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β-catenin regulates *c-Myc* and *CDKN1A* expression in breast cancer cells

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Abstract

We previously reported that the Wnt pathway is preferentially activated in basal-like breast cancer. However, the mechanisms by which the Wnt pathway regulates down-stream targets in basal-like breast cancer, and the biological significance of this regulation, are poorly understood. In this study, we found that c-Myc is highly expressed in the basal-like subtype by microarray analyses and immunohistochemical staining. After silencing β-catenin using siRNA, c-Myc expression was decreased in non-basal-like breast cancer cells. In contrast, *c-Myc* mRNA and protein expression was up-regulated in the basal-like breast cancer cell lines. Decreased *c-Myc* promoter activity was observed after inhibiting β-catenin by siRNA in non-basal-like breast cancer cells; however, inhibition of β-catenin or over-expression of dominant-negative LEF1 had no effect on *c-Myc* promoter activity in basal-like breast cancer cell lines. In addition, *CDKN1A* mRNA and p21 protein expression were significantly increased in all breast cancer cell lines upon β-catenin silencing. Interestingly, inhibiting β-catenin expression alone did not induce apoptosis in breast cancer cell lines despite c-Myc regulation, but we observed a modest increase of cells in the G1 phase of the cell cycle and decrease of cells in S phase upon β-catenin silencing. Our findings suggest that the regulation of c-Myc in breast cancer cells is dependent on the molecular subtype, and that β-catenin-mediated regulation of c-Myc and p21 may control the balance of cell death and proliferation in breast cancer.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Keywords

basal-like breast cancer; β-catenin; *c-Myc*; *CDKN1A*; Wnt

Introduction

Triple negative breast cancer (TNBC, estrogen receptor-negative, progesterone receptor-negative and Her2/Neu-negative) is well known for its poor prognosis and lack of therapeutic targets [1]. However, TNBC is quite heterogeneous. In a recent study with gene expression profiles from 21 breast cancer data sets and 587 TNBC cases, TNBC was classified into 6 subtypes, including two basal-like, an immunomodulatory, a mesenchymal, a mesenchymal stem-like, and a luminal androgen receptor subtype [2]. In immunohistochemical (IHC) staining, basal-like breast cancers usually are triple negative, cytokeratin 5/6 positive, and/or epidermal growth factor receptor positive [3]. Basal-like breast cancer constitutes one of the most challenging subtypes of breast cancers. Although it only accounts for about 10–15% of breast cancer cases, basal-like breast cancer is responsible for a disproportionate number of breast cancer deaths [4]. This subtype of tumors typically has an early age of onset [5, 6], a strong tendency to metastasize to other organs such as the brain and lung [7, 8] and a lack of therapeutic targets[9, 10]. The molecular pathways leading to the development of basal-like breast cancer are not well understood.

Wnt/β-catenin signaling is deregulated in most human cancers [11]. However, the role of Wnt/β-catenin in breast cancer is not as clear as it is in colon cancer and hepatocarcinoma. Mutations of Wnt pathway components, such as *APC, CTNNB1* and *Axin* are rare in breast cancer [12–14]. In addition, results of IHC staining with β-catenin antibodies in breast tumors were inconsistent [15–18]. Moreover, no endogenous TCF reporter activity was detected in breast cancer cell lines [19, 20]. Much of the work has focused upstream of β-catenin, especially at the ligands level. Autocrine Wnt signaling was identified in breast cancer cell lines [21, 22]. In recent years, there is accumulation of subtype-based analysis of the Wnt pathway in breast cancer. We reported that nuclear and cytosolic accumulation of β-catenin was enriched in basal-like breast cancer and correlated with poor prognosis and metastasis, suggesting robust Wnt pathway activation in this specific subtype [23]. Reis-Filho's group described that Wnt pathway activation in breast cancer is associated with the triple negative phenotype but not with *CTNNB1* mutation [24]. Yang *et al* reported that Wnt component FZD7 over-expression is essential for tumorigenesis of TNBC [25]. Most recently, Dey et al. demonstrated that there is a subtype-specific up-regulation of the Wnt pathway in TNBC as compared to luminal (HR+) or HER2+ tumors. In contrast to *CTNNB1* mRNA levels, β-catenin protein expression was significantly higher in TNBC cell lines compared with the other two subtypes [26, 27].

The proto-oncogene *c-Myc* is a potent activator of tumorigenesis and is deregulated in a variety of cancers [28]. The *c-Myc* gene is highly expressed in basal-like breast tumors based on gene expression analysis [29–31]. This suggests that *c-Myc* may play an important role in defining basal-like breast cancer. *c-Myc* is a downstream effector of β-catenin in

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colorectal cancer [32]. A study showed that c-Myc activates Wnt in breast cancer by suppressing the Wnt inhibitors DKK1 and SFRP1, which are strongly repressed in breast cancer cell lines [33]. However, exactly how the Wnt pathway regulates *c-Myc* and other down-stream targets in breast cancer and the biological significance are still unclear. In this study, we found that the regulation of c-Myc in breast cancer cells is dependent on the molecular subtype, and that β-catenin-mediated control of c-Myc and p21 may determine the balance of cell death and proliferation in breast cancer by TCF-independent mechanisms.

Materials and Methods

Patient materials

The research protocols were approved by the Institutional Review Board of the University of Chicago and University of North Carolina. 168 sporadic breast cancer cases were selected from the tumor bank based on tissue availability from cases diagnosed between 1992 and 2002. Tissue sections containing >50% tumor cells were selected after microscopic examination.

cDNA microarrays

RNAs were extracted from 168 fresh frozen invasive breast carcinomas. Microarray was performed in Dr Perou's laboratory at University of North Carolina, Chapel Hill using Agilent oligo microarrays (Agilent Technologies, United States). All primary microarray data are in the Gene Expression Omnibus (GEO) under the accession number of GSE1992. Gene expression data were retrieved from the microarray database where the Lowess normalization procedure and data filtering was performed. In order to identify genes whose expression distinguishes basal-like tumors from all other tumor subtypes combined, we performed a two-class unpaired significance analysis of microarrays (SAM, http://www-stat.stanford.edu/~tibs/SAM/) with a false discovery rate (FDR) <5%.

