c-Src has a role in Wnt1-mediated EGFR transactivation. Initially, we tested whether Wnt1-expressing cells

Figure 5



Wnt1-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation depends on epidermal growth factor receptor (EGFR) activity. **(a)** EGFR was immunoprecipitated from 2 mg of whole cell lysate from T47D/Wnt1, T47D/vector, SkBr3/Wnt1, and SkBr3 vector cells and analyzed by SDS-PAGE/immunoblotting for p-Tyr and EGFR. The p-Tyr signal corresponding to EGFR was quantified using the ImageQuant software. **(b)** T47D/Wnt1 cells were pre-treated for 1 hour with monoclonal antibody 225 containing conditioned medium (CM), and lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2. **(c)** T47D/Wnt1 cells were pre-treated for 1 hour with the metalloprotease inhibitor CGS27023A (50 μM) or dimethyl sulfoxide as control. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2. **(d)** T47D cells were pre-treated for 1 hour with 2 μM PKI166 before treatment with Wnt1 CM or control CM. T47D/Wnt1 and SkBr3/Wnt1 cells were treated for 1 hour with 2 μM PKI166 prior to lysing the cells. Lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2. Tyr, tyrosine.

Figure 6



Src kinase is required for Wnt1-mediated extracellular signal-regulated kinase 1/2 (ERK1/2) activation. **(a)** c-Src was immunoprecipitated from lysates of Wnt1-expressing or vector control-expressing SkBr3 cells. The immunoprecipates were analyzed by SDS-PAGE/immunoblotting for p-Src and Src, and the signals were quantified versus control levels using the ImageQuant program. **(b)** T47D cells were pre-treated with the Src kinase inhibitor CGP77675 (2 μM) or dimethyl sulfoxide (DMSO) for 1 hour before treatment for 20 minutes with Wnt1 conditioned medium (CM). T47D/Wnt1 and SkBr3/Wnt1 cells were treated for 1 hour with CGP77675 or DMSO. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2. **(c)** $Src^{-/-}$ mouse embryonic fibroblasts transfected with a control plasmid or a wild type Src-expressing vector were treated with Wnt1 CM or control CM for 20 minutes. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2. A representative result of three independent experiments is shown. EGF, epidermal growth factor; IP, immunoprecipitation; WB, Western blotting.

have elevated c-Src kinase activity by examining phosphorylation of the regulatory p-Tyr 416 in c-Src IPs. In SkBr3/Wnt1 cells, c-Src activity was increased two-fold over SkBr3/vector cells (Figure 6a). T47D cells have high levels of active c-Src, and no differences were observed between control and Wnt1-expressing cells (data not shown).

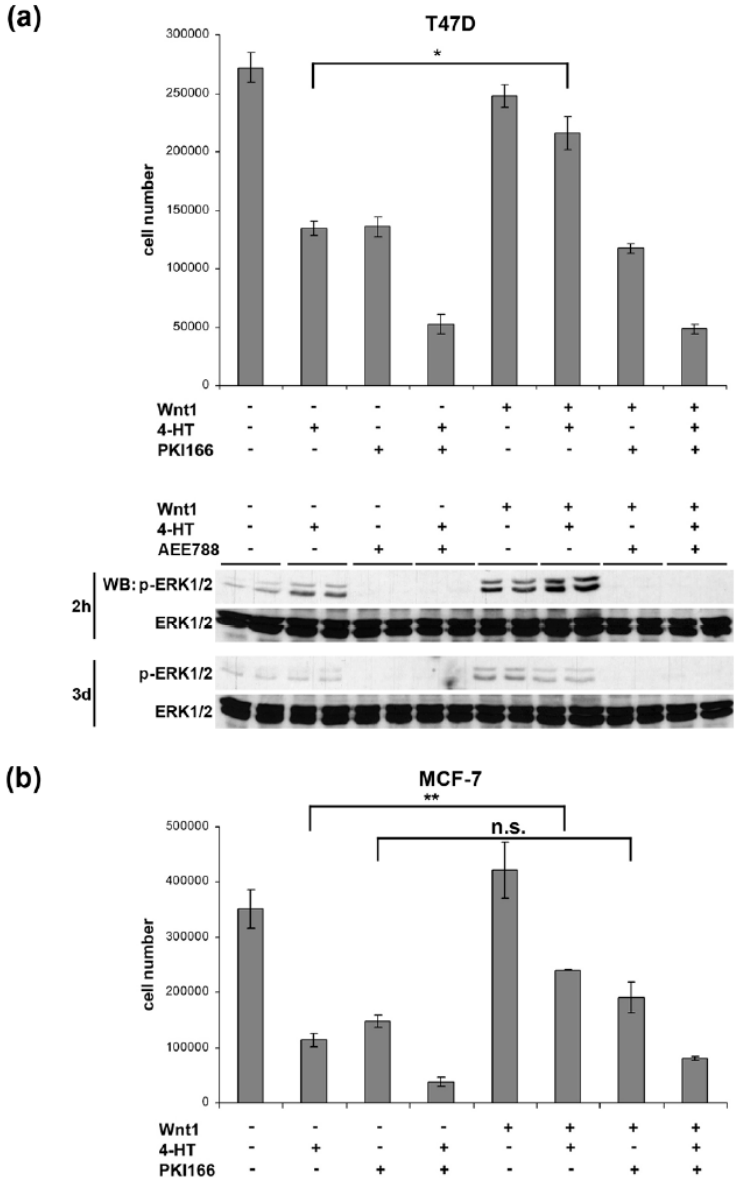
Next, we examined the effects of CGP77675, an Src kinase-selective TKI [38,39]. Treatment of T47D/Wnt1 and SKBR3/Wnt1 cells with CGP77675 lowered ERK1/2 activity. Moreover, induction of p-ERK1/2 mediated by Wnt1 CM was blocked by CGP77675 pre-treatment (Figure 6b). Since CGP77675 blocks the activity of multiple Src family members [38], we used MEFs from c-Src knockout mice that were transfected with a c-Src-expressing vector or a control vector to directly test the role of c-Src. Whereas EGF stimulated ERK1/2 activity in both cell lines (although slightly less in c-Src knockout fibroblasts) [39], Wnt1 treatment increased ERK1/2 activity in c-Src-transfected MEFs, but not in control MEFs (Figure 6c). Interference with intracellular Ca^{2+} levels, PKC signaling, or $G\alpha_{i/o}$ signaling, each of which is known to impact on GPCR-induced EGFR transactivation, did not affect Wnt1-induced ERK1/2 phosphorylation (data not shown). These observations suggest that, as observed for many GPCR-activating ligands) [19], c-Src is also required for Wnt1-mediated EGFR transactivation.

Wnt1 rescues breast cancer cells from growth arrest induced by anti-estrogen therapy

Ligand-mediated autocrine ERBB activation confers resistance to anti-cancer agents [22], including the ER antagonist 4-HT [40]. Based on the ability of Wnt1 to activate EGFR and ERK1/2 signaling in the ER^+ T47D and MCF-7 breast tumor cells (Figure 4a), we examined the effect of Wnt1 treatment on their response to 4-HT.

Treatment of T47D and MCF-7 cells with 4-HT and PKI166 blocked proliferation by approximately 50% and 60%, respectively; the combination of inhibitors is essentially additive in both cell lines (Figure 7a,b). In T47D cells, Wnt1 treatment almost completely rescued the anti-proliferative effect of 4-HT (79% of control level, Figure 7a, upper panel). MCF-7 cells were also significantly rescued from the anti-proliferative activity of 4-HT by Wnt1 (32% of control level, Figure 7b). PKI166-treated T47D and MCF-7 cultures were both insensitive to Wnt1 addition, showing the dominance of EGFR blockade. Importantly, addition of PKI166 completely suppressed the ability of Wnt1 to overcome the anti-proliferative activity of 4-HT in both cell lines, showing the importance of autocrine EGFR activation in the Wnt1-induced rescue. In line with this, Western blot analysis reveals that the slight increase in p-ERK1/2 levels upon Wnt1 treatment observed after 2 hours of incubation (Figure 4e) is completely blocked using the more potent dual EGFR/ERBB2 kinase inhibitor AEE788 [41] while

Figure 7



Wnt1 rescues breast tumor cells from the anti-proliferative effects of 4-hydroxytamoxifen (4-HT). T47D **(a)** or MCF-7 **(b)** cells were treated with 4-HT (5 μM), PKI166 (5 μM), or a combination of both drugs for 7 or 6 days, respectively, in the presence or absence of Wnt1 conditioned medium (CM) or control CM. For control experiments, the solvents ethanol and dimethyl sulfoxide, respectively, were added in corresponding concentrations. Cultures were re-fed with fresh medium and inhibitors after 3 days, before cell number (± standard error) was determined. For biochemical analysis, T47D cells were treated under corresponding conditions with 4-HT (5 μM), AEE788 (2 μM), or a combination of both in the absence or presence of Wnt1 CM or control CM for 2 hours or 3 days. Total lysates were analyzed by SDS-PAGE/immunoblotting for p-ERK1/2 and ERK1/2. *$p < 0.05$, **$p < 0.005$, unpaired Student *t* test. ERK1/2, extracellular signal-regulated kinase 1/2; n.s., difference not significant; WB, Western blotting.

4-HT treatment even enhances the activation of the ERK1/2 pathway slightly (Figure 7a, lower panel). After long-term treatment (3 days) with 4-HT in the presence of Wnt1, p-ERK1/2 levels are still elevated over basal levels, but ERK1/2 phospho-

rylation remains completely blocked by AEE788. These results imply that Wnt1 overcomes the anti-proliferative effect of anti-ER treatment in a manner that depends on EGFR activity.

Discussion

De-regulation of WNT signaling is a well-established hallmark of certain types of human cancer, such as CRC and melanoma, in which a high percentage of mutations in the β-catenin destruction complex components *APC* and *AXIN* or in *β-catenin* itself have been described [42]. Although mutations of this type are rarely observed in breast cancer, we show here that many breast cancer cell lines have autocrine activity of WNT signaling and that blocking this pathway has multiple biological effects. In breast cancer, activation of the Wnt pathway is likely due to co-expression of WNT ligands and FZD receptors (T Schlange, unpublished observations) [7,12-14]. WNT ligands play different roles in cancer biology depending on the downstream pathways activated. Whereas canonical Wnt signaling is required for G_1 cell cycle progression in CRC [43], the non-canonical ligand WNT5A negatively regulates proliferation [44,45] but promotes migration in various cancer types [46,47]. One potential mechanism contributing to pathway activity might be loss of negative modulators of WNT signaling [48], as decreased expression of sFRP1 is well documented in human breast cancer [10,11,29]. Furthermore, the loss of sFRP1 expression was recently shown to synergize with c-MYC-induced tumorigenesis [49]. Extending the analysis of Bafico and colleagues [21], we assayed the activation of WNT signaling by DVL phosphorylation, the most proximal read-out of FZD receptor activation, and found autocrine WNT activity in a panel of human breast cancer cells with diverse genetic alterations.

We show here that treatment of many breast tumor cell lines with sFRP1 has a consistently negative effect on their proliferation by affecting the canonical WNT pathway. In addition, ERK1/2 pathway activity is also decreased by sFRP1 treatment in the majority of the cancer cells, with SkBr3 cells being particularly sensitive. SkBr3 cells have high levels of ERBB activity. The fact that sFRP1 decreases p-ERK1/2 levels suggests that WNT-mediated ERBB transactivation has an important role in maintaining ERK1/2 signaling in these tumor cells. As SkBr3 cells have essentially no active β-catenin, sFRP1 effects on ERK1/2 activity might be the main cause for their decreased proliferation in response to sFRP1 treatment. A similar dependence on a non-canonical WNT signal was observed in β-catenin-deficient mesothelioma cells [50], in which siRNAs against WNT1 and DVL induced apoptosis in a JNK (c-jun N-terminal kinase)-dependent manner. This finding is particularly interesting given the inhibition of proliferation and induction of apoptosis we observe in response to the knockdown of all three DVL homologues in different breast cancer cell lines. Interfering with WNT signaling at the DVL level should block all autocrine activation [5]. sFRP1, on the other hand, most likely binds only a subset of WNT ligands [30,51], which might explain why sFRP1 treatment could not completely block β-catenin stabilization or WNT-induced ERK1/2 activity. In fact, compared with sFRP1 treatment, DVL knockdown elicited a stronger negative effect on ERK1/2 activity in the breast cancer cell lines. BT474 and MCF-7 cells are most resistant to both sFRP1 treatment and DVL knockdown when compared with the other cell lines analyzed. In the case of BT474, this is in line with relatively low levels of DVL phosphorylation, indicating that this cell line is mostly independent of autocrine WNT signaling. This shows that there is differential sensitivity of human breast cancer cells with different oncogenic pathways activated (for example, ERBB2 overexpression, estrogen dependence) to inhibition of autocrine WNT signaling.

Recently, blocking the FZD/DVL interaction using a small molecule targeting the PDZ domain of DVL was explored and shown to inhibit the proliferation of cancer cell lines derived from different types of cancer [52]. Our observations imply that targeting this interaction or the use of a 'ligand trap' like sFRP1 might be a valid approach to treat breast cancer by interfering with the canonical WNT pathway as well as the EGFR/ERK1/2 pathway. Inhibition of more than just one WNT ligand or FZD receptor may overcome the problem of functionally redundant expression of several family members when specific antibodies are used [53-57]. In summary, our observations on blocking autocrine WNT activity in human breast cancer cells suggest an important role for WNT-induced EGFR transactivation in the control of ERK1/2 signaling and of proliferation.

It is also noteworthy that there is differential phosphorylation of DVL isoforms in the panel of breast cancer cell lines. Perhaps DVL family members are not redundant and might be activated by different WNT/FZD complexes. Furthermore, DVL isoform levels vary substantially in different breast cancer cell lines. Therefore, it might be worth analyzing whether aspects of tumor biology like proliferation and migration are differentially regulated by these scaffolding proteins, potentially providing a paradigm for the differentiation of non-canonical versus canonical WNT signaling.

We show here that, in addition to activating the canonical Wnt/β-catenin pathway, Wnt1 transactivates EGFR and stimulates ERK1/2 activity in many human breast cancer cells. This Wnt1-mediated response is similar to EGFR transactivation induced by many GPCRs)[19]. In fact, various lines of evidence, including the GPCR-like heptahelical structure of the FZD receptor family and genetic data from *Drosophila*, suggest that these receptors have biological similarities [58-60]. Although we could not block Wnt1-induced ERK1/2 activation using pertussis toxin (PTX) to block $G\alpha_{i/o}$ proteins, this still leaves the possibility that PTX-insensitive $G\alpha$ proteins mediate the effects of WNT/FZD signaling. Indeed, it was

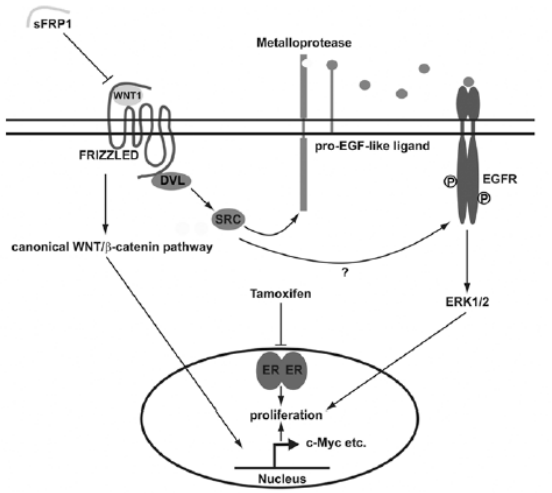
shown that $G\alpha_{q/11}$ group proteins contribute to the activation of canonical WNT signaling [60,61].

Our results also show that c-Src has an important role in Wnt1-driven EGFR transactivation. Wnt1 was able to transactive EGFR in Src-expressing MEFs, but not in Src knockout MEFs. Furthermore, an Src kinase inhibitor abolished the effects of Wnt1 on ERK1/2 activation in human breast cancer cell lines and Src kinase activation was increased in SkBr3/Wnt1 cells. Src kinase has also been implicated in GPCR-mediated EGFR transactivation)[19]. Src kinase might act directly downstream of GPCRs and FZD receptors via its interaction with ADAMs and MMPs [62-65]. Association of Src kinases with these enzymes might regulate their proteolytic activity and subcellular localization [66], leading to an increase in ERBB ligand shedding and autocrine receptor activation. Since we observed that neither metalloprotease inhibitors nor an EGFR-blocking antibody completely blocked Wnt1-induced ERK1/2 activation, this might reflect a direct effect of Src kinase on EGFR activity due to its ability to phosphorylate the receptor at Tyr 845 [67]. The involvement of WNT-induced Src activity on EGFR activation is corroborated by our observation that the knockdown of DVL decreased the level of Tyr 845 phosphorylation in several breast cancer cell lines.

WNT signaling has previously been linked to the activation of Src and ERK1/2 in NIH3T3 cells and in osteoblast progenitors [68-70], and recently EGFR was shown to be involved in ERK1/2 activation downstream of purified Wnt3a [71]. However, these studies rely on overexpression or treatment with recombinant proteins and did not link the transactivation to autocrine signaling processes. It was recently shown that Wnt1 is induced by progesterone receptor (PgR) signaling in T47D breast cancer cells and that it is required for EGFR transactivation by a PgR agonist in an Src- and metalloprotease-dependent manner [72]. These results are interesting to consider in light of the data presented in this paper. It is possible that the rapid effects of steroid hormones leading to sustained proliferation or survival of breast tumor cells proceed by establishing an autocrine loop of EGFR activity that is linked, in part, to Wnt1 production. It will be important to see whether results from the T47D breast cancer model are clinically relevant in primary breast tumors, many of which overexpress Wnt1 [13].

EGFR activity is known to play a role in endocrine therapy resistance (for example, in MCF-7 cells) [73]. In fact, there are increased β-catenin levels and increased expression of WNT pathway target genes in these resistant cells [74], further implicating WNT pathway activity in endocrine resistance. Our data also show the potential importance of autocrine WNT signaling in response to anti-hormonal therapies. Wnt1 treatment of the ER^+ MCF-7 and T47D cells rescued them from the antiproliferative action of 4-HT, and this was blocked by treatment with an EGFR TKI, showing the importance of autocrine EGFR signaling in the Wnt1 rescue.

Figure 8



Schematic representation of wingless and integration site growth factor (WNT)-induced epidermal growth factor receptor (EGFR) transactivation. Our results show that Wnt1 induces a signaling cascade that links the activation of EGFR in a manner dependent on Dishevelled, SRC, metalloprotease, and EGF-like ligand to the sFRP1-sensitive activation of Frizzled receptors. The activation of EGFR, which may occur via phosphorylation of Y845, an SRC phosphorylation site, triggers activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway. Together with the canonical WNT/β-catenin pathway and its target genes, including *c-Myc*, the ERK1/2 pathway promotes proliferation and survival of breast cancer cells. Furthermore, activation of the ERK1/2 signaling by Wnt1 may contribute to the development of anti-estrogen resistance. DVL, Dishevelled; EGF, epidermal growth factor; ER, estrogen receptor; sFRP1, secreted Frizzled-related protein 1.

Conclusion

Our results support the concept that therapeutic interference with autocrine WNT signaling might be a useful strategy for targeting breast cancer. Furthermore, blocking the pathway at the level of WNT/FZD/DVL, in contrast to targeting the β-catenin/TCF complex [75], would not only impact on canonical signaling but also provide a novel interface for interfering with autocrine EGFR activity, an important target in breast cancer [22,32]. In Figure 8, we propose a model that incorporates the data presented in this paper.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TS designed and carried out the experiments, unless otherwise specified, and wrote the manuscript. YM analyzed the specificity of the act. β-catenin antibody. SL assisted TS in carrying out the experiments. AH purified sFRP1 protein. NEH

participated in designing the experiments and in writing the manuscript. All authors read and approved the final manuscript.

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References

1. Cong F, Schweizer L, Varmus H: **Casein kinase I epsilon modulates the signaling specificities of dishevelled.** *Mol Cell Biol* 2004, **24:**2000-2011.
2. Willert K, Brink M, Wodarz A, Varmus H, Nusse R: **Casein kinase 2 associates with and phosphorylates dishevelled.** *EMBO J* 1997, **16:**3089-3096.
3. Kühl M, Geis K, Sheldahl LC, Pukrop T, Moon RT, Wedlich D: **Antagonistic regulation of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signaling.** *Mech Dev* 2001, **106:**61-76.
4. Gonzalez-Sancho JM, Brennan KR, Castelo-Soccio LA, Brown AM: **Wnt proteins induce dishevelled phosphorylation via an LRP5/6-independent mechanism, irrespective of their ability to stabilize beta-catenin.** *Mol Cell Biol* 2004, **24:**4757-4768.
5. Sheldahl LC, Slusarski DC, Pandur P, Miller JR, Kuhl M, Moon RT: **Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos.** *J Cell Biol* 2003, **161:**769-777.
6. Clevers H: **Wnt/beta-catenin signaling in development and disease.** *Cell* 2006, **127:**469-480.
7. Howe LR, Brown AM: **Wnt signaling and breast cancer.** *Cancer Biol Ther* 2004, **3:**36-41.
8. van de Wetering M, Barker N, Harkes IC, van der Heyden M, Dijk NJ, Hollestelle A, Klijn JG, Clevers H, Schutte M: **Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling.** *Cancer Res* 2001, **61:**278-284.
9. Ugolini F, Adelaide J, Charafe-Jauffret E, Nguyen C, Jacquemier J, Jordan B, Birnbaum D, Pebusque MJ: **Differential expression assay of chromosome arm 8p genes identifies Frizzled-related (FRP1/FRZB) and Fibroblast Growth Factor Receptor 1 (FGFR1) as candidate breast cancer genes.** *Oncogene* 1999, **18:**1903-1910.
10. Veeck J, Niederacher D, An H, Klopocki E, Wiesmann F, Betz B, Galm O, Camara O, Durst M, Kristiansen G, *et al.*: **Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis.** *Oncogene* 2006, **25:**3479-3488.
11. Klopocki E, Kristiansen G, Wild PJ, Klaman I, Castanos-Velez E, Singer G, Stohr R, Simon R, Sauter G, Leibiger H, *et al.*: **Loss of SFRP1 is associated with breast cancer progression and poor prognosis in early stage tumors.** *Int J Oncol* 2004, **25:**641-649.
12. Milovanovic T, Planutis K, Nguyen A, Marsh JL, Lin F, Hope C, Holcombe RF: **Expression of Wnt genes and frizzled 1 and 2 receptors in normal breast epithelium and infiltrating breast carcinoma.** *Int J Oncol* 2004, **25:**1337-1342.
13. Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, Mandinova A, Raffoul W, Fiche M, Dotto GP, Brisken C: **Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism.** *Proc Natl Acad Sci USA* 2006, **103:**3799-3804.
14. Benhaj K, Akcali KC, Ozturk M: **Redundant expression of canonical Wnt ligands in human breast cancer cell lines.** *Oncol Rep* 2006, **15:**701-707.
15. Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, Pestell RG, Hung MC: **Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression.** *Proc Natl Acad Sci USA* 2000, **97:**4262-4266.
16. Civenni G, Holbro T, Hynes NE: **Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells.** *EMBO Rep* 2003, **4:**166-171.
17. Blobel CP: **ADAMs: key components in EGFR signalling and development.** *Nat Rev Mol Cell Biol* 2005, **6:**32-43.
18. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A: **EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF.** *Nature* 1999, **402:**884-888.
19. Ohtsu H, Dempsey PJ, Eguchi S: **ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors.** *Am J Physiol Cell Physiol* 2006, **291:**C1-C10.
20. Roelle S, Grosse R, Aigner A, Krell HW, Czubayko F, Gudermann T: **Matrix metalloproteinases 2 and 9 mediate epidermal growth factor receptor transactivation by gonadotropin-releasing hormone.** *J Biol Chem* 2003, **278:**47307-47318.
21. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA: **An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells.** *Cancer Cell* 2004, **6:**497-506.
22. Hynes NE, Lane HA: **ERBB receptors and cancer: the complexity of targeted inhibitors.** *Nat Rev Cancer* 2005, **5:**341-354.
23. Hart S, Fischer OM, Prenzel N, Zwick-Wallasch E, Schneider M, Hennighausen L, Ullrich A: **GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR-independent pathways.** *Biol Chem* 2005, **386:**845-855.
24. Motoyama AB, Hynes NE, Lane HA: **The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides.** *Cancer Res* 2002, **62:**3151-3158.
25. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE: **The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation.** *Proc Natl Acad Sci USA* 2003, **100:**8933-8938.
26. van De Wetering M, Oving I, Muncan V, Pon Fong MT, Brantjes H, Van Leenen D, Holstege FC, Brummelkamp TR, Agami R, Clevers H: **Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector.** *EMBO Rep* 2003, **4:**609-615.
27. Holbro T, Civenni G, Hynes NE: **The ErbB receptors and their role in cancer progression.** *Exp Cell Res* 2003, **284:**99-110.
28. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, *et al.*: **A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.** *Cancer Cell* 2006, **10:**515-527.
29. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB: **A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer.** *Nat Genet* 2002, **31:**141-149.
30. Galli LM, Barnes T, Cheng T, Acosta L, Anglade A, Willert K, Nusse R, Burrus LW: **Differential inhibition of Wnt-3a by Sfrp-1, Sfrp-2, and Sfrp-3.** *Dev Dyn* 2006, **235:**681-690.
31. Harris RC, Chung E, Coffey RJ: **EGF receptor ligands.** *Exp Cell Res* 2003, **284:**2-13.
32. Salomon DS, Brandt R, Ciardiello F, Normanno N: **Epidermal growth factor-related peptides and their receptors in human malignancies.** *Crit Rev Oncol Hematol* 1995, **19:**183-232.
33. Hynes NE, Schlange T: **Targeting ADAMS and ERBBs in lung cancer.** *Cancer Cell* 2006, **10:**7-11.
34. Tanner M, Kapanen AI, Junttila T, Raheem O, Grenman S, Elo J, Elenius K, Isola J: **Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer.** *Mol Cancer Ther* 2004, **3:**1585-1592.
35. Lev DC, Kim LS, Melnikova V, Ruiz M, Ananthaswamy HN, Price JE: **Dual blockade of EGFR and ERK1/2 phosphorylation potentiates growth inhibition of breast cancer cells.** *Br J Cancer* 2004, **91:**795-802.
36. Kozma SC, Bogaard ME, Buser K, Saurer SM, Bos JL, Groner B, Hynes NE: **The human c-Kirsten ras gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231.** *Nucleic Acids Res* 1987, **15:**5963-5971.
37. Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ: **Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma.** *Cancer Res* 2000, **60:**2926-2935.

38. Susa M, Missbach M, Green J: **Src inhibitors: drugs for the treatment of osteoporosis, cancer or both?** *Trends Pharmacol Sci* 2000, **21**:489-495.
39. Olayioye MA, Badache A, Daly JM, Hynes NE: **An essential role for Src kinase in ErbB receptor signaling through the MAPK pathway.** *Exp Cell Res* 2001, **267**:81-87.
40. Nicholson RI, Hutcheson IR, Hiscox SE, Knowlden JM, Giles M, Barrow D, Gee JM: **Growth factor signalling and resistance to selective oestrogen receptor modulators and pure anti-oestrogens: the use of anti-growth factor therapies to treat or delay endocrine resistance in breast cancer.** *Endocr Relat Cancer* 2005, **12(Suppl 1)**:S29-36.
41. Traxler P, Allegrini PR, Brandt R, Brueggen J, Cozens R, Fabbro D, Grosios K, Lane HA, McSheehy P, Mestan J, *et al.*: **AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity.** *Cancer Res* 2004, **64**:4931-4941.
42. Ilyas M: **Wnt signalling and the mechanistic basis of tumour development.** *J Pathol* 2005, **205**:130-144.
43. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, *et al.*: **The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells.** *Cell* 2002, **111**:241-250.
44. Liang H, Chen Q, Coles AH, Anderson SJ, Pihan G, Bradley A, Gerstein R, Jurecic R, Jones SN: **Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue.** *Cancer Cell* 2003, **4**:349-360.
45. Cappellen D, Schlange T, Bauer M, Maurer F, Hynes NE: **Novel c-MYC target genes mediate differential effects on cell proliferation and migration.** *EMBO Rep* 2007, **8**:70-76.
46. Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM: **Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma.** *Cancer Cell* 2002, **1**:279-288.
47. Kurayoshi M, Oue N, Yamamoto H, Kishida M, Inoue A, Asahara T, Yasui W, Kikuchi A: **Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion.** *Cancer Res* 2006, **66**:10439-10448.
48. Shulewitz M, Soloviev I, Wu T, Koeppen H, Polakis P, Sakanaka C: **Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer.** *Oncogene* 2006, **25**:4361-4369.
49. Cowling VH, D'Cruz CM, Chodosh LA, Cole MD: **c-Myc transforms human mammary epithelial cells through repression of the Wnt inhibitors DKK1 and SFRP1.** *Mol Cell Biol* 2007, **27**:5135-5146.
50. You L, He B, Uematsu K, Xu Z, Mazieres J, Lee A, McCormick F, Jablons DM: **Inhibition of Wnt-1 signaling induces apoptosis in beta-catenin-deficient mesothelioma cells.** *Cancer Res* 2004, **64**:3474-3478.
51. Kawano Y, Kypta R: **Secreted antagonists of the Wnt signalling pathway.** *J Cell Sci* 2003, **116**:2627-2634.
52. Fujii N, You L, Xu Z, Uematsu K, Shan J, He B, Mikami I, Edmondson LR, Neale G, Zheng J, *et al.*: **An antagonist of Dishevelled protein-protein interaction suppresses beta-catenin-dependent tumor cell growth.** *Cancer Res* 2007, **67**:573-579.
53. He B, You L, Uematsu K, Xu Z, Lee AY, Matsangou M, McCormick F, Jablons DM: **A monoclonal antibody against Wnt-1 induces apoptosis in human cancer cells.** *Neoplasia* 2004, **6**:7-14.
54. You L, He B, Xu Z, Uematsu K, Mazieres J, Fujii N, Mikami I, Reguart N, McIntosh JK, Kashani-Sabet M, *et al.*: **An anti-Wnt-2 monoclonal antibody induces apoptosis in malignant melanoma cells and inhibits tumor growth.** *Cancer Res* 2004, **64**:5385-5389.
55. He B, Reguart N, You L, Mazieres J, Xu Z, Lee AY, Mikami I, McCormick F, Jablons DM: **Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations.** *Oncogene* 2005, **24**:3054-3058.
56. Mikami I, You L, He B, Xu Z, Batra S, Lee AY, Mazieres J, Reguart N, Uematsu K, Koizumi K, Jablons DM: **Efficacy of Wnt-1 monoclonal antibody in sarcoma cells.** *BMC Cancer* 2005, **5**:53.
57. Nagayama S, Fukukawa C, Katagiri T, Okamoto T, Aoyama T, Oyaizu N, Imamura M, Toguchida J, Nakamura Y: **Therapeutic potential of antibodies against FZD10, a cell-surface protein, for synovial sarcomas.** *Oncogene* 2005, **24**:6201-6212.
58. Malbon CC: **Frizzleds: new members of the superfamily of G-protein-coupled receptors.** *Front Biosci* 2004, **9**:1048-1058.
59. Katanaev VL, Ponzielli R, Semeriva M, Tomlinson A: **Trimeric g protein-dependent frizzled signaling in Drosophila.** *Cell* 2005, **120**:111-122.
60. Liu X, Rubin JS, Kimmel AR: **Rapid, Wnt-induced changes in GSK3beta associations that regulate beta-catenin stabilization are mediated by Galpha proteins.** *Curr Biol* 2005, **15**:1989-1997.
61. Gao Y, Wang HY: **Casein kinase 2 is activated and essential for Wnt/beta-catenin signaling.** *J Biol Chem* 2006, **281**:18394-18400.
62. Poghosyan Z, Robbins SM, Houslay MD, Webster A, Murphy G, Edwards DR: **Phosphorylation-dependent interactions between ADAM15 cytoplasmic domain and Src family protein-tyrosine kinases.** *J Biol Chem* 2002, **277**:4999-5007.
63. Seals DF, Courtneidge SA: **The ADAMs family of metalloproteases: multidomain proteins with multiple functions.** *Genes Dev* 2003, **17**:7-30.
64. Mori S, Tanaka M, Nanba D, Nishiwaki E, Ishiguro H, Higashiyama S, Matsuura N: **PACSIN3 binds ADAM12/meltrin alpha and up-regulates ectodomain shedding of heparin-binding epidermal growth factor-like growth factor.** *J Biol Chem* 2003, **278**:46029-46034.
65. Tanaka M, Nanba D, Mori S, Shiba F, Ishiguro H, Yoshino K, Matsuura N, Higashiyama S: **ADAM binding protein Eve-1 is required for ectodomain shedding of epidermal growth factor receptor ligands.** *J Biol Chem* 2004, **279**:41950-41959.
66. Wild-Bode C, Fellerer K, Kugler J, Haass C, Capell A: **A basolateral sorting signal directs ADAM10 to adherens junctions and is required for its function in cell migration.** *J Biol Chem* 2006, **281**:23824-23829.
67. Ishizawar R, Parsons SJ: **c-Src and cooperating partners in human cancer.** *Cancer Cell* 2004, **6**:209-214.
68. Yun MS, Kim SE, Jeon SH, Lee JS, Choi KY: **Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation.** *J Cell Sci* 2005, **118**:313-322.
69. Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S: **Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT.** *J Biol Chem* 2005, **280**:41342-41351.
70. Kim SE, Lee WJ, Choi KY: **The PI3 kinase-Akt pathway mediates Wnt3a-induced proliferation.** *Cell Signal* 2007, **19**:511-518.
71. Kim SE, Choi KY: **EGF receptor is involved in WNT3a-mediated proliferation and motility of NIH3T3 cells via ERK pathway activation.** *Cell Signal* 2007, **19**:1554-1564.
72. Faivre EJ, Lange CA: **Progesterone receptors upregulate Wnt-1 to induce EGFR transactivation and c-Src dependent sustained activation of Erk1/2 MAP kinase in breast cancer cells.** *Mol Cell Biol* 2007, **27**:466-480.
73. McClelland RA, Barrow D, Madden TA, Dutkowski CM, Pamment J, Knowlden JM, Gee JM, Nicholson RI: **Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex).** *Endocrinology* 2001, **142**:2776-2788.
74. Hiscox S, Jiang WG, Obermeier K, Taylor K, Morgan L, Burmi R, Barrow D, Nicholson RI: **Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation.** *Int J Cancer* 2006, **118**:290-301.
75. Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW, Shivdasani RA: **Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex.** *Cancer Cell* 2004, **5**:91-102.

