WNT4 mediates the autocrine effects of growth hormone in mammary carcinoma cells

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Abstract

The expression of Wingless and Int-related proteins (Wnt) ligands is aberrantly high in human breast cancer. We report here that WNT4 is significantly upregulated at the mRNA and protein level in mammary carcinoma cells expressing autocrine human growth hormone (hGH). Depletion of WNT4 using small-interfering (si) RNA markedly decreased the rate of human breast cancer cell proliferation induced by autocrine hGH. Forced expression of WNT4 in the non-malignant human mammary epithelial cell line MCF-12A, stimulated cell proliferation in low and normal serum conditions, enhanced cell survival, and promoted anchorage-independent growth and colony formation in soft agar. The effects of sustained production of WNT4 were concomitant with up-regulation of proliferative markers (*c-Myc, Cyclin D1*), the survival marker *BCL-XL*, the putative WNT4 receptor *FZD6*, and activation of ERK1 and STAT3. Forced expression of WNT4 resulted in phenotypic conversion of MCF-12A cells such that they exhibited the molecular and morphological characteristics of mesenchymal cells with increased cell motility. WNT4 production resulted in increased mesenchymal and cytoskeletal remodeling markers, promoted actin cytoskeleton reorganization and led to dissolution of cell-cell contacts. In xenograft studies, tumors with autocrine hGH expressed higher levels of *WNT4* and *FZD6* when compared to control tumors. In addition, Oncomine data indicated that *WNT4* expression is increased in neoplastic compared to normal human breast tissue. Accordingly, immunohistochemical detection of WNT4 in human breast cancer biopsies revealed higher expression in tumor tissue vs. normal breast epithelium. WNT4 is thus an autocrine hGH-regulated gene involved in the growth and development of the tumorigenic phenotype.

**Keywords:** Autocrine Growth Hormone, WNT4, FZD6, Breast Cancer,
The human growth hormone \((hGH)\) gene is expressed by normal and neoplastic mammary epithelial cells and its level of expression correlates positively with increased proliferative disorders of the human breast (Raccurt et al. 2002). Autocrine hGH induces phenotypic conversion of mammary carcinoma cells, promotes dissolution of adherens junctions and increases cell migration and invasion (Mukhina et al. 2006; Perry et al. 2006, 2008). Similarly, expression of hGH and its receptor in human mammary epithelial cells enhances cell proliferation, protects against apoptotic cell death, promotes abnormal acinar development \textit{in vitro} and tumor formation \textit{in vivo} (Zhu et al. 2005a) and stimulates the proliferation of breast stem and early progenitor cells (Lombardi et al. 2014). Autocrine hGH promotes mammary neoplastic progression through coordinated activation of angiogenic and lymphangiogenic signaling pathways (Brunet-Dunand et al. 2009). Concordantly, mammary carcinoma cells expressing autocrine hGH exhibit sustained transcriptional regulation of genes involved in cell growth (Xu et al. 2005; Zhu et al. 2005a), apoptosis (Mertani et al. 2001; Zhang et al. 2003) and chemoresistance (Zhu et al. 2005b; Perry et al. 2008; Zatelli et al. 2009). Finally, elevated level of autocrine hGH expression is associated with poor overall survival in breast cancer patients (Wu et al. 2011), and functional antagonism of hGH-positive primary breast cancer cells impairs their proliferation (Chiesa et al. 2011).

Wnt signaling controls embryonic development, cell proliferation/migration and stem cell maintenance (Reya & Clevers 2005). The Wnt ligands form a family of nineteen homologous members of secreted glycoproteins (38-45 kDa, cysteine-rich) (Kikuchi et al. 2009) which exert paracrine effects through activation of the cognate seven-domain transmembrane G-protein-coupled receptor, Frizzled (FZD) (Wang et al. 2006). Wnt ligands recruit a large receptor complex formed by FZD, the low-density lipoprotein (LDL) receptor-related protein (LRP), and additional cell surface molecules such as Receptor Tyrosine Kinase-like Orphan Receptor (Ror) 2 (Minami et al. 2010) and Related to receptor tyrosine (Ryk) kinase (Green et al. 2014). Intracellular signaling is transmitted through the recruitment of the scaffold protein Disheveled (DVL) and can activate either the canonical or noncanonical Wnt pathways (Gao & Chen 2010). The canonical Wnt pathway is characterized by stabilization of \(\beta\)-catenin, a multifunctional intracellular protein involved in cell architectural cohesion and transcriptional regulation (MacDonald et al. 2009). Prototypic canonical ligands such as WNT1, 3a, 8a-b induce nuclear accumulation of \(\beta\)-catenin and increase the association of this protein with the DNA-bound T cell factor (TCF)/lymphoid enhancer protein (LEF) family of
transcription factors, and subsequent activation of target genes (e.g. c-myc, Axin2, Cyclin D) (Mikels & Nusse 2006; MacDonald et al. 2009). This pathway has been extensively investigated in various cancers (Polakis 2000; Reya & Clevers 2005; Paul & Dey 2008; MacDonald et al. 2009; Camilli & Weeraratna 2010). The activation of the noncanonical Wnt signaling pathway, as described for WNT4, WNT5a, WNT11 and WNT7a, is important in embryonic development, asymmetric cell division, planar cell polarity (PCP) and patterning of the central nervous system (Veeman et al. 2003; Katoh 2005; Wang et al. 2006; Camilli & Weeraratna 2010). Ligands of the noncanonical Wnt pathway bind FZD receptors and activate the calcium/protein kinase C (PKC) (Kühl et al. 2000) or the PCP pathway (Katoh 2005). Activation of the noncanonical Wnt signaling pathway in tumorigenesis is not completely understood and has been implicated either as an inducer or repressor of carcinogenesis (Katoh 2005; MacDonald et al. 2009; Clevers & Nusse 2012).