IHC assays

IHC assays were performed as described [23], using a DAKO immunostainer (DAKO, Carpinteria, CA) with antibodies against c-Myc (N262, Santa Cruz Biotechnology, Santa Cruz, CA). 41 patients sample with gene expression data were used for the analysis. Archival formalin-fixed and paraffin-embedded tissues of breast cancer patients were obtained from the surgical pathology archive of the University of Chicago for tissue microarrays (TMA) construction. Cell lines HCC38 and UACC3199 were pelleted and processed by formalin-fixation and paraffin-embedding, then stained with c-Myc antibody and served as positive control. Immunostaining was scored semi-quantitatively by two observers (G. Khramtsova and A. Khramtsov). Scoring was based on intensity and percentage of positive stained cells (nuclei, cytoplasm and membrane respectively). All discrepancies were resolved by a second examination by two observers simultaneously using a multiheaded microscope. The results were scored using a scale from 0 to 3. Weak staining in <15% of tumor cell nuclei was considered as 0 ("negative"), whereas a score of 1 ("weak") was assigned when >15% of the nuclei were weak positive. Scores 2 and 3 (strong immunoreactivity in ≥15% of the nuclei) were considered together as "strong" signal.

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Cell lines

MCF-7, MDA-MB-231, HCC1937 and HCC38 cell lines were obtained from the American Type Culture Collection (Rockville, MD). UACC3199 was obtained from the University of Arizona Cancer Center (Tucson, AZ). MCF-7 was cultured in DMEM (Fisher Scientific, Hanover Park, IL). All other cell lines were grown in RPMI 1640 (Invitrogen, Grand Island, NY). The media were supplemented with 10% FBS and 1% penicillin/streptomycin.

siRNA transfection and cell growth assay

MDA-MB-231 or UACC3199 cells were plated at 1×10^5 per well in 12-well plate. The cells were transfected with si-β-catenin (Hs_CTNNB1_5_HP validated siRNA) or si-control using the HiPerfect transfection reagent (all from Qiagen, Valencia, CA), three days and six days after transfection, cell growth was measured by counting live cells after staining with trypan blue.

Cell cycle analysis

MDA-MB-231 or UACC-3199 cells were seeded at 5×10^5 cells/well in 6-well plates. Cells were transfected with si-β-catenin or si-control. 48 hours after transfection, the cells were harvested for cell cycle analysis in trypsin and washed twice with cold phosphate buffered saline (PBS). Cells were fixed in 100% ethanol for at least 24 hrs and stored at −20°C. Cells were centrifuged at 2000 rpm for 10mins at 4°C, washed twice with cold PBS, re-suspended in propidium iodide (PI) solution (0.1% triton X, 20 μg/ml PI and 0.4mg RNaseA), incubated at room temperature for 30mins. DNA content was measured using the LSRII by the Flow Cytometry Facility at the University of Chicago.

Apoptosis assay

Cells in 6-well plate were transfected with either si-β-catenin or si-control for 24 hrs. Cells were transferred to 96-well plates at the density of 10,000 cells/well. Apoptosis was measured 48 hrs later using the Caspase–glo 3/7 assay system (Promega, Madison, WI). 100 μL of caspase reagent was added directly to the treated cells, followed by one hour incubation, and luminescence activity was measure on a Bio-Tek plate reader.

RNA isolation and quantitative RT-PCR

RNA extraction and cDNA synthesis were carried out as described [34]. Real time PCR was performed on an ABI 7900 instrument with power SYBR Green (Applied Biosystems). The primer sequences for *c-Myc*: Forward 5'-CAG CTG CTT AGA CGC TGG ATT T-3'; Reverse 5'-ACC GAG TCG TAG TCG AGG TCA T-3'.The primer sequences for *CTNNB1*: Forward 5'-AAT ACC ATT CCA TTG TTT GTG CAG-3'; Reverse 5'-AGC TCA ACT GAA AGC CGT TT-3'. The primer sequences for *CDKN1A*: Forward 5'-GCG ATG GAA CTT CGA CTT TGT-3'; Reverse 5'-GGG CTT CCT CTT GGA GAA GAT-3'. The primer sequences for *ACTB:* Forward: 5′-CGG TCA GGT CAT CAC TAT CGG-3′; Reverse: 5′-CAC AGG ATT CCA TAC CCA GGA-3′. *ACTB* was used as a control for normalization.

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Protein extraction and Western blot

Protein extraction and Western blot were carried out as previously described [34]. Western blot was repeated at least one time. Anti-β-catenin antibody was from BD Transduction (Cat. No. 610154, Franklin Lakes, NJ). Anti-c-Myc (N262) antibody was purchased from Santa Cruz Biotechnology (Cat. No. Sc-764, Santa Cruz, CA). Anti-p21 monoclonal antibody was from Millipore (Cat. No. 05–345, Temecula, CA). Anti-β-actin antibody was from Sigma-Aldrich (Cat. No. A2228, Santa Louis, MO)

Luciferase assay

Luciferase activity was measured with the Dual Luciferase Assay system (Promega, WI). A renilla luciferase reporter construct, pGL4.75 (Promega), was used as a transfection efficiency control. FuGENE HD (Roche Molecular Biochemicals) was used as the transfection reagent. The cell lysate was prepared using Passive Lysis Buffer (Promega). The Luc-*c-Myc* promoter was kindly provided by Dr. Kato of Tsukuba University, Japan [35]. Mutant β-cateninS37A was from Stephen Byers at Georgetown University.

