

Research Paper

Silencing of *Wnt-1* by siRNA induces apoptosis of MCF-7 human breast cancer cells

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Abbreviations: APC, adenomatous polyposis coli gene; BSA, bovine serum albumin; CK-1, casein kinase-1; DOC, docetaxel; Dvl, dishevelled; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GSK-3 β , glycogen synthase kinase 3 beta; H3F3A, histon H3A; IAP, inhibitors of apoptosis proteins; OD, optical density; Lef, lymphoid enhancer factor; LRP-5, low-density lipoprotein-receptor related protein 5; LRP-6, low-density lipoprotein-receptor related protein 6; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate buffer saline; PBST, PBS with Tween 20; RPLP0, 60S acidic ribosomal protein P0; SC, scrambled siRNA; siTOX, siCONTROL TOX; siRNA, short interfering RNA; Tcf, T-cell factor; CTRL, untreated cells; Wnt, wingless-type MMTV integration site family; Wnt-1, wingless-type MMTV integration site family member 1; WP, sequence from literature data

Key words: Wnt-1, siRNA, breast cancer, apoptosis

Objective: Wnt family of secreted-type glycoproteins plays key role in carcinogenesis and embryogenesis. Signals of Wnts are transduced through seven-transmembrane-type Wnt receptors encoded by *Frizzled* (*Fzd*) genes to the β -catenin-Tcf pathway, the c-Jun-N-terminal kinase (JNK) pathway or the Ca²⁺-releasing pathway. Aberrant activation of the Wnt/ β -catenin signaling pathway is associated with a variety of human cancers. In human breast cancer, evidence of β -catenin accumulation implies that the canonical Wnt signaling pathway is active in over 50% of carcinomas.

Results: We found that in breast cancer cells overexpressing Wnt-1 siRNA anti-*Wnt-1* induced apoptosis and caused changes in downstream proteins levels. Among treated cells there were 71% apoptotic cells in comparison to cells treated with scrambled siRNA (6%) and control cells (6%) after 48 h ($p < 0.01$).

Methods: To examine if Wnt-1 signal is essential for cancer cell survival, we investigated the effect of *Wnt-1* gene silencing in triggering of apoptosis in MCF-7 breast cancer cell line. Light microscopy, viability/cytotoxicity tests, flow cytometry, real-time PCR and western blotting were used for evaluation of the morphological features of cell death, percentage of apoptotic cells, *Wnt-1* mRNA and protein level.

Conclusion: Our results significantly indicate that anti-*Wnt-1* siRNA inhibits Wnt-1 signaling, inducing apoptosis in human breast cancer MCF-7 cells and thus may serve as a potential anti-cancer drug.

Introduction

Wnts (wingless-type MMTV integration site family) are secreted glycoproteins which are produced by different cell types and are thought to act mostly in a paracrine fashion.¹ In the mammary gland, Wnt signals are strongly implicated in initial development of the mammary rudiments, and in the ductal branching and alveolar morphogenesis that occurs during pregnancy.² In the absence of Wnt signals, free cytosolic β -catenin is incorporated into a complex consisting of Axin, the adenomatous polyposis coli (APC) gene product, and glycogen synthase kinase 3 β (GSK-3 β). Phosphorylation of Axin, APC, and β -catenin by GSK-3 β designates β -catenin for the ubiquitin pathway, where ubiquitination of β -catenin targets it for degradation by proteasomes.³⁻⁷ Binding of Wnt ligands to seven transmembrane receptors called Frizzelds and to coreceptors LRP-5 and LRP-6 (low-density lipoprotein-receptor related protein 5 and 6), which are essential for signal transmission leads to phosphorylation and increased activity of Dishevelled (Dvl).⁸ Phosphorylated Dvl inhibits the phosphorylating activity of GSK-3 β . The regulation of GSK-3 β is mediated through casein kinase-1 (CK-1), which phosphorylates Dvl. This, in turn, prevents GSK-3 β from phosphorylating its substrates, critically decreasing the binding affinities of the negative regulators Axin and APC to the β -catenin.⁹ Unphosphorylated β -catenin escapes recognition by a β -transducing repeat-containing protein, a component of an E3 ubiquitin ligase.¹⁰ This leads to β -catenin stabilization and translocation to the nucleus, where it binds transcription factors T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family and form a complex that activates transcription of downstream target genes.^{11,12} This pathway is a driving force in the development of various human cancers such as breast cancer and melanomas.¹³

Wnt-1 (wingless-type MMTV integration site family member 1) was first identified as a protooncogene activated by viral insertion in mouse mammary tumors.¹⁴ Upregulation of Wnt-1 in different

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Submitted: 09/24/07; Revised: 11/05/07; Accepted: 11/14/07

Previously published online as a *Cancer Biology & Therapy* E-publication: www.landesbioscience.com/journals/cbt/article/5300

Figure 1. *Wnt-1* expression in human breast cancer cell lines. Detection of *Wnt-1* protein in breast cancer cell lines with an anti-*Wnt-1* antibody. Western blot analysis of *Wnt-1* expression in various breast cancer cell lines, including MCF-7, T47D, SKBR-3 and JIMT-1.