WNT signaling enhances breast cancer cell motility and blockade of the WNT pathway by sFRP1 suppresses MDA-MB-231 xenograft growth

Abstract

Introduction

In breast cancer deregulation of the WNT signaling pathway occurs by autocrine mechanisms. WNT ligands and Frizzled (FZD) receptors are coexpressed in primary breast tumors and breast cancer cell lines. Moreover, many breast tumors show hypermethylation of the promoter region of secreted Frizzled-related protein 1 (sFRP1), causing low expression of this WNT antagonist. We have previously shown that the WNT pathway influences proliferation of breast cancer cell lines via activation of canonical signaling and via EGFR transactivation. Furthermore, interference with WNT signaling in breast cancer cell lines reduces their proliferative ability. Here we examine the role of WNT signaling in breast tumor cell migration and on xenograft outgrowth.

Methods

Two sFRP1-negative human breast cancer cell lines, T47D and MDA-MB-231, were used to study WNT signaling. We examined the effects of activating or blocking the WNT pathway on tumor cell motility by treatment with WNT ligands or with sFPR1, respectively. Furthermore, the ability of MDA-MB-231 cells ectopically expressing sFRP1 to grow as xenografts in nude mice was tested. Finally, a microarray analysis was carried out in order to identify targets with roles in MDA-MB-231/sFRP1 tumor growth inhibition.

Results

We show here that WNT stimulates the migratory ability of T47D cells, while ectopic expression of sFRP1 in MDA-MB-231 cells blocks canonical WNT signaling and decreases the migratory potential of these cells. Moreover, outgrowth of MDA-MB-231/sFRP1 expressing cells as xenografts in nude mice is dramatically impaired compared to outgrowth of control tumors. A microarray analysis led to the identification of two genes, *CCND1* and *CDKN1A*, whose expression level is selectively altered *in vivo* in sFRP1 expressing tumors. The encoded proteins, Cyclin D1 and $p21^{Cip1}$ were down- and up-regulated, respectively, in sFRP1 expressing tumors, suggesting that they are downstream mediators of WNT signaling.

Conclusions

Our results show that the WNT pathway influences multiple biological properties of breast cancer cell lines. WNT stimulates tumor cell motility; conversely sFRP1 mediated WNT pathway blockade reduces motility. Moreover, ectopic sFRP1 expression in MDA-MB-231 cells has a strong negative impact on tumor outgrowth. These results suggest that interference with WNT signaling at the ligand-receptor level may be a valid therapeutic approach in breast cancer.

Introduction

The WNT signaling network is complex with 19 WNT ligands, 10 FZD receptors, as well as the co-receptors, low density lipoprotein receptor-related protein 5 (LRP5) and LRP6. WNT receptor binding stimulates intracellular signaling promoting stabilization and nuclear translocation of the key effector of the canonical pathway, β-catenin [101] [178]. In many human cancers, intracellular mediators of the WNT pathway are mutated. Inactivating mutations in the *APC* or the *AXIN* genes, as well as activating *CTNNB1* (encoding β-catenin) mutations all cause β-catenin stabilization and nuclear accumulation in the absence of WNT ligands. In the nucleus, β-catenin forms functional complexes with transcription factors of the LEF-1/T-cell factor (TCF) family, activating expression of target genes with cancer promoting roles [153]. In addition to activation of the canonical pathway by engagement of FZD and LRP receptors, WNT ligands bind the Ror2 or Ryk receptors to stimulate β-catenin independent pathways that have been involved with cytoskeletal reorganization and cell migration [178] [179].

In breast cancer, deregulation of WNT signaling appears to occur by autocrine mechanisms [180] [181] [177]. Multiple WNT ligands and FZD receptors are expressed in primary human breast tumors and in breast cancer cell lines [153, 177] [154, 156]. Furthermore, most breast tumors show hypermethylation of the promoter region of sFRP1 and low expression of this negative WNT pathway regulator [170, 171, 173].

WNT signaling influences biological processes ranging from cell fate to cell motility, proliferation and apoptosis. We have previously shown that interference with autocrine WNT signaling blocks *in vitro* proliferation of many human breast cancer cell lines [177]. We have extended these studies

and show here that blocking the WNT pathway in T47D and MDA-MB-231 breast cancer cells not only decreases proliferation, but also impairs motility of the tumor cells. Furthermore, stable expression of sFRP1 in MDA-MD-231 cells has a dramatic effect on the ability of the cells to grow as tumor xenografts in nude mice. A microarray analysis led to the identification of two genes, *CCND1* and *CDKN1A*, whose expression level is selectively altered *in vivo* in sFRP1 expressing tumors. The encoded proteins, Cyclin D1 and $p21^{Cip1}$ were down- and up-regulated, respectively, in sFRP1 expressing tumors, suggesting that they are downstream mediators of WNT signaling involved in growth inhibition. These results provide further evidence supporting approaches to target WNT pathway activity in breast cancer.

Results

Wnt1 stimulates migration of T47D breast cancer cells

The WNT signaling network controls many biological processes through canonical and noncanonical signaling, via β-catenin stabilization and RhoA activation, respectively. We have previously shown that autocrine WNT signaling stimulates proliferation of breast cancer cell lines via the canonical signaling pathway [177]. In the following experiments we explored the role of WNT signaling in breast tumor cell migration in a wound healing assay. Confluent monolayers of T47D breast tumor cells were scratched and the medium was changed to Wnt1 conditioned medium (CM) or control CM. Cultures treated 3 hours with Wnt1 CM showed elongated cells at the wound edges (Fig 1A, top panel). An analysis of closure kinetics of the wounded area, which was monitored 3 and 7 hr later, revealed that Wnt1 treated cultures closed the wound significantly more rapidly than cultures treated with control medium (Fig 1A lower panel). The effect was Wnt1 specific since addition of the WNT antagonist sFRP1 to the Wnt1 CM reversed its effects so that the time for recovery of the wounded area was the same as measured in control cells (Fig 1A lower panel).

Wnt1 was also stably expressed in the T47D breast cancer cells. We have previously shown that ectopic Wnt1 expression in these cells stimulates canonical signaling and induces an increase in level of active β-catenin [177]. We examined the migratory ability of the Wnt1 expressing T47D cells in a clone with high Wnt1 levels (clone 1), and in a pool of T47D cells with lower levels of Wnt1 (P1) (Fig 1B). Confluent cultures of T47D/Wnt1-clone1 and T47D/Wnt1-P1 as well as

control cultures were scratched and recovery of the wounded area was monitored 3 hr later. In comparison to the control cultures, both the clone and the pool of Wnt1 expressing cells migrated significantly more rapidly (Fig 1C). These results suggest that activation of WNT signaling promotes motility of T47D breast cancer cells.

Elevated RhoA activity in T47D/Wnt1 cells

Remodeling of the actin cytoskeleton is a crucial step in cell migration [182]. Indeed, T47D cultures treated with Wnt1 CM have more prominent actin stress fibers compared to control cultures (Fig 2A). Prompted by this morphological change and the known ability of some WNTs to stimulate RhoA [183, 184], RhoA activity was measured in lysates made from control T47D cultures and from T47D/Wnt1-P1 cultures. The active GTP-bound form of RhoA was captured in a pull-down assay with the GST-C21 fusion protein and examined by a western analysis for RhoA levels using a RhoA specific antiserum. Both cell lines have similar overall RhoA levels, however the Wnt1 expressing cells have 3.9-fold more active, GTP-bound RhoA compared to control cells (Fig 2B). These results suggest that one of the mediators of Wnt1 stimulated tumor cell migration is RhoA.

Ectopic sFRP1 expression in MDA-MB-231 cells blocks WNT signaling

In breast cancer, low levels of sFRP1 combined with co-expression of WNT ligands and FZD receptors contribute to WNT pathway activation [154, 177]. MDA-MB-231 cells, a model of basal breast cancer [72], do not express sFRP1 mRNA as determined by a qRT-PCR analysis (data not shown). Furthermore, active, stabilized β-catenin is present in MDA-MB-231 cells [177], suggesting that WNT signaling is constitutively activated. We examined the effects of blocking WNT pathway by restoring sFRP1 expression in the MDA-MB-231 breast tumor cells. Vectors encoding Myc-tagged sFRP1 and the empty control were transfected into MDA-MB-231 cells and stable clones were selected in G418-containing medium. Three MDA-MB-231/sFRP1 clones expressing moderate to strong levels of the Myc-tagged sFRP1, as well as control clones were selected for further analyses (Fig 3A).

WNT pathway activity was examined in the cells using various markers. As a consequence of WNT binding to FZD, cytoplasmic scaffolding proteins of the Dishevelled family (DVL1, -2 and -3) become phosphorylated on serine and threonine residues. DVL phosphorylation, which is the most proximal signaling event downstream of WNT-mediated FZD activation, can be monitored by a

decrease in the electrophoretic mobility of p-DVL [106, 185]. DVL1, -2 and -3 and stabilized, unphosphorylated active β-catenin, were examined in a western analysis in the individual clones. Each of the 3 DVLs showed an enhancement in the lower non-phosphorylated form in the sFRP1 expressing cells, compared to the vector control cells. Furthermore, a band of active β-catenin is readily detectable in the control cells, while in the two clones expressing the highest level of sFRP1 there was a strong decrease in active β-catenin levels (Fig 3A). The activity of other signaling pathways was also examined in the sFRP1 expressing MDA-MB-231 cells. These cells display high levels of phosphorylated ERK1/2, very likely due to the fact that they possess an activating *K-Ras* mutation [186]. sFRP1 mediated inhibition of the WNT pathway did not alter phosphorylated ERK1/2 levels (Fig3A).

For further studies, the three sFRP1 expressing clones were pooled (P1), and a second pool of sFRP1-expressing MDA-MB-231 cells, consisting of >100 clones was generated (P2). A quantification of the western analysis carried out on both pools shows that P1 has 2.8-fold higher levels of sFRP1 than does P2 (Fig 3B). The lower level of sFRP1 in P2 is likely due to the fact that these cells was prepared from retrovirally infected MDA-MB-231 cultures, while the P1 clones originated from a transfection.

We have previously shown that treatment of various human breast cancer cell lines with purified sFRP1 or with sFRP1 CM lowers their proliferative ability[177]; proliferation of MDA-MB-231 cells were also inhibited by treatment with sFRP1 CM (Supp Fig 1). Furthermore, ectopic expression of sFRP1 in MDA-MB-231 cells also decreases the proliferative ability of the cells in comparison to control cells (Fig 3C). This effect appears to be dependent on sFRP1 expression levels since, in comparison to controls, there is a 31% and a 16% reduction in proliferation of MDA-MB-231/sFRP1-P1 and -P2 cells, respectively (Fig 3C).

We also examined the ability of MDA-MB-231/sFRP1-P1 cultures and control P1 cultures to migrate in a wound healing assay. In response to Wnt1 CM, the control MDA-MB-231 cells migrated significantly more rapidly into the wounded area compared to cultures treated with control CM (Fig 3D gray bars). These results are similar to what was observed for the T47D cultures treated with Wnt1 CM (Fig 1A). In contrast, Wnt1 treatment of MDA-MB-231/sFRP1-P1 cells did not significantly stimulate migration, reflecting the ability of sFRP1 to block Wnt1 mediated FZD

activation (Fig 3D black bars). In summary, ectopic expression of sFRP1 in MDA-MB-231 cells down-regulates WNT pathway activity, leading to a decrease in proliferation and in the ability of Wnt1 to stimulate migration.

Xenografts of sFRP1 expressing MDA-MB-231 cells show reduced growth in nude mice

To test the *in vivo* effects of sFRP1, control and sFRP1 expressing MDA-MB-231 cells (P1 and P2) were injected into the mammary fat pads of female nude mice and tumor growth was monitored (Fig 4 and Supp Fig 2). There was a significant reduction in tumor outgrowth in mice injected with MDA-MB-231/sFRP1-P1 cells ($p<0.01$, Two-way RM ANOVA) (Fig 4A left panel). Furthermore, the time to detection of the first tumors was significantly shorter following injection of control MDA-MB-231 cells, compared to MDA-MB-231/sFRP1-P1 cells (23 days vs 35 days, respectively) (Fig 4A right panel). Moreover, in this experiment, 3 mice injected with the MDA-MB-231/sFRP1-P1 cells remained tumor-free at day 45, when the experiment was terminated. In contrast, all of the mice injected with control MDA-MB-231 cells had tumors (Fig 4A, right panel). MDA-MB-231/sFRP1-P1 cells were tested in three additional independent experiments (Supp Fig 2 and data not shown). While there was some variation in the time of tumor onset in the independent experiments, the time to appearance of the first tumor was consistently longer following injection of the MDA-MB-231/sFRP1 cells, in comparison to control cells. Furthermore, MDA-MB-231/sFRP1 tumors grew more slowly than control tumors, and the number of tumor-free mice at the end of each experiment was always higher (Supp Fig 2).

Interestingly, MDA-MB-231/sFRP1-P2 cells that express almost 3-fold less sFRP1 compared to P1 cells (Fig 3B) also grew more slowly than control cells. Although the effect on tumor growth did not reach significance using Two-way RM ANOVA (Fig 4B left panel), tumor onset was delayed significantly in the cohort injected with MDA-MB-231/sFRP1-P2 cells ($p=0.026$, log-rank test) (Fig 4B right panel). Furthermore, all of the mice had tumors at the end of the experiment (Fig 4B, right panel). The *in vivo* results together with the data on *in vitro* proliferation (Fig 3C) suggest that higher levels of sFRP1 lead to a stronger blockade of WNT pathway activity and to a stronger effect on proliferation and tumor onset.

Mechanisms contributing to the *in vivo* anti-tumor effects of sFRP1

Our next goal was to reveal the mechanism underlying the ability of sFRP1 to decrease the mammary tumor-forming potential of MDA-MB-231 cells. Initially we tested the tumors for continued sFRP1 expression. A western analysis carried out on lysates from tumors arising from MDA-MB-231/sFRP1-P1 cells revealed that sFRP1 levels remained high. Furthermore, WNT signaling was strongly down-regulated, as shown by a decrease in the P-DVL3 levels in the sFRP1 expressing tumors compared to control tumors (Fig 4A left panel, insert). These results suggest that the slow outgrowth of sFRP1 expressing MDA-MB-231 xenografts does not reflect a selection for tumor cells that down-regulated sFRP1 expression.

The mechanisms underlying the ability of sFRP1 to impair tumor growth might be tumor cell intrinsic, resulting from down-regulation of WNT signaling, and/or extrinsic via effects of secreted sFRP1 on tumor-associated cells. We tested both possibilities in the following experiments. *In vivo* tumor cell proliferation was evaluated by examining bromodeoxyuridine (BrdU) incorporation in control and sFRP1 expressing tumors. Incorporated BrdU was detected with a specific antiserum (Fig 5A left panel) and staining was quantified. There was a 70% reduction in BrdU staining in tumors arising from MDA-MB-231/sFRP1-P1 cells compared to control tumors (Fig 5A right panel). Apoptosis, as measured by western blotting for cleaved caspase-3, was low in the MDA-MB-231 control tumor lysates and was not increased in the MDA-MB-231/sFRP1-P1 tumor lysates (data not shown). These results suggest that sFRP1 downregulation of WNT signaling has a strong effect on tumor cell proliferation, but not survival.

sFRP1 has been reported to block *in vivo* neovascularization [187]. Thus, we considered the possibility that the density or the functionality of the tumor-associated vessels might be impaired in sFRP1 expressing tumors. Vasculature was visualized by FITC-labeled *L. esculentum* lectin [188], which was injected in the tail vein of tumor-bearing mice 5 min before the animals were sacrificed. Only functional vessels will be perfused with lectin in this experiment. Tumor sections were prepared and the associated endothelial cells were stained for CD31, while functional vessels were visualized via the FITC signal. There was no significant difference in the total vessel area or the ratio of FITC positive/CD31 positive vessels in sFRP1 expressing tumors compared to control tumors (Fig 5B left panel and right panels, respectively). Thus sFRP1 expressed by the tumor cells does not appear to influence the number or the functionality of tumor-associated blood vessels. In summary, these

results suggest that sFRP1 mediated blockade of WNT pathway activity in tumor cells is the most important factor contributing to the slower outgrowth of the MDA-MB-231/sFRP1 tumors in mammary glands.

Analysis of c-Myc in the MDA-MB-231/sFRP1 tumor model

Based on our previous results showing that siRNA mediated knock-down of c-Myc in MDA-MB-231 cells lowers their *in vitro* proliferation [189] and the fact that c-Myc is a target gene of the WNT pathway in colon cancer models [190], we examined c-Myc in MDA-MB-231/sFRP1 cultured cells and derived tumors. qRT-PCR analyses revealed that there was no significant difference in c-Myc RNA levels in MDA-MB-231/sFRP1 tumor cells or cultured cells in comparison to control tumor cells or control cultured cells (Fig 6A). Interestingly, western analyses carried out on tumor lysates revealed a strong decrease in c-Myc protein levels in MDA-MB-231/sFRP1-P1 tumors compared to control tumors (Fig 6B left panel); while the level of c-Myc was not altered in the cultured MDA-MB-231/sFRP1-P1 cells compared to control cells (Fig 6B right panel).

These results suggest that in the MDA-MB-231 cells, c-Myc is not a WNT target gene. Moreover, c-Myc protein appears to be subjected to different control mechanisms in the tumor environment compared to *in vitr*o cultured cells. Nevertheless, we considered it possible that the lower level of c-Myc might be at least partially responsible for the slower tumor outgrowth kinetics of the MDA-MB-231/sFRP1 cells. Thus, we tested whether ectopic c-Myc expression might alter the growth-suppressed phenotype of MDA-MB-231/sFRP1 tumors. Accordingly, MDA-MB-231/sFRP1-P1 cells were infected with a c-Myc-encoding retroviral vector or a control vector and following selection in antibiotic-containing medium, pools of clones were generated. c-Myc infected MDA-MB-231/sFRP1 cells have elevated c-Myc levels compared to cells infected with the control vector (Supp Fig 3A). *In vitro* proliferation was monitored 3 and 4 days after seeding, revealing a slight, but non-significant increase in cell number in the MDA-MB-231/sFRP1/Myc cells compared to the MDA-MB-231/sFRP1/control cells (Supp Fig 3B). In the final experiment we tested the *in vivo* tumor forming ability of the cell lines. MDA-MB-231/sFRP1/Myc cells and MDA-MB-231/sFRP1/control cells were injected into fat pads of nude mice and tumor outgrowth kinetics was monitored. Forty-one days later, 2/6 mice injected with MDA-MB-231/sFRP1/Myc cells had tumors whereas none of the MDA-MB-231/sFRP1/control cells gave rise to tumors (Supp Fig 3C). Although we observed a tendency that ectopic c-Myc expression rescues the growth potential *in*

vitro and tumor forming potential *in vivo* of MDA-MB-231/sFRP1 cells, the results did not reach significance, suggesting that additional targets might have roles in the growth suppression phenotype.

Identification of genes whose expression level is selectively altered *in vivo* in sFRP1 expressing tumors

In order to identify WNT pathway targets that are controlled by sFRP1 expression and influence proliferation of the MDA-MB-231 cells, we undertook a genome-wide transcriptome analysis using microarrays. RNA isolated from individual tumors arising after injection of MDA-MB-231/sFRP1-P1 cells and MDA-MB-231/control-P1 cells, as well as RNA from *in vitro* cultured MDA-MB-231/sFRP1-P1 cells and MDA-MB-231/control-P1 cells was analyzed.

Considering data generated from the tumors (6 sFRP1 tumors and 5 control tumors), there were 1753 probesets (1246 genes) whose signals were changed more than 1.5 fold (p-value < 0.01 using a 1-way ANOVA) in the tumors arising from MDA-MB-231/sFRP1-P1 cells compared to tumors arising from MDA-MB-231/control-P1 cells. The same analysis performed on *in vitro* cultured samples revealed 428 probesets (332 genes) that had a 1.5 fold difference (p-value < 0.01 using a 1-way ANOVA). Only 69 probesets (54 genes) overlapped between the two analyses showing that gene expression profiles generated from *in vivo* tumors differ substantially from those of *in vitro* cultured cells. A functional analysis using the Ingenuity's Pathway Analysis suite was also performed on the 1753 probesets, in order to identify the biological functions and diseases that were significantly represented in the *in vivo* data set (Supp Fig 4). This analysis revealed a significant enrichment in the group of genes categorized as "cancer" (Fisher's probability p=2.48E-12 to 1.41E-02) (Supp Fig 4A). Within this group, the most significant sub-group (266 genes) is "tumorigenesis" (Supp Fig 4B) (Fisher's probability, p=2.48E-12).

Cell cycle regulators are altered in sFPR1 expressing MDA-MB-231 xenografts

In vitro proliferation of MDA-MB-231/sFRP1-P1 cells was decreased by 30% compared to control cultures (Fig 3C). In comparison, the *in vivo* effects of WNT pathway blockade appeared to be stronger, e.g, outgrowth of MDA-MB-231/sFRP1-P1 tumors was significantly slower and in each experiment tumor-free mice remained in this cohort. Thus, we also screened the 1753 probesets for genes whose expression was significantly altered only in tumors arising from MDA-MB-231/sFRP1-P1 cells compared to: tumors arising from MDA-MB-231/control-P1 cells,

cultured MDA-MB-231/sFRP1-P1 cells or cultured MDA-MB-231/control-P1 cells. Using the "Profile Distance Search" function of Gendata's Analyst 4.5 tool, probes were sorted out of the 1753 probesets. This resulted in 135 probesets (106 genes) that were down-regulated (Fig 7A) and 84 probesets (62 genes) that were up-regulated (Fig 7B), in tumors arising from MDA-MB-231/sFRP1-P1 cells (Supp Table 1 lists the genes and their fold change). Two of the identified genes, *CCND1* and *CDKN1A* were further analyzed based on their known roles in cell cycle regulation and proliferation.

The microarray analysis showed that the signal from one probeset for *CCND1* was down-regulated and the probeset for *CDKN1A* was up-regulated *in vivo* in tumors resulting from MDA-MB-231/sFRP1-P1 cell injection (Fig 7C). Next, we examined protein expression for each. Cyclin D1 was examined by immunohistochemistry (IHC) in tumor sections using a specific antiserum. Quantification of the staining showed a 30% decrease in Cyclin D1 levels in the sFRP1 expressing tumors compared to control tumors (Fig 8A), results that agree well with the microarray analysis (Fig 7C). A western analysis for Cyclin D1, carried out on lysates prepared from MDA-MB-231/sFRP1-P1 cultures and control cultures, revealed no significant difference in expression between the two cell lines (Fig 8B). Next, $p21^{Cip1}$ protein levels were measured by western analysis on lysates from MDA-MB-231/sFRP1-P1 tumors and control tumors, as well as lysates from the corresponding *in vitro* cultured cells. $p21^{Cip1}$ was readily detected in tumors resulting from injection of MDA-MB-231/sFRP1-P1 cells. A representative tumor is shown in Fig 8C and quantification revealed a 3-fold increase in $p21^{Cip1}$ levels in the sFRP1 expressing tumors compared to control tumors (Fig 8D). Neither of the *in vitro* cultured cell lines (MDA-MB-231/sFRP1-P1 or MDA-MB-231/control-P1) had detectable levels of $p21^{Cip1}$ (Fig 8C). For this experiment, the $p21^{Cip1}$ positive MCF7 cell line (+CTRL) and a siRNA mediated $p21^{Cip1}$ KD MCF7 cell line (-CTRL) served as antibody controls (Fig 8C). Thus $p21^{Cip1}$ protein levels also reflect data generated from the microarray analyses. In summary, the *in silico* analysis of tumors arising from the MDA-MB-231/sFRP1 cells revealed target genes that very likely contribute to the strong anti-proliferative effects of sFRP1 expression. Moreover, this analysis also shows the strong influence of the *in vivo* tumor environment on gene expression.

Supplemental experiments

sFRP1 expression and promoter methylation status of sFRP1 genes in breast cancer cell lines

sFRP1 is a WNT antagonist which can compete with Frizzled receptors in binding to WNT ligands [123, 125, 129, 130, 191, 192]. Many primary breast tumors have little or no sFRP1 expression, likely due to promoter hypermethylation [170] [173] [193]. To study sFRP1 in breast cancer, we examined mRNA levels and promoter methylation of *SFRP1* genes in seven human breast cancer cell lines.

sFRP1 RNA expression was examined in a panel of breast cancer cell lines and a normal breast cell line using qRT-PCR. sFRP1 RNA was expressed in the normal breast cell line, MCF10A and HER2 over-expressing breast cancer cell lines, JIMT1 and BT20, basal type breast cancer cell lines, HCC1937, BT549 and MDA-MB-468. On the other hand, sFRP1 RNA was not detected in the ER+ breast cancer cell lines, T47D and MCF7, HER2 over-expressing breast cancer cell lines, SkBr3 and BT474, basal type breast cancer cell line, MDA-MB-231 (Fig 9A), suggesting that sFRP1 expression status is independent of ER+ and HER2 over-expression status. Next we examined the methylation status of *SFRP1* gene promoter region. The promoter region of *SFRP1* gene was methylated in MDA-MB-231 and BT474, half-methylated in T47D. These three cell lines were all sFRP1 RNA negative. On the other hand, the sFRP1 positive cell lines, MDA-MB-468, HCC1937, BT549, BT20 and MCF10A had unmethylated sFRP1 promoter region (Fig 9B). These results suggest that the suppression of sFRP1 expression in breast cancer cell lines mostly results from the promoter hypermethylation of *SFRP1* gene.

Ectopic expression of sFRP1 has effects on cancer stem cell phenotype.

Recently, studies of neoplastic tissues have provided evidence of self-renewing, stem-like cells within tumors, which have been called cancer stem cells (CSCs). In most of the cases CSCs constitute a small minority of neoplastic cells within a tumor and are defined operationally by their ability to seed new tumors [194]. To date, studies using several immortalized cancer cell lines, including the human breast cancer cell line MCF-7, have indicated that they contain a subpopulation of highly tumorigenic cells which retain stem/progenitor-like properties [195-197].

On the other hand, studies in choronic myelogenous leukemia indicate that the elevated levels of nuclear β-catenin exist in a minor population of progenitor cells, resulting in their enhanced capacity for self-renewal and increased leukemic potential [198]. Further evidence of dysregulation of stem/progenitor cell self-renewal and maintenance by the WNT/ β-catenin pathway have been demonstrated in lung cancer, colorectal cancer and gastrointestinal cancer [113, 199-202].

In the breast, a resent report showed that overexpression of β-catenin increased the mammosphere-forming efficiency and depleting endogenous β-catenin by using the dominant negative molecule of β-catenin decreased mammosphere forming efficiency [203]. Since mammosphere assay is an assay for self-renewal based on the hypotheses that stem cells may survive in anchorage-independent conditions whereas differentiated cells need attachment to survive and die by anoikis when they lose contact with extracellular matrix, the report from Chen et al suggests that β-catenin is required in the self-renewal process.

Together with our result showing that tumor xenograft formation is dramatically suppressed by the ectopic expression of sFRP1 in MDA-MB-231 cells, these reported observations prompted us to check whether the ectopic expression of sFRP1 reduces CSC population. For this, we first checked the cell surface protein, CD44 and CD24.

CD44 and CD24 are two cell-surface markers whose expression in the $CD44^{high}/CD24^{low}$ configuration is associated with both human breast CSCs and normal mammary epithelial stem cells [204, 205]. In fact, a subpopulation from human mammary epithelial cells (HMLEs) showing the $CD44^{high}/CD24^{low}$ antigenic phenotype have increased ability to form mammospheres in comparison to the subpopulation showing $CD44^{low}/CD24^{high}$ antigenic phenotype [206]. Since mammospheres are enriched in stem cells and can seed entire mammary epithelial trees when implanted into cleared mammary fat pads [207-209], $CD44^{high}/CD24^{low}$ antigenic phenotype appears to be a reliable marker of breast CSCs. Furthermore, CD44 has been reported to be a target gene of WNT/β-catenin pathway [210].

Microarray analysis on MDA-MB-231 cells showed that CD44 levels were decreased and CD24 levels were increased upon the ectopic expression of sFRP1 both *in vitro* cultured P1 cells and *in*

vivo P1 tumor lysates (Fig 10A). We confirmed the result in cultured cells by FACS analysis to see the cell surface protein level of CD44/24. FACS analysis showed $CD44^{high}/CD24^{low}$ antigenic phenotype was suppressed upon sFRP1 expression in P1 cultured cells (Fig 10B).

The report showing that the $CD44^{high}/CD24^{low}$ antigenic phenotype is enriched in basal-like breast tumors [211] prompted us to check the expression levels of basal cytokeratins, 5/14 and 17. Interestingly, the expression of KRT14 and KRT17 were suppressed upon sFRP1 expression specifically *in vivo*, while the expression level of KRT5 was too low for further analyses. On the other hand, the expression of luminal cytokeratines, KRT8 and KRT18 were increased upon ectopic sFRP1 expression both *in vitro* and *in vivo* (Supp Fig 6).