Here, we sought to investigate the involvement of Wnt ligands in mediating the autocrine effects of hGH in human mammary carcinoma cell progression. Autocrine hGH upregulated expression of WNT4 and its cognate receptor FZD6 in breast cancer cells, while depletion of WNT4 inhibited cellular proliferation induced by hGH. Increasing the level of WNT4 in nonmalignant breast cells stimulated growth, protection against apoptosis, epithelial to mesenchymal transition and increased cell migration. WNT4 expression is upregulated in human breast cancer as compared to normal breast and in tumor xenografts expressing autocrine hGH. Collectively, these findings demonstrate that autocrine hGH regulates WNT4 expression and that WNT4 is a potential therapeutic target in human breast cancer.
MATERIALS AND METHODS

Cell culture
The human mammary carcinoma cell line MCF-7 was obtained from the American Type
Culture Collection (ATCC, Manassas, VA) and maintained at 37°C in a 5% CO₂ atmosphere.
MCF-7 were cultured in RPMI 1640 medium (Invitrogen) supplemented with streptomycin
(100 mg/ml), penicillin (100 U/ml) and 10% fetal bovine serum (FBS). The mammary
epithelial cell line MCF-12A was obtained from ATCC and the culture conditions are
described in the Supplementary legends. The experiments described below were performed
between passages 51 and 58 for MCF-7 and between passages 18 and 26 for MCF-12A. A
stably transfected cell line expressing the wild-type hGH gene (MCF7-hGH) and a control
cell line transfected with pcDNA3 (MCF7-VEC) were established as described (Brunet-
Dunand et al. 2009). JAK2 protein kinase inhibitor AG490 (Tyrphostin B42®, Calbiochem)
was added to the medium at 20µM for 12h before experimentation. Characteristics of the
human breast cancer cell lines used are described in Supplementary Data 1.

Plasmids and transfection
The WNT4 expression plasmid was previously generated by PCR amplification of the coding
sequence of human WNT4 (Jordan et al. 2003). Transfection of pcDNA3-WNT4 (24µg) or
the empty pcDNA3 vector (24µg) were performed using Lipofectamine 2000 (Invitrogen)
and stable transfectants selected using Geneticin (800µg/ml, Invitrogen). For siRNA
experiments, MCF-12A cells were plated in six- or ninety-six well plates and transfected
with either WNT4 siRNA or a scrambled control RNA (0.1nM) (Santa Cruz, CA, USA).

Cellular biology and biochemistry
Real time qRT-PCR: Total RNA was isolated using Trizol (Invitrogen), treated with
TURBO™ DNase (Ambion, Austin, TX USA) and 500ng was reverse transcribed using a
mMLV enzyme kit (Invitrogen). PCR amplification of cDNA was performed using a
synthetic external and non-homologous poly(A) standard RNA (smRNA) to calibrate the
efficiency of the reverse-transcriptase (RT) step (Morales et al. 2006). Quantitative
amplification of target cDNAs was determined using a QuantiTect SYBR Green PCR kit
(Qiagen, Hilden, Germany) on the Lightcycler 1.2 (Roche Diagnostics, Meylan, France) as
described (Marcel et al. 2013). Primers are listed in Supplementary Tables 2 and 3.
**Immunofluorescence**: Cells grown on glass coverslips were fixed with paraformaldehyde (PFA 4%, pH 7.4), permeabilised (Triton X100, 0.5%) and blocked (BSA, 1%). β-catenin (1:300) and WNT4 (1:100) primary antibodies were from Santa Cruz. Alexa488 (Invitrogen), Alexa633 (Interchim) and 4-N-diamidino-2-phenylindole (VECTOR Laboratories, Ublingame, CA) were visualized by confocal microscopy (Carl Zeiss 510®).

**Western Blot Analysis**: total protein extraction and western blot were performed as described (Mertani et al. 2001). Immunoblotting was carried out with the following antibodies: phospho-GSKα/β (Tyr 216) (1:500), GSK3α/β (1:1000), β-catenin (1:2000), β-actin (1:500), STAT3 (1:500) (Santa Cruz biotechnology), GH (DAKO) (1:2000), ERK1/2 (1:1000), phospho-ERK1/2 (1:500), phospho-STAT3 (1:500) (Cell Signaling Technology, Danvers, MA, USA), WNT4 (1:200) (R&D System Minneapolis, MN, USA).

**Cell proliferation and apoptosis**: Cells were seeded in 96-well plates at a density of 2500 or 5000 cells/well for the indicated time periods and medium conditions. Cell viability was measured by dimethylthizol-diphenyl tetrazolium bromide (MTT) viability assay as described (Kaulsay et al. 1999). Measurement of cytoplasmic histone-associated-DNA-fragments as mono- and oligonucleosomes was determined using Cell Death Detection ELISA PLUS (Roche, Germany) as per the manufacturer’s instructions.

**Cell Migration and Soft Agar Colony Formation**: Cell migration was determined using Transwell® migration chambers (Corning, NY, USA) as described (Mukhina et al. 2004). Stably transfected MCF-12A cells (50,000 cells/well) were plated in the upper chamber for 6h and cells migrating through the pores were fixed in 4% PFA, stained with crystal violet and counted. For wound healing migration assays, cells were incubated in growth restrictive medium (2.5% HS, no EGF) for 24h before microscopic analysis. A soft agar colony formation assay was performed as described (Zhang et al. 2003). Cells (5×10^3) were cultured in 0.35% agar incubated for 10 days in growth-restrictive media and photographed.

**xCELLigence cell migration assay**: Cell migration was also assessed using the xCELLigence Real-Time Cell Analysis (RTCA) system (ACEA Biosciences, CAL, USA) using 8µm pore migration plates as described (Marcel et al. 2013). This technology based on real-time measurement of electrical impedance permits continuous measurement of cell-microelectrode contacts and is interpreted as cell density or cell index. Cells were plated in the upper chamber in serum-free medium, whereas the lower chamber was filled with 2.5% FBS-
containing media as chemoattractant. The acquisition of the electrical signal was recorded every 15 minutes for 24 h.