human cancers has also been reported.^{15,16} Increasing evidence indicates that aberrant activation of the *Wnt* signaling pathway is associated with tumor development and/or progression.^{17,18} Recently, it has been shown that *Wnt-1*/ β -catenin signaling promoted cell survival in various cell types, inhibited apoptosis through survivin expression and induced resistance to chemotherapy.¹⁹ In addition, *c-Myc*, *Cyclin D1* and growth factors were identified as one of the transcriptional targets of β -catenin/*Tcf* in colorectal cancer cells,^{20,21} suggesting that *Wnt* signaling functions in oncogenesis, in part, is through the growth promoting activity of *c-Myc* and *Cyclin D1*.¹⁵ Human breast cancer displays nuclear accumulation of β -catenin, induction of *Cyclin D1* and *c-Myc* expression, which suggests that canonical *Wnt*/ β -catenin signaling is activated and plays important role in tumorigenesis.^{20,22}

In the present study, we found that blocking *Wnt-1* signaling by using a siRNA (short interfering RNA) anti-*Wnt-1* can induce rapid and significant apoptosis in MCF-7 breast cancer cell line with *Wnt-1* overexpression. Our results show that siRNA against the *Wnt-1* ligand can induce specifically selective apoptosis in breast cancer cells, and may be relevant as a therapeutic strategy for the treatment of breast cancer.

Results

Detection of *Wnt-1* expression in different human Breast cancer cell lines. We examined *Wnt-1* expression using monoclonal antibody in few human breast cancer cell lines, including MCF-7, SKBR-3, T47D and JIMT-1. We found high level of *Wnt-1* expression only in MCF-7 cell line. Rest of the cell lines (T47D, JIMT-1 and SKBR-3) had no *Wnt-1* expression (Fig. 1).

Designed siRNAs against *Wnt-1* mRNA inhibit cell growth. Cell proliferation of MCF-7 cells was measured over a 24 h treatment of 50 nM siRNAs specific to *Wnt-1* gene, using MTS assay to determine cell growth rates. The growth kinetics of cells treated with siRNA was compared to: untreated cells (CTRL), cells treated with SC siRNA, cells treated with siTOX and cells treated with docetaxel.

To determine nonspecific growth inhibition caused by cytotoxicity of transfection reagent or unspecific siRNA and to check transfection efficiency, SC siRNA and siTOX were used. Values shown on Figure 2A indicate the percentage of proliferation rate with respect to nontransfected control cells. Noncoding siRNA had almost no effect on cell proliferation and transfection efficiency in these experiment was roughly 70%. Few of tested siRNA sequences showed great ability to reduce cell proliferation, in some cases over 50% that was higher than cytostatic drug (DOC). The sequence that has the highest cell growth inhibition potential was W15 which inhibited proliferation by 75% related to untreated cells and was much more effective than docetaxel and WP siRNA known from literature (He et al., 2004).

MCF-7 cell line dose response after anti *Wnt-1* siRNA transfection. We also examined if there is any correlation between dose of siRNA administration and cellular response. MCF-7 cells were