We also used CD29 (β1-integrin) as an indicator of tumor stem cells. CD29 is a stem-cell marker in the skin [212]. Furthermore, cells expressing high level of CD29 are reported to have a mammary stem cell potential and the population of $CD29^{high}$ cells is expanded in the mammary tissue from MMTV-WNT-1 mice [213].

Microarray analysis showed that CD29 mRNA level was suppressed upon the ectopic expression of sFRP1 both *in vitro* cultured P1 cells and *in vivo* P1 tumor lysates (Fig 11A). We confirmed the *in vitro* result by FACS analysis to see the cell surface protein level of CD29. FACS analysis using P1 pools also showed number of CD29 positive cells decreased to 87% upon the ectopic expression of sFRP1 (Fig 11B).

SOX2 is a marker of embryonic stem cells [214, 215]. It is not merely a synergistic factor in the regulatory network to maintain stemness, but a factor whose requirement alone is needed to preserve self-renewal and pluripotency in human ES cells. It has been recently shown that SOX2 regulates self-renewal and pluripotency in human ES cells [216].

Canonical WNT signaling was shown to be an upstream regulator of SOX2 expression during vertebrate development and potentially in embryonic stem cells [217] [218]. There are potential TCF/LEF-binding sites in some of the multiple SOX2 enhancer regions that are conserved between vertebrate species, suggesting that SOX2 is a direct target for Wnt/β-catenin signaling [219, 220].

In addition, SOX2 was recently shown to be expressed in a subset of basal-like breast cancers in a scattered fashion that suggests that it may demarcate a stem-like population [221]. The PCR analysis using the MDA-MB-231/sFRP1 clones and a control clone showed that SOX2 expression was low in MDA-MB-231 cells and down-regulated by sFRP1 (Fig 10C). This result suggests that the blockade of WNT pathway by sFRP1 expression suppress the SOX2 expression, leading to suppression of tumor stem cell phenotype.

Increasing evidence suggests that stem/progenitor cells evade cell death by a number of mechanisms, such as quiescence and drug-efflux conferred by the ABC-family of membrane transporters [222]. A ´side population` was defined based on the efflux capability of a subpopulation of tumor cells for Hoechst dye. This efflux activity requires ATP-dependant transporters and can be blocked by specific inhibitors such as reserpine [223], which was shown previously to be an inhibitor of the Bmr efflux pump of *Bacillus subtilis*[224]. Tumor initiating cells were shown to possess an elevated activity of drug efflux transporters that may be responsible for their increased resistance towards chemotherapeutics [222, 225].

Therefore, we asked whether this additional property of tumor initiating cells is affected by inhibition of autocrine WNT signaling by the treatment of sFRP1 CM. The side population of MDA-MB-231 cells is reduced more than 6-fold from 0.77% in control CM treated cells vs. 0.12% in sFRP1 CM treated cells after 7d (Fig 10D). Furthermore, the expression of *ABCA2*, a member of the ABC super family whose expression has been correlated with drug resistance [226] [227] [228], was suppressed upon sFRP1 expression both *in vitro* and *in vivo* (Fig 10E). This implies that WNT signaling controls the ability of tumor initiating cells to efflux Hoechst dye and may therfore be involved in the development of drug resistance by regulating ATP dependant drug transporters.

Taken together, these findings suggest that autocrine WNT signaling regulates some of the characteristics of tumor initiating cells by 1) regulating extracellular matrix molecule expression that may be required to form a tumor ´stem cell niche`, 2) regulating the expression of differentiation/stemness factors that maintain a multipotent state of the tumor initiating cells and 3) by controlling the drug efflux capability of tumor initiating cells and thereby a possible mechanism of multi-drug resistance."

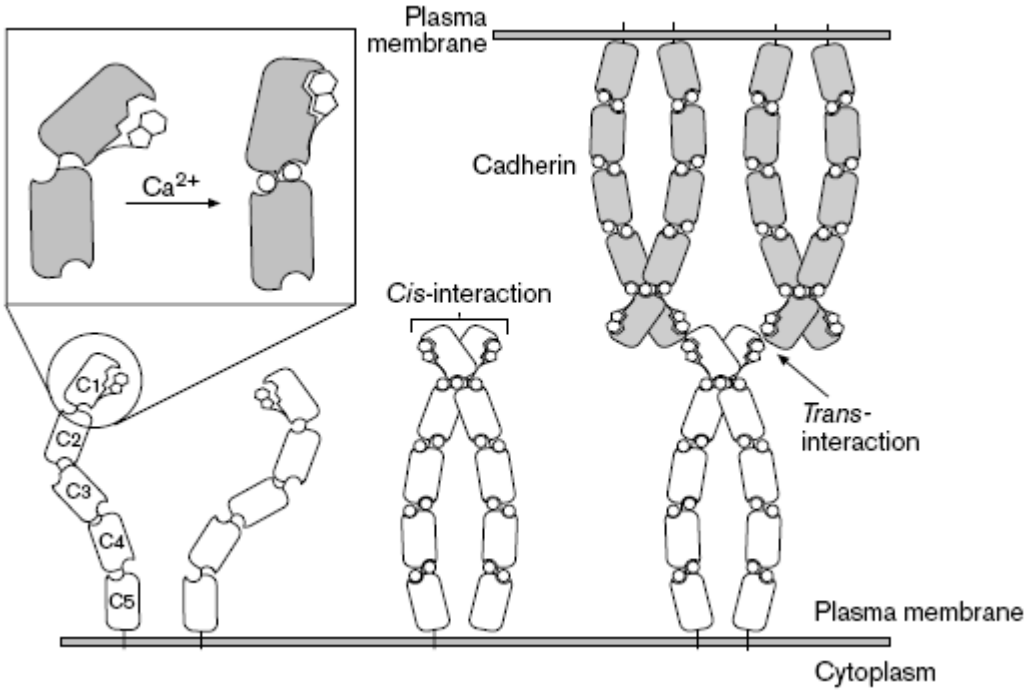
P-cadherin level was up-regulated by WNT signaling in T47D cells

Cadherins were first classified as a family of single-pass transmembrane glycoproteins mediating Ca^{2+}-dependent cell–cell adhesion [229]. Members of Cadherin superfamily are classified into four groups: classical cadherins, desmosomal cadherins, protocadherins and other cadherin-related proteins [230]. Among them, the classical cadherins are present throughout metazoan [231].

Classical cadherins have the signal sequence at the N-terminal site which is flanked by a prosequence that contains a protease processing signal sequence. The proteolytic cleavage at this site appears to be necessary for the activation of the classical cadherins. The extracellular domain is located next to this prosequence followed by single transmembrane segment and the cytoplasmic domain, which is located at the C-terminal side. The cadherin extracellular domain consists of five repeats of a cadherin motif, C1-5, where C1 is the most distant from the membrane and harbors an HAV (histidine-alanine-valine) tripeptide. Each of C1-C5 motif have Ca^{2+}-binding site.

In the absence of Ca^{2+}, the cadherin structure is disorganized and incapable of participating in adhesion. As Ca^{2+} concentrations are increased, the C1-C5 subdomains start to bind Ca^{2+} ions and these subdomains become organized (C1, which has the lowest affinity for calcium, becomes organized last) and the cadhein ectodomain becomes rigid and competent to participate in *cis*-dimerization. When C1 becomes organized at higher calcium concentrations, steric rearrangements shift Trp (W) at the amino acid residue 2 to duck into the intramolecular hydrophobic pocket formed in part by the high conserved HAV sequence. The HAV-W structure is essential for *trans*-dimerization but not *cis*-dimerization [232] (Figure below).

In the cytoplasmic domain, at least two functional sequences have been identified: a well characterized carboxy-terminal 25 amino-acid region that exclusively binds β-catenin or plakoglobin (γ-catenin) and a membrane-proximal region that binds several members of the p120 subfamily. In general, the cytoplasmic domain of classical cadherin interacts with β-catenin or plakoglobin (γ-catenin), which in turn bind α-catenin. α-catenin is attached to the actin cytoskeleton directly and/or indirectly.



Extracellular structure of classical cadherins *Steinberg and McNutt, 1999*

In the breast, one of the classical cadherins, P-cadherin (encoded by the gene, *CDH3*) is expressed by myoepithelial cells and these cells adhere to each other via P-cadherins [233]. P-cadherin$^{-/-}$ mice undergo precocious development [234], which suggests that loosing of myoepithelial cell junctions triggers ductal branching.

Interestingly, the expression of P-cadherin in human breast cancer has been associated with aggressive tumor behavior. Around 30% of breast carcinomas show up-regulation of P-cadherin [235, 236]. Specifically, P-cadherin is frequently over-expressed in high-grade invasive breast carcinomas and enhances migration and invasion of breast cancer cells [237, 238].

Since we observed increased motility in T47D breast cancer cells when treated with Wnt1, the reported evidences that up-regulated expression of P-cadherin associates with aggressive breast tumor behavior prompted us to examine whether P-cadehrin expression is altered upon Wnt stimulation in breast cancer cells. To examine this, T47D cells were ectopically transfected with Wnt1-encoding plasmid or empty vector as a control. More than 100 clones from each group were pooled after the selection. Interestingly, T47D expressing Wnt1 showed up-regulated P-cadherin

RNA level compared to control T47D cells according to microarray analysis (Fig 12A). P-cadherin protein level was also confirmed by western blotting. For western experiments, Wnt1- or Wnt5a-transfected pools of T47D cell were additionally established. Each pools consists of more than 100 clones. Western blotting on three independent T47D/Wnt1 pool, three T47D/Wnt5a pool and three T47D/control pool showed that P-cadherin level was constantly up-regulated in ectopically Wnt1-expressing T47D cells, while control and ectopically Wnt5a-expressing cell did not show up-regulated P-cadherin (Fig 12B). This tendency was observed both in confluent cultured cells and sparcely spread cells. However the effect was more striking in confluent monolayers (Fig.12C).

P-cadherin level was also examined in ectopically sFRP1-expressing T47D cells. sFRP1-encoding vector or empty vector was transfected to T47D cells and two independent sets of pool were made. Each pool contains more than 100 clones. Western blotting analysis showed P-cadherin level was suppressed upon ectopic sFRP1 expression (Fig.12D).

IV. DISCUSSION

Possibility of targeting WNT signaling pathway in breast cancer

Aberrant activation of the WNT pathway plays an important role in many types of human cancer. Thus, approaches to target the pathway are warranted, although its complexity makes this a difficult undertaking [239]. Wnt1 was the first identified oncogene activated by mouse mammary tumor virus (MMTV) insertional mutagenesis [240], establishing the potential of aberrant WNT ligand expression to promote mammary cancer. We now know that multiple WNT ligands and FZD receptors are expressed in primary human breast tumors and breast cancer cell lines [153, 177] [154, 156], making it difficult to identify an individual ligand/receptor complex that could serve as a therapeutic target. However, by using broad antagonists to interfere with WNT/FZD binding, such as the cysteine rich domain (CRD) of the FZD8 receptor [241] or sFRP1 [177, 181], the potential of targeting WNT binding as a therapeutic approach in breast [177, 181] and other cancers [241, 242] has been demonstrated. The results we present here showing that ectopic sFRP1 expression in MDA-MB-231 cells significantly blocks their *in vivo* tumor forming ability support the proposal that extracellular antagonists of the WNT pathway might have a role in treating human cancer.

Suppressed sFRP1 expression in breast tumor

Expression of sFRP1 is reported to be lost in a high percentage of primary breast tumors, both at the RNA level (>75%) [171, 172] and at the protein level (>40%) [173]. Moreover, methylation of the promoter region of the *SFRP1* gene was reported both in primary breast tumors (>60%) and breast cancer cell lines [170] [174] [175]. Interestingly, loss of sFRP1 protein expression and promoter hypermethylation is associated with disease progression and poor prognosis [173] [170]. Since aberrant hypermethylation of gene promoters is a major mechanism associated with inactivation of tumor-suppressor genes in cancer [162], promoter hypermethylation of *SFRP1* gene suggests one mechanism that might contribute to WNT pathway activity.

In the current study, we used T47D and MDA-MB-231 human breast cancer cell lines as experimental models. It has been reported that *SFRP1* is not expressed in these cells by semi quantitative real time PCR. Moreover, the promoter region of *SFRP1* is reported to be fully methylated in MDA-MB-231 and partially methylated in T47D cells [193] [175]. We confirmed that T47D, MCF7, BT474 and MDA-MB-231 do not express sFRP1 RNA using quantitative RT-PCR and the promoter region of *SFRP1* gene in MCF7, BT474 and MDA-MB-231 was 100% methylated and that in T47D was partially methylated (Fig 9). These results suggest that suppressed sFRP1 expression in breast cancer cell lines is at least partially because of the promoter methylation of *SFRP1* gene.

WNT signaling and breast tumor cell motility

We show here that the WNT pathway influences breast tumor cell motility. Various WNT ligands, including Wnt1 and Wnt3a have been shown to stimulate cellular migration [183, 184]. Our results show that Wnt1 stimulates T47D breast tumor cell motility; conversely sFRP1 mediated blockade of endogenous WNT signaling reduces motility of the MDA-MB-231 cells (Figs 1 and 3). To provide mechanistic insight into the effects on motility, we show that the activity of RhoA, a GTPase that has been implicated in WNT signaling and cell movement [183, 184] is elevated in Wnt1 expressing T47D cells (Fig 2).

Rho-family GTPases, including Cdc42, Rac1 and RhoA, are known to play a central role in establishing cell polarization and migration [182]. Microinjection of active forms of RhoA rapidly induces stress fiber formation in mammalian cells [243] [244]. Stress fibers are bundles of actin

filaments that traverse the length of a cell. Stress fibers regulate cell shape by providing mechanical rigidity and help to control cell migration and motility by connecting the cytoplasm to the extracellular matrix (ECM) via focal adhesions. Namely, Rho regulates eukaryotic cell polarity, shape, and migration by controlling stress fibers and focal adhesion formation [245]. RhoA is reported to be activated upon canonical WNT stimulation in some contexts. For example, HEK 293T cells show elevated level of activated RhoA upon treatment with Wnt1 CM [183] and CHO cells show pronounced stress fiber formation, RhoA activation and promotion of cell motility upon Wnt3a stimulation. Furthermore, the elevated motility induced by Wnt3a was blocked respectively by sFRP1 or Rho kinase inhibitor [184].

In view of cancer cell motility, we also checked the level of P-cadherin as an interesting molecule related to cell invasiveness in breast cancer. P-cadherin is one of the classical cadherins, which mediate calcium-dependent cell-cell binding when they are localized to the adherents-type junctions. In normal adult nonlactating breast tissue, P-cadherin expression is restricted to myoepithelial cells. It remains unknown what biological function P-cadherin has in tumor lesions, however, particularly in breast tumor, P-cadherin is reported to be able to enhance cell invasion and tumor aggressiveness. Several reports have indicated that P-cadherin expression in mammary carcinomas associated with poor prognosis in patients [238]. We showed that P-cadherin protein levels were elevated upon the ectopic expression of Wnt1 in T47D cells (Fig 12), and cell motility was also promoted in these cells (Fig 1). These results suggest that the expression level of P-cadherin is regulated by the activation of WNT signaling. These results suggest the possibility that WNT signaling stimulates expression of mediators which leads to increased cell motility.

It has been reported that transgenic expression of Wnt1 in the mammary gland of mice results in mammary adenocarciomas with metastasis to lymph nodes and lungs [246]. This metastasic phenotype might come from the promoted cell motility by activated WNT signaling because WNT activation promotes cell motility and P-cadherin expression. Our results suggest the possibility that blockade of canonical WNT signaling might be an effective way to target metastatic breast cancer.

Cell proliferation and tumor growth in view of WNT signaling

We have previously shown that proliferation of the estrogen receptor (ER) positive MCF7 and T47D breast tumor cells, and the ErbB2-overexpressing JIMT-1, SKBR3 and BT474 breast tumor

cells is decreased following treatment with sFRP1 [177]. It has been reported that many primary tumors have low sFRP1 levels [171-173]. Here we tested the impact of ectopic sFRP1 expression in the basal-like [72] MDA-MB-231 breast tumor cells. We show that sFRP1 expressing MDA-MB-231 display down-regulation of WNT signaling activity, reduced *in vitro* proliferation and a dramatic impairment in their ability to grow as mammary tumor xenografts in nude mice (Figs 3, 4). Taken together, these results suggest that blockade of WNT signaling might be a general approach to target breast cancer.

Our results suggest that the *in vivo* effects of sFRP1 on WNT pathway blockade are more striking than its *in vitro* activity. In four independent experiments with the highly sFRP1-expressing MDA-MB-231/sFRP1-P1 cells, the time to appearance of the first tumor following their injection was consistently longer, in comparison to control MDA-MB-231 cells. Furthermore, tumors generated by MDA-MB-231/sFRP1-P1 cells grew more slowly than control tumors, and the number of tumor-free mice at the end of each experiment was consistently higher. The mechanisms underlying the ability of sFRP1 to impair tumor growth might be tumor cell intrinsic or extrinsic, via effects of secreted sFRP1 on tumor-associated cells. We examined tumor associated vessels, which are obvious candidates based on the reported ability of sFPR1 to block *in vivo* neovascularization [187], however, we have no evidence that vessel number or functionality are affected by expression of sFRP1 in the MDA-MB-231 cells (Fig 5). We favor the hypothesis that the effects of sFRP1 are mainly on the tumor cells themselves via blockade of WNT pathway activity.

Our in depth transcriptome analysis resulted in the identification of a panel of genes whose expression is altered by blockade of WNT signaling. The genes were classified as those that are altered *in vivo* and *in vitro* by sFRP1 expression, and those that are only affected *in vivo* in sFRP1-expressing tumors. Considering the first category, there were 1753 probesets (1246 genes) whose signals were significantly changed in the tumors arising from MDA-MB-231/sFRP1 cells compared to tumors arising from control cells. The same analysis performed on *in vitro* cultured samples revealed 428 probesets (332 genes). Only 69 probesets (54 genes) overlapped between the two analyses clearly demonstrating the important effect of the cellular environment on the transcriptome.

After filtering out genes whose expression was only altered *in vivo*, in the sFRP1 expressing tumors,

we analyzed 106 down-regulated genes and 62 up-regulated genes for targets that might contribute to tumor growth suppression (Supp Table 1 lists the genes and their fold change). Two of them, *CCND1* and *CDKN1A*, that were down-regulated and up-regulated, respectively, were also validated at the protein level and are very likely contributing to the suppressed tumor outgrowth.

Cyclin D1, which is encoded by the gene *CCND1*, plays an important role in G1-S transition during cell cycle progression. Binding of Cyclin D1 to its kinase partners, the Cyclin dependent kinases 4 and 6 (CDK4/6), results in the formation of active complexes that phosphorylate the Retinoblastoma tumor suppressor protein (RB). Hyperphosphorylation of RB results in the release of RB-sequesterd E2F transcription factors and the subsequent expression of genes required for entry into S-phase. Furthermore, Cyclin D1 has also been shown to act as a cofactor for several transcription factors [247] [248]. Importantly, *CCND1* is one of the WNT target genes [109, 110, 249]. Namely, the *CCND1* promoter has a consensus LEF-1 binding site [110] and in some colon cancer cell lines [109], but not others [250], its transcription is responsive to β-catenin/TCF activation. It is possible that in the MDA-MB-231 model Cyclin D1 is a direct WNT pathway target. In our experiment, one of the *CCND1* probesets revealed less *CCND1* RNA in both *in vivo* and *in vitro* MDA-MB-231/sFRP1 cells (Supp Table 2 and Supp Fig 5). With the other probeset, *CCND1* was only decreased *in vivo* in MDA-MB-231/sFRP1 tumors (Fig 7C). However, Cyclin D1 protein was only down-regulated *in vivo* in MDA-MB-231/sFRP1 tumors (Fig 8), suggesting that transcriptional as well as post-transcriptional regulatory mechanisms play a role in influencing the final Cyclin D1 protein levels (Fig 8).

Cyclin D1 can also be induced by extracellular signal-regulated kinases through the cascade composed of Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) [251]. Conversely, inhibition of the Ras pathway inhibited *CCND1* gene expression [252]. Since MDA-MB-231 cell line has an activating *k-ras* mutation [186], the "Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) cascade" should theoretically be constitutively activated in MDA-MB-231 cells. In agreement with this, we have observed that sFRP1 expressing cells maintain high P-ERK levels (Fig 3A). The fact that Cyclin D1 expression can still be suppressed by sFRP1 in MDA-MB-231 cells implies the dominancy of WNT signaling to induce *CCND1* in breast cancer cell lines.

On the other hand, $p21^{Cip1}$, a protein encoded by *CDKN1A* gene, is a well-characterized CDK inhibitor that belongs to the Cip/Kip family. $p21^{Cip1}$ mainly inhibits the activity of Cyclin/CDK2 complexes and negatively modulates cell cycle progression [253]. In addition, $p21^{Cip1}$ can bind to proliferating cell nuclear antigen thereby blocking DNA synthesis [254]. Based on the positive role of Cyclin D1 in cell cycle progression and the function of $p21^{Cip1}$ to negatively modulate cell cycle progression, the suppression of tumor growth *in vivo* upon sFRP1 expression is likely to be caused by cell cycle arrest.

We also looked specifically at known WNT pathway target genes (http://www.stanford.edu/~rnusse/pathways/targets.html). Among these, there are 16 genes where at least one of the probesets shows a tendency for suppression in MDA-MB-231/sFRP1 cells both *in vitro* and *in vivo* (Supp Table 2 and Supp Fig 5). c-Myc, a well described target of canonical WNT signaling [111, 190, 250], was not decreased in the MDA-MB-231/sFRP1 cells. However, tumors resulting from injection of MDA-MB-231/sFRP1 cells have low c-Myc protein levels (Fig 6).

The protooncogene *c-MYC*, which encodes a transcription factor playing a major role in the regulation of normal cell proliferation, is aberrantly expressed in many human cancers [255]. Myc is a member of the bHLHZip4 family of transcription factors which, when dimerized with its partner Max, binds to specific DNA sequences resulting in the transcriptional regulation of target genes involved in the control of many aspects of cellular physiology including growth and proliferation [256-262].

In normal cells, *c-MYC* mRNA and c-Myc protein are both short-lived [258]. Thus, Myc expression is highly regulated at multiple levels in a normal cell. In contrast, deregulated c-Myc expression is quite prevalent in human tumors. In breast cancer, it has been reported that approximately 40-45% of primary breast tumors show overexpression of c-MYC protein [263, 264] and around 20% of all breast tumors show amplification of the *c-MYC* gene [264, 265]. Furthermore, examination of *c-MYC* mRNA has suggested the possibility of aberrant regulation of *c-MYC* expression on the transcriptional level [266]. Interestingly, although our experiments showed that c-Myc protein was decreased in tumors arising from MDA-MB-231/sFRP1 cells, qRT-PCR analysis and microarray analysis revealed that the regulation of c-Myc protein level upon the ectopic expression of sFRP1 in the tumor was not accompanied with changes in the *c-MYC* mRNA level. This suggests that

activating WNT signaling pathway might not only target c-Myc transcription at least in some models, but could also protect the c-Myc protein from degradation and/or might be implicated in the translational control of c-Myc.

It is also reported that c-Myc can stimulate the transcription of D-type cyclins [267, 268]. Together with these reports, our data suggest that there might be a synergistic effect of Myc-mediated Cyclin D1 transcription and β-catenin mediated Cyclin D1 transcription by the activation of WNT signling pathway in MDA-MB-231 breast cancer cells. On the other hand, it is known that *CDKN1A* expression is suppressed by the c-Myc/Miz-1 heterodimer [269-271]. Furthermore, in breast cancer, c-Myc has been shown to be a negative regulator of $p21^{Cip1}$ [272]. Thus, the increase in $p21^{Cip1}$ that is only observed *in vivo* in the sFRP1 expressing tumors might be due to the dramatic c-Myc reduction observed in the same tumors (Fig 8).

Since c-Myc can induce the synthesis of CyclinD1 and suppress the transcription of *CDKN1A*, the decrease of c-Myc protein might be a pivotal event caused by ectopic sFRP1 expression and might contribute to the dramatic suppression of tumor formation and tumor growth observed *in vivo*. Furthermore, c-Myc has recently been shown to be the crucial mediator of early stage intestinal neoplasia arising in APC mutant mice [190], implying that c-Myc plays critical role downstream of Wnt/β-catenin signaling leading to tumorigenesis. Nevertheless our Myc-rescue experiment did not result in total recovery of tumor formation, over-expression of c-Myc partially rescued the impaired tumorigenesis resulting from MDA-MB-231/sFRP1 cells (Suppl Fig 3). It is possible that the ectopic expression level of c-Myc was not high enough to rescue tumor formation completely. However, the fact that only a slight over-expression of c-Myc rescued the tumorigenesis in some of the cases suggests the importance of the role played by c-Myc under the autocrine activation of WNT signaling in breast cancer.

Further experiments will be needed to confirm whether c-Myc plays a pivotal role in the tumor growth suppression in MDA-MB-231/sFRP1 xenograft model. However, it is clear that all three proteins, Cyclin D1, p21 and c-MYC, have important roles in cell cycle progression. In colorectal cancer models, it has been reported that sustained down-regulation of canonical Wnt/β-catenin

signaling results in a cell cycle arrest [250]. Our results in the MDA-MB-231 breast tumor model are similar since sFPR1-expressing tumors showed less BrdU incorporation in comparison to controls tumors. The results from our study suggest the decrease in S-phase activity is caused by the regulated levels of Cyclin D1, $p21^{Cip1}$ and c-Myc.

In addition to these molecules known to be cell cycle modulators, we identified additional transcripts in the microarray analyses that might also contribute to the decreased proliferation of sFRP1 expressing cells, including β1 integrin (CD29) and its ligand, fibronectin (FN1). The RNA levels of both CD29 and FN1, the latter a known WNT pathway target (Supp Table 2) are suppressed in MDA-MB-231/sFRP1 cells, *in vitro* and *in vivo*. We have previously shown that siRNA mediated knock-down of β1 integrin in MDA-MB-231 cells causes an increase in $p21^{Cip1}$ expression and a decrease in proliferation [273]. FN1, which was shown to be down-regulated following expression of sFRP3 in a prostate cancer model [242], has also been shown to affect cancer cell proliferation [274]. This growth signaling via FN1 and β1 integrin might also a mediator of the growth promotion triggered by the activated autocrine WNT pathway.

Apoptosis is not important in MDA-MB-231/sFRP1 experimental model

Despite the fact that the microarray analysis revealed that proapoptotic genes are up-regulated in sFRP1 expressing cells, including the FAS receptor and its ligands, TNFSF12/13 and TNFSF15, we could not detect increased levels of apoptotic MDA-MB-231/sFRP1 tumor cells. Since many inhibitors of apoptosis (IAP) were expressed in these tumors, including BIRC2 (cIAP1), BIRC3 (cIAP2), BIRC4 (XIAP), BIRC5 (survivin), BIRC6 (apollon) and BIRC7, and only BIRC6 (apollon) levels decreased in sFRP1 expressing cells (from microarray analysis), it is possible that the multiple IAPs contribute to the survival of the MDA-MB-231/sFRP1 tumors.

WNT signaling and tumor progenitor cell phenotype

CD44, CD24 and CD29 are surface markers used for the selection of progenitor cells [204-206, 213]. The $CD44^{high}/CD24^{low}$ population is associated with breast tumor progenitor cells and normal mammary epithelial stem cells [204, 205]. Our microarray analysis showed that CD44, a WNT pathway target gene [210] was down-regulated (Supp Table 2 and Supp Fig 5), while CD24 was increased in MDA-MB-231/sFRP1 cells, *in vitro* and *in vivo*. This was confirmed by FACS analysis

of protein expression on cultured cells (Fig 10). As mentioned above, CD29 (β1 integrin) was also decreased in the sFRP1 expressing cells. High CD29 levels have been associated with mammary stem cell potential, and interestingly, $CD29^{high}$ population is expanded in mammary tissue from MMTV-WNT mice [213].

There is increasing evidence that the WNT pathway regulates self-renewal and fate of breast cancer stem cells [275]. The results we present here suggest that autocrine WNT signaling might regulate the tumor initiating cells in the MDA-MB-231 model of basal breast cancer and this may be one of the reasons that ectopic sFRP1 expression has such a striking effect on the tumor outgrowth potential of the cells.

Link between EMT and tumor initiating cells

Interestingly, there is a report showing that the expression of stem cell markers and the increased ability to form mammospheres result from the induction of epithelial-mesenchymal transition (EMT) in human mammary epithelial cells [206]. On the other hand, stem-cell like cells isolated from human mammary epithelial cell culture had undergone EMT [206]. These observations illustrate a direct link between the EMT and the gain of epithelial stem cell properties.

EMT, which is the loss of epithelial and the gain of mesenchymal characteristics, is an important step leading to invasive cancer cells. Epithelial cells undergo a developmental switch from the polarized, epithelial phenotype to a highly motile fibroblastoid phenotype [276]. The process of EMT, whereby epithelial cells lose cell–cell contact, undergo remodeling of the cytoskeleton, and manifest a migratory phenotype has been implicated in the conversion of early-stage tumors to invasive malignancy [277]. A defining feature of EMT is the loss of E-cadherin expression and gain of fibronectin and vimentin expression [278], [279]. In addition to the disorganization of E-cadherin-ß-catenin complexes, the *de novo* expression of vimentin is a mechanism frequently associated with an EMT and the metastatic conversion of epithelial cells.

Vimentin is a type III intermediate filament normally expressed in cells of mesenchymal origin [280]. On the other hand, vimentin can also be expressed in epithelial cells involved in physiological or pathological processes requiring epithelial cell migration, suggesting that the expression of vimentin is required for epithelial cells to accomplish EMT. Vimentin has indeed been described in

migratory epithelial cells involved in embryological and organogenesis processes, in placentation, in wound healing, or in tumor invasion [281] [282] [283] [284]. Also, vimentin antisense transfection in vimentin-expressing breast cell lines including MDA-MB-231 was shown to reduce their *in vitro* invasiveness or migration, strongly emphasizing a functional contribution of vimentin to epithelial cell invasion/migration [284, 285]. In the same way, an impaired wound healing has been observed in vimentin knockout mice [286] [287]. These observations suggest that vimentin expression promotes EMT and cell motility.

The MDA-MB-231 cell line is highly motile and invasive, carries an activated Ki-ras allele and appears phenotypically to have undergone EMT [186] [288] [289]. However, this mesenchymal phenotype of MDA-MB-231 cells seems to be reverted upon the ectopic expression of sFRP1. Namely, in our study, although E-cadherin mRNA levels were not changed, the mRNA level of the other EMT markers, fibronectin and vimentin were suppressed upon the ectopic expression of sFRP1 both in cultured MDA-MB-231 cells and xenograft tumors (Supp Fig 7). We also observed the down regulated cell motility following ectopic sFRP1 expression in MDA-MB-231 cells (Fig 3D). These observations suggest that the ectopic expression of sFRP1 might let MDA-MB-231 cells undergo mesenchymal-epithelial transition (MET) and eventually suppress cell motility.

Possible mechanisms behind breast tumor growth suppression by interfering with antocrine WNT pathway

We observed that the ectopic expression of sFRP1 in MDA-MB-231 cells has a big impact on xenograft tumor outgrowth and this effect is most likely via cell cycle arrest. However, I think one of the most pivotal mechanisms behind this effect might be the fact that the interference with WNT signaling via sFRP1 expression affects tumor initiating cell phenotype.