Xenograft analyses: mammary tumors were established using MCF7-hGH and MCF7-VEC cells as described (Mukhina et al. 2004; Zhu et al. 2005a; Brunet-Dunand et al. 2009) with approval from the Animal Ethics Committee of the University Claude Bernard. Tumors were dissected at day 35 post-implantation, halved and either frozen or fixed in 4% PFA, embedded in paraffin and sectioned. Immunohistochemistry and analysis was carried out as described (Brunet-Dunand et al. 2009).

Immunohistochemical staining of WNT4 in breast cancer biopsies: tissue samples from five normal breast biopsies and nine cases of primary resections from different breast tumors were retrieved from the Anatomy and Pathology files of the University Hospital Centre of Santiago (Spain) and were a kind gift of Pr. T.G Caballero. The detection method used was performed as described previously (Raccurt et al. 2002; Brunet-Dunand et al. 2009; Vidal et al. 2010).

Statistics
All data is expressed as means ± SEM of triplicate determinants. Data were analyzed using the unpaired two-tailed t test. Experiments were repeated at least three times.
RESULTS

Autocrine hGH regulation of WNT4

To determine the relevance of the Wnt pathway in breast cancer cell progression induced by autocrine hGH, we first examined the expression profile of members of this gene family in MCF7-hGH and MCF7-VEC cells using qRT-PCR. As shown in Figure 1A, autocrine hGH significantly increased the expression of $\text{WNT4}$ (2.6 fold), $\text{WNT10b}$ (7.8 fold), $\text{LRP5}$ (3.4 fold), $\text{FZD6}$ (21.7 fold) and $\text{DVL1}$ (1.8 fold) (Fig. 1A). In subsequent experiments we focused our study on the regulation of $\text{WNT4}$ since it was also found to be upregulated with its receptor $\text{FZD6}$ in vivo, in solid hGH-tumors. Also, in normal human mammary epithelial cell lines $\text{WNT4}$ is expressed at a low level (Supplementary Data 1). We analyzed whether autocrine hGH altered the expression of $\text{WNT4}$ protein. Western blot analysis demonstrated that $\text{WNT4}$ was processed and secreted as a single protein with the appropriate molecular weight of 40kDa (Fig. 1B) and autocrine hGH increased the level of $\text{WNT4}$ produced and secreted by MCF-7 cells (Fig. 1B). $\text{WNT4}$ protein expression was also investigated by immunofluorescent staining and was localized in the cytoplasm of both MCF7-hGH cells and MCF7-VEC cells, and autocrine hGH was clearly associated with increased $\text{WNT4}$ signal (Fig. 1C). Autocrine hGH binding to its receptor activates the protein kinase JAK2 in mammary carcinoma cells (Kaulsay et al. 2001). The pharmacological inhibitor of JAK2, AG490 (12h, 20µM), abrogated the effect of autocrine hGH on the expression level of $\text{WNT4}$ in MCF7-hGH cells, as demonstrated by immunofluorescence and western blot analysis (Fig. 1C). Thus, the effects of autocrine hGH on $\text{WNT4}$ expression and the secretion of $\text{WNT4}$ are mediated through JAK2 activation in mammary carcinoma cells.

WNT4 enhances mammary carcinoma cell proliferation

To determine the functional significance of $\text{WNT4}$ induction by autocrine hGH in mammary carcinoma cells we used a specific siRNA construct targeting human $\text{WNT4}$. Robust depletion of $\text{WNT4}$ protein was achieved 24h post-transfection and maintained for up to 72h post-transfection (Fig. 2A). We analyzed the effect of $\text{WNT4}$ depletion on MCF-7 cell proliferation (MTT assay) and apoptosis (cytoplasmic histone-associated-DNA assay). Depletion of $\text{WNT4}$ in both MCF-7 wild-type and MCF7-VEC cells decreased cell proliferation by approximately 30% at 72h as compared to cells transfected with the scrambled control siRNA (Fig. 2B). Following $\text{WNT4}$ depletion in MCF7-hGH cells, a significant decrease in cell proliferation was observed at 48h and 72h (40%) when compared...
to cells transfected with control siRNA (Fig. 2B). The percentage of cells undergoing apoptosis was not altered by the depletion of WNT4 in MCF-7 wild-type, MCF-hGH or MCF-VEC cells (data not shown). Therefore, depletion of WNT4 reduces the growth of MCF-7 wild-type cells and drastically hampers the proliferative effects of autocrine hGH in human mammary carcinoma cells.

**WNT4 enhances proliferation and survival in human mammary epithelial cells**

To study the role of WNT4 in the initial steps of mammary cancer progression, we used the non-malignant breast epithelial cell line MCF-12A which exhibited low levels of WNT4 compared to mammary carcinoma cell lines (Supplementary Data 1). MCF-12A cells were stably transfected with the WNT4 expression vector (cell line designated 12A-WNT4) or an empty vector as control (designated 12A-pcDNA3). Three stable MCF-12A cell lines were established for each vector. Forced expression of WNT4 in MCF-12A cells was confirmed by qRT-PCR, western blot, and immunofluorescence analysis (Supplementary Data 1). We first determined whether forced expression of WNT4 played a role in MCF-12A cell proliferation and apoptotic cell death. Since the growth of MCF-12A cells is serum and EGF-dependent (Schedin et al. 2004), we performed our experiments in growth-restrictive media (no EGF) containing either normal (5%) or reduced (2.5%) horse serum concentration. Forced expression of WNT4 significantly increased cell proliferation in growth-restrictive media when compared to the control cell line (Fig. 3A). The proliferative effect of WNT4 was detected as early as 24h after EGF deprivation in reduced serum conditions (2.5%) and 48h after EGF deprivation in normal serum conditions (5%) (Fig. 3A). Transient transfection of siRNA for WNT4 was also performed in MCF12A-pcDNA3 and MCF12A-WNT4 cell lines (Supplementary Data 1). In both stable cell lines, proliferation was significantly decreased at 72h and 96h post transfection as compared to cells transfected with the scrambled control siRNA (Supplementary Data 1). The decrease in cell growth induced by transfection with WNT4 siRNA was greater when cells were grown in growth restrictive medium with 2.5% horse serum as compared to cells maintained in 5% horse serum (data not shown) further confirming the importance of WNT4 on human mammary epithelial cell growth. Forced expression of WNT4 significantly protected MCF-12A cells from apoptotic cell death induced by EGF-deprivation as compared to the control (Fig. 3B). WNT4 decreased the rate of apoptosis by 30% in both 2.5% and 5% horse serum, suggesting that WNT4 itself rather than serum is involved in mammary epithelial cell survival (Fig. 3B). The increased proliferation and survival capacities afforded by WNT4 in MCF-12A cells prompted us to test
the effect of WNT4 on the ability of MCF-12A cells to grow as colonies in semi-solid agar in
growth-restrictive medium. Although MCF-12A cells are non-tumorigenic they have been
reported to form a limited number of colonies in semi-solid agar (Banwell et al. 2006). Forced
expression of WNT4 enhanced colony formation by 45% as compared to the control cell line
(Fig. 3C) and also increased individual colony size by 36% (Fig. 3C). Thus, sustained
expression of WNT4 confers human mammary epithelial cells with a significant proliferative
and survival advantage in growth-restrictive conditions and supports resistance to anoikis.