Statistical analyses

Two-factor analysis of variance (ANOVA) was used for gene expression analysis with real-time PCR. Fisher's exact test was used to examine whether there is difference in IHC staining of c-Myc between basal-like breast cancer and non-basal-like breast cancer. The Student's t-test was used for the cell growth and apoptosis assay. $P<0.05$ is considered statistically significant.

Results

c-Myc is highly expressed in basal-like breast cancer

By analyzing the gene expression profiles of 168 breast tumors using DNA microarrays, we found that *c-Myc* was highly expressed in the basal-like subtype in comparison to the other four breast cancer subtypes [31] (Figure 1A). In addition, *CCNE1* and *CDKN2A* were up-regulated in basal-like tumors as previously reported [36]. In contrast, *CDKN1A* was down-regulated. To validate this observation, we analyzed c-Myc protein expression by IHC in 41 primary breast tumors that were classified with DNA microarray analysis (Fig. 1B). Cell lines HCC38 and UACC3199 (Fig. 1B a and b) were used as positive control. As shown in Table 1, c-Myc was highly expressed in 79% of basal-like tumors; in contrast, only 26% of non-basal-like tumors showed high level c-Myc expression. This difference was statistically significant ($p=0.005$ by Fisher's exact test). In addition, we confirmed the concordant expression of beta-catenin and c-Myc in 9 of 14 basal-like breast tumors although it did not reach statistical significance due to small sample size.

Protein expression of c-Myc was analyzed in five cell lines (MCF-7, MDA-MB-231, HCC1937, UACC3199 and HCC38) by Western blot. As shown in Figure 2A, c-Myc was highly expressed in the four TNBC cell lines (three basal-like cell lines HCC1937, UACC3199 and HCC38, and a claudin-low line MDA-MB-231) in comparison with the luminal cell line MCF-7. While, β-catenin proteins was highly expressed in the basal-like cell lines in comparison to non-basal-like cell lines MDA-MB-231and MCF-7, and the

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active form of β-catenin (unphosphorylated protein) was only detected in the basal-like breast cancer cells (Figure 2A and 2B). In contrast, no or very little p21 protein expression was detected in the three basal-like breast cancer cell lines and the claudin-low line MDA-MB-231compared to MCF-7 cells (Fig. 2A).

β-catenin regulates *c-Myc* and *CDKN1A* gene expression

To understand how β-catenin regulates *c-Myc* expression in breast cancer, we examined *c-Myc* mRNA and protein expression after knocking down β-catenin using siRNA transfection. *c-Myc* expression was decreased in MCF-7 and MDA-MB-231 cells by depletion of β-catenin. Surprisingly, *c-Myc* mRNA and protein expression were up-regulated in the three basal-like breast cancer cell lines after knocking down β-catenin (Figure 2C, 2B; Figure S1, S2). The difference between basal-like and non-basal-like was statistically significant ($p<0.001$). These data suggest that β-catenin functions as a repressor of *c-Myc* in basal-like breast cancer cells, but perhaps as an activator of *c-Myc* expression in non-basal-like breast cancer cells.

To examine how β-catenin regulates *CDKN1A* expression, we analyzed mRNA and protein expression of *CDKN1A* after depleting β-catenin in the same experimental setting. As shown in Fig. 2D and 2B, *CDKN1A* mRNA and protein expression levels were increased in all cell lines tested after the β-catenin knocking down, suggesting that β-catenin inhibits *CDKN1A* gene expression in breast cancer cells (Fig. 2B, Fig. S1 and S2). UACC3199 cells showed the least increase of *CDKN1A* mRNA and protein expression.

β-catenin regulates gene expression in a TCF-independent manner

To assess more completely the mechanism(s) by which β-catenin affects *c-Myc* transcription in more detail, luciferase assays were conducted with a *c-Myc* promoter construct in the five breast cancer cell lines (MCF-7, MDA-MB-231, HCC1937, UACC3199, and HCC38). We observed that inhibiting β-catenin expression caused reduction of *c-Myc* promoter activity in non-basal-like breast cancer cells but had no effect on *c-Myc* promoter activity in basal-like breast cancer cells (Fig. 3A). To understand whether β-catenin regulates *c-Myc* transcription through TCF/LEF activity, a dominant-negative form of LEF1 (dnLEF1) was co-transfected with the *c-Myc* promoter. Surprisingly, dnLEF1 had little effect on *c-Myc* promoter activity in all five cell lines that had been tested (Fig. 3B). This indicates that endogenous β-catenin regulates *c-Myc* transcription in a TCF-independent pathway in breast cancer. To further support this conclusion, luciferase assays were carried out in UACC3199 cells, with ectopic over-expression of S37A β-catenin, a stabilized mutant of β-catenin, we observed a four-fold increase of *c-Myc* promoter activity (Fig. 3C). Introduction of dnLEF1 suppressed S37A β-catenin induced-*c-Myc* promoter activity, but had no effect on the promoter activity if only endogenous β-catenin were present.

β-catenin inhibition does not induce c-Myc-dependent apoptosis in cultured breast cancer cells

Based on the observations that 1) β-catenin and *c-Myc* are highly expressed in basal-like breast cancer cells and 2) β-catenin functions as a repressor of *c-Myc* in basal-like breast cancer cells, we hypothesized that there was an antagonism between endogenous β-catenin

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and *c-Myc* in regulating apoptosis in basal-like breast cancer cells. We tested whether inhibiting β-catenin expression could sensitize the basal-like breast cancer cell lines to apoptosis. Apoptosis was measured with the caspase glo 3/7 assay after knocking down β-catenin by siRNA in the five breast cancer cell lines. As shown in Figure 4, suppressing β-catenin expression did not induce activation of caspase 3/7 in these breast cancer cell lines, suggesting that β-catenin inhibition alone was insufficient to induce c-Myc-dependent apoptosis in cultured breast cancer cells.