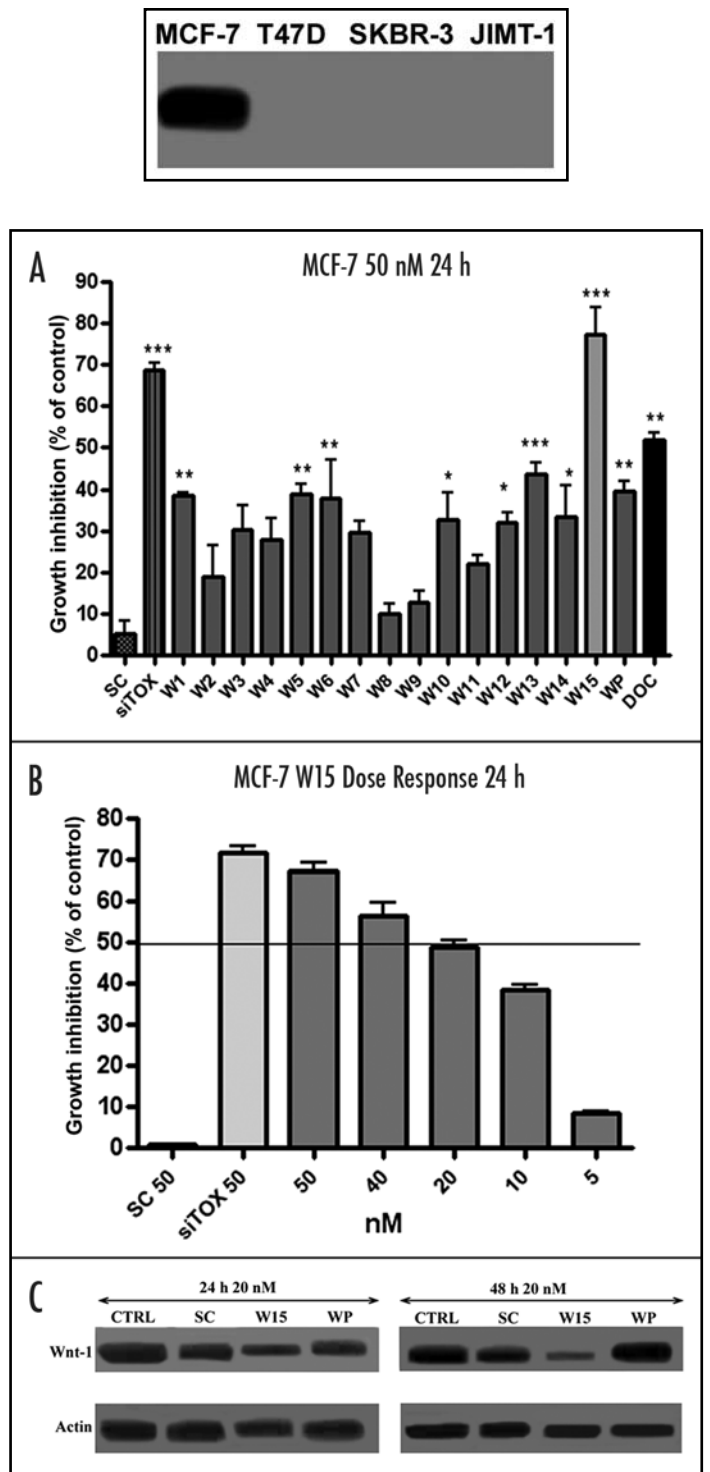


Figure 2. Screening of fifteen siRNAs against *Wnt-1* and improvement of sequence with best efficiency in silencing. (A) Antiproliferative effect of fifteen siRNA sequences against *Wnt-1*. Cell proliferation was measured using MTS viability test 24 h after 50 nM siRNA treatment. Each point represents the mean (\pm SEM) from three replications of three different experiments. *significant differences ($p < 0.05$), **highly significant differences ($p < 0.01$) and ***very highly significant differences ($p < 0.001$). (B) W15 sequence dose response of MCF-7 after 24 h siRNA exposition. Effect of each dose on cell viability was measured using MTS test. Each point represents the mean (\pm SEM) from three replications of three different experiments. Differences between means representing each dose were statistically significant ($p < 0.01$). (C) Effect of W15 and WP sequence on 41kDa *Wnt-1* protein expression in MCF-7 24 and 48 h after 20 nM siRNA treatment.

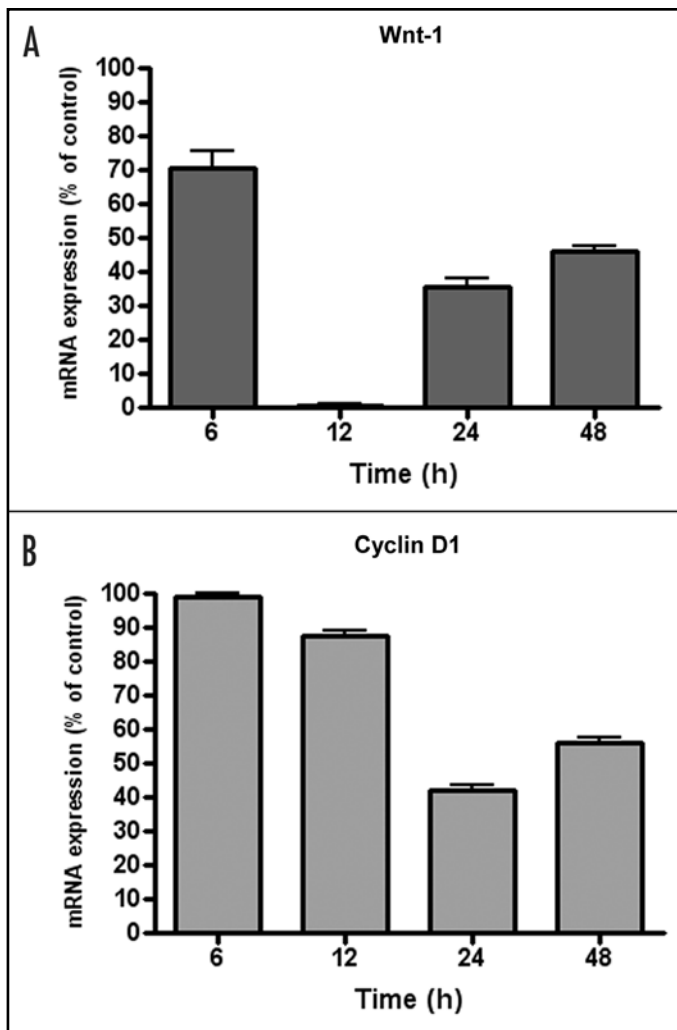


Figure 3. Real time PCR analysis of *Wnt-1* and *Cyclin D1* expression. Effect of 20 nM W15 treatment on *Wnt-1* (A) and *Cyclin D1* (B) mRNA expression in MCF-7 breast cancer cells. Each point represents the mean (\pm SEM) from three different experiments. Differences between means representing each time point were statistically significant ($p < 0.01$) except differences in *Wnt-1* mRNA expression between 24 and 48 h after treatment.

transfected with different concentrations of siRNA sequence No. W15 and inhibition of cells growth was assayed after 24 h. Concentration range was 5–50 nM and control siRNA (SC) and siTOX were used at 50 nM concentration. We obtained 50% in growth inhibition with siRNA concentration of 20 nM (Fig. 2B). There was almost no silencing at 5 nM concentration. Based on this results we decided to use 20 nM concentration for further analyses.