WNT4 initiates a proliferative signaling program

To understand the molecular mechanisms mediating the effects of forced expression of
WNT4 in MCF-12A cell proliferation and resistance to apoptosis, we analyzed the
expression of genes involved in cell cycle progression and survival. Forced expression of
WNT4 significantly increased the mRNA levels of genes critical for cell proliferation (i.e. c-
Myc, Cyclin D1) and for cell survival (i.e. BCL-XL) as compared to the control cell line (Fig.
4A). The expression of FZD6 was also increased by 2.5 fold (p < 0.01) in 12A-WNT4 cells
vs. control cells, demonstrating the positive regulation induced by WNT4 on its cognate
receptor FZD6. We also found that forced expression of WNT4 significantly increased the
mRNA levels of JAK2 and STAT3 (Fig. 4B). The relative level of phosphorylated STAT3,
ERK1 and ERK2 was higher in 12A-WNT4 as compared to 12A-pcDNA3 cells (Fig. 4C).
We next examined whether WNT4 activates the canonical Wnt/β-catenin pathway in MCF-
12A cells and found that forced expression of WNT4 in MCF-12A cells neither altered
GSK3β phosphorylation nor changed the transcriptional activity of β-catenin (Supplementary
Data 2). Activation of β-catenin-mediated TCF/LEF transcriptional activity was efficiently
induced in both cell lines with LiCl, an inhibitor of GSK3β phosphorylation (Supplementary
Data 2). However, forced expression of WNT4 significantly decreased LiCl-induced
luciferase activity vs. control cells (Supplementary Data 2) suggesting that WNT4 partially
antagonizes the canonical Wnt/β-catenin pathway in MCF12-A. Moreover, transcriptional
activation of the c-Jun amino-terminal kinase gene (2.1 fold, p < 0.01) further suggested that
forced expression of WNT4 in MCF-12A cells may preferentially signal through the
noncanonical WNT pathway. These data indicate that JAK/STAT, MAPK and the non-
canonical Wnt/β-catenin pathway signaling are involved in WNT4-mediated mammary
epithelial cell proliferation and survival.
WNT4 induces phenotypic conversion of mammary epithelial cells and enhances cell migration

To determine the effects of WNT4 on the mammary epithelial phenotype of MCF-12A cells, we performed qRT-PCR analysis of mesenchymal and cytoskeleton genes. Forced expression of WNT4 significantly increased the mRNA level of the intermediate filament genes *Vimentin* and *Fibronectin* in comparison to the control cell line (Fig. 5A). The expression of genes coding for α6-integrin and RHOA was also significantly increased (Fig. 5A), whereas the expression of adherens junction markers β-catenin and plakoglobin (*JUP*) was not altered (Fig. 5A). To further investigate the effects of WNT4 on cytoskeletal reorganization, we analyzed the spatial distribution of proteins controlling intercellular adhesion and cell shape using immunofluorescence and confocal microscopy. β-catenin was mainly localized at cellular adherens junctions in 12A-pcDNA3 cells and forced expression of WNT4 increased expression and cytoplasmic accumulation of β-catenin (Fig. 5B). The organization of the actin cytoskeleton visualized using rhodamine-phalloidin showed a regular and intense localization at the plasma membrane borders of 12A-pcDNA3 cells (Fig. 5B). Forced expression of WNT4 resulted in a reorganization of the actin cytoskeleton and disruption of cell-cell contacts (Fig. 5B). The signal of longitudinal F-actin stress fibers was decreased by WNT4 whereas cortical-actin-complex increased with several images of lamellipodia (yellow arrows) and filopodia (green arrows) (Fig. 5B). Finally, the fluorescence signal of Vimentin increased in 12A-WNT4 cells as compared to the control (Fig. 5B bottom panel), further supporting the role of WNT4 in the establishment of a mesenchymal phenotype.