β-catenin controls breast cancer cell cycle and growth

Because knocking down β-catenin induces up-regulation of *CDKN1A* gene expression, we tested the effect of β-catenin knockdown on cell cycle progression using MDA-MB-231 and UACC3199 cells. As shown in Table 2, in concordance with p21 up-regulation, we observed a subtle increase of cells in G1 and decrease of cells in S phase in both cell lines. This result indicates that inhibiting β-catenin partially blocks G1-S cell cycle progression.

Cell proliferation was analyzed by cell quantification three days and six days after siRNA transfection. There was no statistic difference of cell numbers with three days inhibition of β-catenin. We observed a 30% reduction of proliferation six days after β-catenin down-regulation (Fig. 5).

Discussion

In this study, using cell line models, we confirmed our previous observation that the Wnt pathway is preferentially activated in basal-like breast cancer [23]. We demonstrated that c-Myc is highly expressed in the basal-like subtype using microarray and IHC analyses. In contrast to the observation that β-catenin positively regulates *c-Myc* expression in colorectal cancer and hepatocarcinoma, β-catenin regulates *c-Myc* expression in a subtype dependent manner in breast cancer. After silencing β-catenin using siRNA, *c-Myc* expression was decreased in non-basal-like breast cancer cells. However, *c-Myc* mRNA and protein expression was up-regulated in the basal-like breast cancer cell lines. Interestingly, β-catenin regulates *c-Myc* in a TCF-independent manner. Furthermore, we found that β-catenin represses *CDKN1A* expression in a subtype-independent manner.

In general, c-Myc promotes cellular proliferation, which is necessary for tumorigenesis. However, over-expression of c-Myc causes apoptosis. Abrogation of c-Myc-induced apoptosis is crucial for cellular transformation and tumorigenesis. Basal-like tumor cell lines express high levels of both β-catenin and c-Myc. When β-catenin was knocked down with siRNA, we observed an unexpected increase of c-Myc expression. For that reason, we propose that β-catenin antagonizes c-Myc expression. While appearing contradictive in some regards, one could view this as a survival mechanism for tumor cells such that c-Myc level is tightly controlled so as not to be too high in basal-like tumor cells; otherwise, the cells would undergo apoptosis. Consistent with this interpretation, it has been reported that Wnt/β-catenin signaling suppressed apoptosis by inhibiting c-Myc-induced caspase activation when over-expressing both Wnt1 and c-Myc in Rat-1 and RIE cell lines [37]. On the other hand, we could not exclude the possibility that the antagonism only occurs when the expression of both β-catenin and c-Myc has reached certain levels. Since silencing β-catenin

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caused up-regulation of *c-Myc* expression, we hypothesized that inhibition of β-catenin in basal-like breast cancer may promote c-Myc-induced apoptosis. However, inhibition of endogenous β-catenin expression does not induce apoptosis in basal-like breast cancer cell lines as measured by caspase 3/7 activation. This suggests that alternative pathways exist to prevent c-Myc-induced apoptosis and inhibiting β-catenin alone is not enough to induce apoptosis. There are multiple mechanisms involved in c-Myc deregulation in breast cancer including gene amplification, transcriptional regulation, and mRNA and protein stabilization [31]. In addition, high level of c-Myc expression is associated with poor outcomes [31]. However, the mechanism by which c-Myc is highly expressed in basal-like breast tumors is still unknown. Furthermore, there are many genes expressed along with c-Myc, and comprehensive transcriptome analysis is needed in future to fully appreciate the upstream regulators and downstream effectors of c-Myc.

p21 has been shown to be a direct target of c-Myc, and c-Myc promotes cell proliferation through inhibition of p21 [38, 39]. In this study, we observed that β-catenin represses p21 transcription in addition to c-Myc. Inhibition of β-catenin caused up-regulation of p21 at mRNA and protein levels even though the c-Myc expression level was increased in basal-like breast cancer after treatment, resulting in a G1 cell cycle arrest and a reduction of cell proliferation. UACC3199 cells showed the most modest increase of *CDKN1A* mRNA and protein expression after β-catenin knockdown, probably due to repression of high levels of c-Myc in the cells. It has been reported that p21 was up-regulated in sFRP1-expressing tumors, suggesting that it is a downstream mediator of WNT signaling [40]. Our results demonstrate that β-catenin can directly regulate p21 expression. p21 has also been shown to have anti-apoptotic activity and probably plays a key role in cell survival following DNA damage by promoting the growth arrest that permits DNA repair [41]. This may partially explain why β-catenin knockdown did not influence apoptosis.

The central dogma of the canonical Wnt pathway is cytosolic β-catenin stabilization, nuclear translocation and transactivation of downstream gene targets after binding to TCF/LEF transcriptional factors. Surprisingly, our data suggest that β-catenin regulates *c-Myc* in a TCF-independent manner. Unlike most colon cancer cells, there was no TCF/LEF reporter activity in breast cancer cell lines [19, 20] irrespective of the presence of “active β-catenin” or not. Since silencing β-catenin in non-basal-like cells affects both endogenous *c-Myc* and *c-Myc* reporter activity, we suspect that a pool of β-catenin other than so-called “active β-catenin” is responsible for the regulation. These findings suggest that Wnt pathway regulation may be unique in breast cancer cells. Because the *c-Myc* reporter activity was extremely high (i.e., perhaps saturated) in basal-like cancer cells compared with non-basal-like, silencing β-catenin increased endogenous *c-Myc* expression in basal-like cells without increasing reporter activity. Exogenous S37A mutant activated the *c-Myc* reporter in basal-like UACC3199 cells in a TCF-dependent fashion, which could be antagonized by dnTCF1. These data collectively illustrate the complexity of β-catenin-mediated gene regulation and indicate that further study is warranted to understand the mechanism of TCF-independent regulation of gene expression via β-catenin, particularly if considering β-catenin as an anticancer therapeutic target.