Designed siRNA is specific and potent in decreasing level of *Wnt-1* and *Cyclin D1* mRNA. Decreasing in mRNA level is the most direct result of siRNA action. Thus we determined whether MCF-7 cells transfection with siRNA against *Wnt-1* mRNA would cause decrease in mRNA level. Analysis was performed 6, 12, 24 and 48 h after transfection. The highest decrease in *Wnt-1* mRNA, by 96% in comparison to untreated control, we observed after 12 h MCF-7 cells treatment with W15 siRNA. Next, after 24 and 48 hrs treatment, we observed increased mRNA level, up to 47% of control (Fig. 3A). To determine specificity of our siRNA, we performed experiment with A549 cells. It is known that there is no expression

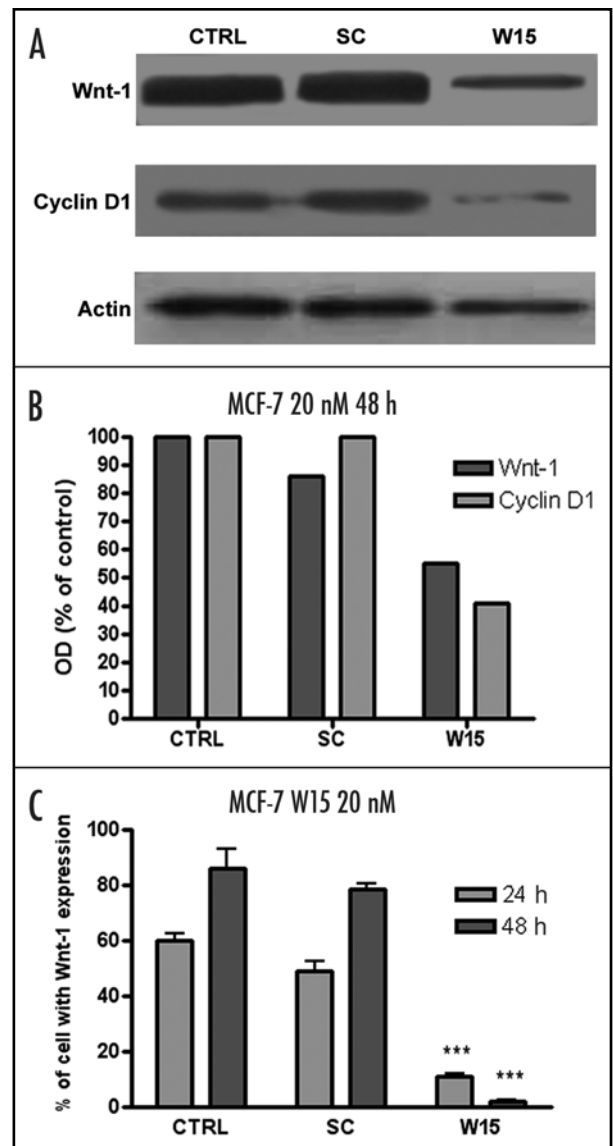


Figure 4. *Wnt-1* and *Cyclin D1* expression in MCF-7 cells treated with W15 siRNA. (A) Western blot analysis of *Wnt-1* and *Cyclin D1* protein expression 48 h after 20 nM W15 exposure. (B) Expression of actin was measured as a control of equal protein concentration. Optical density (OD) of bands presented as a percent of control obtained after western blot analysis. (C) Flow cytometry analysis of *Wnt-1* expression in MCF-7 cells treated with W15 siRNA for 24 and 48 h. Each point represents the mean (\pm SEM) from three replications of three different experiments. Differences between means representing each point were statistically significant ($p < 0.01$).

of *Wnt-1* in A549 cells (He et al 2004). We observed no changes in proliferation and mRNA level over 48 h treatment of A549 cells with W15 sequence (data not shown). We also measured mRNA level of *Cyclin D1* which is downstream target gene of *Wnt-1* pathway and is upregulated by β -catenin. Expression was measured 12, 24 and 48 h after transfection and we obtained silencing levels at 12%, 58% and 44% respectively (Fig. 3B).

siRNA specific to *Wnt-1* provokes decrease of protein level. Western blotting analysis of MCF-7 cells transfected with siRNA against *Wnt-1* were performed to determine protein level of *Wnt-1* (Fig. 2C). There was decrease in *Wnt-1* level in cells treated with