The loss of cell–cell contacts is often associated with a migratory phenotype (Thiery *et al.* 2009), we therefore examined the effect of forced expression of WNT4 on motility of MCF-12A cells. Wound-healing assay demonstrated that WNT4 stimulates MCF-12A cell migration leading to a faster closing of the wound as compared to the control (Fig. 5C). Forced expression of WNT4 in MCF-12A cells significantly increased motility by 60% as quantitated in transwell Boyden chamber assay (Fig. 5D upper panel) and in real time impedance-based system xCELLigence (ACEA Biosciences, CA, USA) (Fig. 5E). Thus, forced expression of WNT4 in mammary epithelial cells promotes expression of mesenchymal and cytoskeletal remodeling markers and enhances cell migratory capacities.
We next wanted to determine whether \textit{WNT4} expression was detectable \textit{in vivo} in solid human tumors. First, we orthotopically injected MCF7-hGH and MCF7-VEC cells into the mammary fat pad of nude mice as previously described (Mukhina \textit{et al.} 2004). Solid mammary tumors formed by MCF7-hGH cells exhibited enhanced tumor growth over 35 days and a higher proliferative index compared to MCF7-VEC cells (Supplementary Data 3). qRT-PCR of sixteen gene members of the WNT pathway was performed using RNA extracted from fresh tumors. Increased expression of \textit{WNT4} (2.9 fold), \textit{LRP5} (1.6 fold), \textit{FZD6} (1.3 fold), \textit{DVL1} (6.8 fold) and \textit{DVL3} (4.3 fold) induced by autocrine hGH was confirmed \textit{in vivo} (Fig. 6A). Immunohistochemical detection of WNT4 showed an intense signal localized in the cytoplasm and at the plasma membrane of tumor cells (Fig. 6B). WNT4 immunoreactive signal was more abundant in tumors formed by MCF7-hGH cells as compared to MCF7-VEC cells (Fig. 6B). Quantification of the staining intensity demonstrated that MCF7-hGH tumors exhibited significantly increased level of WNT4 protein as compared to control (Fig. 6C).

The relevance of \textit{WNT4} expression to human breast cancer was determined through data mining of the gene-expression database Oncomine (Supplementary Table 3). Analysis of the Oncomine datasets showed that \textit{WNT4} mRNA analysis was reported in 496 high-throughput transcriptomic studies. By filtering the data to only select experiments where \textit{WNT4} was in the top 10\% of all genes measured, with a \textit{p}-value cut-off \leq 0.005 and with a fold change in expression \geq 2, we found 85 studies in which \textit{WNT4} was up-regulated and 30 studies where \textit{WNT4} was down-regulated. This included more than 20 different types of cancers. Six transcriptomic studies performed in human breast cancer reported the expression of \textit{WNT4} and all showed significantly higher expression in breast carcinoma compared to normal tissue and also higher expression in ductal carcinoma \textit{in situ} as compared to invasive ductal carcinoma (Supplementary Table 3). We also determined the significance of WNT4 in human breast cancer progression by qRT-PCR analysis of established human immortalized mammary carcinoma cells. The expression level of \textit{WNT4} was low in two normal human breast epithelial cell lines MCF-12A and HBL-100 as compared to the carcinoma cell lines MCF-7, CAL-51, MDA-MB-231, BT-20, MDA-MB549 (Supplementary Data 1).
To further validate these findings, we examined the level of WNT4 protein by immunohistochemistry on biopsies obtained from breast cancer patients. The clinical informations concerning these samples are indicated in supplementary data 4. In normal human breast (n=5) the presence of moderate amounts of WNT4 was detected in the luminal epithelial cells and in myoepithelial cells of the ducts. No signal was evidenced in the fibroblasts of the connective tissue compartment (Fig. 6D). In patients with intraductal *in situ* carcinoma (n=5), intense immunoreactivity for WNT4 was detected in the carcinomatous cells within the enlarged ducts (Fig. 6D). In addition, a positive signal for WNT4 was present within cells of the stromal compartment surrounding the ducts filled with carcinoma cells (Fig. 6D). On tissue sections obtained from patients with invasive ductal breast carcinoma (n=4), a strong immunoreactive signal for WNT4 was localized in strands of tumor cells extending into the stroma (Fig. 6D). Taken together, these results indicate that WNT4 mRNA synthesis is frequently upregulated in various types of human cancers and this regulation also occurs at the protein level in breast cancer.
DISCUSSION

This study is the first demonstration that a subset of WNT ligands and receptors are upregulated in vitro by autocrine production of hGH in human mammary carcinoma cells and that WNT4, its receptor FZD6 and their associated signaling scaffold DVL1 are also upregulated in vivo in mice with hGH-mammary tumors. Our experiments focused on WNT4 expression to demonstrate its role in hGH-induced mammary carcinoma cell progression and as a direct inducer of proliferation, phenotypic conversion and migration of non-malignant human mammary epithelial cells. Interestingly, we found that components of both the canonical (i.e. WNT10b, DVL1, LRP5) and noncanonical (i.e. WNT4, FZD6) Wnt pathways are upregulated by autocrine hGH. Compelling evidence has demonstrated the importance of the canonical Wnt/β-catenin pathway during the development of several human cancers, whereas the association between noncanonical Wnt/PKC/PCP pathway and carcinogenesis is gradually emerging (Camilli & Weeraratna 2010; Yu et al. 2012). The transcriptional control of WNT ligand expression by local production of growth factors or hormones during carcinogenesis is still poorly defined. It has been demonstrated that WNT2 which is functionally related to WNT4 is an activity-dependent c-fos cAMP-response element binding protein (CREB)-responsive gene in neurons (Wayman et al. 2006). The level of CREB protein and its activating kinases ERK1/2 are high in MCF-7 cells (Seoane et al. 2007). Our present demonstration that WNT4 activates ERK1 along with our previous work showing that MCF-7 cells expressing hGH exhibit a sustained activation of ERK1/2 (Zhu et al. 2005b) suggest a positive regulatory loop between WNT4 transcription and ERK1/CREB activation. The transcription of WNT4 is activated by the oncogenic transcription/translation factor Y-box binding protein-1 (YB-1) in basal-like breast cancer cell lines (Finkbeiner et al. 2009). YB-1 is also activated by the serine/threonine kinase Akt in wild type (Evdokimova et al. 2006) and autocrine hGH-expressing MCF-7 cells (Mertani et al. unpublished data) and thus represents a possible inducer of WNT4.