MDA-MB-231 is a basal type breast cancer cell line [72]. In the breast, the term “basal” has acquired two meanings. In one context it has become the word having the same meaning of “breast myoepithelium” and in the other context, it defines a specific subpopulation of “basal” cytokeratins, CK5, CK14 and CK17, expressing cells [6]. However, in the latter meaning, the origin of “basal” cell is still controversial. Because most basal-like breast tumors still express luminal cytokeratin 8/18, suggesting that basal-like cancers also possess characteristics of luminal lineage. Furthermore, several classic myoepithelial markers were rarely expressed in basal-like tumors. These findings

provide evidence against the hypothesis that basal-like breast cancers are derived directly from myoepithelial cells. Instead, these findings could suggest that basal-like breast cancer cells are differentiated directly from a stem cell [67].

According to our microarray experiment, MDA-MB-231 cells also express high levels of luminal cytokeratines 8/18 (Supp Fig 6), which is in line with the hypothesis that the origin of these cells is a stem cell. This hypothesis is currently still under the discussion, however, is very interesting together with our observations from MDA-MB-231 cells. We observed two important facts, 1; interfering with autocrine WNT signaling pathway in MDA-MB-231 cells suppresses the cancer stem cell phenotype and 2; at the same time, interfering with autocrine WNT signaling pathway in these cells leads to the phenotype which seems to have undergone MET. Since there is a direct link between cancer stem cell phenotype and EMT [206], these two observations suggest that interfering with autocrine WNT pathway "reverts" cancer stem cell phenotype of MDA-MB-231 cells or "decreases" the cancer stem cell population in MDA-MB-231 cells.

Reverting cancer stem cell phenotype and/or decreasing cancer stem cell population by interfering with autocrine WNT pathway could be broadly efficient to treat breast cancer patients because suppressing cancer stem cell phenotype can allow cancer cells to become more sensitive to chemotherapeutics. Cancer stem cells are hard to eliminate by chemotherapeutics due to their elevated activity of drug efflux [222, 225]. Increasing evidences suggest that stem/progenitor cells evade cell death by a number of mechanisms, such as quiescence and drug-efflux conferred by the ABC-family of membrane transporters [222]. In our experiments, the drug-efflux activity was suppressed upon the sFRP1 CM treatment in MDA-MB-231 cells (Fig.10D) and the expression of *ABCA2*, a member of the ABC super family whose expression has been correlated with drug resistance [226] [227] [228], was suppressed upon sFRP1 expression both *in vitro* and *in vivo* (Fig 10E).

It is difficult to target cancer stem cells not only because of their efflux activity, but also due to the fact that they are de-differenciated. For example, after the tamoxifen treatment to target ER+ breast tumors, even if a turmor seems to have regressed, it can relapse after a while because there might had been a small number of cancer stem cells which can grow in an ER signaling independent manner. MDA-MB-231 cells are known to be ER negative [72]. However, according to our

microarray analysis, one of the probesets showed slightly increased signal of the gene coding ERα upon the ectopic expression of sFRP1 in MDA-MD-231 cells (data not shown). Furthermore, ectopic expression of Wnt1 in T47D cells, which is a ER+ breast cancer cell line [72], leads to a slight decrease of ERα RNA expression according to the microarray analysis (data not shown). These transcriptional alterations of ERα are very slight and further experiments will be needed to confirm them. However, it is quite interesting because it might be showing the fact that activating WNT signaling can de-differenciate breast cancer cell phenotype and interfering with the autocrine WNT signaling activity can re-differenciate breast cancer cell phenotype.

Final statements

To conclude, interfering with autocrine WNT pathway by sFRP1 not only suppresses breast tumor outgrowth by cell cycle arrest, but also might cause breast tumor cells to become more sensitive to chemotherapies by suppressing their drug-efflux ability and by leading them to re-differenciate. One important question which should be answered is whether established breast tumors regress upon *de novo* expression of sFRP1 or sFRP1 treatment. For this, MDA-MB-231 cells infected with sFRP1 coding sequence under tetracycline responsible promoter will be needed. Without doxycycline, these cells do not express sFRP1. After letting these cells grow in immunocompromised mice as xenograft, sFRP1 expression can be induced by feeding them with doxycycline. If tumor regresses after they have doxycycline, it confirms the hypothesis that treating breast cancer patients with sFRP1 is a good target therapy. Furthermore, our observations suggest the efficiency of interfering with autocrine WNT signaling in combination with chemotherapy to treat breast tumor patients, because interfering with autocrine WNT pathway seems to cause breast cancer cells to become more sensitive to chemotherapeutics. For example, treatment with purified sFRP1 or humanized antibody which can block WNT receptors at the same time of treatment with tamoxifen for ER+ breast cancer patients might bring better outcome compared to tamoxifen treatment alone.

In this thesis, I showed that sFRP1 mediated WNT pathway blockade strongly blocks the *in vivo* mammary tumor forming potential of MDA-MB-231 breast cancer cells. The results presented here suggest that interference with WNT signaling at the ligand-receptor level may be a valid therapeutic approach in breast cancer. It is just a starting point to establish or to make a way to establish a novel

approach to target breast tumor, however, I wish this study becomes a start for me to contribute to release patients who are suffering from various cancers some day in the future. Every year, many people are losing their lives because of cancer including breast cancer. This is not only a tragedy, but also a considerable loss for our society. I have been researching on breast cancer throughout my PhD study and I am also going to continue cancer research in the future. Throughout my research life, I wish I can contribute to solving at least one piece of the complicated puzzle of tumor biology. I would be eventually very happy if this one piece could help us humans with overcoming cancer.

V. FIGURES AND TABLES

Fig.1

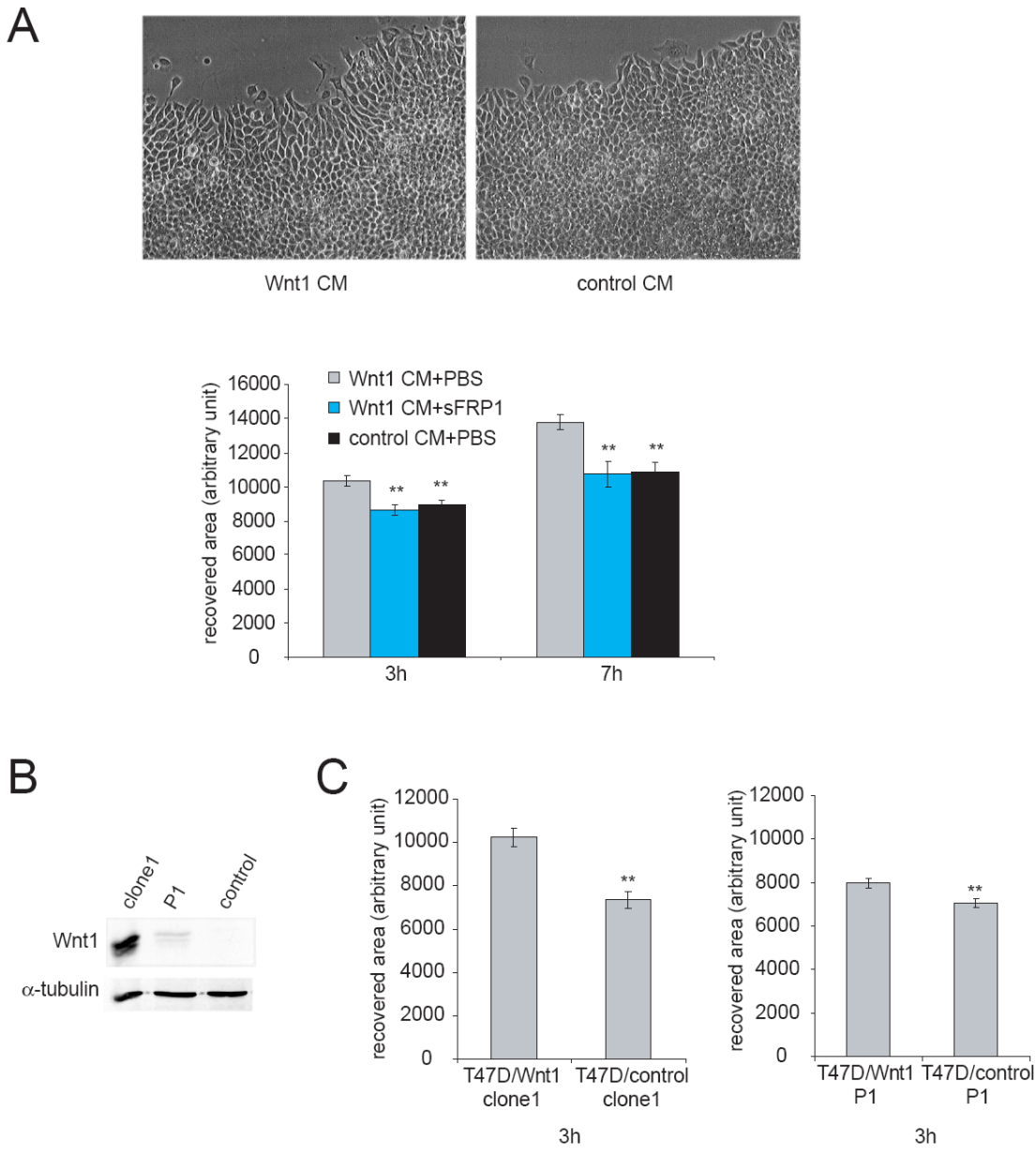
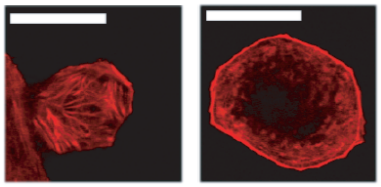


Fig.2

A



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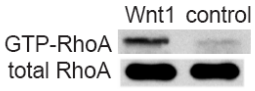


Fig.3

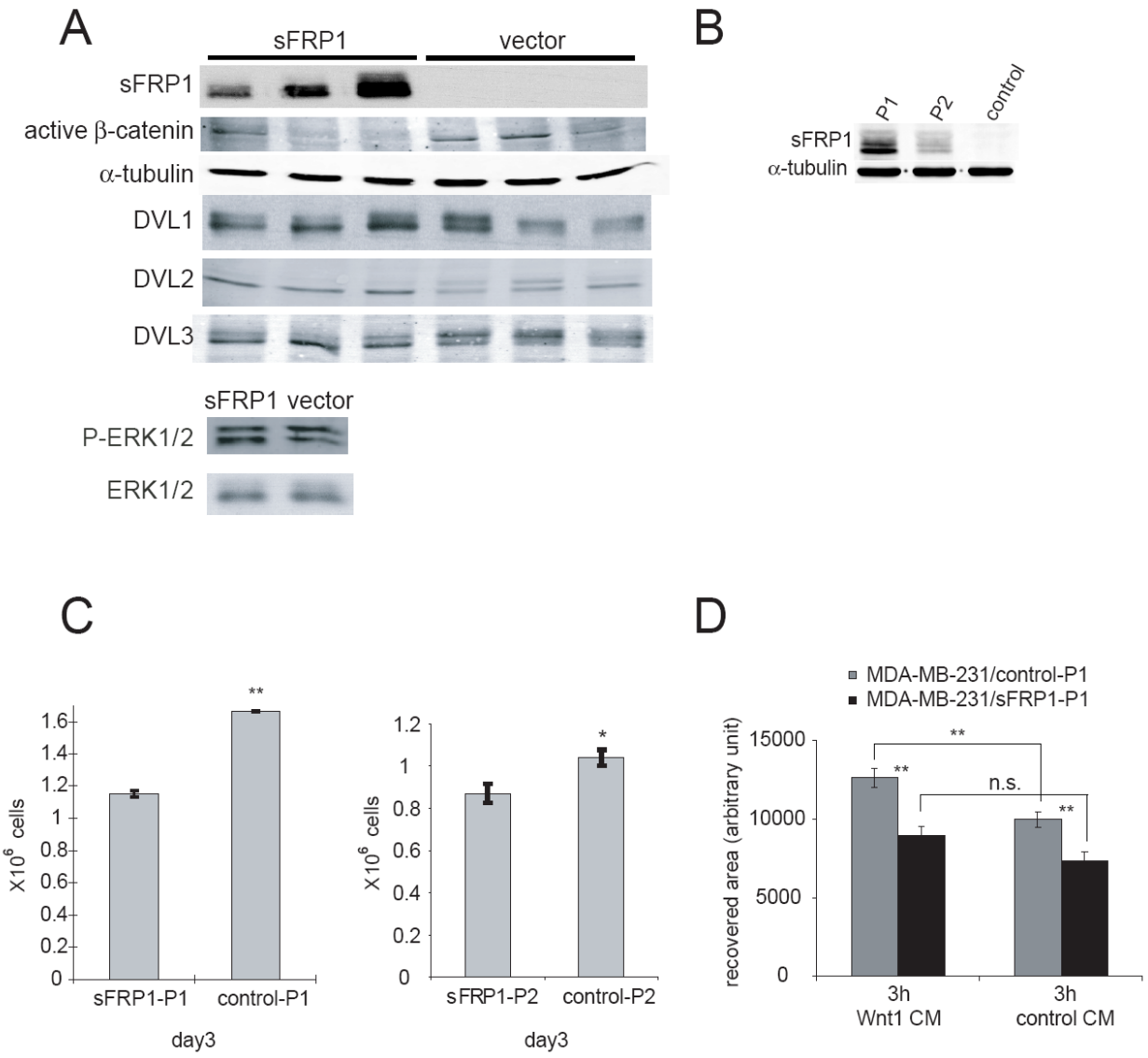

A
sFRP1
vector
sFRP1
active β-catenin
α-tubulin
DVL1
DVL2
DVL3
sFRP1 vector
P-ERK1/2
ERK1/2
B
P1
P2
control
sFRP1
α-tubulin
C
X10^6 cells
sFRP1-P1
control-P1
day3
sFRP1-P2
control-P2
day3
D
MDA-MB-231/control-P1
MDA-MB-231/sFRP1-P1
recovered area (arbitrary unit)
n.s.
3h
Wnt1 CM
3h
control CM

Fig.4

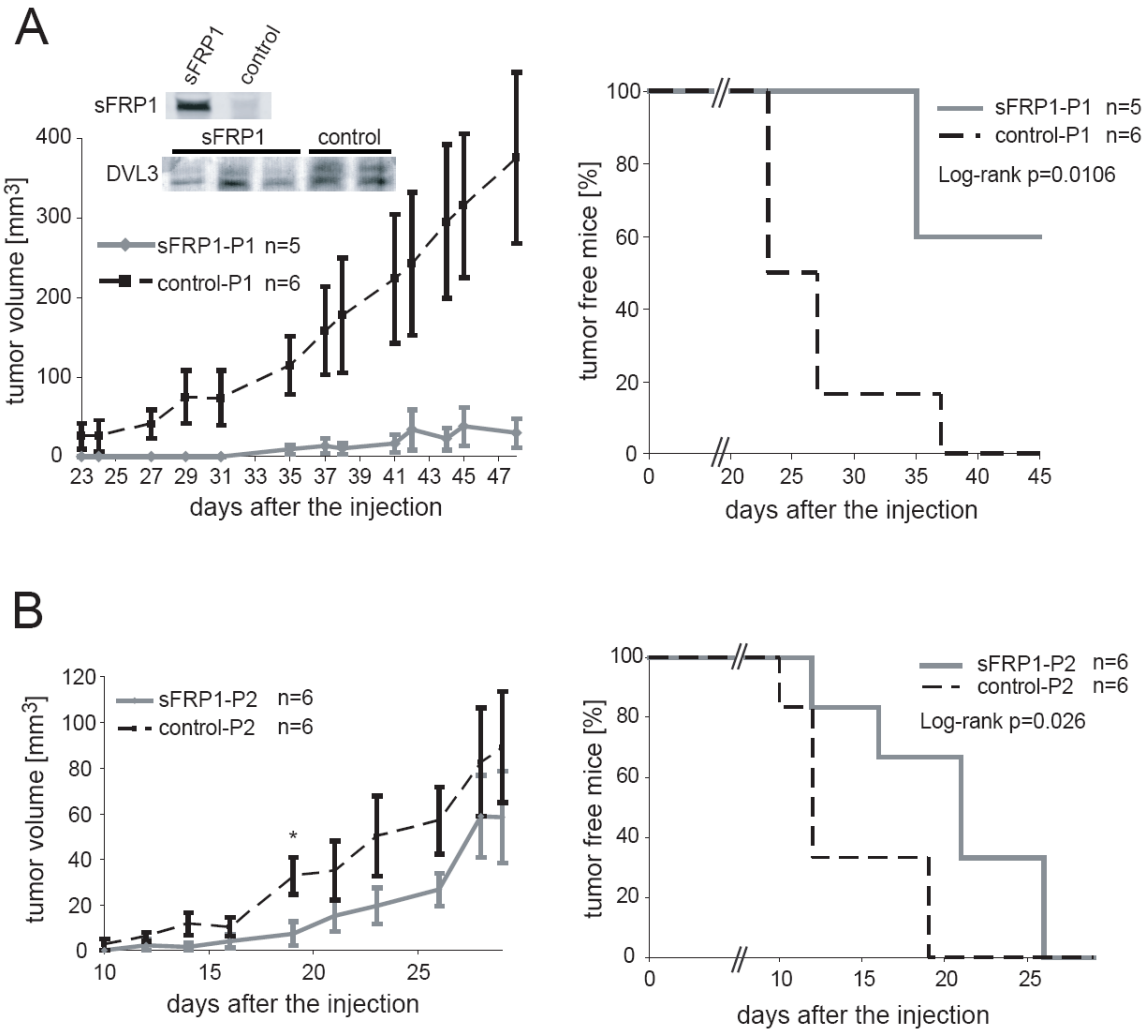

A
sFRP1
control
sFRP1
control
DVL3
tumor volume [mm3]
sFRP1-P1 n=5
control-P1 n=6
days after the injection
tumor free mice [%]
sFRP1-P1 n=5
control-P1 n=6
Log-rank p=0.0106
days after the injection
B
tumor volume [mm3]
sFRP1-P2 n=6
control-P2 n=6
*
days after the injection
tumor free mice [%]
sFRP1-P2 n=6
control-P2 n=6
Log-rank p=0.026
days after the injection

Fig.5

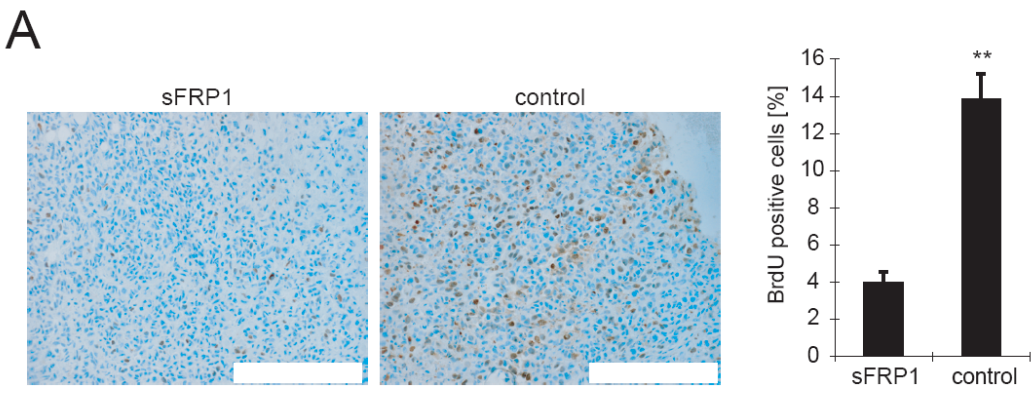

A
sFRP1
control
BrdU positive cells [%]
16
14
12
10
8
6
4
2
0
**
sFRP1
control
B
CD31 positive area / total area [%]
10
9
8
7
6
5
4
3
2
1
0
n.s.
sFRP1
control
lectin / CD31 [%]
40
35
30
25
20
15
10
5
0
n.s.
sFRP1
control

Fig.6

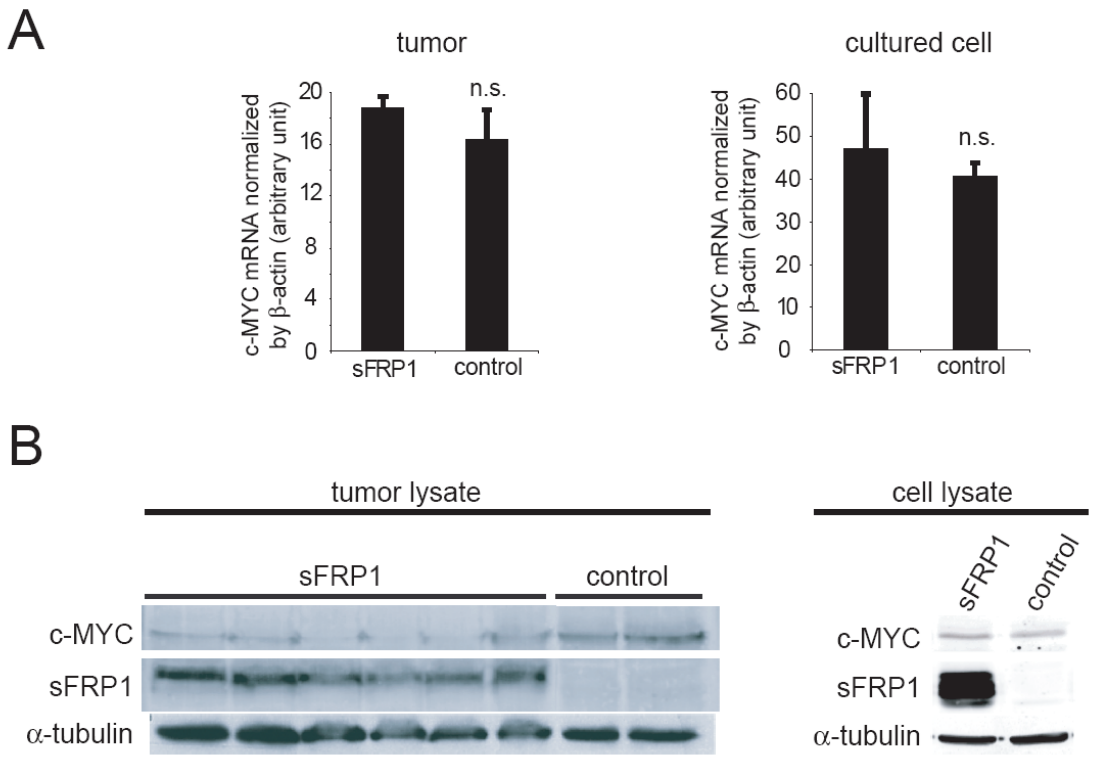

A
tumor
cultured cell
c-MYC mRNA normalized by β-actin (arbitrary unit)
20
16
12
8
4
0
n.s.
sFRP1
control
60
50
40
30
20
10
0
B
tumor lysate
cell lysate
sFRP1
control
c-MYC
sFRP1
α-tubulin

Fig.7

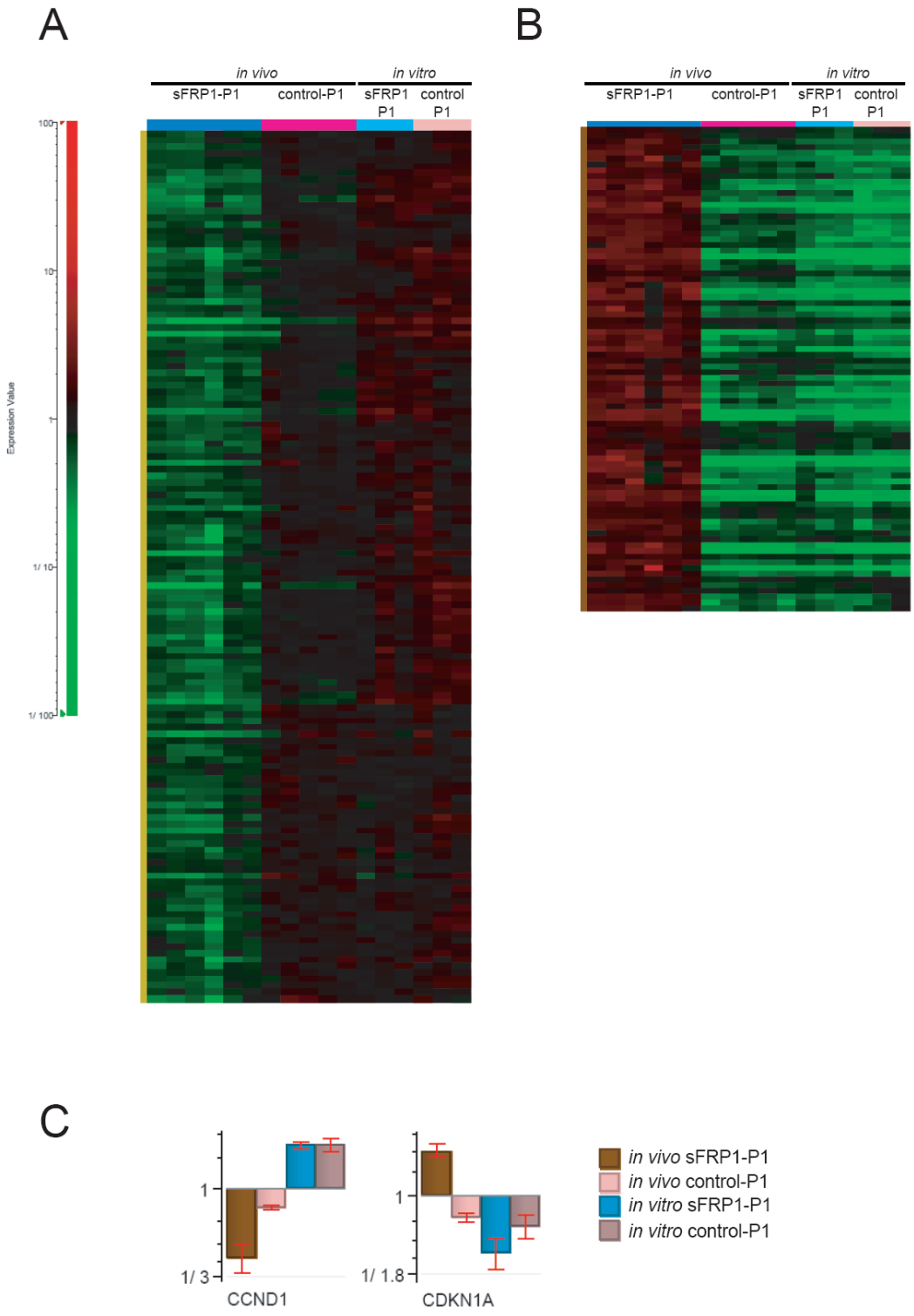

A
B
C
in vivo
in vitro
sFRP1-P1
control-P1
sFRP1 P1
control P1
Expression Value
100
10
1
1/ 10
1/ 100
1
1/ 3
CCND1
1
1/ 1.8
CDKN1A
in vivo sFRP1-P1
in vivo control-P1
in vitro sFRP1-P1
in vitro control-P1

Fig.8

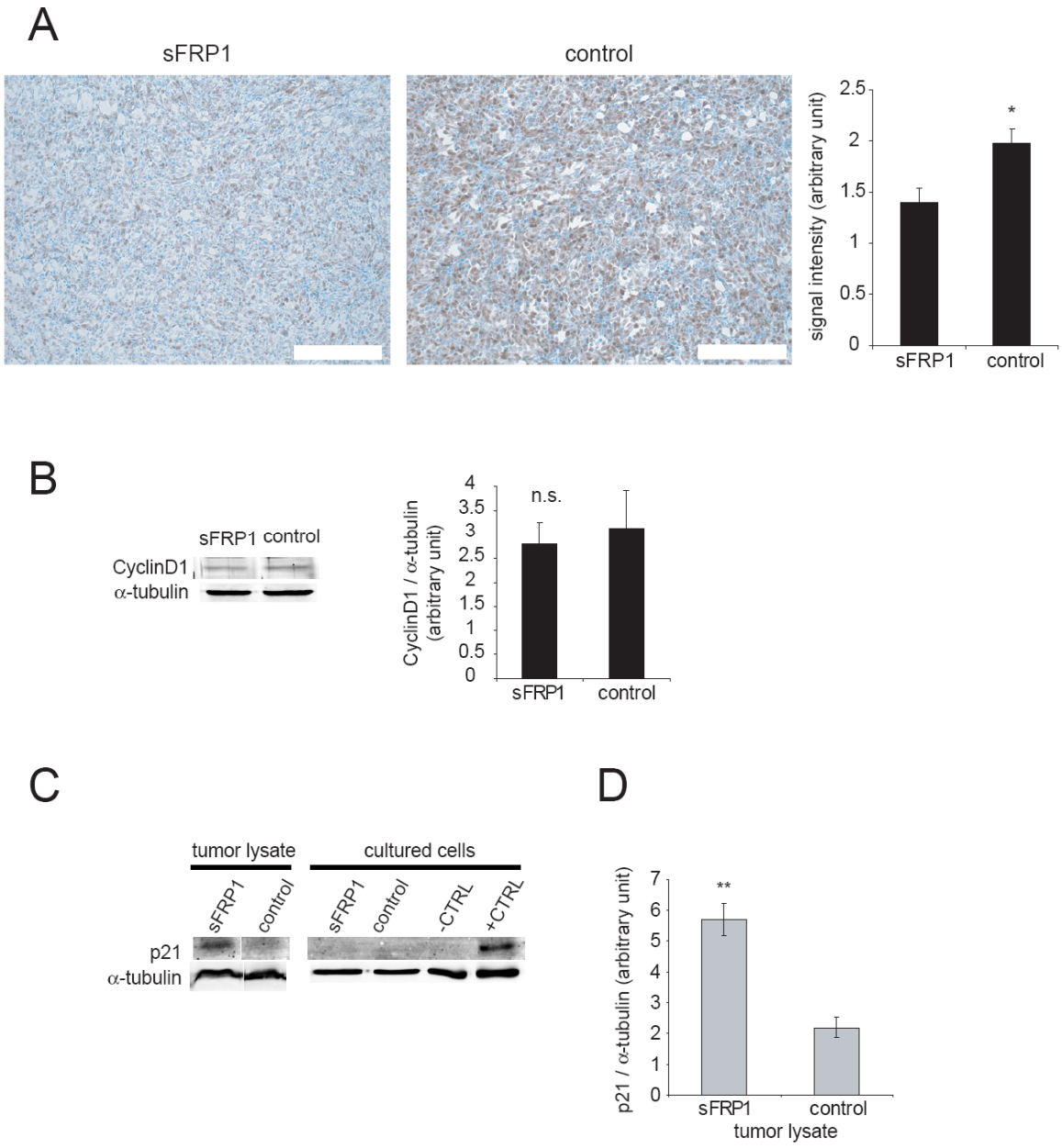
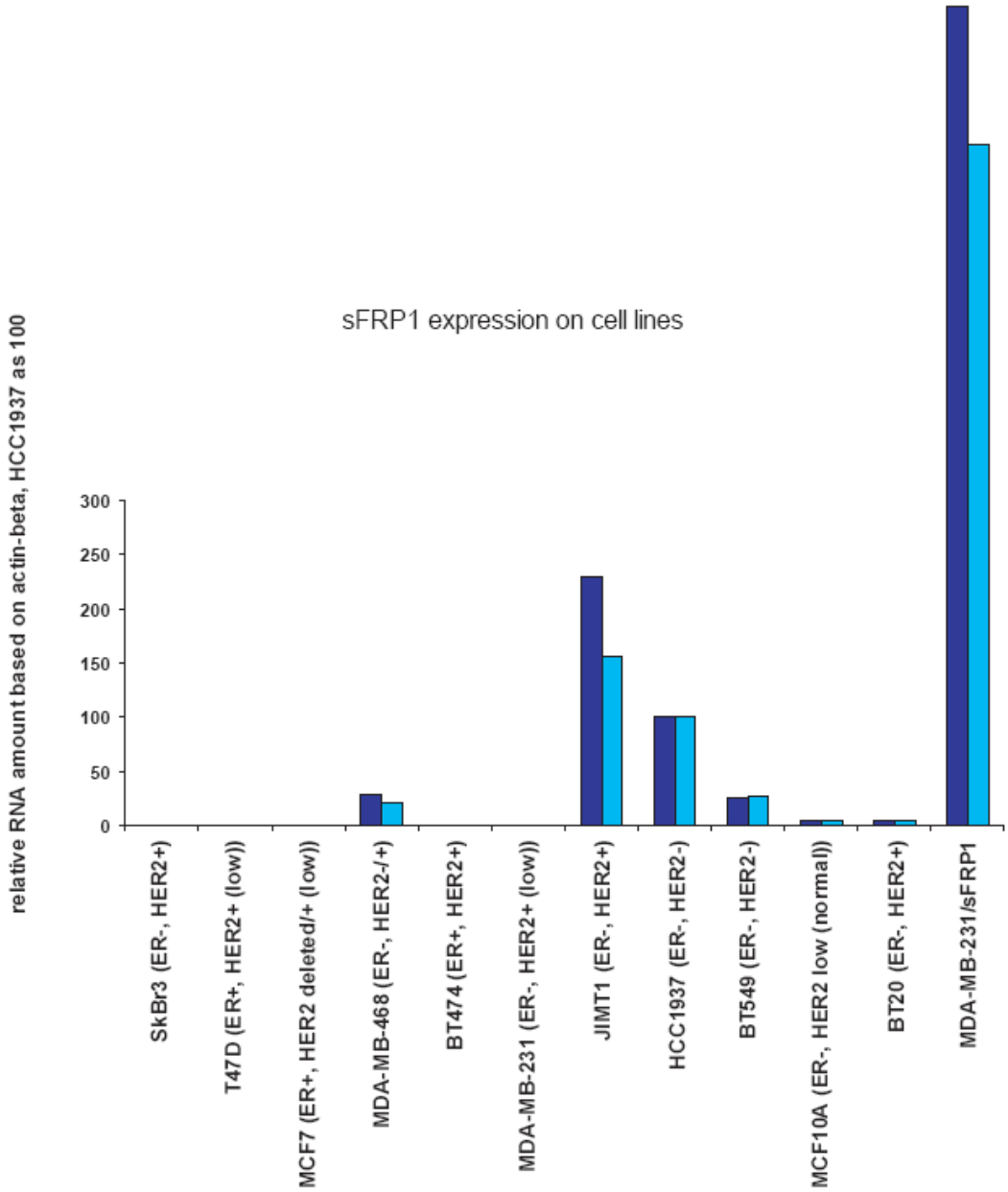