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In addition to recent reports concerning Wnt activation in basal-like and TNBC, Schade et al. reported that β-catenin signaling is a critical event in the basal category of ERBB2-mediated mammary tumor progression [42]. They proposed that targeting of β-catenin–dependent signaling may hold potential therapeutic value in the treatment of the basal category of ERBB2-positive breast cancer. Furthermore, MDA-MB-231 is the representative of the claudin-low breast cancer subtype, characterized by the enrichment of EMT and stem cell-like features, and significantly associated with disease recurrence [43]. Further underscoring the complexity regarding Wnt signalling in breast cancer and urgent need for personalized medicine for breast cancer treatment, we found that this cell line has a drastic difference in terms of Wnt pathway activation compared with basal-like breast cancer. And finally, while this work primarily focused on cell line models, it will be necessary and important to further validate the findings using primary tumors from patients, including patient-derived xenografts, in future work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Definitions for abbreviations

ANOVA	analysis of variance
APC	Adenomatosis polyposis coli
CCNE1	Cyclin E1
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog
CTNNB1	Catenin (cadherin-associated protein), beta 1
FBS	fetal bovine serum
FDR	false discovery rate
FZD7	Frizzled family receptor 7
G1 phase	gap phase 1
G2 phase	gap phase 2
GEO	Gene Expression Omnibus

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IHC	immunochemistry
PBS	phosphate buffered saline
PI	propidium iodide
S phase	DNA synthesis phase
SAM	significant analysis of microarrays
TCF4/LEF1	T cell specific factor 4/ Lymphoid enhancer-binding factor 1
TNBC	Triple negative breast cancer (Estrogen receptor-negative, Progesterone receptor-negative and Her2/Neu-negative breast cancer)
Wnt	Wingless-type MMTV integration site family

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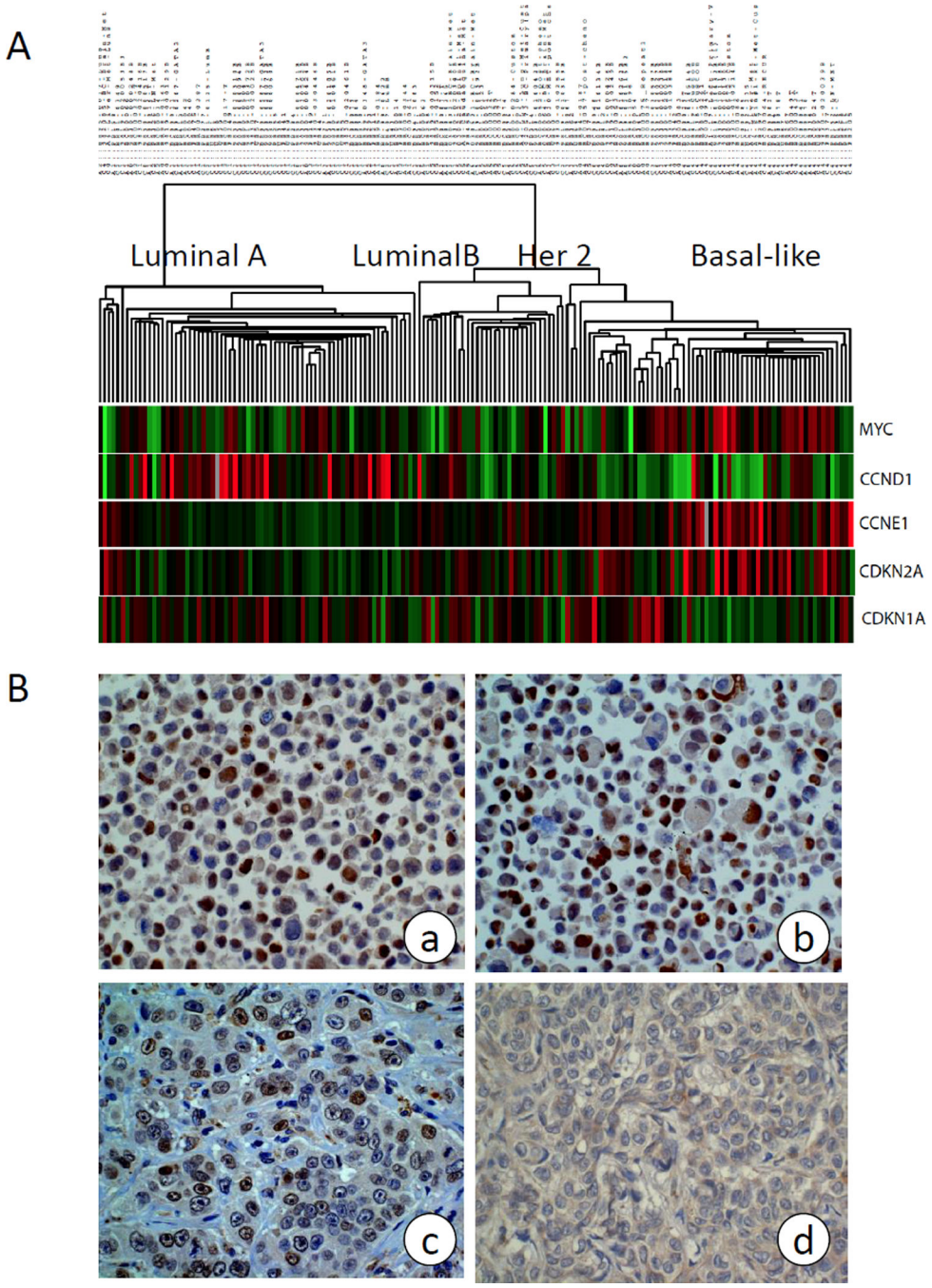

Figure 1. c-Myc is highly expressed in basal-like breast cancer

(A) Microarray analysis of 168 primary tumors. Supervised clustering shows *c-Myc, CCNE1* and *CDKN2A* are highly expressed, while expression of *CCND1* and *CDKN1A* is low in basal-like breast cancer compared with other subtypes.