Concomitant to increased WNT4 expression, autocrine production of hGH also induced a significant increase in WNT10b, DVL1, and LRP5 expression that could likewise mediate carcinoma cell progression. Indeed, high level of WNT10b is associated with breast cancer progression (Bui et al. 1997) and forced expression of WNT10b in mammary epithelium activates the degradation of CDK inhibitor p27KIP1 and consequent neoplastic transformation (Miranda-Carboni et al. 2008). The expression of DVL1 is similarly high in primary breast cancers (Nagahata et al. 2003) and promotes proliferative and anti-apoptotic effects in MCF-
7 cells (Schlange et al. 2007). Finally, the demonstration that coreceptor LRP5 deficiency in MMTV-WNT1 transgenic mice hampers tumor formation (Liu et al. 2005; Lindvall et al. 2006) and that proliferation of MCF-7 cells by WNT3 is mediated by a truncated LRP5 (Björklund et al. 2009) form, suggest that hGH-induced mammary cancer progression also involves LRP5 activation.

In order to determine whether autocrine hGH exerted a regulatory role on Wnt signaling in vivo, we conducted qRT-PCR and IHC analyses of solid mammary tumors expressing hGH formed in nude mice. We found a significant increase of WNT4, LRP5, FZD6 and DVL1 expression, similar to our in vitro results. In addition we found a significant increase in DVL3 expression whereas WNT7b and WNT10b expression was not altered in solid tumors vs. in vitro. These results further support the importance of WNT4 in human mammary carcinoma cell progression. Acting through its cognate receptor FZD6, WNT4 controls embryonic development of various organs (Maurus et al. 2005; Yuzugullu et al. 2009) including the mammary gland (Peltoketo et al. 2004). The secretion of WNT4 by mouse mammary epithelial cells mediates progesterone-induced ductal side branching during pregnancy (Brisken et al. 2000). WNT4 is also a progesterone target gene in basal mammary stem/progenitor cells controlling self-renewal properties (Lamb et al. 2007). We have demonstrated that transient introduction of WNT4 siRNA partially abrogated the proliferative effects of autocrine hGH in mammary carcinoma cells. To our knowledge, this is the first time that WNT4 production has been directly linked to human mammary carcinoma cell proliferation. Previous work identified WNT4 as a major upregulated genes in spontaneous versus radiation-induced mammary gland carcinoma in rats (Imaoka et al. 2008) and a high level of WNT4 expression was described in human breast cancer (Huguet et al. 1994; Milovanovic et al. 2004) and cancer cell lines (Benhaj et al. 2006; Finkbeiner et al. 2009) (Supplementary Data 1, Supplementary Table 3). Our results complete these findings at the protein level, showing that WNT4 production is low in normal human breast and elevated in patients with intraductal in situ and invasive carcinoma. Also of interest, upregulation of WNT4 is a molecular characteristic of lung (Garnis et al. 2005), skin (Devgan et al. 2005), liver (Bengochea et al. 2008) and pituitary (Miyakoshi et al. 2008) tumors (Supplementary Table 3) suggesting a broader implication of WNT4 in cancer. Consistently, high throughput analysis demonstrated that WNT4 expression is higher in breast cancer vs. other types of human adenocarcinoma (Ramaswamy et al. 2003).

We addressed the role of WNT4 in breast cancer progression using siRNA and overexpression of WNT4 in two immortalized human mammary cell lines. In MCF-7 cells,
the depletion of WNT4 clearly inhibited cell proliferation, and in non-malignant MCF-12A cells, expression of WNT4 promoted a hyperproliferative phenotype, enhanced survival and colony formation. The acquisition of proliferative and survival characteristics of WNT4-expressing MCF-12A is associated with transcriptional upregulation of cell cycle activators (c-myc, cyclin D1), the JAK2 kinase and the anti-apoptotic factor Bcl-xl. We also demonstrated that WNT4 signaling in MCF-12A regulates the phosphorylation of STAT3 and ERK1/2. The activation of the JAK/STAT3 pathway by Wnt/β-catenin pathway has been described in mice as a critical event in embryonic stem cells renewal and maintenance (Hao et al. 2006). In human mammary epithelial cells, the interplay between MAPK and JAK/STAT pathways is well described (Frank 2008), but the relationship between MAPK and WNT/β-catenin signaling has never been addressed. The precise link between noncanonical WNT4 production, ERK1/2 activation and the establishment of a pre-tumorigenic phenotype in mammary epithelial cells requires further investigations. We also found that transcriptional activity of β-catenin, the classical activator of oncogenic program is partially counteracted in WNT4-expressing MCF-12A cells (Supplementary Data 2). Functional antagonism between β-catenin dependent and independent pathways has been reported in human embryonic kidney (HEK) 293T cell line stimulated by WNT4 (Bernard et al. 2008). Understanding the signaling intermediates activated by WNT4 in mammary epithelial cell also needs further investigation.

We found that forced expression of WNT4 stimulates MCF-12A cell migration, a process accompanied by up-regulation of mesenchymal and cytoskeletal markers. A study conducted on human prostate cancer cells similarly demonstrated that WNT4 and WNT5a activate cell invasive and migratory capacities (Yamamoto et al. 2010). In contrast, WNT4 has been shown to decrease cell migration of rat thyroid cells FRTL-5 suggesting that it can exert different actions depending on the cellular context (De Menna et al. 2013). It was recently shown that WNT4 secretion by human umbilical cord mesenchymal stem cells stimulates their migration, and is activating angiogenesis in vivo in a rat skin burn model (Zhang et al. 2015). Interestingly, exosomal release of WNT4 is responsible for mesenchymal stem cell migration (Zhang et al. 2015), and similar exosome release of WNT5a induces human melanoma cancer cell migration and invasion (Ekström et al. 2014). Whether a similar mechanism is involved for WNT4-induced migration of normal mammary epithelial cells needs to be investigated.