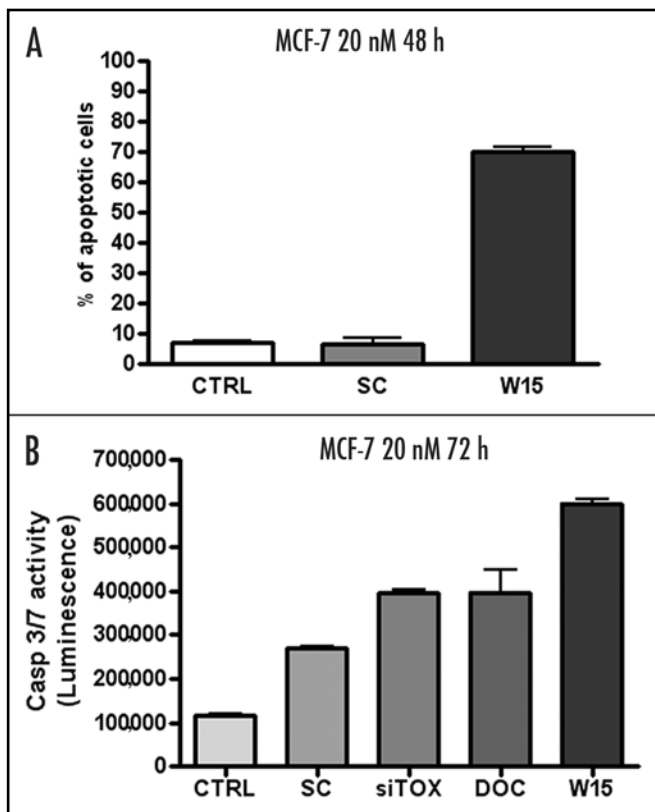


Figure 5. Analysis of apoptosis in MCF-7 cells exposed to W15 siRNA sequence. (A) Effect of 20 nM of W15 on induction apoptosis measured by flow cytometry using Annexin V and Propidium Iodide test in MCF-7 cells 48 h after siRNA treatment. (B) Caspase 3/7 activation in MCF-7 exposed to 20 nM of W15 for 72 h. Each point represents the mean (\pm SEM) from three replications of three different experiments. Differences between means representing each point were statistically significant ($p < 0.01$).

W15 sequence after 48 h comparing to the control. There were slight decrease of Wnt-1 level after WP siRNA treatment of MCF-7.

We observed the correlation between decline in Wnt-1 levels and lowered Cyclin D1 in MCF-7 cells treated with W15 siRNA for 48 hrs (Fig. 4A). In comparison to control there was 14% decrease of Wnt-1 expression after SC siRNA treatment, while there was no differences in Cyclin D1 level. Respectively, after W15 treatment Wnt-1 was lowered by 45% and Cyclin D1 by 59%, in comparison to control (Fig. 4B).

Next, the changes of Wnt-1 expression after siRNA treatment in MCF-7 cells were measured using flow cytometry (Fig. 4C). There were 60% and 86% of control cells expressing Wnt-1 after 24 h and 48 h respectively. Among cells treated with SC siRNA after 24 h there were 49% cells expressing Wnt-1, while after 48 h there were 79% of them. In turn only 11% and 2% of cells treated with W15 sequence had expression of Wnt-1 after 24 h and 48 h respectively.

siRNA against Wnt1 induced apoptosis but not necrosis. We determined the number of apoptotic cells using Annexin V and propidium iodide double staining. Among the control cells 6% were apoptotic after 48 h, while after SC siRNA treatment there were also 6% of apoptotic cells. After transfection with W15 sequence there were 71% apoptotic cells (Fig. 5A).

To verify what kind of cell death is triggered by siRNA treatment we performed caspases activation assay. We observed that after

treatment of MCF-7 cells with W15 sequence there was at least sixfold increase in activation of caspases 3 and 7, while after treatment with cytotoxic docetaxel it was about fourfold increase (Fig. 5B).

Apoptosis induced by siRNA specific to *Wnt-1* is correlated with decrease of protein level. Flow cytometry was used to verify if apoptosis was triggered by Wnt-1 decrease in MCF-7 cells transfected with siRNA against *Wnt-1*. Control cells and cells treated with SC siRNA or W15 siRNA were collected and stained 48 h after transfection (Fig. 6A and B). Accordingly, there were 80%, 70% and 10% living cells expressing Wnt-1 in those groups. The populations of apoptotic cells without Wnt-1 expression were 3%, 13% and 67% respectively. The number of apoptotic cells expressing Wnt-1 was constant (12%), while the population of living cells without Wnt-1 expression increased slightly up to 12%.

Discussion

Wnt proteins represent a growing family, to date 19 members are known, of secreted signaling molecules that are expressed in diverse tissues and have been shown to regulate cell proliferation, growth, and differentiation.^{17,23} Importantly, the deregulation of the Wnt-1 signaling pathway has been linked with tumorigenesis, presumably by promoting cell growth and proliferation. Also as was shown by Chen et al.²⁴ Wnt-1 can potently inhibit chemotherapy-mediated apoptosis indicating that Wnt-1 may transform cells via an anti-apoptotic mechanism, in addition to promoting cell growth and proliferation. Several Wnt proteins, including Wnt-1, have been shown to be overexpressed in a number of cancers.^{4,9} Although *Wnt-1* was first identified as a protooncogene activated by viral insertion in mouse mammary tumors, little is known regarding the role of Wnt ligands in human breast cancerogenesis. Wnt-1 signal may be a key survival and antiapoptotic factor in breast cancer and thus may be a potential cancer therapeutic target.

To test such hypothesis we have developed a siRNA anti-*Wnt-1* and evaluated its effect in breast cancer cell lines. Firstly we examined expression of Wnt-1 protein in different breast cancer cell lines and we found that only MCF-7 cells expressed this protein (Fig. 1). Other tested cell lines (SKBR-3, T47D and JIMT-1) had no Wnt-1 expression. These results confirmed recent data presented by He et al.⁹ that only MCF-7 cells expressed Wnt-1 protein.

Next, the fifteen siRNAs specific to human *Wnt-1*, derived from a mRNA sequence of human *Wnt-1* gene, were tested for ability to inhibit proliferation of MCF-7 cells. As we demonstrated, one siRNA (W15) can inhibit MCF-7 cells proliferation at the level up to 70% (Fig. 2A). This antiproliferative effect was stronger than after treatment with docetaxel a commonly used cytostatic drug, indicating that siRNA can be more efficient in growth inhibition.