A
sFRP1
control
signal intensity (arbitrary unit)
2.5
2
1.5
1
0.5
0
*
sFRP1
control
B
sFRP1 control
CyclinD1
α-tubulin
CyclinD1 / α-tubulin (arbitrary unit)
4
3.5
3
2.5
2
1.5
1
0.5
0
n.s.
sFRP1
control
C
tumor lysate
cultured cells
sFRP1
control
sFRP1
control
-CTRL
+CTRL
p21
α-tubulin
D
p21 / α-tubulin (arbitrary unit)
7
6
5
4
3
2
1
0
**
sFRP1
control
tumor lysate

Fig. 9

A


sFRP1 expression on cell lines
relative RNA amount based on actin-beta, HCC1937 as 100
300
250
200
150
100
50
0
SkBr3 (ER-, HER2+)
T47D (ER+, HER2+ (low))
MCF7 (ER+, HER2 deleted/+ (low))
MDA-MB-468 (ER-, HER2-/+)
BT474 (ER+, HER2+)
MDA-MB-231 (ER-, HER2+ (low))
JIMT1 (ER-, HER2+)
HCC1937 (ER-, HER2-)
BT549 (ER-, HER2-)
MCF10A (ER-, HER2 low (normal))
BT20 (ER-, HER2+)
MDA-MB-231/sFRP1

B

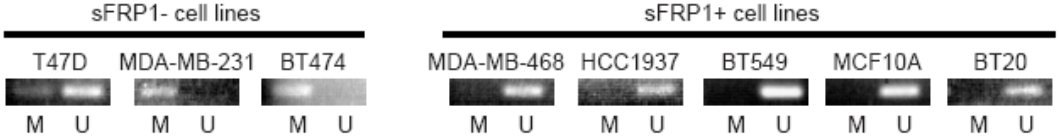

sFRP1- cell lines
T47D
MDA-MB-231
BT474
M U M U M U
sFRP1+ cell lines
MDA-MB-468
HCC1937
BT549
MCF10A
BT20
M U M U M U M U M U

Fig.10

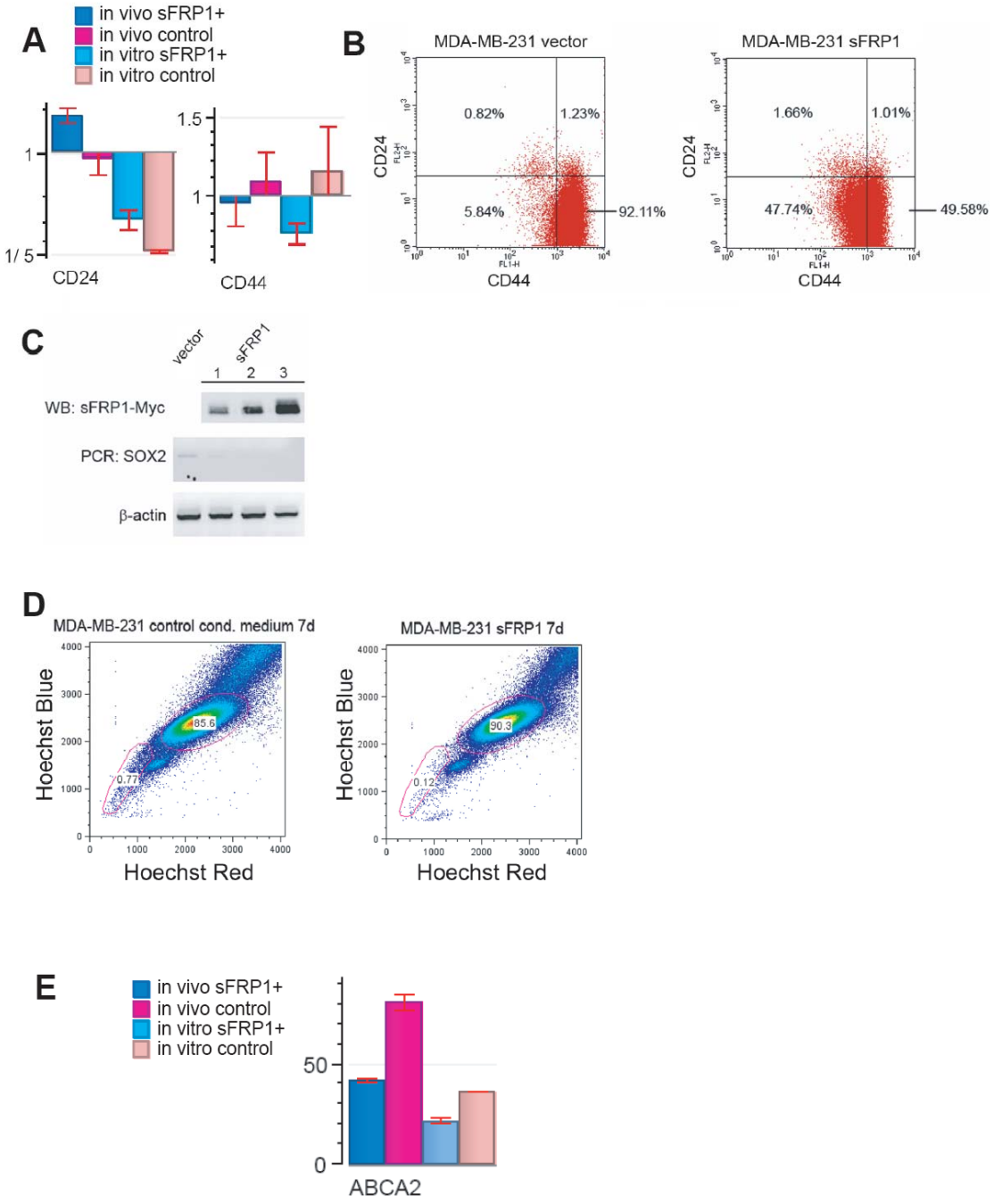

A
in vivo sFRP1+
in vivo control
in vitro sFRP1+
in vitro control
1
1/ 5
CD24
1.5
1
CD44
B
MDA-MB-231 vector
0.82%
1.23%
5.84%
92.11%
CD24
CD44
MDA-MB-231 sFRP1
1.66%
1.01%
47.74%
49.58%
CD24
CD44
C
vector
sFRP1
1 2 3
WB: sFRP1-Myc
PCR: SOX2
β-actin
D
MDA-MB-231 control cond. medium 7d
Hoechst Blue
Hoechst Red
85.6
0.77
MDA-MB-231 sFRP1 7d
Hoechst Blue
Hoechst Red
90.3
0.12
E
in vivo sFRP1+
in vivo control
in vitro sFRP1+
in vitro control
50
0
ABCA2

Fig.11

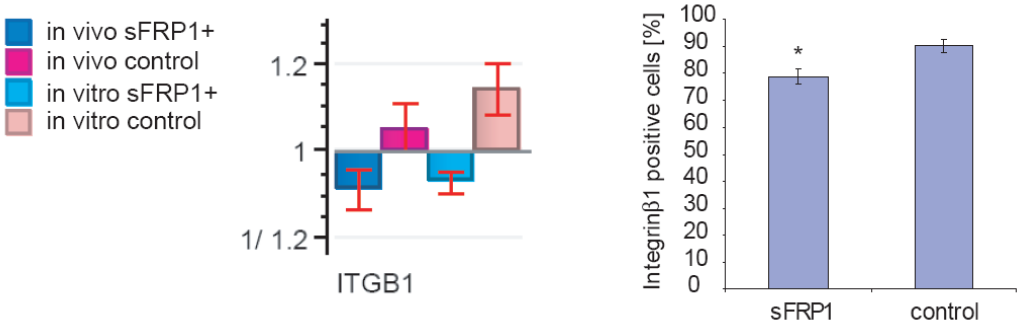
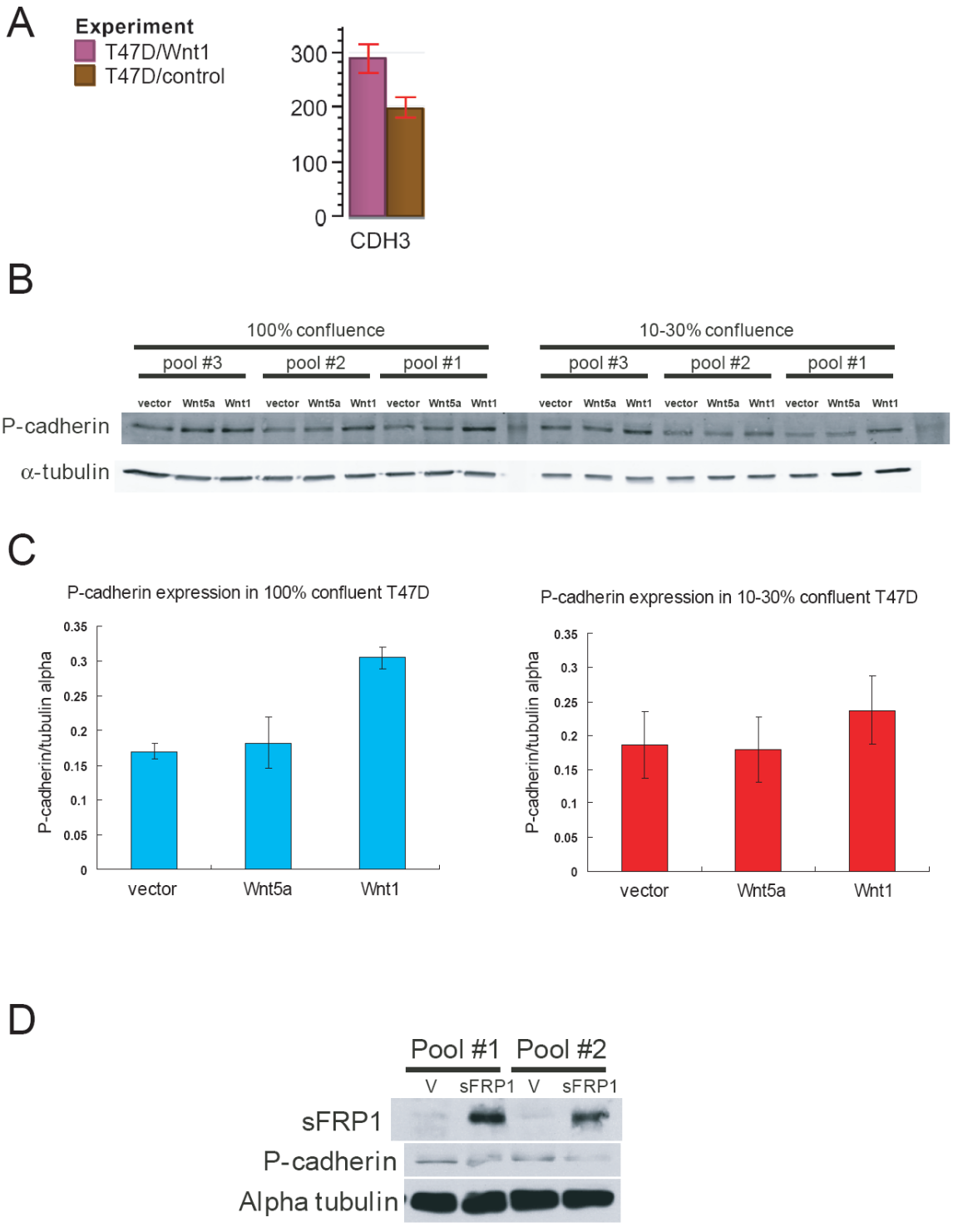
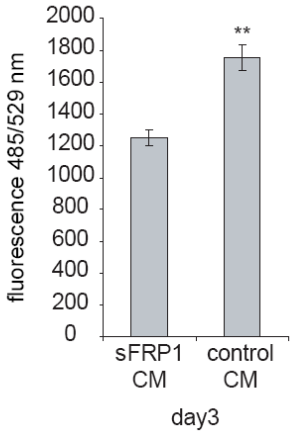

in vivo sFRP1+
in vivo control
in vitro sFRP1+
in vitro control
1.2
1
1/ 1.2
ITGB1
Integrinβ1 positive cells [%]
100
90
80
70
60
50
40
30
20
10
0
*
sFRP1
control

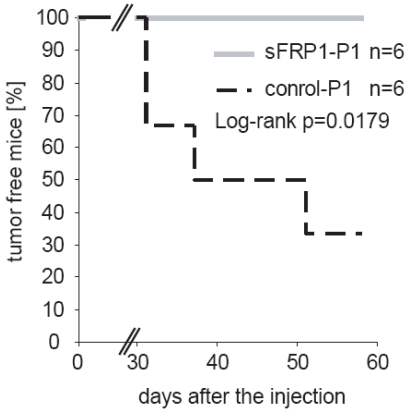
Fig.12

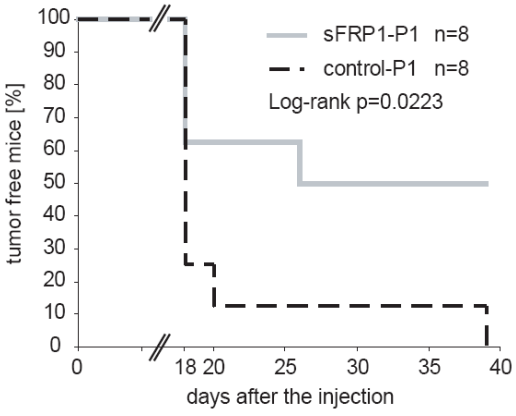

A
Experiment
T47D/Wnt1
T47D/control
300
200
100
0
CDH3
B
100% confluence
10-30% confluence
pool #3
pool #2
pool #1
vector
Wnt5a
Wnt1
P-cadherin
α-tubulin
C
P-cadherin expression in 100% confluent T47D
P-cadherin expression in 10-30% confluent T47D
P-cadherin/tubulin alpha
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
vector
Wnt5a
Wnt1
D
Pool #1
Pool #2
V
sFRP1
sFRP1
P-cadherin
Alpha tubulin

Supplemental figure 1



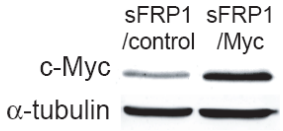
Supplemental figure 2



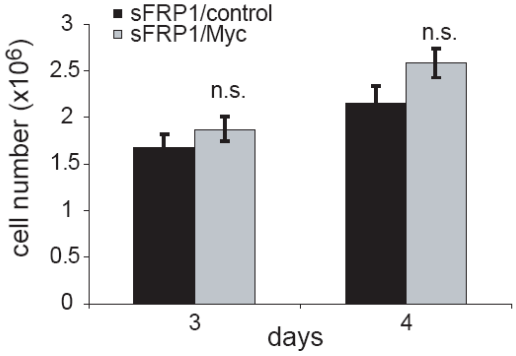


Supplemental figure 3

A



B



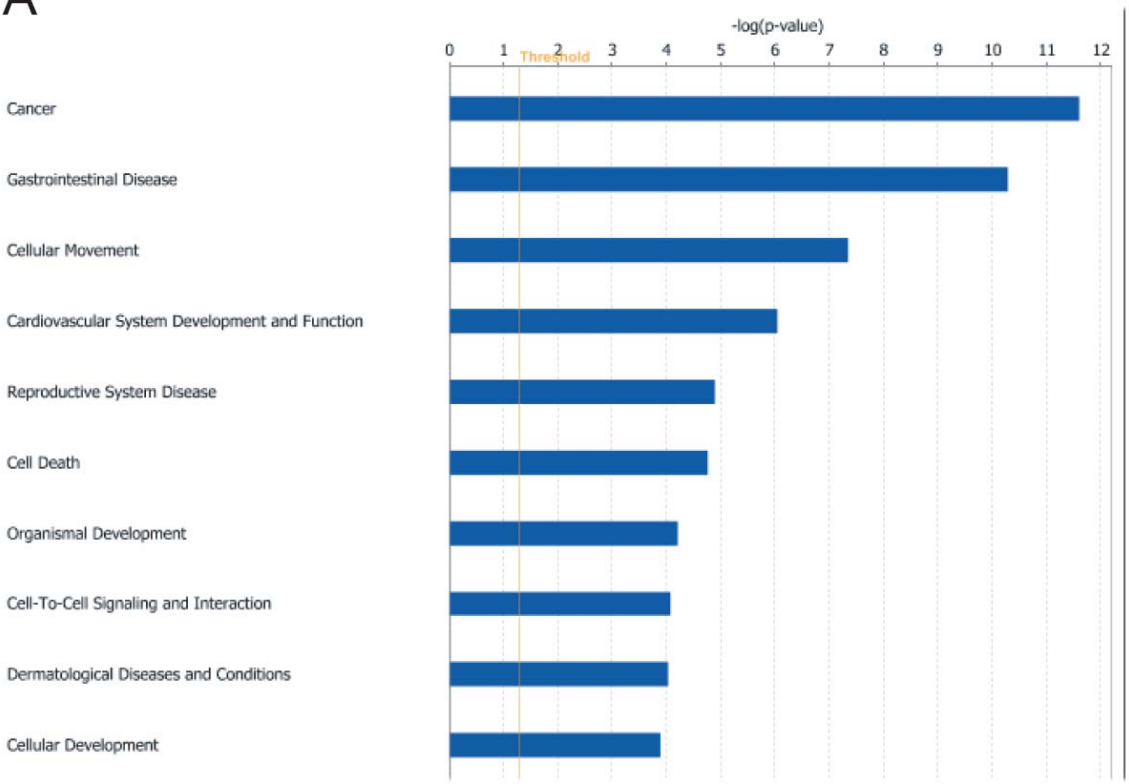
C

	tumor-bearing mice	%
sFRP1/control	0/6	0%
sFRP1/Myc	2/6	33%

Log-rank p=0.138

Supplemental figure 4

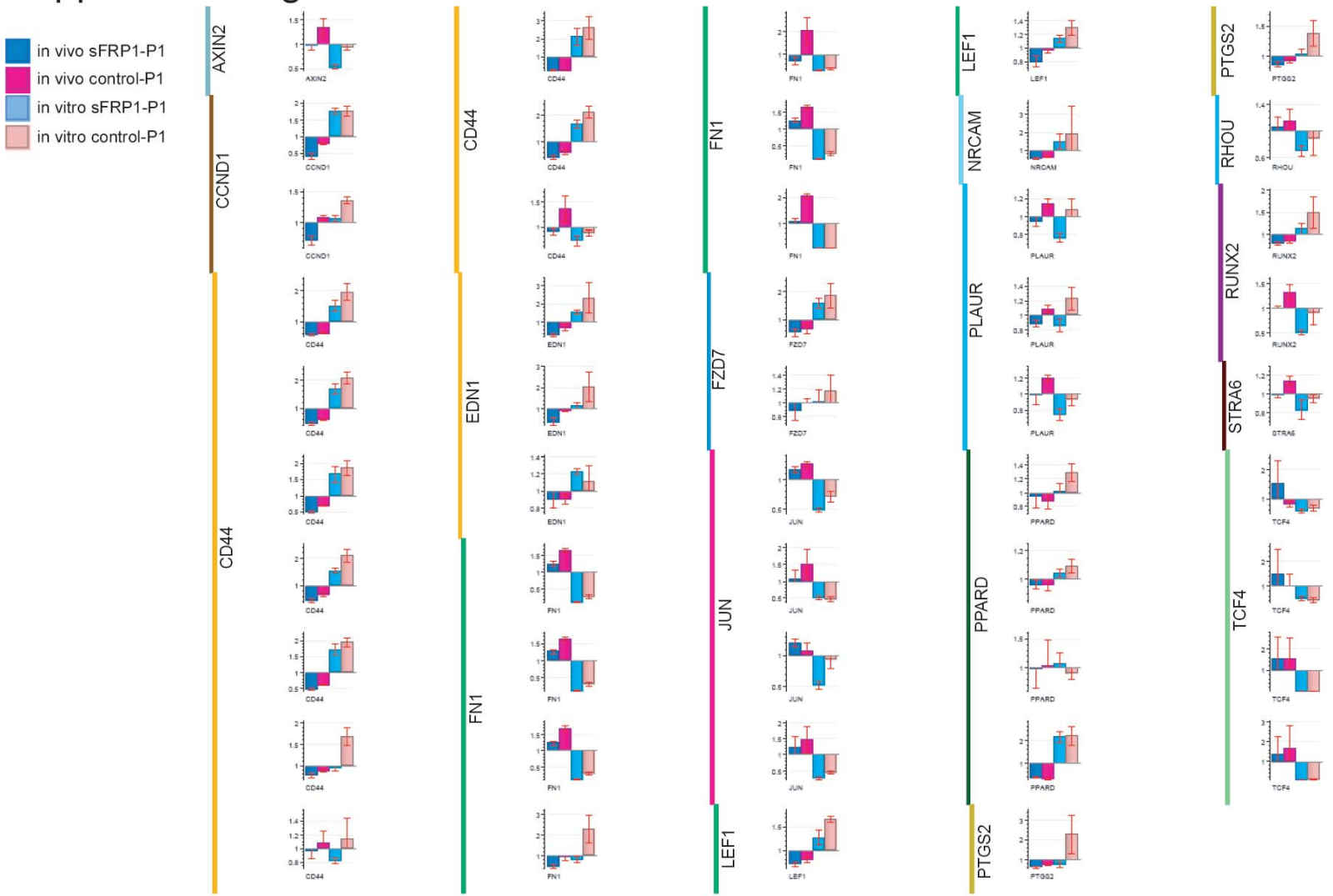
A



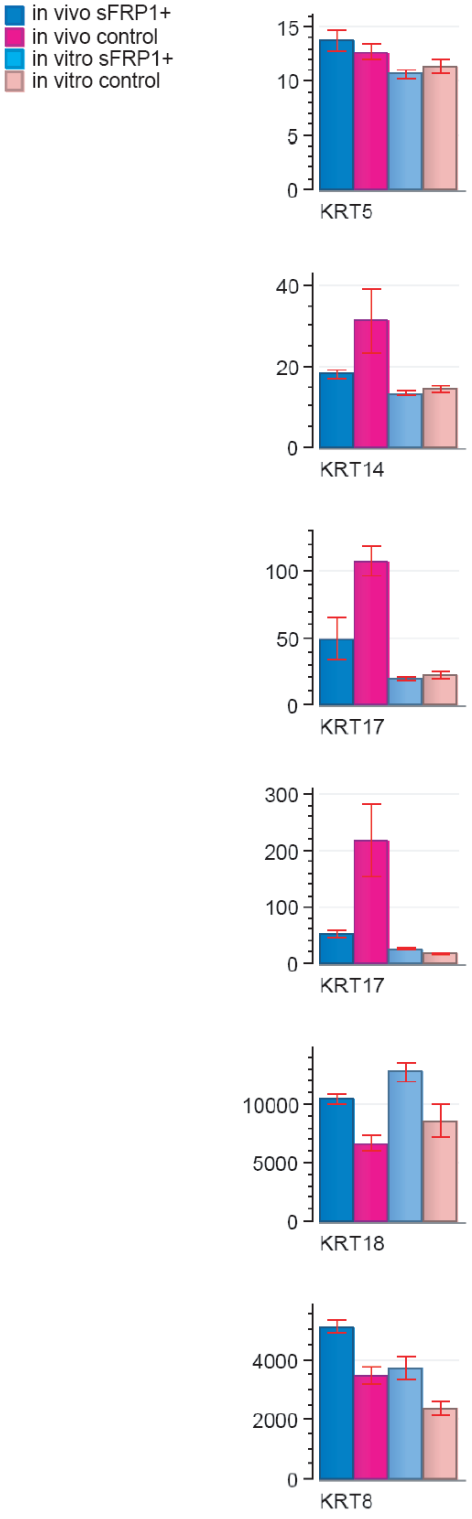
B

↑ABCC3*, ↑ACSL5*, ↑ACVR1B, ↑ADORA2B, ↓ADRB1, ↑AGR2, ↑AIM2, ↓AKAP12*, ↑AKR1B10, ↑AKR1C1*, ↑AKR1C2*, ↑AKR1C3, ↓AKT3, ↑ANK3*, ↓ANTXR1*, ↓ANXA4*, ↑ARHGAP26*, ↓ASH2L, ↑ASS1, ↑B4GALT5*, ↑BAMBI, ↓BMPR2*, ↑BRCA1, ↑BST2, ↑C10ORF116, ↑C11ORF9, ↓C13ORF15, ↑C15ORF48, ↑CALB2, ↓CALD1*, ↓CAPG, ↑CASP1*, ↑CASP4, ↑CCL5, ↑CCL24, ↓CCND1, ↑CCND2, ↑CD24, ↑CD40*, ↓CD59*, ↑CD74, ↓CD151, ↓CDH11, ↑CDKN1A, ↓CDKN1C*, ↑CEACAM1, ↓CENPA, ↑CFI*, ↑CHFR, ↑CLDN1, ↑CLU*, ↓COL12A1*, ↓COL18A1*, ↓COL1A1*, ↓COL1A2, ↓COL3A1*, ↓COL4A1*, ↓COL4A2*, ↑COP1*, ↑COTL1*, ↓CRELD1, ↑CRKRS, ↓CRYL1, ↑CSRP2*, ↓CTSB*, ↑CTSS*, ↑CXCL1, ↑CYP2E1, ↓CYS1, ↓DDB2, ↑DGKA, ↓DLC1, ↓DST, ↓DSTN, ↓DTNA, ↑DUSP1, ↑DYNLL1*, ↑ECM1, ↑EFEMP1, ↑EFNA1, ↑EGF, ↑ENG, ↓ENPP1, ↓EPHA4*, ↑ERAP2, ↑EREG, ↑ETS2, ↑F3, ↑FABP5, ↑FABP6, ↑FBXL7, ↓FDFT1, ↑FGF1*, ↑FKBP1A, ↓FN1, ↓FOXA2, ↓FST*, ↑FYN, ↑GATA6, ↓GCDH, ↓GLRX, ↑GLRX3, ↓GLUD1*, ↓GPC1, ↑GPM6A*, ↓GPR177, ↓GSTP1, ↑HCLS1, ↓HDAC8, ↑HIST1H2BD, ↑HIST1H4H, ↑HLA-B, ↑HLA-DMA, ↑HLA-DRA*, ↑HNRNPA1, ↑HOXB3, ↓HRAS, ↑HSD11B1, ↑IFI27, ↑IFI44*, ↑IFI44L, ↑IFIT1, ↑IFIT3*, ↓IFITM2, ↓IFNGR2, ↓IGF1R*, ↓IL13RA1*, ↑IL13RA2, ↑IL1B*, ↑IL7R*, ↓ILK, ↓INHBA, ↓IQGAP1 (includes EG:8826), ↑ISG20*, ↑ISG15 (includes EG:9636), ↑ITGA5, ↑ITGA6, ↓ITGB5*, ↑JAG1*, ↑KIAA0746, ↑KRT7, ↑LAMA3, ↓LAMA4, ↑LETMD1*, ↓LIMS1*, ↓LIN7C, ↓LOXL1, ↑LRIG1, ↑LY6E (includes EG:4061), ↑MAD1L1, ↓MAGED2, ↑MAP2K3, ↓MBNL1*, ↑METAP2 (includes EG:10988), ↓MFGE8, ↑MGP, ↑MLLT11, ↑MMP2, ↓MMP11, ↑MMP19, ↑MMP1 (includes EG:4312), ↑MXRA8, ↓MYL9 (includes EG:10398), ↓NAP1L2, ↓NCOA1, ↑NEK4, ↓NES, ↑NLRP1, ↑NNMT, ↓NOTCH3, ↓NR2F1*, ↓NRIP1, ↑NUP37, ↑NUPR1, ↑P4HA1, ↑PAEP, ↓PAPPA*, ↑PBX1, ↓PCSK6, ↓PDGFC, ↓PDGFRB, ↓PDLIM3, ↑PECAM1*, ↑PLEK2, ↓PML*, ↑POLE, ↓POLE4*, ↓PPARG, ↑PRKCH, ↑PRKCSH, ↓PSME4, ↑PTN, ↑PTPN11, ↑PTX3, ↓PVRL3, ↑PYCARD, ↑QPCT (includes EG:25797), ↑RAE1, ↑RAGE, ↑RAN, ↓RAP2B*, ↓RHOG, ↑RNF7, ↓RP6-213H19.1, ↓RRM1, ↓RTN1, ↓RUNX3*, ↑S100A2, ↑S100A3, ↑S100P, ↓SCD*, ↓SDC1, ↑SERPINA1*, ↑SERPINA3, ↑SERPINA5, ↑SERPINB9, ↓SERPINE1*, ↓SERTAD2*, ↑SFRP1*, ↓SGK3, ↓SIGIRR, ↓SKP1, ↑SLC12A7, ↑SLC12A8, ↓SLC22A18, ↓SLC26A2, ↓SLC39A4 (includes EG:55630), ↑SLC46A3, ↑SLC5A3, ↓SMPD1, ↓SORBS2*, ↓SORT1, ↓SPAST, ↓SPSB1, ↓SPTBN1, ↑SRD5A1, ↑SRPX, ↑STK31, ↑SULT1A2, ↓SYT12, ↑TARBP2, ↑TBXAS1, ↑TFF1, ↑TFF3, ↑TFRC*, ↑TGFBR3, ↑TIAM1, ↑TLR3, ↓TM4SF1, ↑TMEM106C, ↑TMPRSS3*, ↓TNFRSF11B, ↓TNFSF10*, ↑TNS1, ↑TPD52L1*, ↓TPM1*, ↑TRIO, ↓TSG101, ↓TSPAN4*, ↓TUSC3*, ↓VCAM1, ↑VDR*, ↓WBP1, ↑XAF1*, ↑ZFPM2, ↓ZNF185