In conclusion, we demonstrated that WNT4 expression is increased in breast cancer and mediates the proliferative effects of autocrine hGH in human mammary carcinoma cells.
WNT4 confers a significant growth and survival advantage to non-malignant mammary epithelial cells, changes their phenotype and enhances their migratory capacities. Thus, deregulated production of WNT4 could sensitize mammary epithelial cells to oncogenic signals, and antagonizing WNT4 and hGH signaling could represent a strategy to counteract the early stages of human mammary neoplastic transformation.
AUTHOR CONTRIBUTION


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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary Information accompanies this paper
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FIGURE LEGENDS

Figure 1. WNT pathway activation and WNT4 production by autocrine hGH in human mammary carcinoma cells

(A) Real-time RT-PCR analysis of several genes involved in the Wnt/β-catenin signal pathway was determined with total mRNA purified from MCF7-VEC and MCF7-hGH cells serum-starved overnight. β-actin was used as loading control. A proprietary Sm internal standard RNA was used as the internal control for qRT-PCR (Morales et al. 2006). Results presented are expressed as fold change of expression level in MCF7-hGH compared to MCF7-VEC. Bar, SE; *, P < 0.05; **, P < 0.001; ***, P < 0.0001; Student’s t test, n = 3. (B) Western blot analysis of WNT4 expression and secretion by MCF7-hGH and MCF7-VEC cells serum-starved overnight. 30µg of protein from total cell lysates and from ultrafiltered medium were used for PAGE. Red Ponceau staining (not shown) and β-actin were used as loading controls. WNT4 was identified as a single band with the expected molecular weight of 40kDa and was more abundant in total cell lysates from MCF7-hGH (upper panels) and also in the media collected from MCF7-hGH cells (bottom panels) when compared to the control cell line. Detection of 22 kDa hGH in the medium was used as a control for protein secretion (bottom panel). (C) Detection of WNT4 immunoreactivity by confocal microscopy using a secondary antibody conjugated to fluorescent Alexa 488 (green) in MCF7-hGH and MCF7-VEC cells maintained overnight in serum free media (upper panel). Fluorescent labeling indicated that WNT4 immunoreactivity is localized in the cytoplasm and Golgi area and was more intense in MCF7-hGH cells relative to MCF7-VEC cells. When both cell lines were treated for 12h with a JAK2 inhibitor (AG490, 20µM) in serum free media (lower panels), the immunofluorescence signal was significantly reduced in MCF7-hGH cells and was unchanged in MCF7-VEC cells. Similarly, western blot analysis of WNT4 expression in MCF7-hGH cells showed decreased WNT4 level following the treatment with JAK2 inhibitor AG490 (lower panel). The scale bar represents 25µm.

Figure 2. Depletion of WNT4 decreased the effect of autocrine hGH on mammary carcinoma cell proliferation

(A) Western blot characterization of WNT4 protein depletion using a WNT4 targeted siRNA compared to the scrambled control (Sc RNA) in MCF7-hGH cells at 24, 48 and 72h post transfection. 50µg of protein from whole cell lysate extract was used. The depletion of WNT4 is effective at 24h post transfection and still maintained at 72h. β-actin was used as loading control. (B) WNT4 depletion inhibited cell proliferation in the three cell lines tested.
The inhibitory effect on cell proliferation was observed at 48h post transfection in MCF7-hGH cells, whereas it was detected at 72h post transfection in MCF7-VEC and MCF-7 wild-type cell lines. Cell proliferation was measured as described in the Materials and Methods.

Results are expressed as fold change of cell viability between cell lines. Bars, SE; *, P < 0.05; **, P < 0.001; Student’s t test, n = 3.

Figure 3. Forced expression of WNT4 in MCF-12A cells stimulates their proliferation, survival and anchorage-independent growth

(A) Effects of WNT4 overexpression on human mammary epithelial cell (MCF-12A) proliferation were determined in EGF-deprived medium containing either 2.5% (Left panel) or 5% horse serum (HS) (Right panel). Results are expressed as fold induction in 12A-WNT4 and 12A-pcDNA3 cells of cell number at day 0. Overexpression of WNT4 stimulates MCF-12A cell proliferation as early as 24h in media with low (2.5%) horse serum, whereas the stimulatory effect is seen at 48h in media with 5% horse serum. (B) Effect of WNT4 overexpression on protection from apoptosis in EGF-deprived medium containing either 2.5% or 5% horse serum. Cells were processed as described in Materials and Methods. Results are expressed as the percentage of changes in apoptotic 12A-WNT4 cells as compared to 12A-pcDNA3 control cells measured at 48h. The number of apoptotic cell death was reduced by 30% in MCF-12A cells with overexpression of WNT4. (C) Effect of WNT4 overexpression on colony formation by 12A-WNT4 and 12A-pcDNA3 cells cultured in soft agar. Results are expressed as a percentage of the control cell line (100%). Overexpression of WNT4 significantly increased the number (left) and size (right) of the colonies formed in agar. The photographs below are representative of the colonies formed on soft agar by 12A-pcDNA3 and 12A-WNT4 cell lines. Bars, SE; **, P < 0.05; ***, P < 0.001; Student’s t test, n = 3.

Figure 4. Forced expression of WNT4 in MCF-12A cells stimulates signaling pathways involved in cell proliferation and survival

(A) Real-time qRT-PCR analysis of several genes involved in cell proliferation and apoptotic cell death was performed on mRNA purified from 12A-WNT4 and 12A-pcDNA3 cell lines cultured overnight in growth-restrictive medium (no EGF, 2.5% horse serum) as described in the Materials and Methods. Overexpression of WNT4 statistically increased gene expression of the cell cycle activators c-myc and cyclin D1 and of the anti-apoptotic factor BCL-XL. Results are expressed as fold change of gene expression compared to 12A-pcDNA3 cells. (B) Real-time qRT-PCR analysis demonstrating that overexpression of WNT4 significantly increased gene expression of the tyrosine kinase JAK2 and signal transducer and activator of
transcription STAT3. (C) Western blot analysis demonstrating that overexpression of WNT4 increases the levels of the phosphorylated forms of STAT3 and ERK1 and ERK2 proteins. *Bars*, SE; **, *P* < 0.001; ***, *P* < 0.0001; Student’s *t* test, *n* = 3.