We determined dose effect of W15 siRNA. We showed that cell growth inhibition is significantly associated with W15 concentration and 20 nM can inhibit proliferation in 50% (Fig. 2B). To confirm target specific effect of W15 on Wnt-1 expression, western blotting analysis was performed. We showed that Wnt-1 protein level was significantly decreased 48 h after W15 siRNA transfection (Fig. 2C). In contrast, the WP sequence (tested as a reference sequence) presented by He et al⁹ had minimal effect on Wnt-1 expression after this time.

To confirm effect of W15 siRNA on mRNA expression of *Wnt-1* and *Cyclin D1* (downstream target gene of Wnt/ β -catenin pathway)

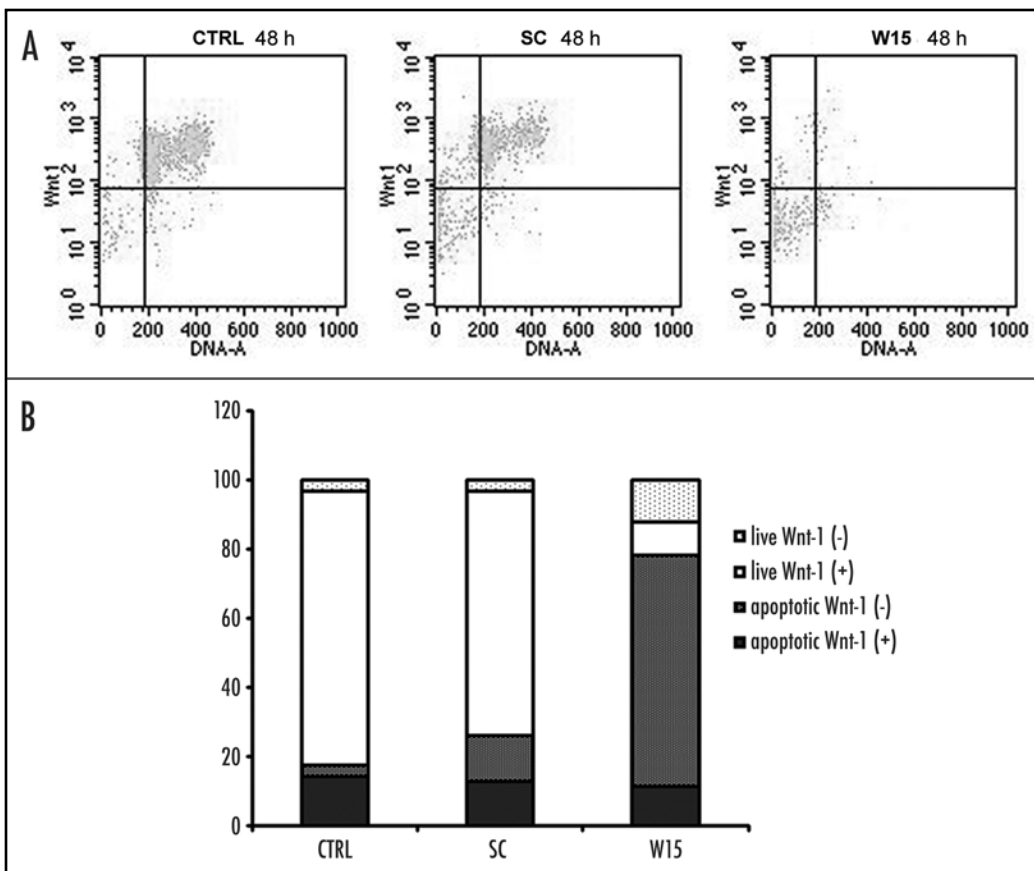


Figure 6. Flow cytometry analysis of Wnt-1 expression and MCF-7 apoptosis after W15 treatment. (A) Cytograms representing changes in cell cycle correlated with Wnt-1 expression in MCF-7 exposed to W15 for 48 h. (B) Percent of cells with or without Wnt-1 expression in alive and apoptotic population of MCF-7 cell line 48 h after siRNA treatment. Each point represents the mean (\pm SEM) from three replications of three different experiments. Differences between means representing each point were statistically significant ($p < 0.01$).

we performed a real-time PCR analysis. Our experiments showed correlation between time depended changes in *Wnt-1* and *Cyclin D1* mRNA expression. 6 h after W15 transfection *Wnt-1* mRNA was reduced by 30% according to control cells and 12 h after treatment *Wnt-1* mRNA was reduced up to 4% of control cells (Fig. 3A). *Wnt-1* mRNA reduction to 50% of control was still observed 48 h after transfection. This effect was correlated with significantly reduction in protein level 48 h after treatment analyzed by western blotting (Fig. 4A and B) and flow cytometry (Fig. 4C). These results indicate that W15 sequence can specifically and very potently silence *Wnt-1* gene expression. In the case of *Cyclin D1* expression was reduced by 60% 24 h after W15 treatment (Fig. 3B). We hypothesizes that 12 h time shift between *Wnt-1* and *Cyclin D1* mRNA reduction strongly indicate that *Cyclin D1* is downstream target of Wnt/ β -catenin¹³ and proliferation of MCF-7 cells is Wnt-1 dependent. Effect of Cyclin D1 silencing was confirmed by western blotting analysis, where we observed significant reduction of protein level 48 h after W15 treatment (Fig. 4A).