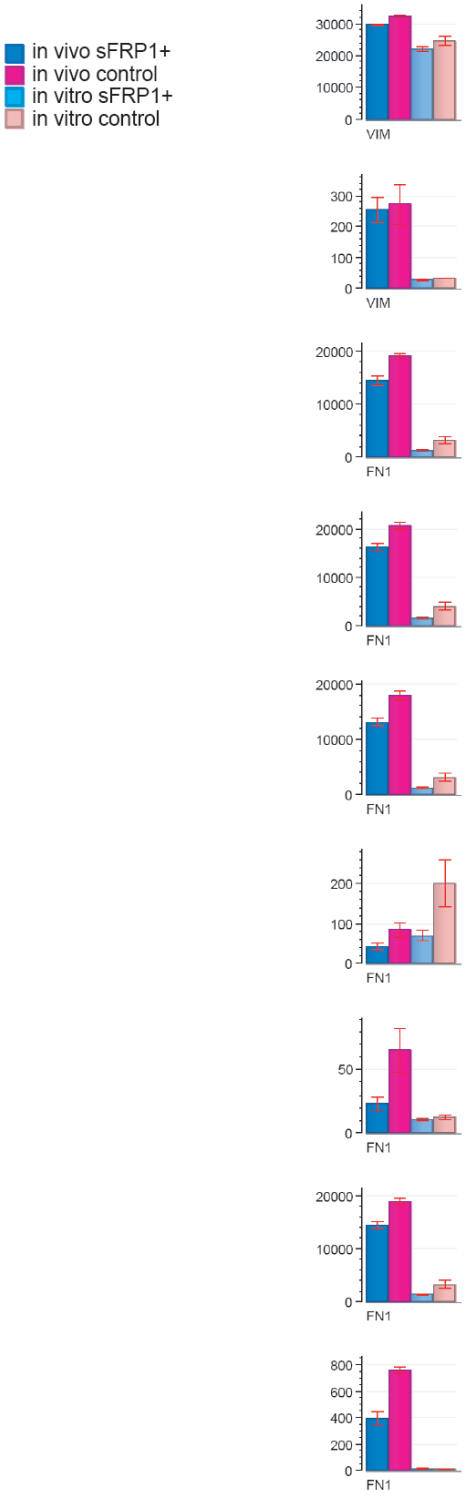
Supplemental figure 5



Supplemental Fig 6



Supplemental figure 7



Supplemental table 1A

Gene	fold down-regulation upon sFRP1 expression
FGF5	4.09
BDNF	4.03
FBXO25	3.84
BDNF	3.30
FGF5	3.28
DEAF1	2.97
DLC1	2.96
DKFZp313A2432	2.77
APEH	2.70
ZNF289	2.69
FGF5	2.65
TEX261	2.65
GALNT10	2.65
SLC39A4	2.60
ARHGEF10	2.60
TSPAN4	2.58
LYPD6	2.54
EPS8L2	2.45
MFHAS1	2.40
KLF11	2.40
FXC1	2.38
PTPMT1	2.30
C9orf140	2.30
MFHAS1	2.26
TOLLIP	2.22
PTPN18	2.21
FLJ11236	2.20
EIF2B4	2.20
ANTXR1	2.19
MAPKAPK3	2.17
HOMER3	2.16

TOLLIP	2.16
MAPKAPK3	2.14
KIAA0652	2.09
HOMER3	2.09
TGOLN2	2.08
CUGBP2	2.07
LOC283267	2.04
RNF141	2.04
MAGEA3	2.03
SCD	2.03
COMTD1	2.02
CCL28	1.99
FLJ13305	1.99
GSTM4	1.98
MAGEA6	1.97
POLE4	1.96
RNH1	1.95
TSTA3	1.95
POLE4	1.95
GRK6	1.94
KBTBD4	1.94
TGOLN2	1.94
NUP98	1.93
NAP1L4	1.93
NPAS2	1.92
SUSD1	1.90
THUMPD3	1.88
NAP1L4	1.85
MCART1	1.85
BOK	1.85
RIC8A	1.85
TALDO1	1.84
MTA3	1.84
B3GNT2	1.83

B3GNT2	1.83
MPHOSPH10	1.83
SGK3	1.83
FAM125A	1.83
CRIM1	1.82
EIF2B4	1.81
HRAS	1.80
PDLIM5	1.80
AP2A2	1.80
PEX13	1.80
ALKBH3	1.79
ARHGAP1	1.78
SERTAD2	1.76
C19orf12	1.76
STOML1	1.76
RAP2B	1.74
ASB1	1.74
SMEK2	1.74
LEPROTL1	1.73
SAAL1	1.73
PSMD13	1.70
C6orf108	1.69
CCDC75	1.67
ASH2L	1.65
ING5 : LOC727773	1.65
MOBKL1B	1.64
SUMO3	1.64
IQGAP1	1.63
PI4K2B	1.63
LOC728944 : THAP4	1.62
ALDH3A2	1.62
PSME4	1.62
RAB1A	1.62
RFXANK	1.61

AKT3	1.61
RNASEH1	1.60
POLR2L	1.60
C9orf142	1.60
MRPL23	1.59
AKTIP	1.59
SETMAR	1.58
C11orf17	1.57
SGCB	1.57
ANAPC1	1.57
MOBKL1B	1.57
SLC4A1AP	1.56
KIAA0652	1.55
TMEM9B	1.55
MRPL53	1.55
C5orf30	1.54
C2orf4P : MEMO1	1.54
PSMC3	1.53
AUP1	1.52
SMEK2	1.51
CCND1	**1.51**
CENPA	1.51

Supplemental table 1B

Gene	fold up-regulation upon sFRP1 expression
SFRP1	51.13
PAEP	30.29
PRSS1 : PRSS2 : PRSS3 : TRY6	29.23
SERPINA3	27.59
ECSM2	26.93
TRY6	26.15
PRSS1	25.21
ECSM2	23.03
SERPINA1	20.26
SLC2A10	19.62
OBP2A : OBP2B	12.72
SERPINA1	11.04
LOC149773	8.94
OAS2	8.12
GPR87	7.35
TMEM178	6.76
FOLR1	6.16
LTBP2	6.06
TIE1	5.81
WFDC10B	5.74
APCDD1L	5.26
RARRES3	5.25
CLU	5.24
METTL7A	5.15
CTSS	5.11
DTX3	5.07
AIM2	4.97
OBP2A	4.91
TMPRSS2	4.85
BHLHB3	4.82
GLDN	4.80

TAGLN3	4.72
CLU	4.62
LOC286299	4.40
HOXB3	4.24
CASP1	4.24
CLU	3.99
APOBEC3F : APOBEC3G	3.91
IGFBP6	3.84
SSPN	3.77
HERC6	3.72
FAM125A	3.70
ANK3	3.66
LTBP2	3.57
CTSS	3.47
CASP1 : COP1	3.20
FLJ20035	3.07
KLHL3	3.03
CYP2E1	3.02
SULT1A3 : SULT1A4	3.02
CDRT4	3.00
ABCC3	2.84
USP52	2.75
PER1	2.72
SLC39A11	2.71
ITGA5	2.56
C17orf60	2.43
FXYD5	2.07
TAPBPL	2.02
ARHGAP26	1.98
FXYD5	1.98
KRT79	1.96
PRIC285	1.90
TIAM1	1.86
PECAM1	1.77

MMD	1.75
CPD	1.70
CDKN1A	**1.66**
B4GALT5	1.64
CPD	1.64

Supplemental table 2

NRCAM
PTGS2 (COX2)
PPARD
RUNX2
FZD7 (Frizzled7)
LEF1
CCND1 (CyclinD1)
EDN1 (Endothelin1)
CD44
RHOU (Ras homolog gene family, member U)
AXIN2
JUN
STRA6
PLAUR (Plasminogen activator)
TCF4
FN1 (Fibronectin1)

VI. FIGURE LEGENDS

Figure 1

Wnt1 promotes T47D cell migration

(A) Upper panel; Confluent monolayers of T47D cells were scratched and the medium was changed to Wnt1 CM or control CM and pictures of wounded edges were taken 3 hours later. Bar-graph; Confluent monolayers of T47D cells were scratched and the medium was changed to Wnt1 CM, Wnt1 CM + sFRP1 or control CM. For Wnt1 CM + sFRP1, ¼ volume of purified sFRP1 [7] was added to the CM. For the other conditions, the same volume of PBS was added to the CM. Nine randomly-chosen wound edges per condition were monitored 3 hours and 7 hours after scratching. The average recovered area (arbitrary unit; pixels on the computer screen) +/- SEM was calculated. **$p<0.01$ (B) Wnt1 levels in a clone and a pool of T47D/Wnt1 expressing cells (clone 1 and P1) and in control cells were measured in a western analysis with a Wnt1 specific antiserum. (C) Confluent monolayers of T47D/Wnt1 clone 1 and a control (left bar graph) or T47D/Wnt1 P1 and a control (right bar graph) were scratched and a wound healing assay was performed in DMEM plus 10% FCS. Nine randomly-chosen wound edges per condition were monitored 3 hours after scratching. Average recovered areas (arbitrary unit) +/- SEM were calculated. ** $p<0.01$

Figure 2

Elevated RhoA activity in Wnt1 expressing T47D cells

(A) T47D cells were seeded on cover glasses and stimulated by Wnt1 CM or control CM for 30 minutes, then fixed and stained with phalloidin to visualize actin. Bars: 20 microns. (B) A GST-C21 (Rhotekin) pull-down assay was performed on lysates of the T47D/Wnt1 P1 and a control pool. The level of GTP-RhoA bound to the beads and the total RhoA in the lysate were determined by a western analysis with a RhoA antibody. The ratio of: GTP-RhoA/total-RhoA is 0.2408 in T47D/Wnt1-P1 and 0.0616 in T47D/control-P1 cells. The quantification was done using ImageQuant TL (GE Healthcare).

Figure 3

Ectopic expression of sFRP1 in MDA-MB-231 breast cancer cells

(A) A western analysis was performed on lysates of three MDA-MB-231/sFRP1 clones and three MDA-MB-231/control clones and the levels of active β-catenin, α-tubulin and DVL 1-3 were determined with specific antisera. Myc-tagged sFRP1 was detected with a Myc specific antiserum.

Lower panel; Level of total ERK1/2 and P-ERK1/2 in MDA-MB-231/sFRP1 clone and in MDA-MB-231/control clone. (B) The level of Myc-tagged sFRP1 in two pools of MDA-MB-231/sFRP1 cells was determined in a western analysis using a Myc specific antiserum. α-tubulin levels served as a control. P1 is a mixture of the 3 clones shown in panel A; P2 was generated from >100 sFRP1 infected clones. (C) MDA-MB-231/sFRP1 P1 and P2 and control pools P1 and P2 were seeded on 6-well dishes (200000 cells/well) in DMEM 10% FCS and cells were counted after 3 days. Left: sFRP1-P1 and control. Right: sFRP1-P2 and control. 3 wells per condition**.** Average cell numbers were calculated +/- SEM. ** $p<0.01$, * $p<0.05$ (D) Confluent monolayers of MDA-MB-231/sFRP1-P1 cells and control-P1 cells were scratched, the medium was changed to Wnt1 CM or control CM and 3 hours later the recovered areas were calculated on 9 randomly chosen wound edges. The results are presented in arbitrary units. Average recovered area were calculated +/- SEM. ** $p<0.01$, n.s.=not significant ($p>0.05$).

Figure 4

Ectopic expression of sFRP1 in MDA-MB-231 cells suppresses *in vivo* tumor formation

MDA-MB-231/sFRP1-P1 cells and control cells ($1x10^6$) (A) and MDA-MB-231/sFRP1-P2 cells and control cells ($1x10^6$) (B) were injected into mammary fat pads of Balb/c nude mice and tumor formation and growth were monitored. Average tumor volume (left graph) and % of tumor free mice (right graph) are shown. P1: Left: $p<0.01$ (Two-way RM ANOVA), Right: $p=0.0106$ (log rank test); P2: left: * $p<0.05$ on day 19, Right: $p=0.026$ (log rank test). Insert; A western analysis was performed on tumor lysates to check the Myc-tagged sFRP1 and endogenous DVL3 status at the end of the experiment. Representative pair of tumor lysates to detect Myc-tagged sFRP1 and lysates of 3 sFRP1-expressing and 2 control tumors to check DVL3 status are shown here.

Figure 5

Analysis of proliferation and tumor angiogenesis in MDA-MB-231/sFRP1 xenografts

(A) Mice bearing sFRP1-P1 or control MDA-MB-231 tumors were injected with BrdU and two hours later they were sacrificed. Tumors of similar weight from each group were sectioned and stained with an anti-BrdU antibody (left panel) and BrdU positive nuclei and total nuclei were counted in nine randomly chosen areas from each section. Bar graph (right panel) shows the quantification +SEM. ** $p<0.01$, Bar=250 microns (B) Functional blood vessels in tumor-bearing mice were visualized by injecting FITC-lectin into tail veins 5 min before sacrificing. Tumor

sections were made and stained for CD31, an endothelial cell marker to visualize whole blood vessels. Total area, FITC+ area and CD31+ area were measured using IMARIS software (Bitplane). Left bar graph: CD31+ area vs total area is presented (5 sFRP1 expressing and 8 control tumors). Right bar graph: FITC+ area vs CD31+ area is shown (3 sFRP1 expressing and 5 control tumors). +SEM, n.s.=not significant

Figure 6

c-Myc expression in MDA-MB-231/sFRP1- and control- tumors and cell lines

(A) qRT-PCR analysis for c-Myc RNA levels in tumor lysates from 6 sFRP1+ tumors and 5 control tumors (left bar graph) and cell lysates from the 3 *in vitro* cultured MDA-MB-231/sFRP1 clones and the 3 control clones shown in Fig 3A (right bar graph). Average is shown. +SEM, n.s.=not significant (B) Left; sFRP1 and c-Myc levels were analyzed in lysates prepared from 6 MDA-MB-231/sFRP1-P1 tumors and 2 MDA-MB-231/control-P1 tumors. Right; Lysates of cultured MDA-MB-231/sFRP1-P1 cells and MDA-MB-231/control-P1 cells were also subjected to a western analysis to check c-Myc levels. Both using c-Myc antiserum. α-tubulin serves as a standard.

Figure 7

Microarray analysis comparing gene expression profiles generated from tumors and from *in vitro* cultured cells

(A) y-axis- 135 probesets (106 genes) showing low expression in sFRP1+ tumors and moderate-high expression in control tumors, and *in vitro* cultured sFRP1+ and control cells. (B) y-axis- 84 probesets (62 genes) showing high expression in sFRP1+ tumors and low expression in control tumors, and *in vitro* cultured sFRP1+ and control cells. (A & B) From left to right, columns represent 6 sFRP1+ tumors, 5 control tumors, 3 MDA-MB-231/sFRP1 clones and 3 MDA-MB-231/control clones (shown in Fig 3A). Highly-expressed genes are indicated in red, moderately-expressed genes are indicated in black and lower-expressed genes are indicated in green. (C) Normalized gene expression of *CCND1* (Cyclin D1) and *CDKN1A* ($p21^{Cip1}$) in MDA-MB-231/sFRP1-P1 tumors, MDA-MB-231/control-P1 tumors, MDA-MB-231/sFRP1-P1 *in vitro* cultured cells and MDA-MB-231/control-P1 cultured cells.

Figure 8

Detection of Cyclin D1 and $p21^{Cip1}$ in tumors and in cultured cells

(A) Tumor sections taken from mice bearing MDA-MB-231/sFRP1-P1 and control tumors were stained with a Cyclin D1 specific antiserum. Signal intensities, shown in the bar graph, reflect data collected from 6 MDA-MB-231/sFRP1-P1 tumors and 8 control tumors. +SEM, * $p<0.05$ (B) Cyclin D1 was detected in lysates from *in vitro* cultured MDA-MB-231/sFRP1-P1 cells and control cells by immunoblotting. Right bar graph; Expression levels of Cyclin D1 in cultured cells were quantified from 3 MDA-MB-231/sFRP1 clones and 3 control clones (shown in Fig 3A). Average is shown. +SEM, n.s.=not significant (C) $p21^{Cip1}$ was detected in tumor lysates prepared from a representative MDA-MB-231/sFRP1-P1 tumor and a control tumor. Lysates from *in vitro* cultured MDA-MB-231/sFRP1-P1 and MDA-MB-231 control cells had no detectable $p21^{Cip1}$. Lysates from MCF7 breast tumor cells and MCF7 with siRNA mediated $p21^{Cip1}$ KD served as positive and negative controls, respectively (+CTRL and -CTRL). All lysates are 40ug per lane. α-tubulin serves as a standard. (D) Bar graph: Expression levels of $p21^{Cip1}$ in tumor lysates were quantified from 5 sFRP1+ tumors and 5 control tumors with Odyssey software (LI-COR biosciences). +/- SEM, ** $p<0.01$

Figure 9

sFRP1 expression and the methylation status of sFRP1 gene promoter in a panel of breast cancer and normal breat cell lines

(A) sFRP1 expression was examined in a panel of breast cancer cell lines and normal MCF10A breast cell line using qRT-PCR. The expression level of sFRP1 was normalized with that of beta-actin and indicated in the relative values in which the sFRP1 expression level of HCC1937 cell is set as "100". The ectopically sFRP1 expressing MDA-MB-231/sFRP1 cell serves as a positive control. Experiment was duplicated (shown in dark blue and light blue). (B) Methylation status of the sFRP1 promoter region was examined by bisulfite sequencing. The three cell lines on the left are sFRP1 negative cell lines and the five cell lines on the right are sFRP1 positive cell lines. If the sFRP1 promoter region is methylated, a band appears in the lane indicated by "M", if unmethylated, a band appears in the lane indicated by "U".

Figure 10

sFRP1 suppresses the tumor initiating cell phenotype

(A) RNA expression of CD24 and CD44 by microarray analysis. From left to right, RNA from tumor lysates arising from MDA-MB-231/sFRP1-P1, tumor lysates arising from MDA-MB-231/control-P1, cultured MDA-MB-231/sFRP1-P1 cells and cultured MDA-MB-231/control-P1 cells. (B) FACS analysis using the specific antisera against CD24 and CD44 to see the surface expression of CD24 and CD44 in cultured MDA-MB-231/sFRP1-P1 cells and cultured MDA-MB-231/control-P1 cells. (C) Sox2 expression was majored in three individual MDA-MB-231/sFRP1 clones and a MDA-MB-231/control clone by RT-PCR using a specific primer set. Expression of beta-actin is served as a standard. On the top, Myc-tagged sFRP1 expression is shown by western blotting. (D) Efflux activity was examined in MDA-MB-231 cells treated with sFRP1 CM and MDA-MB-231 cells treated with control CM for 7 days. Cells were incubated with Hoechst followed by the FACS sorting. The "side population" was 0.77% in the cohort treated with control CM, while 0.12% in the cohort treated by sFRP1 CM. (E) RNA expression of ABCA2 was examined by microarray analysis. The sample order from left to right is the same as shown in (A).

Figure 11

Integrin β1 expression is suppressed upon ectopic sFRP1 expression

Left: RNA expression level of Integrin β1 in *in vivo* tumor lysates and *in vitro* cultured cells. From left to right, RNA from tumor lysates arising from MDA-MB-231/sFRP1-P1, tumor lysates arising from MDA-MB-231/control-P1, cultured MDA-MB-231/sFRP1-P1 cells and cultured MDA-MB-231/control-P1 cells. Right: Integrin β1 positive cells are sorted by FACS analysis using the specific antiserum. Experiment is tripricated. sFRP1: MDA-MB-231/sFRP1-P1 cells, control: MDA-MB-231/control-P1 cells. Y-axis indicates the proportion of Integrin β1 positive cells.

Figure 12

P-cadherin expression was promoted by Wnt1

(A) CDH3 (gene encoding P-cadherin) RNA level in Wnt1 expressing T47D and control T47D cells was analyzed by microarray analysis. (B) P-cadherin expression level was analyzed in T47D/Wnt1, T47D/Wnt5a and T47D/control pools by western blotting. Three independent pools were made for each -/Wnt1, -/Wnt5a and -/control cells (pool#1-pool#3). Each pools consist of more than 100

clones. Cells were let to become confluent (left) or 20-30% confluent (right) and lysed. P-cadherin was detected by western blotting. α-tubulin serves as a standard. (C) P-cadherin expression level shown in (B) was quantified using Odyssey. Y-axsis represents the P-cadherin level normalized by α-tubulin level. (D) Ectopically sFRP1 expressing T47D cell pools were established (pool#1 and pool#2). Each pool constitutes of more than 100 clones. P-cadherin level was examined by western blotting using the specific antiserum. Ectopically expressed Myc-tagged sFRP1 is also detected using an anti Myc antibody. α-tubulin serves as a standard.

Supplemental Figure 1

Proliferation suppression of MDA-MB-231 cells by sFRP1 CM treatment

One thousand MDA-MB-231 cells were seeded per well of a 96-well plate and proliferation was measured with a YOPRO assay after 3 days of treatment with sFRP1 CM or control CM.

Supplemental Figure 2

Two additional xenograft experiments

Onset of tumor appearance in two additional xenograft experiments with MDA-MB-231/sFRP1-P1 and MDA-MB-231/control-P1 cells. Left; 6 mice per group, p=0.0179 (log rank test) Right; 8 mice per group, p=0.0223 (log rank test)

Supplemental Figure 3

Effect of ectopic c-Myc on MDA-MB-231/sFRP1 xenograft growth

(A) Lysates from MDA-MB-231/sFRP1-P1 cells infected with a c-Myc encoding vector and a control vector were analyzed for c-Myc levels with a specific antiserum. (B) $2x10^5$ MDA-MB-231/sFRP1/Myc and MDA-MB-231/sFRP1/control cells were seeded on 6-well plates and cell numbers were counted after 3 days and 4 days. 3 wells per condition. Average cell numbers were calculated +/-SEM. n.s.=not significant (C) $1x10^6$ MDA-MB-231/sFRP1/Myc cells and MDA-MB-231/sFRP1/control cells were injected into the fat pads of 6 nude mice. 2 of 6 mice injected with MDA-MB-231/sFRP1/Myc cells formed tumors on day 26 and on day 41 respectively, whereas no mice injected with MDA-MB-231/sFRP1/control cells formed tumors. p=0.138 (log rank test)

Supplemental Figure 4

Ingenuity Pathway Analysis

(A) Using Affymetrix U133 plus 2.0 human GeneChips™ and the software Genedata's Analyst 4.5, 1753 probesets whose levels were changed more than 1.5 fold between sFRP1 positive tumors and control tumors with the p-values less than 0.01 using 1-way ANOVA were identified in the microarray analysis. These 1753 probes were further analyzed using Ingenuity's Pathway Analysis to identify bio functions most likely to be affected by ectopic sFRP1 expression in the MDA-MB-231 xenograft model. The top 10 candidate bio functions (and diseases) are listed, with the Fisher's p-values. (B) In the bio function "cancer", the sub-category "tumorigenesis" had the lowest p-value. 266 genes listed here are related to the "tumorigenesis" bio function. Genes with red arrows and green arrows were up-regulated and down-regulated, respectively, upon ectopic sFRP1 expression in the xenograft tumors. The cut off for all genes was 1.5 fold.

Supplemental Figure 5

Known WNT target genes whose expression was downregulated upon sFRP1 expression both *in vitro* and *in vivo*

Normalized microarray results of established WNT pathway target genes listed in Supplemental Table 2

Supplemental Figure 6

Expression of basal and luminal keratins

Normalized microarray results of keratin (KRT) 5, 14, 17, 18 and 8

Supplemental Figure 7

Expression of vimentin and fibronectin 1

Normalized microarray results of vimentin (VIM) and fibronectin 1 (FN1)

Supplemental Table 1

List of genes whose expression was altered upon sFRP1 expression only *in vivo*

List of 106 (table 1A) and 62 (table 1B) identified genes shown in Fig 7A and 7B, respectively. Fold-change (down- or up-regulation) refers to expression changes between sFRP1+ and control tumors. Some genes have more than one probesets with different values of fold-change.

Supplemental Table 2

Established WNT pathway target genes whose expression was suppressed upon sFRP1 expression both *in vitro* and *in vivo*

Expressions of established WNT pathway target genes (http://www.stanford.edu/~rnusse/pathways/targets.html) were examined by microarray analysis. Listed here are genes whose expression was suppressed upon sFRP1 expression both *in vitro* and *in vivo*.

VII. MATERIALS AND METHODS

Reagents

The following primary antibodies were used: RhoA (sc-418), c-Myc (9E10), DVL2 and –3 (Santa Cruz Biotechnology, Inc., CA, USA); Wnt1 and DVL1 (R&D, Abingdon, UK); active beta-catenin (anti-ABC, Upstate, VA, USA); α-tubulin (DM1A) (Neomarkers, CA, USA); Cyclin D1 (SP4) (Cell MARQUE, CA, USA) for immunohistochemistry (IHC); Cyclin D1 (Chemicon, MA, USA) for western blotting; BrdU (Roche, Basel Switzerland); $p21^{cip1}$ (OP64-100UG) (Oncogene Research Products, MA, USA); ERK and P-ERK (Thr202/Tyr204) (Cell Signaling Technology, MA, USA); and CD31 (BD Pharmingen, CA, USA); Integrin β1 (MAB2079Z, chemicon). As secondary antibodies we used: anti-rabbit and anti-mouse (GE Healthcare, Little Chalfont Buckinghamshire, UK; LI-COR Bioscience, NE, USA), anti-rat (GE Healthcare) or anti-goat (DAKO A/S, Glostrup, Denmark) coupled to horse raddish peroxidase (HRP) or IRDye 800CW. For IHC we used: Biotin-SP-conjugated affinipure donkey anti-rabbit, anti-mouse, anti-rat IgG (Jackson ImmunoResearch, PA, USA), goat anti-rat ALEXA 568 (Molecular probes, OR, USA) and TRITC- labelled phalloidin (Sigma, MO, USA).

Mouse Wnt1 in the retroviral vector pLNCX was obtained from Andrew McMahon (Harvard University, Cambridge, MA, USA); the cDNA encoding Myc/His-tagged human sFRP1 in pCDNA was provided by Jeffrey Rubin (NCI, Bethesda, MD, USA) and was recloned into the pBabePuro retroviral vector: Human c-Myc from c-Myc/pCDNA3Neo [189] was recloned in pBabePuro. Conditioned media (CM) from Wnt1 and sFRP1 producing cells, and purified sFRP1 were prepared as previously described [177].

Cell culture, transfections, retroviral infections and proliferation assays

The human breast cancer cell lines, T47D and MDA-MB-231 (ATCC, VA, USA) were cultivated in DMEM, 10% heat inactivated FCS (Amimed, Allschwil, Switzerland) supplemented with Penicillin and Streptomycin (Sigma). All transfections were performed using FuGENE 6 Transfection Reagent (Roche) following the manufacturer's guidelines. T47D cells were stably transfected with Wnt1 or empty pLNCX as control, followed by selection with 0.5mg/ml G-418 (Life Technologies, Inc, MD, USA). A clone expressing high levels of Wnt1, T47D/Wnt1 and a control clone were previously described [177]. A pool of >100 T47D/Wnt1 clones (T47D/Wnt1-P1) and a pool of control clones (T47D/control-P1) were also generated. MDA-MB-231 cells were stably transfected with pCDNA3.1(+) (Invitrogen) encoding Myc/His-tagged human sFRP1 or empty pCDNA3.1(+) as control. After selection with 1mg/ml G-418, three clones of MDA-MB-231/sFRP1 and three control clones were isolated. Equal cell numbers of these clones were pooled before some experiments (MDA-MB-231/sFRP1-P1 and MDA-MB-231/control-P1). A second pool of sFRP1 expressing MDA-MB-231 cells (MDA-MB-231/sFRP1-P2) and control cells (MDA-MB-231/control-P2), each representing >100 clones, was generated by infecting the cells with pBabePuro encoding Myc/His-tagged human sFRP1 or empty pBabePuro followed by the selection with 2 µg/ml Puromycin (Sigma). For the c-Myc rescue experiment, MDA-MB-231/sFRP1-P1 cells were infected with a pBabePuro vector encoding human c-Myc or empty pBabePuro as control. These cells were selected with 1mg/ml G-418 and 2µg/ml Puromycin and pooled to make MDA-MB-231/sFRP1/Myc and MDA-MB-231/sFRP1/control. Cell proliferation was measured either by counting cell numbers with a Vi-Cell XR cell viability analyzer (Beckman Coulter, CA, USA) on selected days after seeding 200000 cells on 6-well plates or using the YOPRO cell viability assay (Invitrogen) 3 days after seeding 1000 cells on 96-well plate, according to manufacturer's instructions. Unless otherwise noted, p-values were calculated using Student's t-test.

Protein extraction and western blotting

Cells were lysed in 1% Nonidet P-40, 50mM Tris pH 7.5, 120mM NaCl, 5mM EDTA, 1mM EGTA, 2mM sodium vanadate, 20mM β-glycerophosphate, 10µg/ml aprotinin, 10µg/ml leupeptin, 0.5mM PMSF, 50mM NaF, 1mM DTT for 5min on ice before collecting lysates. Debris was removed by centrifugation at 4°C and protein concentration was determined using the Bradford reagent (BioRad, CA, USA). For western blotting, protein loading buffer was added to 30-50µg of total protein and

the samples were denatured for 10min at 95°C prior to separation on SDS-polyacrylamide gels and blotting by semi-dry transfer for 90min on PVDF membrane (Millipore, MA, USA). Membranes were blocked using 10% horse serum in TBS-T buffer for 1h (0.2M NaCl, 25mM Tris, pH 7.5, 0.5mL/L Tween-20), except for $p21^{Cip}$ detection where PBS-T buffer was used for blocking. Blots were incubated with primary antibodies at room temperature for 1h or at 4°C overnight, followed by a 30 min incubation with secondary antibodies anti-rabbit-, anti-mouse-HRP (1:5000) or anti-goat-HRP (1:5000) either IRDye 800CW Goat anti-rabbit- or anti-mouse-IgG (1:10000) (LI-COR Biosciences). Detection of luminescence was carried out using ECL (GE Healthcare) according to manufacturer's instructions and using X-OMAT LS films (Kodak, NY, USA), or performed using the LI-COR Odyssey system according to manufacturer`s instructions (LI-COR Biosciences). Quantification of protein expression was done using Odyssey 2.1 (LI-COR Biosciences).

Immunofluorescence microscopy

Cells were seeded on glass coverslips (BD Biosciences, CA, USA) in DMEM 10% FCS. 30 minutes after media was changed to Wnt1 CM or control CM, cells were fixed with 4 % paraformaldehyde and 3 % sucrose in PBS, permeabilized in 0.2% Triton X-100 in PBS, and blocked with 10% goat serum in PBS. F-actin was stained at RT with 2 U/ml TRITC-labelled phalloidin (Sigma). Cells were washed with PBS and mounted with a mounting solution (Calbiochem, CA, USA). Mounted samples were examined using an LSM510 microscope (Carl Zeiss, Jena, Germany).

Wound healing assay

Cells were seeded on 6-well plates and grown to confluency. Monolayers were scratched, and in the indicated experiments media was changed to Wnt1 CM or control CM. Pictures of randomly-chosen 9 wound edges per condition were taken at time 0 and at indicated time points using Nikon DIAPHOT (Nikon, Tokyo, Japan). Recovered area was calculated using ImageQuant TL (GE Healthcare). In some experiments, purified sFRP1 [177] was added to the CM.