**Figure 5. Forced expression of WNT4 alters mesenchymal and cytoskeletal remodeling markers and promotes MCF-12A cell migration**

(A) qRT-PCR analysis of genes involved in cytoskeletal remodeling and in the establishment of mesenchymal and motile phenotype. The PCR analysis was conducted on total mRNA extracted from 12A-WNT4 and 12A-pcDNA3 cells cultured overnight in growth-restrictive medium. Results are expressed as fold change of gene expression in 12A-pcDNA3 cells.

Forced expression of WNT4 statistically increased the expression of all genes tested except β-catenin and Junction plakoglobin (JUP). (B) Immunofluorescence analysis by confocal microscopy of β-catenin, rhodamine conjugated phalloidin (actin cytoskeleton) and vimentin distribution in 12A-WNT4 and 12A-pcDNA3 cells. DAPI was used to label the nuclei. Cells were cultured overnight in growth restrictive-medium and immunofluorescence was performed as described in the Materials and Methods. Overexpression of WNT4 induced the redistribution of β-catenin to intracytoplasmic and perinuclear localization (top panel) and the reorganization of the actin cytoskeleton with disruption of cell-cell contacts (middle panel) and formation of numerous lamellipodia (yellow arrows) and filopodia (green arrows).

Forced expression of WNT4 in MCF-12A cells also contained more intracytoplasmic vimentin, forming an extended network of intermediate filaments, as compared to the control cell line (bottom panel). The pictures shown are representative of three different experiments. The scale bar represents 10µm. (C) Wound-healing assay comparing the migratory capacity of the 12A-WNT4 and 12A-pcDNA3 cell lines. The wounded area delimited by the two black lines was examined at 0 and 24h after seeding in growth-restrictive medium (magnification ×20). Forced expression of WNT4 in MCF-12A cells enhanced wound closure when compared to the 12A-pcDNA3 control cell line. (D) Boyden chamber migration assay in 12A-WNT4 and 12A-pcDNA3 cell lines. The cells were allowed to migrate to the lower compartment for 6h in growth restrictive medium. Representative microphotographs (magnification ×20) of the cells fixed and stained with crystal violet are shown. The graphical representation below indicates the percentage of migrating cells with the number of 12A-pcDNA3 cells established as 100%. Forced expression of WNT4 significantly stimulates the number of migrating cells by almost 70% as compared to the control cell line. (E) Cell impedance migration assay in 12A-WNT4 and 12A-pcDNA3 cells. 20,000 cells were seeded in duplicate in the upper chamber of the CIM-16 plate in growth...
restrictive medium, and cell migration was monitored every 15 minutes for 24h using the real
time cell analyzer (RTCA)-DP instrument. The cell index is the unit used to measure the
relative change in electrical impedance generated by the migration of 12A-WNT4 (red line)
and 12A-pcDNA3 (blue line) cells. It represents cell density and is correlated to the number
of cells that migrated through the porous membrane and made contact with the electrodes.
The lower chamber contained 2.5% FBS as a chemoattractant. The mean of three replicates ±
standard deviation are shown. Bar, SE (unless otherwise stated); *, P < 0.05; **, P < 0.001;
***, P < 0.0001; Student’s t test, n = 3.

Figure 6. WNT4 expression in human breast cancer

(A) Real-time qRT-PCR analysis of genes involved in the Wnt/β-catenin signal pathway was
determined in mRNA purified from solid frozen mammary gland tumors formed following
orthotopic implantation of MCF7-hGH and MCF7-VEC carcinoma cells in the mammary fat
pad of nude mice. Experimental details are available in Supplementary Data 3. Results
presented are expressed as fold change of expression level in MCF7-hGH compared to
MCF7-VEC. (B) Immunohistochemical detection of WNT4 and hGH on sections from
paraffin-embedded tumors xenografts 35 days after implantation of MCF7-hGH and MCF7-
VEC cells. The positive signal (brown precipitate) indicating WNT4 immunoreactivity is
localized in strands of human cancer cells, absent in the conjunctive stroma and stronger in
tumors formed by MCF7-hGH cells (right upper panel) compared to tumors from vector cells
(left upper panel). The phenotypic retention of hGH expression in tumors formed by MCF7-
hGH cells was strongly maintained in vivo (right lower panel) and absent in MCF7-VEC
cells. The scale bar represents 30µm. (C) Quantification of WNT4 protein immunoreactivity
on serial sections of solid tumors formed after implantation of MCF7-hGH and MCF7-VEC
cells in the mammary fat pad of nude mice. The graphical representations indicate the mean
staining intensity of WNT4 immunoreactivity (upper panel) and the percentage of tumor area
showing WNT4-labeled cells (lower panel). (D) Immunohistochemical detection of WNT4
protein expression on sections obtained from paraffin-embedded normal human breast tissue,
ductal in situ breast cancer and invasive ductal breast cancer. The labeling is faint and
localized in epithelial and myoepithelial cells forming the duct of the normal human breast.
WNT4 immunoreactivity is strongly detected in cancer cells filling the duct of the ductal in
situ breast cancer and some stromal cells are also labeled. In invasive ductal breast cancer,
WNT4 immunoreactivity is also detected in cancer cells invading the stroma. The scale bar
represents 50µm. Bar, SE; *, P < 0.05; **, P < 0.001; Student’s t test, n = 3.
**Figure 1. Vouyovitch et al.**

(A) Gene expression (% relative to MCF7-VEC) for various proteins:

- WNT7b
- WNT4
- WNT10b
- LRP5
- FZD1
- FZD6
- DVL1
- DVL3
- Axin2
- APC
- GSK3β
- β-catenin

(B) Western blot analysis of WNT4 and β-actin in MCF7-VEC and MCF7-hGH conditions.

(C) Immunofluorescence images of MCF7-VEC and MCF7-hGH in serum-free and AG 490 conditions.

- Control
- AG 490
Figure 2. Vouyovitch et al.
Figure 3. Vouyovitch et al.
Figure 4. Vouyovitch et al.
Figure 5. Vouyovitch et al.
Figure 6. Vouyovitch et al.