(B) c-Myc protein expression analyzed by IHC with basal-like breast cancer cell line UACC3199 (a) and HCC38 (b), and with Myc high (c) and Myc low (d) primary tumors. Cell lines HCC38 and UACC3199 were pelleted and processed by formalin-fixation and paraffin-embedding, then stained with c-Myc antibody and served as positive control.

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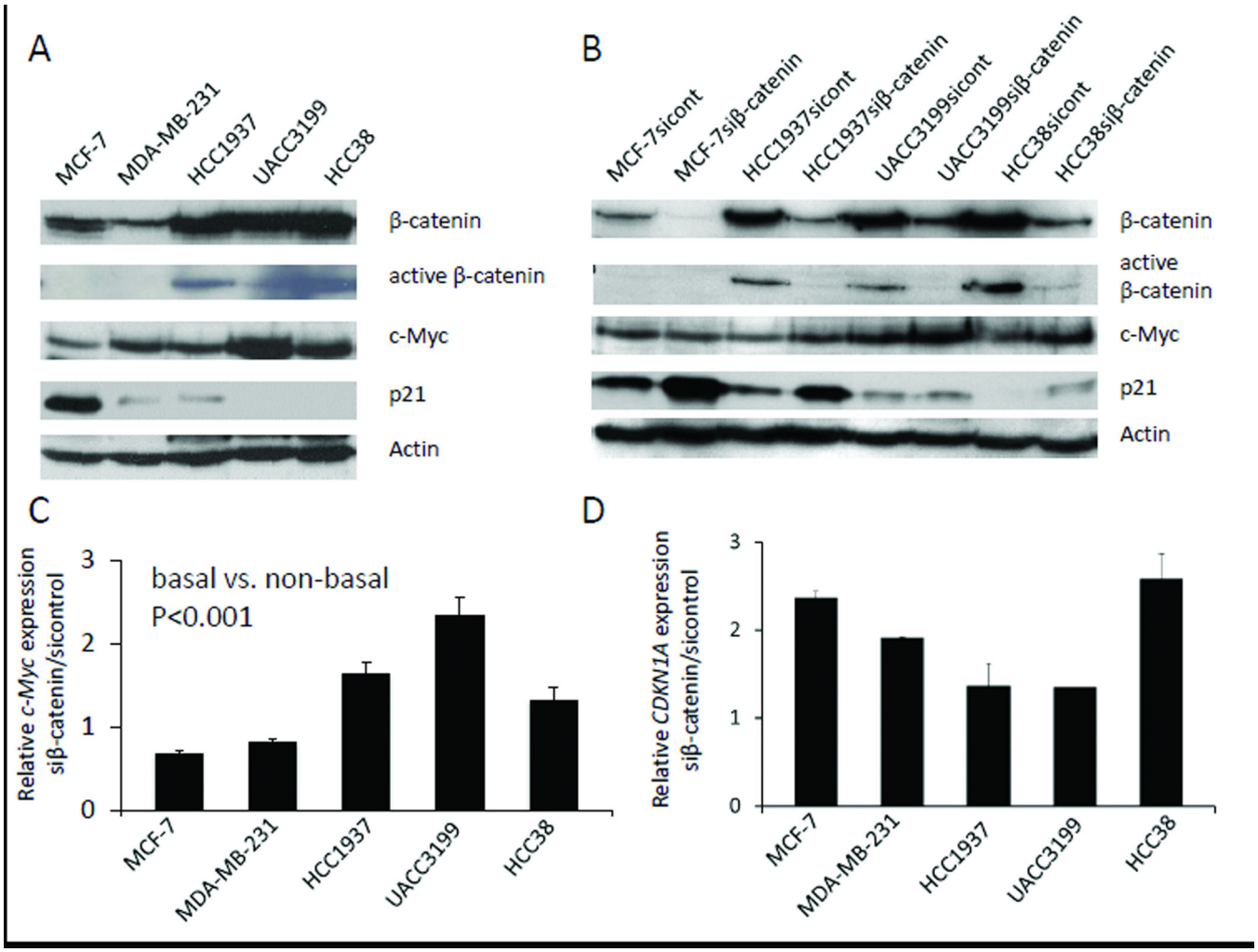

Figure 2. β-catenin regulates *c-Myc* and *CDKN1A* gene expression

(A) Protein expression of β-catenin, active β-catenin, c-Myc and p21 in five breast cancer cell lines analyzed by Western blot. Actin was used as a loading control.

(B) Protein expression after knocking down β-catenin by siRNA in breast cancer cells. Scramble siRNA was used as a control.

(C) *c-Myc* mRNA expression by quantitative RT-PCR after 48 hr si-β-catenin treatment in breast cancer cells (basal vs. non-basal, P<0.001 by Two-factor ANOVA. For each cell lines, si-control vs. si-β-catenin P<0.05 by Two-factor ANOVA). Results are represented as the average of two independent experiments with triplicates. Bars represent mean ± SD.

(D) *CDKN1A* mRNA expression by quantitative RT-PCR after 48 hr si-β-catenin treatment in breast cancer cells. Results are represented as the average of two independent experiments with triplicates. Bars represent mean ± SD.