RhoA activity assay

Active RhoA was detected using a glutathione *S*-transferase (GST) −C21 (Rhotekin) fusion protein, as described previously [290]. Briefly, lysates from cells were incubated with bacterially produced GST−C21 fusion protein bound to glutathione−Sepharose beads. Proteins bound to the fusion

protein were analysed by western blotting using an anti-RhoA antibody (Santa Cruz). Aliquots from the cell lysates were taken to analyse total RhoA levels.

MDA-MB-231 xenograft model, BrdU incorporation and tumor lysis

7-10 weeks old female Balb/c nude mice were obtained from Charles River Laboratories (L'Arbresle, France) and were maintained in accordance with the Swiss guidelines for animal safely. Mammary tumors were established in the mice by injecting 0.5-1.0 x 10^6 control or sFRP1 expressing MDA-MB-231 cells either appropriate cell lines in 100-150 µl PBS into the fourth right-side mammary fat pads of 5-8 mice per group. Tumor size was measured 2-3 times a week using a gage and the volume was calculated considering a tumor as a oval according to the formula, 4π(longer edge/2)(shorter edge/2)^2/3. To assay tumor cell proliferation *in vivo*, mice were injected in the abdomen (Intraperitoneal injection) with 100µg/g body weight BrdU (Cell Proliferation Kit II, Roche) and sacrificed 2h later. Tumors were excised and washed with PBS before fixation in 4% PFA at 4°C for 24 hours. Procedures for detection of BrdU were performed as previously described [273]. Excised tumors were snap frozen and pulverized in liquid nitrogen and lysed in SDS buffer (100mM Tris-HCl pH 7.6, 2% SDS, 10mM DTT, 2mM sodium vanadate, 0.5mM EDTA) by incubation at 95°C for 10 min. Western blot analyses were carried out to detect specific proteins.

RNA isolation, quantitative RT-PCR and semi-quantitative RT-PCR

Cultured cells were collected when plates were 70-80% confluent and RNA was extracted using RNeasy Mini kit (Qiagen, Venlo, The Netherlands). To extract the RNA from tumours, dissected tumours were put in RNAlater (Qiagen) over night at 4°C, followed by RNA extraction using TRIzol reagent (Invitrogen) and washing using the RNAeasy Mini kit according to manufacturer's instructions. For each sample cDNA was made from 2.5µg of RNA using Ready-To-Go™ You-Prime First-Strand Beads (GE healthcare). Semi-quantitative PCR was performed using taq DNA polymerase. Quantitative RT-PCR was performed with ABI prism 7000 (Applied Biosystems, CA, USA) using ABsolute SYBR Green ROX Mix (THERMO scientific, MA, USA) following the manufacturer's guidelines. Primer sequences used are shown in the end of this section.

Methylation analysis

Genomic DNA was extracted from the cell lines and detection of the methylated- or unmethylated promoter sequence was done using EpiTect Bisulfite Kit (QIAGEN) following manufacturer's

instructions with the primers which are shown in the paper published [193]. Promeer sequences used are shown in the end of this section.

Microarray analysis

Total RNA was isolated from individual mammary tumors (6 MDA-MB-231/sFRP1-P1 tumors and 5 control tumors) and cultured cells (3 MDA-MB-231/sFRP1 clones and 3 control clones). All RNAs were individually amplified and labeled using the Ambion MessageAMP III RNA Amplification Kit. Biotinylated, fragmented cRNA was hybridized to Affymetrix U133 plus 2.0 human GeneChips™ (Affymetrix, CA, USA). Expression values were estimated using the GC-RMA implementation found in Genedata's Refiner 4.5 software (Gendata AG, Basel, Switzerland). Quantile normalization and median scaling were performed in order to standardize array signal distributions to facilitate the comparison between *in vitro* cultured cells and *in vivo* tumor samples. Probesets showing statistically different expression profiles (1-way ANOVA with $p<0.01$; Benjamini and Hochberg Q-values were determined to minimize the false discovery rate) and specific pairwise fold changes were clustered by rank correlation with an R value of $R>0.8$ for the first criterion and $R>0.885$ for the second criterion using "Profile Distance Search" function of Genedata's Analyst 4.5 tool. Functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com). The Bio functional Analysis identified the biological functions and diseases that were most significantly enriched within the data set. Genes from the dataset that were associated with biological functions and diseases in the Ingenuity knowledge base were considered for the analysis. Fisher's exact test was used to calculate a p-value determining the probability that each biological function and disease assigned to that data set is due to chance alone.

The entire set of microarray data is in GEO (Gene Expression Omnibus) with accession number GSE13806 (released when paper is published).

Immunohistochemistry and functional vessel analysis on tumor sections

To detect functional vessels in tumors, 100 µl of a 2 µg/µl solution of Fluorescein-labeled *Lycopersicon esculentum* lectin (Vector Labs, CA, USA) was injected into tail veins of tumor-bearing mice [188] and mice were sacrificed 5 min later. Tumors were excised, fixed in 4% paraformaldehyde in PBS for 48 h at 4°C, followed by an overnight incubation in 30% sucrose in

PBS at 4°C then embedded in tissue-Tec O.C.T. compound 4583 (Sakura, Tokyo, Japan). Frozen sections (9 µm) were subjected to IHC analysis to detect tumor-associated vessels using rat anti-mouse CD31 (BD Pharmingen; diluted 1:100) and goat anti-rat ALEXA 568 (Molecular Probes; diluted 1:200). Staining was done using Discovery XT (Ventana Medical Systems, Inc., AZ, USA). Pictures were taken with a Z1 microscope (Carl Zeiss) and analyzed with IMARIS (Bitplane, Zurich, Switzerland) to calculate the co-localized area. For detection of Cyclin D1, frozen tumor sections (9 µm) were subjected to IHC analysis using the SP4 antibody (diluted 1:100) and Biotin-SP-conjugated affinipure donkey anti-rabbit IgG (diluted 1:100). Staining was done using Discovery XT with sCC1 pretreatment. Pictures were taken with Eclipse E600 (Nikon) and analyzed with IMARIS (Bitplane) to calculate signal intensity.

FACS analysis

Following the incubation, cells were tripsinized and 3 x 10^5 cells were re-suspended and incubated with 1µg of 1st Ab in 50µl PBS at 4°C for 60 min followed by the 2nd Ab (dilution=1:250). Afterwards, cells were washed, pelleted and re-suspended in 300µl FACS buffer and sorted by FACS.

Sorting of cancer cell side population

MDA-MB-231 cells were cultured in sFRP1 CM or in control CM for 7 days. Afterwards, cells were tripsinized, pelleted and 10^6 cells were re-suspended in 1ml DMEM 10% FCS. Hoechst 33342 dye was added to a final concentration of 5µg/ml and cells were incubated at 37°C in a water bath for 60 min. After the incubation, cells were centrifuged at 1500rpm at 4°C, washed with PBS/2%FCS and re-suspend in 500-1000µl PBS/2%FCS. Propidium iodide was added prior to FACS analyis to a final concentration of 2µg/ml (stock solution 1mg/ml in EtOH).

Primers used for the experiments

Primers	Sequence (5'-3')	experiment
human sFRP1 forward human sFRP1 reverse	GGTCTTCCTCTGCTCGCTCTTC AGGACACACCGTTGTGCCTT	Quantitative RT-PCR
human Myc forward human Myc reverse	CCTACCCTCTCAACGACAG CTTGTTCCTCCTCAGAGTCG	Quantitative RT-PCR

human β-actin forward human β-actin reverse	TGTCCACCTTCCAGCAGATGT CGCAACTAAGTCATAGTCCGCC	Quantitative RT-PCR
sFRP1 unmrthylated forward sFRP1 unmrthylated reverse	TTTTAGTAAATTGAATTTGTTTGTGA TAAAATACACAAAACTCCTACAAC	methylation alanysis (Lo et al. 2006)
sFRP1 mrthylated forward sFRP1 mrthylated reverse	TTTAGTAAATCGAATTCGTTCGC TAAAATACGCGAAACTCCTACG	methylation analysis (Lo et al. 2006)
human SOX2 forward human SOX2 reverse	CCGCATGTACAACATGATGG CTTCTTCATGAGCGTCTTGG	semi-quantitative RT-PCR

VIII. REFERENCES

1. Lakhani SR, O'Hare MJ: **The mammary myoepithelial cell--Cinderella or ugly sister?** *Breast Cancer Res* 2001, **3**(1):1-4.
2. Bocker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, Burger H, Wai D, Ina Diallo R, Brandt B *et al*: **Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept**. *Lab Invest* 2002, **82**(6):737-746.
3. Longacre TA, Bartow SA: **A correlative morphologic study of human breast and endometrium in the menstrual cycle**. *Am J Surg Pathol* 1986, **10**(6):382-393.
4. Neville MC, Morton J, Umemura S: **Lactogenesis. The transition from pregnancy to lactation**. *Pediatr Clin North Am* 2001, **48**(1):35-52.
5. Zinkernagel RM: **Maternal antibodies, childhood infections, and autoimmune diseases**. *N Engl J Med* 2001, **345**(18):1331-1335.
6. Gusterson BA, Ross DT, Heath VJ, Stein T: **Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer**. *Breast Cancer Res* 2005, **7**(4):143-148.
7. Kelsey JL, Gammon MD, John EM: **Reproductive factors and breast cancer**. *Epidemiol Rev* 1993, **15**(1):36-47.
8. Brinton LA, Schairer C, Hoover RN, Fraumeni JF, Jr.: **Menstrual factors and risk of breast cancer**. *Cancer Invest* 1988, **6**(3):245-254.
9. Hsieh CC, Trichopoulos D, Katsouyanni K, Yuasa S: **Age at menarche, age at menopause, height and obesity as risk factors for breast cancer: associations and interactions in an international case-control study**. *Int J Cancer* 1990, **46**(5):796-800.
10. Trichopoulos D, MacMahon B, Cole P: **Menopause and breast cancer risk**. *J Natl Cancer Inst* 1972, **48**(3):605-613.
11. Alexander FE, Roberts MM: **The menopause and breast cancer**. *J Epidemiol Community Health* 1987, **41**(2):94-100.
12. Henderson BE, Ross RK, Judd HL, Krailo MD, Pike MC: **Do regular ovulatory cycles increase breast cancer risk?** *Cancer* 1985, **56**(5):1206-1208.
13. Layde PM, Webster LA, Baughman AL, Wingo PA, Rubin GL, Ory HW: **The independent associations of parity, age at first full term pregnancy, and duration of breastfeeding with the risk of breast cancer. Cancer and Steroid Hormone Study Group**. *J Clin Epidemiol* 1989, **42**(10):963-973.

14. **Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease**. *Lancet* 2001, **358**(9291):1389-1399.
15. Venkitaraman AR: **Cancer susceptibility and the functions of BRCA1 and BRCA2**. *Cell* 2002, **108**(2):171-182.
16. Yang H, Jeffrey PD, Miller J, Kinnucan E, Sun Y, Thoma NH, Zheng N, Chen PL, Lee WH, Pavletich NP: **BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure**. *Science* 2002, **297**(5588):1837-1848.
17. King MC, Marks JH, Mandell JB: **Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2**. *Science* 2003, **302**(5645):643-646.
18. Lux MP, Fasching PA, Beckmann MW: **Hereditary breast and ovarian cancer: review and future perspectives**. *J Mol Med* 2006, **84**(1):16-28.
19. Esteller M, Herman JG: **Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours**. *J Pathol* 2002, **196**(1):1-7.
20. Lakhani SR, Jacquemier J, Sloane JP, Gusterson BA, Anderson TJ, van de Vijver MJ, Farid LM, Venter D, Antoniou A, Storfer-Isser A *et al*: **Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations**. *J Natl Cancer Inst* 1998, **90**(15):1138-1145.
21. Foulkes WD, Stefansson IM, Chappuis PO, Begin LR, Goffin JR, Wong N, Trudel M, Akslen LA: **Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer**. *J Natl Cancer Inst* 2003, **95**(19):1482-1485.
22. Honrado E, Benitez J, Palacios J: **Histopathology of BRCA1- and BRCA2-associated breast cancer**. *Crit Rev Oncol Hematol* 2006, **59**(1):27-39.
23. Hedenfalk I, Duggan D, Chen Y, Radmacher M, Bittner M, Simon R, Meltzer P, Gusterson B, Esteller M, Kallioniemi OP *et al*: **Gene-expression profiles in hereditary breast cancer**. *N Engl J Med* 2001, **344**(8):539-548.
24. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT *et al*: **Gene expression profiling predicts clinical outcome of breast cancer**. *Nature* 2002, **415**(6871):530-536.
25. Thorpe SM, Rose C, Rasmussen BB, Mouridsen HT, Bayer T, Keiding N: **Prognostic value of steroid hormone receptors: multivariate analysis of systemically untreated patients with node negative primary breast cancer**. *Cancer Res* 1987, **47**(22):6126-6133.
26. Williams MR, Todd JH, Ellis IO, Dowle CS, Haybittle JL, Elston CW, Nicholson RI, Griffiths K, Blamey RW: **Oestrogen receptors in primary and advanced breast cancer: an eight year review of 704 cases**. *Br J Cancer* 1987, **55**(1):67-73.
27. Nomura Y, Miura S, Koyama H, Enomoto K, Kasumi F, Yamamoto H, Kimura M, Tominaga T, Iino H, Morimoto T *et al*: **Relative effect of steroid hormone receptors on the prognosis of patients with operable breast cancer. A univariate and multivariate analysis of 3089 Japanese patients with breast cancer from the Study Group for the Japanese Breast Cancer Society on Hormone Receptors and Prognosis in Breast Cancer**. *Cancer* 1992, **69**(1):153-164.
28. Yager JD, Davidson NE: **Estrogen carcinogenesis in breast cancer**. *N Engl J Med* 2006, **354**(3):270-282.
29. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA: **Mechanisms of estrogen action**. *Physiol Rev* 2001, **81**(4):1535-1565.
30. Pasqualini JR: **The selective estrogen enzyme modulators in breast cancer: a review**. *Biochim Biophys Acta* 2004, **1654**(2):123-143.
31. O'Lone R, Frith MC, Karlsson EK, Hansen U: **Genomic targets of nuclear estrogen receptors**. *Mol Endocrinol* 2004, **18**(8):1859-1875.

32. Levin ER: **Cellular functions of plasma membrane estrogen receptors**. *Steroids* 2002, **67**(6):471-475.
33. Liao JK: **Cross-coupling between the oestrogen receptor and phosphoinositide 3-kinase**. *Biochem Soc Trans* 2003, **31**(Pt 1):66-70.
34. Losel R, Wehling M: **Nongenomic actions of steroid hormones**. *Nat Rev Mol Cell Biol* 2003, **4**(1):46-56.
35. Bjornstrom L, Sjoberg M: **Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes**. *Mol Endocrinol* 2005, **19**(4):833-842.
36. Miller K: **Estrogen and DNA damage: the silent source of breast cancer?** *J Natl Cancer Inst* 2003, **95**(2):100-102.
37. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, Maggi A, Muramatsu M, Parker MG, Gustafsson JA: **International Union of Pharmacology. LXIV. Estrogen receptors**. *Pharmacol Rev* 2006, **58**(4):773-781.
38. Gotteland M, Desauty G, Delarue JC, Liu L, May E: **Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues**. *Mol Cell Endocrinol* 1995, **112**(1):1-13.
39. Poola I, Speirs V: **Expression of alternatively spliced estrogen receptor alpha mRNAs is increased in breast cancer tissues**. *J Steroid Biochem Mol Biol* 2001, **78**(5):459-469.
40. Jensen EV, Jordan VC: **The estrogen receptor: a model for molecular medicine**. *Clin Cancer Res* 2003, **9**(6):1980-1989.
41. Yamamoto T, Nishida T, Miyajima N, Kawai S, Ooi T, Toyoshima K: **The erbB gene of avian erythroblastosis virus is a member of the src gene family**. *Cell* 1983, **35**(1):71-78.
42. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: **Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences**. *Nature* 1984, **307**(5951):521-527.
43. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U *et al*: **Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene**. *Science* 1985, **230**(4730):1132-1139.
44. King CR, Kraus MH, Aaronson SA: **Amplification of a novel v-erbB-related gene in a human mammary carcinoma**. *Science* 1985, **229**(4717):974-976.
45. Semba K, Kamata N, Toyoshima K, Yamamoto T: **A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma**. *Proc Natl Acad Sci U S A* 1985, **82**(19):6497-6501.
46. Ogiso H, Ishitani R, Nureki O, Fukai S, Yamanaka M, Kim JH, Saito K, Sakamoto A, Inoue M, Shirouzu M *et al*: **Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains**. *Cell* 2002, **110**(6):775-787.
47. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Zhu HJ, Walker F, Frenkel MJ, Hoyne PA *et al*: **Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha**. *Cell* 2002, **110**(6):763-773.
48. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW *et al*: **The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors**. *Mol Cell* 2003, **11**(2):495-505.
49. Olayioye MA, Neve RM, Lane HA, Hynes NE: **The ErbB signaling network: receptor heterodimerization in development and cancer**. *Embo J* 2000, **19**(13):3159-3167.
50. Soltoff SP, Cantley LC: **p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other**

cells. *J Biol Chem* 1996, **271**(1):563-567.
51. Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, Lavi S, Seger R, Ratzkin BJ, Sela M *et al*: **Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions**. *Embo J* 1996, **15**(10):2452-2467.
52. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: **Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene**. *Science* 1987, **235**(4785):177-182.
53. Paterson MC, Dietrich KD, Danyluk J, Paterson AH, Lees AW, Jamil N, Hanson J, Jenkins H, Krause BE, McBlain WA *et al*: **Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer**. *Cancer Res* 1991, **51**(2):556-567.
54. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ: **Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005**. *Ann Oncol* 2005, **16**(10):1569-1583.
55. Stephens P, Hunter C, Bignell G, Edkins S, Davies H, Teague J, Stevens C, O'Meara S, Smith R, Parker A *et al*: **Lung cancer: intragenic ERBB2 kinase mutations in tumours**. *Nature* 2004, **431**(7008):525-526.
56. Badache A, Goncalves A: **The ErbB2 signaling network as a target for breast cancer therapy**. *J Mammary Gland Biol Neoplasia* 2006, **11**(1):13-25.
57. Holbro T, Hynes NE: **ErbB receptors: directing key signaling networks throughout life**. *Annu Rev Pharmacol Toxicol* 2004, **44**:195-217.
58. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI: **Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies**. *Cell* 1985, **41**(3):697-706.
59. Austin CD, De Maziere AM, Pisacane PI, van Dijk SM, Eigenbrot C, Sliwkowski MX, Klumperman J, Scheller RH: **Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin**. *Mol Biol Cell* 2004, **15**(12):5268-5282.
60. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT *et al*: **PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients**. *Cancer Cell* 2004, **6**(2):117-127.
61. Crowder RJ, Lombardi DP, Ellis MJ: **Successful targeting of ErbB2 receptors-is PTEN the key?** *Cancer Cell* 2004, **6**(2):103-104.
62. Taylor-Papadimitriou J, Stampfer M, Bartek J, Lewis A, Boshell M, Lane EB, Leigh IM: **Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium**. *J Cell Sci* 1989, **94 (Pt 3)**:403-413.
63. Ronnov-Jessen L, Petersen OW, Bissell MJ: **Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction**. *Physiol Rev* 1996, **76**(1):69-125.
64. Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS: **Progesterone receptors as a prognostic factor in Stage II breast cancer**. *N Engl J Med* 1983, **309**(22):1343-1347.
65. Gelbfish GA, Davidson AL, Kopel S, Schreibman B, Gelbfish JS, Degenshein GA, Herz BL, Cunningham JN: **Relationship of estrogen and progesterone receptors to prognosis in breast cancer**. *Ann Surg* 1988, **207**(1):75-79.
66. Stonelake PS, Baker PG, Gillespie WM, Dunn JA, Spooner D, Morrison JM, Bundred NJ, Oates GD, Lee MJ, Neoptolemos JP *et al*: **Steroid receptors, pS2 and cathepsin D in early clinically node-negative breast cancer**. *Eur J Cancer* 1994, **30A**(1):5-11.

67. Cleator S, Heller W, Coombes RC: **Triple-negative breast cancer: therapeutic options**. *Lancet Oncol* 2007, **8**(3):235-244.
68. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA *et al*: **Molecular portraits of human breast tumours**. *Nature* 2000, **406**(6797):747-752.
69. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS *et al*: **Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications**. *Proc Natl Acad Sci U S A* 2001, **98**(19):10869-10874.
70. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S *et al*: **Repeated observation of breast tumor subtypes in independent gene expression data sets**. *Proc Natl Acad Sci U S A* 2003, **100**(14):8418-8423.
71. Vargo-Gogola T, Rosen JM: **Modelling breast cancer: one size does not fit all**. *Nat Rev Cancer* 2007, **7**(9):659-672.
72. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F *et al*: **A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes**. *Cancer Cell* 2006, **10**(6):515-527.
73. Sharma RP, Chopra VL: **Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster**. *Dev Biol* 1976, **48**(2):461-465.
74. Nusslein-Volhard C, Wieschaus E: **Mutations affecting segment number and polarity in Drosophila**. *Nature* 1980, **287**(5785):795-801.
75. Faschinger A, Rouault F, Sollner J, Lukas A, Salmons B, Gunzburg WH, Indik S: **Mouse mammary tumor virus integration site selection in human and mouse genomes**. *J Virol* 2008, **82**(3):1360-1367.
76. Nusse R, Varmus HE: **Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome**. *Cell* 1982, **31**(1):99-109.
77. Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R: **The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless**. *Cell* 1987, **50**(4):649-657.
78. Kikuchi A, Yamamoto H, Kishida S: **Multiplicity of the interactions of Wnt proteins and their receptors**. *Cell Signal* 2007, **19**(4):659-671.
79. van Amerongen R, Berns A: **Knockout mouse models to study Wnt signal transduction**. *Trends Genet* 2006, **22**(12):678-689.
80. Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R: **A new member of the frizzled family from Drosophila functions as a Wingless receptor**. *Nature* 1996, **382**(6588):225-230.
81. Reichsman F, Smith L, Cumberledge S: **Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction**. *J Cell Biol* 1996, **135**(3):819-827.
82. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR, 3rd, Nusse R: **Wnt proteins are lipid-modified and can act as stem cell growth factors**. *Nature* 2003, **423**(6938):448-452.
83. Kadowaki T, Wilder E, Klingensmith J, Zachary K, Perrimon N: **The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing**. *Genes Dev* 1996, **10**(24):3116-3128.
84. Hausmann G, Banziger C, Basler K: **Helping Wingless take flight: how WNT proteins are secreted**. *Nat Rev Mol Cell Biol* 2007, **8**(4):331-336.
85. Tanaka K, Kitagawa Y, Kadowaki T: **Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the**

endoplasmic reticulum. *J Biol Chem* 2002, **277**(15):12816-12823.
86. Kusserow A, Pang K, Sturm C, Hrouda M, Lentfer J, Schmidt HA, Technau U, von Haeseler A, Hobmayer B, Martindale MQ *et al*: **Unexpected complexity of the Wnt gene family in a sea anemone**. *Nature* 2005, **433**(7022):156-160.
87. Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ: **Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains**. *Nature* 2001, **412**(6842):86-90.
88. Carron C, Pascal A, Djiane A, Boucaut JC, Shi DL, Umbhauer M: **Frizzled receptor dimerization is sufficient to activate the Wnt/beta-catenin pathway**. *J Cell Sci* 2003, **116**(Pt 12):2541-2550.
89. Kaykas A, Yang-Snyder J, Heroux M, Shah KV, Bouvier M, Moon RT: **Mutant Frizzled 4 associated with vitreoretinopathy traps wild-type Frizzled in the endoplasmic reticulum by oligomerization**. *Nat Cell Biol* 2004, **6**(1):52-58.
90. Wong HC, Bourdelas A, Krauss A, Lee HJ, Shao Y, Wu D, Mlodzik M, Shi DL, Zheng J: **Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled**. *Mol Cell* 2003, **12**(5):1251-1260.
91. Saitoh T, Hirai M, Katoh M: **Molecular cloning and characterization of human Frizzled-5 gene on chromosome 2q33.3-q34 region**. *Int J Oncol* 2001, **19**(1):105-110.
92. Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, Vaizel-Ohayon D, Schejter E, Tomlinson A, DiNardo S: **arrow encodes an LDL-receptor-related protein essential for Wingless signalling**. *Nature* 2000, **407**(6803):527-530.
93. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X: **LDL-receptor-related proteins in Wnt signal transduction**. *Nature* 2000, **407**(6803):530-535.
94. Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C *et al*: **Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling**. *Nature* 2002, **417**(6889):664-667.
95. Forrester WC, Dell M, Perens E, Garriga G: **A C. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division**. *Nature* 1999, **400**(6747):881-885.
96. Mikels AJ, Nusse R: **Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context**. *PLoS Biol* 2006, **4**(4):e115.
97. Oishi I, Suzuki H, Onishi N, Takada R, Kani S, Ohkawara B, Koshida I, Suzuki K, Yamada G, Schwabe GC *et al*: **The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway**. *Genes Cells* 2003, **8**(7):645-654.
98. Nishita M, Yoo SK, Nomachi A, Kani S, Sougawa N, Ohta Y, Takada S, Kikuchi A, Minami Y: **Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration**. *J Cell Biol* 2006, **175**(4):555-562.
99. Park M, Moon RT: **The planar cell-polarity gene stbm regulates cell behaviour and cell fate in vertebrate embryos**. *Nat Cell Biol* 2002, **4**(1):20-25.
100. Lu W, Yamamoto V, Ortega B, Baltimore D: **Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth**. *Cell* 2004, **119**(1):97-108.
101. Clevers H: **Wnt/beta-catenin signaling in development and disease**. *Cell* 2006, **127**(3):469-480.
102. McMahon AP, Moon RT: **Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis**. *Cell* 1989, **58**(6):1075-1084.
103. Cong F, Schweizer L, Varmus H: **Casein kinase Iepsilon modulates the signaling specificities of dishevelled**. *Mol Cell Biol* 2004, **24**(5):2000-2011.
104. Willert K, Brink M, Wodarz A, Varmus H, Nusse R: **Casein kinase 2 associates with and phosphorylates dishevelled**. *Embo J* 1997, **16**(11):3089-3096.
105. Kuhl M, Geis K, Sheldahl LC, Pukrop T, Moon RT, Wedlich D: **Antagonistic regulation**

of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signaling. *Mech Dev* 2001, **106**(1-2):61-76.
106. Gonzalez-Sancho JM, Brennan KR, Castelo-Soccio LA, Brown AM: **Wnt proteins induce dishevelled phosphorylation via an LRP5/6- independent mechanism, irrespective of their ability to stabilize beta-catenin**. *Mol Cell Biol* 2004, **24**(11):4757-4768.
107. Sheldahl LC, Slusarski DC, Pandur P, Miller JR, Kuhl M, Moon RT: **Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos**. *J Cell Biol* 2003, **161**(4):769-777.
108. Eastman Q, Grosschedl R: **Regulation of LEF-1/TCF transcription factors by Wnt and other signals**. *Curr Opin Cell Biol* 1999, **11**(2):233-240.
109. Tetsu O, McCormick F: **Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells**. *Nature* 1999, **398**(6726):422-426.
110. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A: **The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway**. *Proc Natl Acad Sci U S A* 1999, **96**(10):5522-5527.
111. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: **Identification of c-MYC as a target of the APC pathway**. *Science* 1998, **281**(5382):1509-1512.
112. Giles RH, van Es JH, Clevers H: **Caught up in a Wnt storm: Wnt signaling in cancer**. *Biochim Biophys Acta* 2003, **1653**(1):1-24.
113. Reya T, Clevers H: **Wnt signalling in stem cells and cancer**. *Nature* 2005, **434**(7035):843-850.
114. Huelsken J, Behrens J: **The Wnt signalling pathway**. *J Cell Sci* 2002, **115**(Pt 21):3977-3978.
115. Habas R, Dawid IB: **Dishevelled and Wnt signaling: is the nucleus the final frontier?** *J Biol* 2005, **4**(1):2.
116. Heisenberg CP, Tada M: **Wnt signalling: a moving picture emerges from van gogh**. *Curr Biol* 2002, **12**(4):R126-128.
117. Kohn AD, Moon RT: **Wnt and calcium signaling: beta-catenin-independent pathways**. *Cell Calcium* 2005, **38**(3-4):439-446.
118. Uren A, Reichsman F, Anest V, Taylor WG, Muraiso K, Bottaro DP, Cumberledge S, Rubin JS: **Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling**. *J Biol Chem* 2000, **275**(6):4374-4382.
119. Kawano Y, Kypta R: **Secreted antagonists of the Wnt signalling pathway**. *J Cell Sci* 2003, **116**(Pt 13):2627-2634.
120. Jones SE, Jomary C: **Secreted Frizzled-related proteins: searching for relationships and patterns**. *Bioessays* 2002, **24**(9):811-820.
121. Leyns L, Bouwmeester T, Kim SH, Piccolo S, De Robertis EM: **Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer**. *Cell* 1997, **88**(6):747-756.
122. Wang S, Krinks M, Lin K, Luyten FP, Moos M, Jr.: **Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8**. *Cell* 1997, **88**(6):757-766.
123. Finch PW, He X, Kelley MJ, Uren A, Schaudies RP, Popescu NC, Rudikoff S, Aaronson SA, Varmus HE, Rubin JS: **Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action**. *Proc Natl Acad Sci U S A* 1997, **94**(13):6770-6775.
124. Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA: **Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling**. *J Biol Chem* 1999, **274**(23):16180-16187.

125. Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J: **A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors**. *Proc Natl Acad Sci U S A* 1997, **94**(7):2859-2863.
126. Lin K, Wang S, Julius MA, Kitajewski J, Moos M, Jr., Luyten FP: **The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling**. *Proc Natl Acad Sci U S A* 1997, **94**(21):11196-11200.
127. Caldwell GM, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, Chughtai S, Wallis Y, Matthews GM, Morton DG: **The Wnt antagonist sFRP1 in colorectal tumorigenesis**. *Cancer Res* 2004, **64**(3):883-888.
128. Chong JM, Uren A, Rubin JS, Speicher DW: **Disulfide bond assignments of secreted Frizzled-related protein-1 provide insights about Frizzled homology and netrin modules**. *J Biol Chem* 2002, **277**(7):5134-5144.
129. Dennis S, Aikawa M, Szeto W, d'Amore PA, Papkoff J: **A secreted frizzled related protein, FrzA, selectively associates with Wnt-1 protein and regulates wnt-1 signaling**. *J Cell Sci* 1999, **112 (Pt 21)**:3815-3820.
130. Xu Q, D'Amore PA, Sokol SY: **Functional and biochemical interactions of Wnts with FrzA, a secreted Wnt antagonist**. *Development* 1998, **125**(23):4767-4776.
131. Jaspard B, Couffinhal T, Dufourcq P, Moreau C, Duplaa C: **Expression pattern of mouse sFRP-1 and mWnt-8 gene during heart morphogenesis**. *Mech Dev* 2000, **90**(2):263-267.
132. Hussain SZ, Sneddon T, Tan X, Micsenyi A, Michalopoulos GK, Monga SP: **Wnt impacts growth and differentiation in ex vivo liver development**. *Exp Cell Res* 2004, **292**(1):157-169.
133. Yoshino K, Rubin JS, Higinbotham KG, Uren A, Anest V, Plisov SY, Perantoni AO: **Secreted Frizzled-related proteins can regulate metanephric development**. *Mech Dev* 2001, **102**(1-2):45-55.
134. Barandon L, Couffinhal T, Ezan J, Dufourcq P, Costet P, Alzieu P, Leroux L, Moreau C, Dare D, Duplaa C: **Reduction of infarct size and prevention of cardiac rupture in transgenic mice overexpressing FrzA**. *Circulation* 2003, **108**(18):2282-2289.
135. Roth W, Wild-Bode C, Platten M, Grimmel C, Melkonyan HS, Dichgans J, Weller M: **Secreted Frizzled-related proteins inhibit motility and promote growth of human malignant glioma cells**. *Oncogene* 2000, **19**(37):4210-4220.
136. Dufourcq P, Couffinhal T, Ezan J, Barandon L, Moreau C, Daret D, Duplaa C: **FrzA, a secreted frizzled related protein, induced angiogenic response**. *Circulation* 2002, **106**(24):3097-3103.
137. Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, Nusse R, Dawid IB, Nathans J: **A new secreted protein that binds to Wnt proteins and inhibits their activities**. *Nature* 1999, **398**(6726):431-436.
138. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C: **Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction**. *Nature* 1998, **391**(6665):357-362.
139. Krupnik VE, Sharp JD, Jiang C, Robison K, Chickering TW, Amaravadi L, Brown DE, Guyot D, Mays G, Leiby K *et al*: **Functional and structural diversity of the human Dickkopf gene family**. *Gene* 1999, **238**(2):301-313.
140. Caricasole A, Ferraro T, Iacovelli L, Barletta E, Caruso A, Melchiorri D, Terstappen GC, Nicoletti F: **Functional characterization of WNT7A signaling in PC12 cells: interaction with A FZD5 x LRP6 receptor complex and modulation by Dickkopf proteins**. *J Biol Chem* 2003, **278**(39):37024-37031.
141. Nakamura T, Aoki S, Kitajima K, Takahashi T, Matsumoto K, Nakamura T: **Molecular cloning and characterization of Kremen, a novel kringle-containing transmembrane protein**. *Biochim Biophys Acta* 2001, **1518**(1-2):63-72.

142. Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM: **Transactivation of vimentin by beta-catenin in human breast cancer cells**. *Cancer Res* 2003, **63**(10):2658-2664.
143. Polakis P: **Wnt signaling and cancer**. *Genes Dev* 2000, **14**(15):1837-1851.
144. Kinzler KW, Vogelstein B: **Lessons from hereditary colorectal cancer**. *Cell* 1996, **87**(2):159-170.
145. Bittner JJ: **The Milk-Influence Of Breast Tumors In Mice**. *Science* 1942, **95**(2470):462-463.
146. Nusse R, Varmus HE: **Wnt genes**. *Cell* 1992, **69**(7):1073-1087.
147. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE: **Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice**. *Cell* 1988, **55**(4):619-625.
148. Abraham SC, Reynolds C, Lee JH, Montgomery EA, Baisden BL, Krasinskas AM, Wu TT: **Fibromatosis of the breast and mutations involving the APC/beta-catenin pathway**. *Hum Pathol* 2002, **33**(1):39-46.
149. Kashiwaba M, Tamura G, Ishida M: **Aberrations of the APC gene in primary breast carcinoma**. *J Cancer Res Clin Oncol* 1994, **120**(12):727-731.
150. Candidus S, Bischoff P, Becker KF, Hofler H: **No evidence for mutations in the alpha- and beta-catenin genes in human gastric and breast carcinomas**. *Cancer Res* 1996, **56**(1):49-52.
151. Schlosshauer PW, Brown SA, Eisinger K, Yan Q, Guglielminetti ER, Parsons R, Ellenson LH, Kitajewski J: **APC truncation and increased beta-catenin levels in a human breast cancer cell line**. *Carcinogenesis* 2000, **21**(7):1453-1456.
152. Jonsson M, Borg A, Nilbert M, Andersson T: **Involvement of adenomatous polyposis coli (APC)/beta-catenin signalling in human breast cancer**. *Eur J Cancer* 2000, **36**(2):242-248.
153. Howe LR, Brown AM: **Wnt signaling and breast cancer**. *Cancer Biol Ther* 2004, **3**(1):36-41.
154. Ayyanan A, Civenni G, Ciarloni L, Morel C, Mueller N, Lefort K, Mandinova A, Raffoul W, Fiche M, Dotto GP *et al*: **Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism**. *Proc Natl Acad Sci U S A* 2006, **103**(10):3799-3804.
155. Benhaj K, Akcali KC, Ozturk M: **Redundant expression of canonical Wnt ligands in human breast cancer cell lines**. *Oncol Rep* 2006, **15**(3):701-707.
156. Milovanovic T, Planutis K, Nguyen A, Marsh JL, Lin F, Hope C, Holcombe RF: **Expression of Wnt genes and frizzled 1 and 2 receptors in normal breast epithelium and infiltrating breast carcinoma**. *Int J Oncol* 2004, **25**(5):1337-1342.
157. Nagahata T, Shimada T, Harada A, Nagai H, Onda M, Yokoyama S, Shiba T, Jin E, Kawanami O, Emi M: **Amplification, up-regulation and over-expression of DVL-1, the human counterpart of the Drosophila disheveled gene, in primary breast cancers**. *Cancer Sci* 2003, **94**(6):515-518.
158. Ryo A, Nakamura M, Wulf G, Liou YC, Lu KP: **Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC**. *Nat Cell Biol* 2001, **3**(9):793-801.
159. Prasad CP, Gupta SD, Rath G, Ralhan R: **Wnt signaling pathway in invasive ductal carcinoma of the breast: relationship between beta-catenin, dishevelled and cyclin D1 expression**. *Oncology* 2007, **73**(1-2):112-117.
160. Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, Pestell RG, Hung MC: **Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression**. *Proc Natl Acad Sci U S A* 2000, **97**(8):4262-4266.

161. Nakopoulou L, Mylona E, Papadaki I, Kavantzas N, Giannopoulou I, Markaki S, Keramopoulos A: **Study of phospho-beta-catenin subcellular distribution in invasive breast carcinomas in relation to their phenotype and the clinical outcome**. *Mod Pathol* 2006, **19**(4):556-563.
162. Baylin SB, Herman JG: **DNA hypermethylation in tumorigenesis: epigenetics joins genetics**. *Trends Genet* 2000, **16**(4):168-174.
163. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M *et al*: **Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer**. *Nat Genet* 2004, **36**(4):417-422.
164. Takada T, Yagi Y, Maekita T, Imura M, Nakagawa S, Tsao SW, Miyamoto K, Yoshino O, Yasugi T, Taketani Y *et al*: **Methylation-associated silencing of the Wnt antagonist SFRP1 gene in human ovarian cancers**. *Cancer Sci* 2004, **95**(9):741-744.
165. Stoehr R, Wissmann C, Suzuki H, Knuechel R, Krieg RC, Klopocki E, Dahl E, Wild P, Blaszyk H, Sauter G *et al*: **Deletions of chromosome 8p and loss of sFRP1 expression are progression markers of papillary bladder cancer**. *Lab Invest* 2004, **84**(4):465-478.
166. Marsit CJ, Karagas MR, Andrew A, Liu M, Danaee H, Schned AR, Nelson HH, Kelsey KT: **Epigenetic inactivation of SFRP genes and TP53 alteration act jointly as markers of invasive bladder cancer**. *Cancer Res* 2005, **65**(16):7081-7085.
167. Lee AY, He B, You L, Dadfarmay S, Xu Z, Mazieres J, Mikami I, McCormick F, Jablons DM: **Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma**. *Oncogene* 2004, **23**(39):6672-6676.
168. Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H: **Functional epigenomics identifies genes frequently silenced in prostate cancer**. *Cancer Res* 2005, **65**(10):4218-4227.
169. Fukui T, Kondo M, Ito G, Maeda O, Sato N, Yoshioka H, Yokoi K, Ueda Y, Shimokata K, Sekido Y: **Transcriptional silencing of secreted frizzled related protein 1 (SFRP 1) by promoter hypermethylation in non-small-cell lung cancer**. *Oncogene* 2005, **24**(41):6323-6327.
170. Veeck J, Niederacher D, An H, Klopocki E, Wiesmann F, Betz B, Galm O, Camara O, Durst M, Kristiansen G *et al*: **Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis**. *Oncogene* 2006, **25**(24):3479-3488.
171. Ugolini F, Adelaide J, Charafe-Jauffret E, Nguyen C, Jacquemier J, Jordan B, Birnbaum D, Pebusque MJ: **Differential expression assay of chromosome arm 8p genes identifies Frizzled-related (FRP1/FRZB) and Fibroblast Growth Factor Receptor 1 (FGFR1) as candidate breast cancer genes**. *Oncogene* 1999, **18**(10):1903-1910.
172. Ugolini F, Charafe-Jauffret E, Bardou VJ, Geneix J, Adelaide J, Labat-Moleur F, Penault-Llorca F, Longy M, Jacquemier J, Birnbaum D *et al*: **WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type**. *Oncogene* 2001, **20**(41):5810-5817.
173. Klopocki E, Kristiansen G, Wild PJ, Klaman I, Castanos-Velez E, Singer G, Stohr R, Simon R, Sauter G, Leibiger H *et al*: **Loss of SFRP1 is associated with breast cancer progression and poor prognosis in early stage tumors**. *Int J Oncol* 2004, **25**(3):641-649.
174. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB: **A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer**. *Nat Genet* 2002, **31**(2):141-149.
175. Suzuki H, Toyota M, Caraway H, Gabrielson E, Ohmura T, Fujikane T, Nishikawa N, Sogabe Y, Nojima M, Sonoda T *et al*: **Frequent epigenetic inactivation of Wnt antagonist**

genes in breast cancer. *Br J Cancer* 2008, **98**(6):1147-1156.
176. Shulewitz M, Soloviev I, Wu T, Koeppen H, Polakis P, Sakanaka C: **Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer**. *Oncogene* 2006, **25**(31):4361-4369.
177. Schlange T, Matsuda Y, Lienhard S, Huber A, Hynes NE: **Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation**. *Breast Cancer Res* 2007, **9**(5):R63.
178. van Amerongen R, Mikels A, Nusse R: **Alternative wnt signaling is initiated by distinct receptors**. *Sci Signal* 2008, **1**(35):re9.
179. Katoh M, Katoh M: **WNT signaling pathway and stem cell signaling network**. *Clin Cancer Res* 2007, **13**(14):4042-4045.
180. Huguet EL, McMahon JA, McMahon AP, Bicknell R, Harris AL: **Differential expression of human Wnt genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue**. *Cancer Res* 1994, **54**(10):2615-2621.
181. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA: **An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells**. *Cancer Cell* 2004, **6**(5):497-506.
182. Raftopoulou M, Hall A: **Cell migration: Rho GTPases lead the way**. *Dev Biol* 2004, **265**(1):23-32.
183. Habas R, Kato Y, He X: **Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1**. *Cell* 2001, **107**(7):843-854.
184. Endo Y, Wolf V, Muraiso K, Kamijo K, Soon L, Uren A, Barshishat-Kupper M, Rubin JS: **Wnt-3a-dependent cell motility involves RhoA activation and is specifically regulated by dishevelled-2**. *J Biol Chem* 2005, **280**(1):777-786.
185. Lee JS, Ishimoto A, Yanagawa S: **Characterization of mouse dishevelled (Dvl) proteins in Wnt/Wingless signaling pathway**. *J Biol Chem* 1999, **274**(30):21464-21470.
186. Kozma SC, Bogaard ME, Buser K, Saurer SM, Bos JL, Groner B, Hynes NE: **The human c-Kirsten ras gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231**. *Nucleic Acids Res* 1987, **15**(15):5963-5971.
187. Ezan J, Leroux L, Barandon L, Dufourcq P, Jaspard B, Moreau C, Allieres C, Daret D, Couffinhal T, Duplaa C: **FrzA/sFRP-1, a secreted antagonist of the Wnt-Frizzled pathway, controls vascular cell proliferation in vitro and in vivo**. *Cardiovasc Res* 2004, **63**(4):731-738.
188. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM: **Openings between defective endothelial cells explain tumor vessel leakiness**. *Am J Pathol* 2000, **156**(4):1363-1380.
189. Cappellen D, Schlange T, Bauer M, Maurer F, Hynes NE: **Novel c-MYC target genes mediate differential effects on cell proliferation and migration**. *EMBO Rep* 2007, **8**(1):70-76.
190. Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR, Vass JK, Athineos D, Clevers H, Clarke AR: **Myc deletion rescues Apc deficiency in the small intestine**. *Nature* 2007, **446**(7136):676-679.
191. Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei LD, Umansky SR: **SARPs: a family of secreted apoptosis-related proteins**. *Proc Natl Acad Sci U S A* 1997, **94**(25):13636-13641.
192. Hoang B, Moos M, Jr., Vukicevic S, Luyten FP: **Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis**. *J Biol Chem* 1996, **271**(42):26131-26137.
193. Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: **Epigenetic suppression of secreted frizzled related protein 1 (SFRP1) expression in**

human breast cancer. *Cancer Biol Ther* 2006, **5**(3):281-286.
194. Reya T, Morrison SJ, Clarke MF, Weissman IL: **Stem cells, cancer, and cancer stem cells**. *Nature* 2001, **414**(6859):105-111.
195. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG: **Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties**. *Cancer Res* 2005, **65**(13):5506-5511.
196. Kondo T, Setoguchi T, Taga T: **Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line**. *Proc Natl Acad Sci U S A* 2004, **101**(3):781-786.
197. Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG: **Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic**. *Cancer Res* 2005, **65**(14):6207-6219.
198. Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A *et al*: **Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML**. *N Engl J Med* 2004, **351**(7):657-667.
199. Brabletz T, Hlubek F, Spaderna S, Schmalhofer O, Hiendlmeyer E, Jung A, Kirchner T: **Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin**. *Cells Tissues Organs* 2005, **179**(1-2):56-65.
200. He B, Barg RN, You L, Xu Z, Reguart N, Mikami I, Batra S, Rosell R, Jablons DM: **Wnt signaling in stem cells and non-small-cell lung cancer**. *Clin Lung Cancer* 2005, **7**(1):54-60.
201. Mishra L, Shetty K, Tang Y, Stuart A, Byers SW: **The role of TGF-beta and Wnt signaling in gastrointestinal stem cells and cancer**. *Oncogene* 2005, **24**(37):5775-5789.
202. Yardy GW, Brewster SF: **Wnt signalling and prostate cancer**. *Prostate Cancer Prostatic Dis* 2005, **8**(2):119-126.
203. Chen MS, Woodward WA, Behbod F, Peddibhotla S, Alfaro MP, Buchholz TA, Rosen JM: **Wnt/beta-catenin mediates radiation resistance of Sca1+ progenitors in an immortalized mammary gland cell line**. *J Cell Sci* 2007, **120**(Pt 3):468-477.
204. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells**. *Proc Natl Acad Sci U S A* 2003, **100**(7):3983-3988.
205. Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ: **CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells**. *Breast Cancer Res* 2006, **8**(1):R7.
206. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M *et al*: **The epithelial-mesenchymal transition generates cells with properties of stem cells**. *Cell* 2008, **133**(4):704-715.
207. Liao MJ, Zhang CC, Zhou B, Zimonjic DB, Mani SA, Kaba M, Gifford A, Reinhardt F, Popescu NC, Guo W *et al*: **Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity**. *Cancer Res* 2007, **67**(17):8131-8138.
208. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW, Suri P, Wicha MS: **Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells**. *Cancer Res* 2006, **66**(12):6063-6071.
209. Moraes RC, Zhang X, Harrington N, Fung JY, Wu MF, Hilsenbeck SG, Allred DC, Lewis MT: **Constitutive activation of smoothened (SMO) in mammary glands of transgenic mice leads to increased proliferation, altered differentiation and ductal dysplasia**. *Development* 2007, **134**(6):1231-1242.
210. Wielenga VJ, Smits R, Korinek V, Smit L, Kielman M, Fodde R, Clevers H, Pals ST: **Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT**

pathway. *Am J Pathol* 1999, **154**(2):515-523.
211. Honeth G, Bendahl PO, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K, Grabau D, Ferno M, Borg A, Hegardt C: **The CD44+/CD24- phenotype is enriched in basal-like breast tumors**. *Breast Cancer Res* 2008, **10**(3):R53.
212. Jones PH, Harper S, Watt FM: **Stem cell patterning and fate in human epidermis**. *Cell* 1995, **80**(1):83-93.
213. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE: **Generation of a functional mammary gland from a single stem cell**. *Nature* 2006, **439**(7072):84-88.
214. Takahashi K, Yamanaka S: **Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors**. *Cell* 2006, **126**(4):663-676.
215. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG *et al*: **Core transcriptional regulatory circuitry in human embryonic stem cells**. *Cell* 2005, **122**(6):947-956.
216. Fong H, Hohenstein KA, Donovan PJ: **Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells**. *Stem Cells* 2008, **26**(8):1931-1938.
217. Van Raay TJ, Moore KB, Iordanova I, Steele M, Jamrich M, Harris WA, Vetter ML: **Frizzled 5 signaling governs the neural potential of progenitors in the developing Xenopus retina**. *Neuron* 2005, **46**(1):23-36.
218. Okubo T, Pevny LH, Hogan BL: **Sox2 is required for development of taste bud sensory cells**. *Genes Dev* 2006, **20**(19):2654-2659.
219. Uchikawa M, Ishida Y, Takemoto T, Kamachi Y, Kondoh H: **Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals**. *Dev Cell* 2003, **4**(4):509-519.
220. Takemoto T, Uchikawa M, Kamachi Y, Kondoh H: **Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1**. *Development* 2006, **133**(2):297-306.
221. Rodriguez-Pinilla SM, Sarrio D, Moreno-Bueno G, Rodriguez-Gil Y, Martinez MA, Hernandez L, Hardisson D, Reis-Filho JS, Palacios J: **Sox2: a possible driver of the basal-like phenotype in sporadic breast cancer**. *Mod Pathol* 2007, **20**(4):474-481.
222. Dean M, Fojo T, Bates S: **Tumour stem cells and drug resistance**. *Nat Rev Cancer* 2005, **5**(4):275-284.
223. Garvey MI, Piddock LJ: **The efflux pump inhibitor reserpine selects multidrug-resistant Streptococcus pneumoniae strains that overexpress the ABC transporters PatA and PatB**. *Antimicrob Agents Chemother* 2008, **52**(5):1677-1685.
224. Ahmed M, Borsch CM, Neyfakh AA, Schuldiner S: **Mutants of the Bacillus subtilis multidrug transporter Bmr with altered sensitivity to the antihypertensive alkaloid reserpine**. *J Biol Chem* 1993, **268**(15):11086-11089.
225. Eyler CE, Rich JN: **Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis**. *J Clin Oncol* 2008, **26**(17):2839-2845.
226. Boonstra R, Timmer-Bosscha H, van Echten-Arends J, van der Kolk DM, van den Berg A, de Jong B, Tew KD, Poppema S, de Vries EG: **Mitoxantrone resistance in a small cell lung cancer cell line is associated with ABCA2 upregulation**. *Br J Cancer* 2004, **90**(12):2411-2417.
227. Laing NM, Belinsky MG, Kruh GD, Bell DW, Boyd JT, Barone L, Testa JR, Tew KD: **Amplification of the ATP-binding cassette 2 transporter gene is functionally linked with enhanced efflux of estramustine in ovarian carcinoma cells**. *Cancer Res* 1998, **58**(7):1332-1337.
228. Vulevic B, Chen Z, Boyd JT, Davis W, Jr., Walsh ES, Belinsky MG, Tew KD: **Cloning and characterization of human adenosine 5'-triphosphate-binding cassette, sub-family A,**

transporter 2 (ABCA2). *Cancer Res* 2001, **61**(8):3339-3347.
229. Takeichi M: **Morphogenetic roles of classic cadherins**. *Curr Opin Cell Biol* 1995, **7**(5):619-627.
230. Suzuki ST: **Structural and functional diversity of cadherin superfamily: are new members of cadherin superfamily involved in signal transduction pathway?** *J Cell Biochem* 1996, **61**(4):531-542.
231. Gallin WJ: **Evolution of the "classical" cadherin family of cell adhesion molecules in vertebrates**. *Mol Biol Evol* 1998, **15**(9):1099-1107.
232. Steinberg MS, McNutt PM: **Cadherins and their connections: adhesion junctions have broader functions**. *Curr Opin Cell Biol* 1999, **11**(5):554-560.
233. Cowin P, Rowlands TM, Hatsell SJ: **Cadherins and catenins in breast cancer**. *Curr Opin Cell Biol* 2005, **17**(5):499-508.
234. Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, Takeichi M, Hynes RO: **Precocious mammary gland development in P-cadherin-deficient mice**. *J Cell Biol* 1997, **139**(4):1025-1032.
235. Gamallo C, Moreno-Bueno G, Sarrio D, Calero F, Hardisson D, Palacios J: **The prognostic significance of P-cadherin in infiltrating ductal breast carcinoma**. *Mod Pathol* 2001, **14**(7):650-654.
236. Peralta Soler A, Knudsen KA, Salazar H, Han AC, Keshgegian AA: **P-cadherin expression in breast carcinoma indicates poor survival**. *Cancer* 1999, **86**(7):1263-1272.
237. Paredes J, Albergaria A, Oliveira JT, Jeronimo C, Milanezi F, Schmitt FC: **P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation**. *Clin Cancer Res* 2005, **11**(16):5869-5877.
238. Paredes J, Correia AL, Ribeiro AS, Albergaria A, Milanezi F, Schmitt FC: **P-cadherin expression in breast cancer: a review**. *Breast Cancer Res* 2007, **9**(5):214.
239. Takahashi-Yanaga F, Sasaguri T: **The Wnt/beta-catenin signaling pathway as a target in drug discovery**. *J Pharmacol Sci* 2007, **104**(4):293-302.
240. Nusse R, van Ooyen A, Cox D, Fung YK, Varmus H: **Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15**. *Nature* 1984, **307**(5947):131-136.
241. DeAlmeida VI, Miao L, Ernst JA, Koeppen H, Polakis P, Rubinfeld B: **The soluble wnt receptor Frizzled8CRD-hFc inhibits the growth of teratocarcinomas in vivo**. *Cancer Res* 2007, **67**(11):5371-5379.
242. Zi X, Guo Y, Simoneau AR, Hope C, Xie J, Holcombe RF, Hoang BH: **Expression of Frzb/secreted Frizzled-related protein 3, a secreted Wnt antagonist, in human androgen-independent prostate cancer PC-3 cells suppresses tumor growth and cellular invasiveness**. *Cancer Res* 2005, **65**(21):9762-9770.
243. Ridley AJ, Hall A: **The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors**. *Cell* 1992, **70**(3):389-399.
244. Burridge K, Wennerberg K: **Rho and Rac take center stage**. *Cell* 2004, **116**(2):167-179.
245. Wherlock M, Mellor H: **The Rho GTPase family: a Racs to Wrchs story**. *J Cell Sci* 2002, **115**(Pt 2):239-240.
246. Li Y, Hively WP, Varmus HE: **Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer**. *Oncogene* 2000, **19**(8):1002-1009.
247. Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, Sherr CJ: **Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins**. *Cell* 1992, **71**(2):323-334.
248. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G: **Cyclin D1 is a nuclear protein required for cell cycle progression in G1**. *Genes Dev* 1993, **7**(5):812-821.

249. Sansom OJ, Reed KR, van de Wetering M, Muncan V, Winton DJ, Clevers H, Clarke AR: **Cyclin D1 is not an immediate target of beta-catenin following Apc loss in the intestine**. *J Biol Chem* 2005, **280**(31):28463-28467.
250. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP *et al*: **The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells**. *Cell* 2002, **111**(2):241-250.
251. Roovers K, Assoian RK: **Integrating the MAP kinase signal into the G1 phase cell cycle machinery**. *Bioessays* 2000, **22**(9):818-826.
252. Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, Pestell RG: **Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions**. *J Biol Chem* 1995, **270**(40):23589-23597.
253. Brugarolas J, Moberg K, Boyd SD, Taya Y, Jacks T, Lees JA: **Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation**. *Proc Natl Acad Sci U S A* 1999, **96**(3):1002-1007.
254. Waga S, Hannon GJ, Beach D, Stillman B: **The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA**. *Nature* 1994, **369**(6481):574-578.
255. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme MH, Gest J, Callahan R: **Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas**. *Proc Natl Acad Sci U S A* 1986, **83**(13):4834-4838.
256. Cole MD, McMahon SB: **The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation**. *Oncogene* 1999, **18**(19):2916-2924.
257. Dang CV: **c-Myc target genes involved in cell growth, apoptosis, and metabolism**. *Mol Cell Biol* 1999, **19**(1):1-11.
258. Henriksson M, Luscher B: **Proteins of the Myc network: essential regulators of cell growth and differentiation**. *Adv Cancer Res* 1996, **68**:109-182.
259. Luscher B, Larsson LG: **The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncoproteins: function and regulation**. *Oncogene* 1999, **18**(19):2955-2966.
260. Bouchard C, Staller P, Eilers M: **Control of cell proliferation by Myc**. *Trends Cell Biol* 1998, **8**(5):202-206.
261. Amati B, Alevizopoulos K, Vlach J: **Myc and the cell cycle**. *Front Biosci* 1998, **3**:d250-268.
262. Prendergast GC: **Mechanisms of apoptosis by c-Myc**. *Oncogene* 1999, **18**(19):2967-2987.
263. Naidu R, Wahab NA, Yadav M, Kutty MK: **Protein expression and molecular analysis of c-myc gene in primary breast carcinomas using immunohistochemistry and differential polymerase chain reaction**. *Int J Mol Med* 2002, **9**(2):189-196.
264. Chrzan P, Skokowski J, Karmolinski A, Pawelczyk T: **Amplification of c-myc gene and overexpression of c-Myc protein in breast cancer and adjacent non-neoplastic tissue**. *Clin Biochem* 2001, **34**(7):557-562.
265. Nass SJ, Dickson RB: **Defining a role for c-Myc in breast tumorigenesis**. *Breast Cancer Res Treat* 1997, **44**(1):1-22.
266. Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R, Vidaud M: **Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay**. *Cancer Res* 1999, **59**(12):2759-2765.
267. Perez-Roger I, Kim SH, Griffiths B, Sewing A, Land H: **Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1)**. *Embo J* 1999, **18**(19):5310-5320.
268. Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, Reed S, Sicinski P, Bartek J, Eilers M: **Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27**. *Embo J* 1999, **18**(19):5321-5333.

269. Mitchell KO, El-Deiry WS: **Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells**. *Cell Growth Differ* 1999, **10**(4):223-230.
270. Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR: **Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion**. *Proc Natl Acad Sci U S A* 2000, **97**(7):3260-3265.
271. Gartel AL, Ye X, Goufman E, Shianov P, Hay N, Najmabadi F, Tyner AL: **Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3**. *Proc Natl Acad Sci U S A* 2001, **98**(8):4510-4515.
272. Musgrove EA, Sergio CM, Loi S, Inman CK, Anderson LR, Alles MC, Pinese M, Caldon CE, Schutte J, Gardiner-Garden M *et al*: **Identification of functional networks of estrogen- and c-Myc-responsive genes and their relationship to response to tamoxifen therapy in breast cancer**. *PLoS ONE* 2008, **3**(8):e2987.
273. Li N, Zhang Y, Naylor MJ, Schatzmann F, Maurer F, Wintermantel T, Schuetz G, Mueller U, Streuli CH, Hynes NE: **Beta1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli**. *Embo J* 2005, **24**(11):1942-1953.
274. Han S, Sidell N, Roman J: **Fibronectin stimulates human lung carcinoma cell proliferation by suppressing p21 gene expression via signals involving Erk and Rho kinase**. *Cancer Lett* 2005, **219**(1):71-81.
275. Charafe-Jauffret E, Monville F, Ginestier C, Dontu G, Birnbaum D, Wicha MS: **Cancer stem cells in breast: current opinion and future challenges**. *Pathobiology* 2008, **75**(2):75-84.
276. Huber MA, Kraut N, Beug H: **Molecular requirements for epithelial-mesenchymal transition during tumor progression**. *Curr Opin Cell Biol* 2005, **17**(5):548-558.
277. Thiery JP, Sleeman JP: **Complex networks orchestrate epithelial-mesenchymal transitions**. *Nat Rev Mol Cell Biol* 2006, **7**(2):131-142.
278. Hendrix MJ, Seftor EA, Chu YW, Trevor KT, Seftor RE: **Role of intermediate filaments in migration, invasion and metastasis**. *Cancer Metastasis Rev* 1996, **15**(4):507-525.
279. Shook D, Keller R: **Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development**. *Mech Dev* 2003, **120**(11):1351-1383.
280. Steinert PM, Roop DR: **Molecular and cellular biology of intermediate filaments**. *Annu Rev Biochem* 1988, **57**:593-625.
281. Ramaekers FC, Haag D, Kant A, Moesker O, Jap PH, Vooijs GP: **Coexpression of keratin- and vimentin-type intermediate filaments in human metastatic carcinoma cells**. *Proc Natl Acad Sci U S A* 1983, **80**(9):2618-2622.
282. Guarino M: **Epithelial-to-mesenchymal change of differentiation. From embryogenetic mechanism to pathological patterns**. *Histol Histopathol* 1995, **10**(1):171-184.
283. Gilles C, Polette M, Piette J, Delvigne AC, Thompson EW, Foidart JM, Birembaut P: **Vimentin expression in cervical carcinomas: association with invasive and migratory potential**. *J Pathol* 1996, **180**(2):175-180.
284. Gilles C, Polette M, Zahm JM, Tournier JM, Volders L, Foidart JM, Birembaut P: **Vimentin contributes to human mammary epithelial cell migration**. *J Cell Sci* 1999, **112 (Pt 24)**:4615-4625.
285. Hendrix MJ, Seftor EA, Seftor RE, Trevor KT: **Experimental co-expression of vimentin and keratin intermediate filaments in human breast cancer cells results in phenotypic interconversion and increased invasive behavior**. *Am J Pathol* 1997, **150**(2):483-495.
286. Eckes B, Dogic D, Colucci-Guyon E, Wang N, Maniotis A, Ingber D, Merckling A, Langa F, Aumailley M, Delouvee A *et al*: **Impaired mechanical stability, migration and**

contractile capacity in vimentin-deficient fibroblasts. *J Cell Sci* 1998, **111 (Pt 13)**:1897-1907.
287. Eckes B, Colucci-Guyon E, Smola H, Nodder S, Babinet C, Krieg T, Martin P: **Impaired wound healing in embryonic and adult mice lacking vimentin**. *J Cell Sci* 2000, **113 (Pt 13)**:2455-2462.
288. Dandachi N, Hauser-Kronberger C, More E, Wiesener B, Hacker GW, Dietze O, Wirl G: **Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors, and oncoproteins**. *J Pathol* 2001, **193**(2):181-189.
289. Pulyaeva H, Bueno J, Polette M, Birembaut P, Sato H, Seiki M, Thompson EW: **MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells**. *Clin Exp Metastasis* 1997, **15**(2):111-120.
290. Marone R, Hess D, Dankort D, Muller WJ, Hynes NE, Badache A: **Memo mediates ErbB2-driven cell motility**. *Nat Cell Biol* 2004, **6**(6):515-522.

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Wnt pathway activation may play an important role in tumor formation, growth and metastasis in breast cancer

Yutaka Matsuda, Thomas Schlange and Nancy E. Hynes

XXXI Symposium on Hormones and Regulation: Cancer Cell Signalling

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Publications

Autocrine WNT signaling contributes to breast cancer cell proliferation via the canonical WNT pathway and EGFR transactivation

Thomas Schlange, Yutaka Matsuda, Susanne Lienhard, Alexandre Huber and Nancy E. Hynes

Breast Cancer Research 2007, 9:R63

WNT signaling enhances breast cancer cell motility and blockade of the WNT pathway by sFRP1 suppresses MDA-MB-231 xenograft growth

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