Taken together, these results indicate that W15 siRNA sequence can strongly silence Wnt-1 expression and in consequence influence on MCF-7 proliferation by reduction of Cyclin D1 expression.

Our data demonstrate that W15 siRNA against *Wnt-1* induces apoptosis in MCF-7 breast cancer cells. The siRNA-induced apoptotic

cell death (Fig. 5) not only correlates with Wnt-1 expression, but also is consistent with decreased Cyclin D1 (Fig. 4A). Apoptosis activation was confirmed by morphological changes typical to apoptosis (cell shrinkage—data not shown) and significantly increased binding of Annexin V to treated cells 48 h after W15 treatment (Fig. 5A). Also flow cytometry analysis showed strong correlation between Wnt-1 protein reduction and increasing population of dead cells (Fig. 6).

Taken together, our results suggest a strong linkage between Wnt-1 signaling and tumor cell survival. These results provide evidence that Wnt-1 silencing, can induce apoptosis in MCF-7 breast cancer cells. Therefore, we postulate that Wnt-1 upregulation may function as a survival mechanism in cancer cells by inhibiting apoptotic machinery in the MCF-7 tumor cells. The link between Wnt-1 signaling and apoptosis has been studied by several groups.²⁴⁻²⁶ Yet, the mechanism through which blocking Wnt-1 signaling induces apoptosis in human cancer cells remains unclear. Chen et al²⁴ have demonstrated that Wnt-1 signaling can inhibit induced apoptosis in

Rat-1 cells stably expressing Wnt-1. They have reported that cells expressing Wnt-1 are resistant to cancer therapy-mediated apoptosis and they have also shown that Wnt-1 signaling can inhibit cytochrome c release and caspase activation induced by the chemotherapeutic drugs or c-Myc.^{24,27} These results are consistent with our current findings that anti *Wnt-1* siRNA downregulates the Wnt/ β -catenin signaling pathway by reduction of Cyclin D1, and induces apoptosis through and caspase activation in human cancer cells. Chen et al results suggest that the Wnt-1-mediated cell survival is dependent on the canonical pathway, no difference was found in the Bcl-2 and IAP (inhibitors of apoptosis proteins) family proteins in the cells expressing Wnt-1 in their experiments.²⁴ Our findings also suggest, in theory, that silencing Wnt-1 expression may help abrogate chemotherapy resistance in tumors and/or potentate chemotherapeutic effect.

In summary, our results indicate that the siRNA specific to *Wnt-1* gene induces apoptosis in human breast MCF-7 cancer cells. Our data suggest that silencing of Wnt-1 may be a novel therapeutic strategy for breast cancer and other malignancies dependent of Wnt/ β -catenin pathway.

Materials and Methods

Cell culture. Human breast cancer SKBR-3 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA), MCF-7 and JIMT-1 cell lines were obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). T47D were kindly provided by Anna Laskowska (Institute of Immunology and Experimental Therapy, Wroclaw, Poland). MCF-7 and JIMT-1 were maintained in DMEM supplemented with 10% (v/v) FBS (fetal bovine serum), 50 µg/ml gentamycin, 2.5 µg/ml fungizone, 50 UI/ml penicillin and 50 µg/ml streptomycin (Invitrogen Carlsbad, USA). SKBR-3 and T47D were maintained in RPMI1640 supplemented with 10% (v/v) FBS, 50 µg/ml gentamycin, 2.5 µg/ml fungizone, 50 UI/ml penicillin and 50 µg/ml streptomycin (Invitrogen). All cell lines were cultured in an atmosphere of 5% CO₂/95% humidified air at 37°C, and routinely subcultured every 2 or 3 days.

RNA interference. The siRNAs-targeted human *Wnt-1* are derived from a mRNA sequence of human *Wnt-1* gene (GenBank: NM_005430). The ion-exchange high-performance liquid chromatography-purified siRNAs: W1-W15, scrambled siRNA (SC) as nonsilencing control, and literature data sequence (WP)⁹ as a positive control, ≥97% pure were purchased from Genepharma (Shanghai, China). The lyophilized siRNAs were dissolved in RNase-free water (Fermentas, Hanover MD, USA) to final concentration 20 µM. siRNA transfection was performed using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's protocol. Next cells were incubated for 6–48 h in an atmosphere of 5% CO₂/95% humidified air at 37°C before additional analysis.

Cell proliferation analysis. For proliferation tests cells were plated in Opti-MEM (Invitrogen) at 7 × 10³ cells per well in 96-well plates one day before experiments. Next day cells were transfected with fifteen siRNAs specific to *Wnt-1* mRNA and SC sequence in concentration 50 nM for 24 h using Lipofectamine RNAi MAX according to manufacturer's protocol. siCONTROL TOX (siTOX) (Dharmacon, USA) was used as a control of transfection efficiency. As a reference treatment we used cytostatic drug docetaxel (DOC) (Sigma Aldrich, St. Louis, USA) in concentration of 15 nM. After 24 h of transfection proliferation inhibition was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay [Promega, Madison, USA]—MTS (3-(4,5-dimethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] based test.