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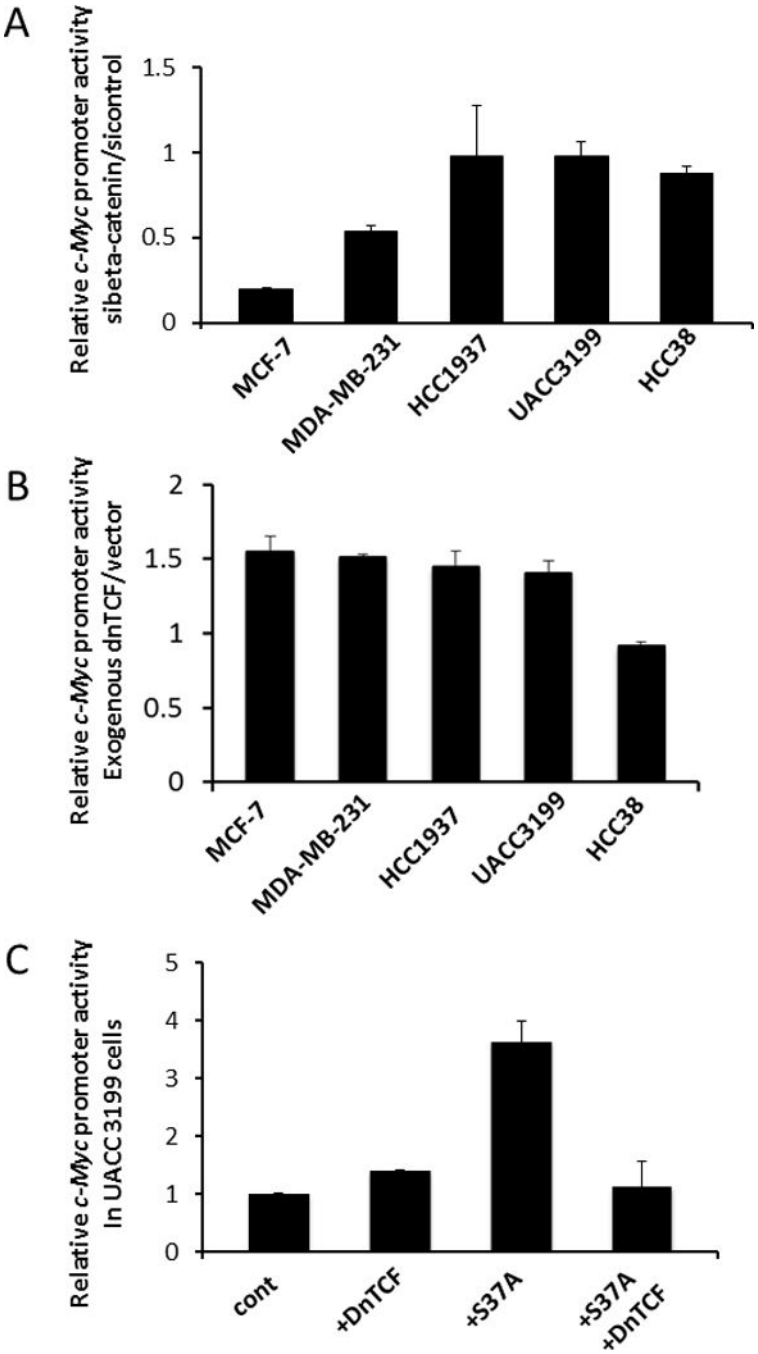

Figure 3. Luciferase promoter assay in breast cancer cell lines

Luciferase activity was measured using a dual-luciferase reporter assay system. Results are represented as the average of two independent experiments with triplicates. Bars represent mean ± SD.

(A) *c-Myc* promoter assay after inhibiting β-catenin by siRNA for 48hr.

(B) Effect of dnLEF1 on *c-Myc* promoter activity.

(C) Effect of exogenous mutant β-catenin on *c-Myc* promoter activity in UACC3199 cells.

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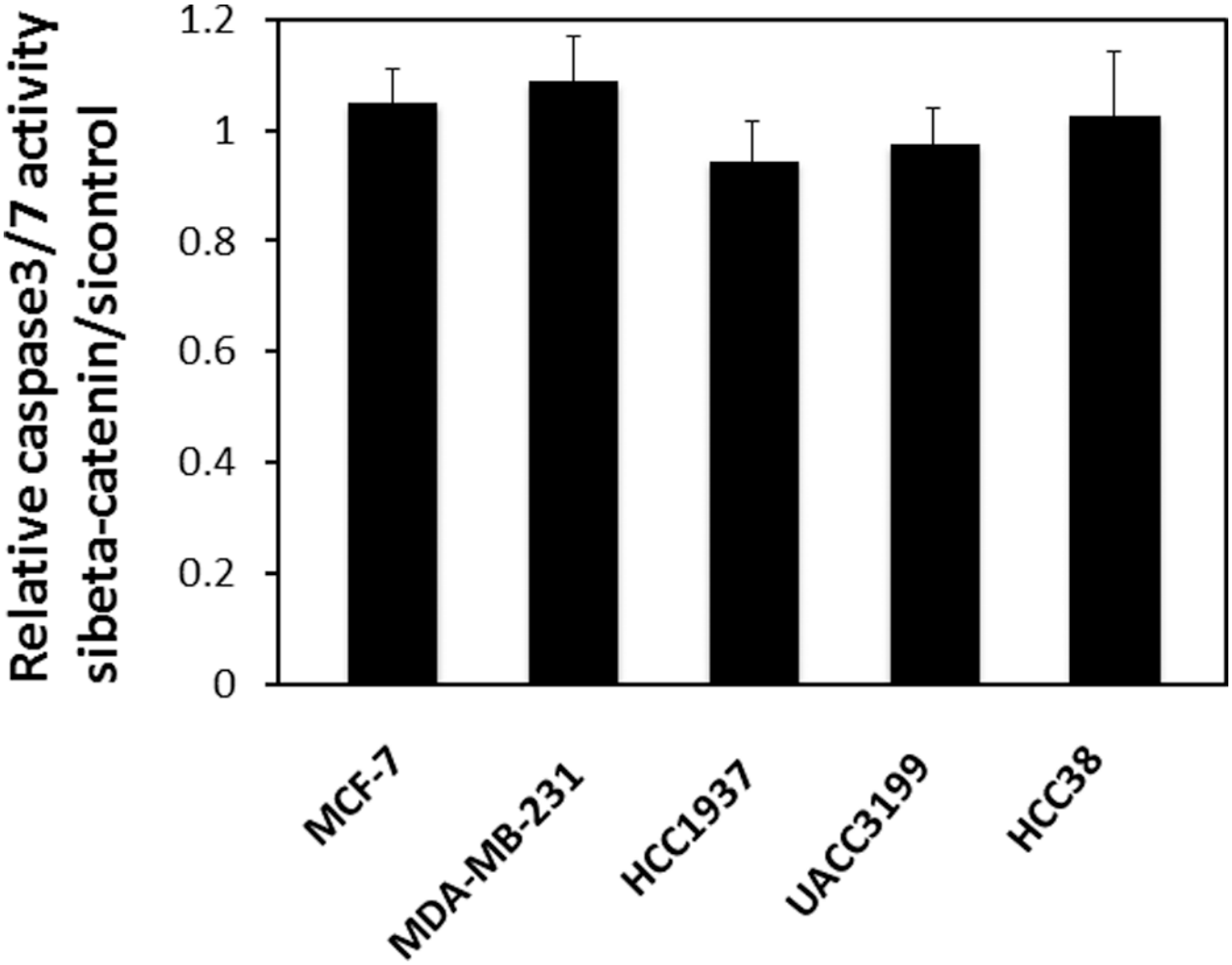