Western blot analysis. Reagents for western blotting were purchased from BioRad (Hercules, USA), anti-Wnt1 antibody was from Zymed-Invitrogen, anti-actin and anti-Cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, USA). Western blotting detection reagents were from Roche Diagnostics (Indianapolis, USA) and Light Film BioMax was from Kodak (Rochester, USA). Cells were plated at density 250 × 10³ cells per 25 cm² conical flasks and incubated in Opti-MEM medium for 24 hrs to 60% confluence. To knock-down the *Wnt-1* gene, medium was removed and replaced with the transfection medium with siRNAs complexed with Lipofectamine RNAi MAX. Cells were harvested after 24 h and 48 h by trypsinization and centrifuged at 2000 g, for 5 min, at 4°C and the cells pellet was washed twice with ice-cold PBS (phosphate buffer saline). Then the cell pellet was resuspended in 0.5 ml Total Lysis Buffer RIPA (Santa Cruz Biotechnology, Santa Cruz, CA, USA),

and incubated at 4°C for 30 min. The cell lysate were centrifuged at 9000 g, 10 min, at 4°C, then the supernatant (containing the soluble protein fraction) was carefully removed and passed six times through a 20-gauge syringe needle. The lysates were mixed 1:2 (v/v) with Laemmli sample buffer (BioRad) containing 2.5% 2-mercaptoethanol and boiled for 3 min. Samples containing identical quantities of proteins were subjected to SDS-PAGE (10% gel) together with a Kaleidoscope Marker (BioRad). The electrophoresis was run for 90 min at 100 V using a Mini Protean III cell (BioRad). The separated proteins were electroblotted on a PVDF membrane (Biorad) for 60 min at 100 V using the Mini Protean III. The membranes were blocked 1 h with 5% w/v solution of nonfat powdered milk in PBST (PBS with Tween 20) (pH 7.5). Next the membranes were incubated overnight at 4°C with the primary antibodies diluted 1:500 in 2.5% w/v solution of nonfat powdered milk in PBST. The membranes were then rinsed four times for 10 min in PBST and incubated with diluted 1:1000 in 2.5% w/v solution of nonfat powdered milk in PBST secondary antibodies conjugated with horseradish peroxidase (Sigma Aldrich) for another 1 h at room temperature. Finally, the membranes were rinsed three times for 10 min in PBST, and labeled proteins were visualized using the LumiLight (Roche) western blotting detection reagent on a high performance chemiluminescence BioMAX light film (Kodak). The image was then analyzed with a Kodak Edas System and the optical density (OD) was measured.

Real Time-PCR. Cells were plated at density 250 × 10³ cells per 25 cm² conical flasks and incubated in Opti-MEM medium for 24 hrs to 60% confluence. To knock-down the *Wnt-1* gene, medium was removed and replaced with the transfection medium with siRNAs. After 6–48 h the cultured cells were harvested by trypsinization and centrifuged at 2000 g, for 5 min, at 4°C and the cells pellet was washed twice with ice-cold PBS. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. cDNA was synthesized using ImProm-II Reverse Transcriptase kit (Promega, Madison, USA), according to manufacturer's protocol. Changes in mRNA expression of target genes were measured using Rotor-Gene™6500 (Corbett Research, Mortlake, Australia) and calculated as relative expression using Relative Expression Software Tool for Rotor-Gene® (REST-RG®). Expression was normalized to that of house-keeping genes *H3F3A* (histon H3A) and *RPLP0* (60S acidic ribosomal protein P0). As a reference-calibrator mRNA we used Stratagene QPCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA). Reactions were performed using qPCR core kit for SYBR Green I® (Eurogentec, Seraing, Belgium). All primers were purchased from Qiagen.

Immunofluorescence Staining for Flow Cytometry. Cells were plated at density 250 × 10³ cells per 25 cm² conical flasks and incubated in Opti-MEM medium for 24 hrs to 60% confluence. To knock-down the *Wnt-1* gene, medium was removed and replaced with the transfection medium with siRNAs. After 24 h and 48 h the cultured cells were harvested by trypsinization and centrifuged at 2000 g, for 5 min, at 4°C and the cells pellet was suspended in ice-cold PBS. Then cells were fixed in 1% formaldehyde for 15 min, washed twice with PBS, suspended in ice-cold 70% ethanol and stored at -20°C for 24 h. Next the cells were washed twice with PBS-1% BSA (bovine serum albumin) and incubated for 1 h with either primary antibody anti-Wnt1 (Zymed-Invitrogen) diluted 1:250 with PBS. After primary incubation the cells were washed twice with PBS, and

incubated for 1 h with 1:500 secondary antibodies labeled with Alexa Fluor 488 (Molecular Probes, Eugene, USA). The cells were then washed twice in PBS and finally incubated with a 10 µg/ml solution propidium iodide with RNase A for 15 min to counterstain the DNA. Then the cells were measured using BD FACS Calibur Flow Cytometry (Becton Dickinson, Franklin Lake, USA).

Apoptosis analysis. To analyze apoptosis, cells transfected with siRNA were harvested by trypsinization and stained using an Annexin V FLUOS Staining Kit (Roche), according to the manufacturer's protocol. Then stained cells were immediately analyzed by flow cytometry (BD FACS Calibur). Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bound to Annexin V-FITC but excluded propidium iodide. Cells in late apoptotic stages were labeled with both Annexin V-FITC and propidium iodide.

Statistical evaluation. The results were statistically evaluated by ANOVA and Tukey's multiple range tests using Prism version 4.0 software (GraphPad Software, San Diego, CA); $p \leq 0.05$ was regarded as significant and $p \leq 0.01$, as highly significant.

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