Figure 4. Suppression of β-catenin expression alone is insufficient to induce apoptosis in breast cancer cells

Apoptosis was analyzed by caspase-glo 3/7 assay. After siRNA treatment, the cells were starved in serum-free media for 48 hrs before apoptosis detection. The experiments were repeated three times. Bars represent mean ± SD.

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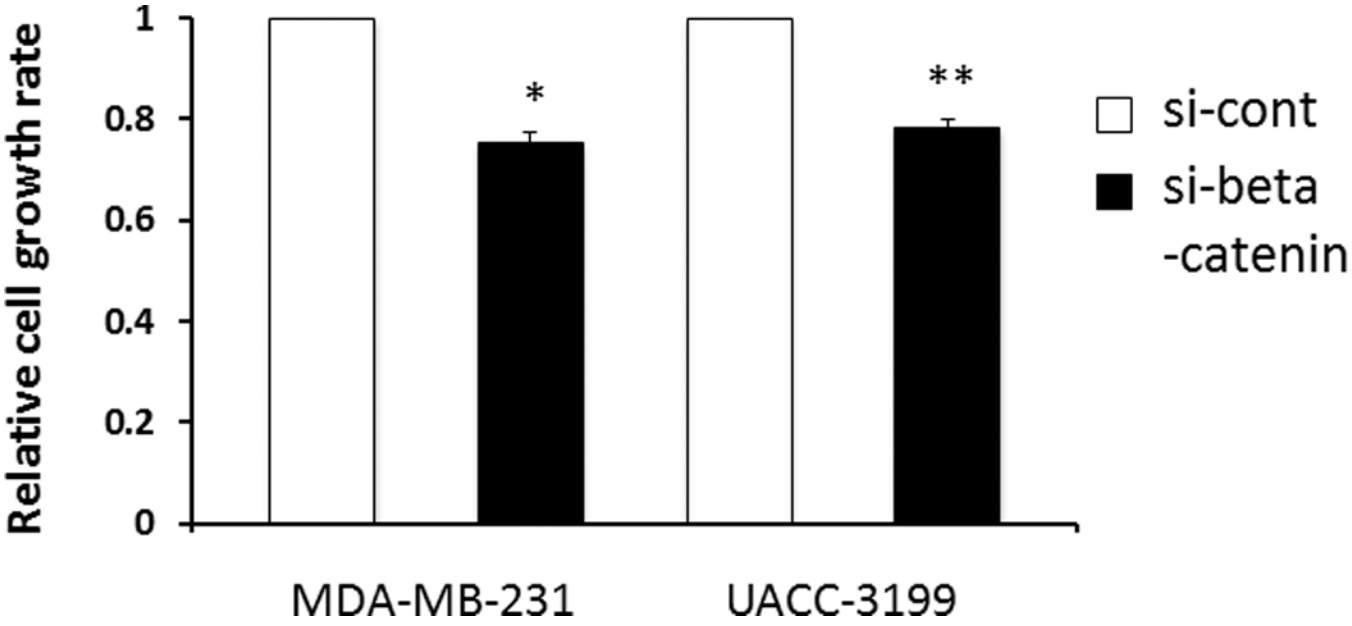

Figure 5. β-catenin knockdown by siRNA partially inhibits breast cancer cell proliferation

Cell proliferation was analyzed by cell counting at day 3 and day 6 after siRNA treatment. Relative growth at day 6 is shown (si-β-catenin vs. si-control, $P<0.01$ with the Student's t-test). Results are represented as the average of two independent experiments performed in triplicate. Bars represent mean ± SD.

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Table 1

c-Myc expression by IHC in 41 primary tumors with subtype status by microarray

	Non-basal-like	**Basal-like**	**Total**
Myc-low	20 [LumA: 9 ; LumB: 3 ; Her2: 8] (74%)	3 (21%)	23 (56%)
Myc-high	7 [LumA: 4 ; LumB: 0 ; Her2: 3] (26%)	11 (79%)	18 (44%)
Total	27 [LumA: 13 ; LumB: 3 ; Her2: 11] (66%)	14 (34%)	41 (100%)

Basal-like vs. non-basal-like: P=0.005 by Fisher's exact test.

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Table 2

Cell cycle analysis by FACS after 48 hr siRNA treatment

	MDA-MB-231 si-Control	MDA-MB-231 si-β-catenin	UACC3199 si-control	UACC3199 si-β-catenin
G1%	50.25±0.67	55±1.84	28.25±0.64	35.2±2.83
S%	28.25±3.46	22.3±4.1	42.8±1.56	35.4±2.4
G2%	15.3±0.57	17.7±1.84	21.15±0.49	20.7±2.4

Results are represented as the mean ± SD (Standard deviation) of two independent experiments.